Essential amino acids and muscle protein recovery from resistance exercise

ELISABET BÖRSHEIM, KEVIN D. TIPTON, STEVEN E. WOLF, AND ROBERT R. WOLFE
Metabolism Unit, Department of Surgery, Shriners Hospital for Children/Galveston,
University of Texas Medical Branch, Galveston, Texas 77550

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MUSCLE PROTEIN SYNTHESIS is stimulated in the recovery period after resistance exercise (4, 8). However, the rate of muscle protein breakdown is also increased, thereby blunting the change in the net balance between synthesis and breakdown. Although net muscle protein balance is generally improved after resistance exercise, it remains negative. Therefore, nutrient intake is necessary to achieve positive net muscle protein balance.

The optimal composition and amount of nutrient ingestion to maximally stimulate muscle protein synthesis after resistance exercise are not known. It is clear that amino acids or protein should be a component, as we have previously shown that either the infusion (5) or ingestion of a large amount (17) (30–40 g) of amino acids after exercise stimulates muscle protein synthesis. Furthermore, muscle protein synthesis was increased 3.5-fold when only a small amount (6 g) of a mixture of essential amino acids (EAAs) was given along with 35 g of carbohydrate after resistance exercise (16). The latter results (16) suggest that a relatively small amount (i.e., 6 g) of EAAs can effectively stimulate muscle protein synthesis, but the independent effects of amino acids and carbohydrate were not assessed. Thus a principal goal of this study was to determine the independent effect of ingestion of a bolus of 6 g of EAAs on net muscle protein synthesis after resistance exercise.

The proportional contributions of individual amino acids to a mixture ingested after resistance exercise can potentially affect the response. In an earlier study (17), we concluded that the ingestion of only EAAs was necessary for stimulation of muscle protein synthesis, because the effect on net muscle protein synthesis was similar when subjects were given either 40 g of a balanced mixture (21.4 g EAAs and 18.6 g nonessential amino acids (NEAAs), roughly in proportion to their relative contributions to muscle protein), or 40 g of only EAAs. However, if the NEAAs are not necessary for stimulation of synthesis, it is unclear why ingestion of the EAAs alone did not stimulate muscle protein synthesis to a greater extent than did the balanced mixture. It could be that, in fact, the NEAAs served no function, but the amount of EAAs in the balanced mixture (21 g) exceeded the maximal effective dose. In this case, intake of more than 21 g of EAAs (i.e., 40 g) would have no further effect than that already elicited by 21 g. If true, then comparison of less than the maximally effective dose of EAAs with a comparable amount of a balanced mixture of EAAs + NEAAs should reveal a significantly greater effect of the EAAs. Thus a secondary goal of this project was to compare the response to 6 g of an EAA mixture with the response to 6 g of a balanced mixture of amino acids, which included ~3 g of EAAs and 3 g of NEAAs. We speculated that 6 g of EAA would be less than the maximally effective dose of EAA, and thus there would be a greater response to the EAAs than to the mixture of EAAs and NEAAs. The results of the response to 6 g of the balanced mixture have been published previously (14).

The composition of the mixture of EAAs we have tested in this and previous studies (16) was originally based on the composition of muscle protein, with the

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idea being to increase the availability of each EAA in proportion to its requirement for the synthesis of muscle protein. However, different clearance rates of individual amino acids could result in rates of uptake that do not directly correspond to the composition of the ingested mixture. This would be reflected by disproportionate changes in concentrations of blood amino acids compared with the composition of the ingested mixture. Therefore, another goal of the current study was to measure blood and intramuscular concentrations of all amino acids before and after ingestion of the mixture to determine whether the mixture achieved the goal of causing proportional increases in all EAAs, and if any NEAA dropped sufficiently to become potentially rate limiting for protein synthesis. This information could help to formulate a new mixture that might be more effective than the EAA mixture we have used.

Not only is the composition of nutrient ingestion after exercise potentially important, but also the pattern of ingestion may affect the response of muscle protein synthesis. We have previously shown that amino acid concentration, per se, is not a direct determinant of muscle protein synthesis. Thus, when blood amino acid concentrations were elevated to a steady-state level about twice the basal values for 6 h, muscle protein synthesis was stimulated over the first 2 h but thereafter returned to the resting level despite persistent elevations in blood amino acid concentrations (6). Similarly, in our earlier study after exercise (16), the rate of net muscle protein synthesis returned to the basal level 60 min after the ingestion of a bolus of EAAs + carbohydrate, even though the blood amino acid concentrations were still approximately double the basal level. These observations are best explained by the muscle becoming refractory to a persistent elevation in amino acid concentrations. If true, it follows that the synthetic response to ingestion of a bolus of amino acids would be refractory to a second dose until the concentrations from the first dose returned to the basal level. It was therefore a further goal of this study to determine whether the response of net muscle protein synthesis to a second dose of amino acids would be affected by the ingestion of an initial dose 1 h earlier.

MATERIALS AND METHODS

Subjects. Six healthy subjects (3 men and 3 women), 19–25 yr of age, participated in the study (Table 1). Subjects were recreationally active but were not involved in a consistent resistance or endurance-training program. They were fully informed about the purpose and procedures of the study before written consent was obtained. Before participation in the experiments, each subject had a complete medical screening, including vital signs, blood tests, urine tests, and a 12-lead electrocardiogram, for determination of health status at the General Clinical Research Center (GCRC) of the University of Texas Medical Branch (UTMB) at Galveston, TX. The protocol was approved by the Institutional Review Board of the UTMB.

Preexperimental procedures. At least 1 wk before an experiment, subjects were familiarized with the exercise protocol, and their one repetition maximum (1RM, the maximum weight that can be lifted for one repetition) was determined by the procedure described by Mayhew et al. (13) (Table 1). The leg volume of each subject was estimated from anthropometric measures of leg circumference and height at multiple points down the length of the leg (Table 1).

Experimental protocol. Each subject was studied once. The subjects were instructed not to exercise for 2 days before an experiment, not to make any changes in their dietary habits, and not to use tobacco or alcohol during the last 24 h before an experiment. The subjects reported to the GCRC in the evening before an experiment for an overnight stay and were fasted from 10:00 PM.

The experimental protocol is shown schematically in Fig. 1. At ~6:00 AM, an 18-gauge polyethylene catheter (Cook, Bloomington, IN) was inserted into an antecubital arm vein for the primed continuous infusion of stable isotopes of amino acids. After obtaining a blood sample for measurement of background amino acid enrichment, a primed, constant infusion of [15N2]urea was started at ~180 min (~6:30 AM). At ~120 min (~7:30 AM), a primed, constant infusion of L-[ring-2H5]phenylalanine and L-[1-13C]leucine was started. The following infusion rates (IR) and priming doses (PD) were used:

- L-[1-13C]leucine: IR = 0.12 μmol·kg⁻¹·min⁻¹,
  PD = 2 μmol/kg
- L-[15N2]urea: IR = 0.2267 μmol·kg⁻¹·min⁻¹,
  PD = 88 μmol/kg

Isotopes were purchased from Cambridge Isotopes (Andover, MA). They were dissolved in 0.9% saline and were filtered through a 2-μm filter before infusion. The infusion protocol was designed to allow the quantification of the effect of the drink on muscle protein synthesis and breakdown. The net balance between protein synthesis and breakdown (net muscle protein synthesis) was considered to be the primary end point of the study, and urea production was measured.

Table 1. Physical characteristics and exercise data for all subjects

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Gender, F/M</th>
<th>Age, yr</th>
<th>Height, m</th>
<th>Weight, kg</th>
<th>Leg Volume, l</th>
<th>1RM Leg Press, kg</th>
<th>1RM Leg Extension, kg</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>25</td>
<td>1.72</td>
<td>57.2</td>
<td>7.48</td>
<td>130.5</td>
<td>113.5</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>24</td>
<td>1.76</td>
<td>71.4</td>
<td>11.87</td>
<td>85.0</td>
<td>79.5</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>24</td>
<td>1.90</td>
<td>90.7</td>
<td>11.65</td>
<td>146.5</td>
<td>130.5</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>25</td>
<td>1.83</td>
<td>82.0</td>
<td>10.51</td>
<td>141.5</td>
<td>113.5</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>23</td>
<td>1.63</td>
<td>56.0</td>
<td>7.54</td>
<td>79.5</td>
<td>60.0</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>19</td>
<td>1.63</td>
<td>56.8</td>
<td>6.91</td>
<td>136.0</td>
<td>136.0</td>
</tr>
</tbody>
</table>

Means ± SD: 23 ± 2, 1.74 ± 0.11, 69.0 ± 14.9, 9.33 ± 2.27, 119.8 ± 29.6, 105.5 ± 29.7

1RM, one repetition maximum.
isotopically to assess short-term changes in total amino acid oxidation.
At ~7:30 AM, 3-Fr 8-cm polyethylene catheters (Cook) were inserted into the femoral vein and the femoral artery with the subject under local anesthesia. Both femoral catheters were used for blood sampling, and the femoral arterial catheter was also used for indocyanine green dye (ICG) infusion for determination of leg blood flow (3). A constant infusion of ICG (0.5 mg/min) was given at intervals during the experiment (Fig. 1). The infusion ran for ≥10 min before peripheral and femoral venous blood samples were drawn for measurement of blood flow. The peripheral venous blood samples were drawn from an 18-gauge polyethylene catheter inserted into an antecubital vein of the arm opposite that into which the amino acids were infused. Patency of catheters was maintained by saline infusion.

Subjects rested in bed until the exercise started at ~45 min (8:45 AM). Subjects performed 10 sets of 10 repetitions of leg presses and 8 sets of 8 repetitions of leg extensions at 80% of the 1RM. Each set was completed in ~30 s with a 2-min rest between sets, and the entire bout was completed in ~40 min. This exercise bout was difficult for all subjects to complete. The exercise ended 3 h after the start of the urea infusion and 2 h after the start of the amino acid infusion. Subjects then returned to bed, and the first samples were taken 30 min after the end of exercise.

At 1 and 2 h postexercise, the subjects were given an oral supplement of 0.087 g of essential amino acids (EAA)/kg body weight. The nutritional composition was designed to increase intramuscular availability of EAA in proportion to the composition of muscle protein (Table 2). Each supplement solution was composed of 425 ml of double-distilled water, the appropriate mixture of EAA, and an artificial sweetener. L-[ring-2H5]phenylalanine and L-[13C]leucine were added to the drink in amounts to equal 8% enrichment, to allow maintenance of isotopic equilibrium during ingestion of the AA drinks.

To measure the isotopic enrichment of free amino acid tracers in the muscle, muscle biopsies were sampled at 30, 90, 150, and 240 min after exercise (Fig. 1). With subjects under local anesthesia, the biopsies were taken from the lateral portion of the vastus lateralis ~10–15 cm above the knee. A 5-mm Bergstrom biopsy needle (Depuy, Warsaw, IN) was used to sample ~30–50 mg of mixed muscle tissue. The sample was quickly rinsed, blotted, immediately frozen in liquid nitrogen, and stored at ~80°C for later analysis.

Blood samples were drawn for determination of net muscle protein balance from the femoral artery and venous catheters at 30 min after exercise, at 70, 80, 90, 105, 130, 140, 150, and 165 min after exercise (corresponding to 10, 20, 30, and 45 min after each drink), and at 180, 210, 220, and 240 min after exercise (Fig. 1). The samples were analyzed for phenylalanine and leucine enrichments and concentrations. To allow sampling from the femoral artery, the dye infusion was stopped for <10 s and then quickly resumed.

**Sample analyses.** Blood samples for determination of amino acid enrichment and concentrations were immediately precipitated in preweighed tubes containing 15% sulfosalicylic acid (SSA), and a weighed amount of an appropriate internal standard consisting of amino acids labeled differently from the infused amino acids was added (3, 4, 15). The supernatant was passed over a cation exchange column (Dovex AG 50W-8X, 100–200 mesh H+ form; Bio-Rad Laboratories, Richmond, CA) and dried under vacuum with a Speed Vac (Savant Instruments, Farmingdale, NY). Enrichments of intracellular free amino acids were then determined on the tertiary-butyldimethylsilyl (t-BDMS) derivatives using GC-MS (Hewlett-Packard 5973, Palo Alto, CA) and selected ion monitoring (21). Enrichments were expressed as tracer-to-tracee ratios. Appropriate corrections were made for overlapping spectra (21).

To determine muscle intracellular enrichment of infused tracers, muscle tissue was weighed and the protein precipitated with perchloroaeetic acid. The tissue was then homogenized and centrifuged, and the supernatant was collected. The procedure was then repeated, and the pooled supernatant was processed in the same way as the supernatant from the blood samples.

Urea production was calculated from enrichment and tracer infusion rates, as described previously (20). ICG concentration in serum for the determination of leg blood flow was measured spectrophotometrically at λ = 805 nm (10, 19). Plasma samples and muscle intracellular fluid were also analyzed for amino acid concentrations by high-performance liquid chromatography (Waters Alliance HPLC System 2690, Milford, MA). Plasma glucose concentration was determined enzymatically by an automated system (YSI 1500, Yellow Spring Instruments, Yellow Springs, OH). Plasma insulin concentration was determined by a radioimmunoassay method (Diagnostic Products, Los Angeles, CA).

**Calculations.** Net muscle phenylalanine balance, which was considered as the primary end point, was calculated as follows: (phenylalanine arterial concentration – venous concentration) × blood flow. Because phenylalanine is neither produced nor metabolized in muscle, net phenylalanine balance reflects net muscle protein synthesis, provided there are no significant changes in the free intracellular pool of phenylalanine. Area under the curve (AUC) of net phenylalanine uptake was determined for each individual hour after drink ingestion, with net uptake at t = 30 min postexercise used as the zero point for each hour of the recovery period. This approach assumes a

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**Table 2. Amino acid composition of drink**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>% of Total AAs</th>
<th>Grams in EAA Drink</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>10.9</td>
<td>0.6540</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>10.1</td>
<td>0.6060</td>
</tr>
<tr>
<td>Leucine</td>
<td>18.6</td>
<td>1.1160</td>
</tr>
<tr>
<td>Lysine</td>
<td>15.5</td>
<td>0.9300</td>
</tr>
<tr>
<td>Methionine</td>
<td>3.1</td>
<td>0.1860</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>15.5</td>
<td>0.9300</td>
</tr>
<tr>
<td>Threonine</td>
<td>14.7</td>
<td>0.8820</td>
</tr>
<tr>
<td>Valine</td>
<td>11.5</td>
<td>0.6900</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>99.9</td>
<td>5.994</td>
</tr>
</tbody>
</table>

AA, amino acid; EAA, essential amino acids. Composition is based on a 70-kg person. Drink was given at 1 and 2 h after completion of exercise.
constant net balance after the basal sample if amino acids are not given. The basis for this assumption is the relatively constant net balance for 3 h after exercise in the absence of nutrient intake that we have previously observed when using the same exercise protocol (16).

Leg amino acid kinetics were calculated according to a three-pool compartment model previously presented (2, 3). Hourly averages for blood flow and blood and muscle amino acid concentrations and enrichments were used in the calculation of leg amino acid kinetics. Kinetic parameters calculated for both amino acid tracers included intracellular de novo appearance, irreversible disappearance from the intracellular compartment, and the rate of transport from blood into muscle. In the case of phenylalanine, irreversible loss from the intracellular pool can only be to protein synthesis, because it is not oxidized in the muscle. Because leucine can be oxidized in muscle, the irreversible loss is due to synthesis + oxidation. Neither leucine nor phenylalanine can be synthesized in muscle, so de novo appearance of both amino acids is due entirely to breakdown. We also calculated the rate of release of phenylalanine and leucine from protein breakdown into blood (Rd) and incorporation of phenylalanine from blood into muscle protein (Ra). Calculation of R and Rd, as well as the three-pool kinetic factors, requires an isotopic, but not physiological, steady state. By adding an appropriate amount of tracer to the ingested amino acids, we were able to maintain a relatively stable isotopic steady state, despite changing concentrations of plasma amino acids (see below). Thus the difference between total protein synthesis and Rd is the amount of recycling of phenylalanine that was released from breakdown and directly incorporated into protein without entering the blood. Similarly, the difference between total protein breakdown and Ra is the amount of phenylalanine from breakdown that was directly reincorporated into protein, rather than being released into blood.

Statistical methods. Overall significance of differences in response with time was tested by repeated-measures analysis of variance followed by Dunnett’s test (SigmaStat 2.03, SPSS, Chicago, IL). The response to the second drink was compared with the response to the first drink by Tukey’s test. Results were considered significant if P < 0.05. The results are presented as means ± SE unless otherwise noted.

RESULTS

Phenylalanine concentration and balance. At 30 min after exercise, the arterial blood phenylalanine concentration was 59 ± 4 nmol/ml. The concentration rose significantly within 10 min of ingestion of the EAA drink (Fig. 2). Phenylalanine concentration declined before the second drink but stayed significantly above predrink values until 240 min after exercise. The response to the second drink was comparable to that of the first.

Phenylalanine net balance followed the same time pattern as the blood concentration changes, with rapid changes in response to arterial blood phenylalanine changes (Fig. 2). However, the early increase of net uptake was proportionately greater than the change in arterial concentration, and the rate of net balance returned to the basal value 40 min after ingestion of the first drink, despite persistent elevation of arterial phenylalanine concentration. Net balance also increased significantly in response to the second drink (Fig. 2) and returned to the basal value during the 3rd h. The AUC for net uptake of phenylalanine above the basal value was similar for the 1st h after the first drink (67 ± 17 mg/leg) and the 1st h after the second drink (77 ± 20 mg/leg). However, the AUC decreased significantly by the 3rd h (~5 ± 20 mg/leg), despite the fact that the arterial concentration was significantly elevated until 240 min after the first drink.

Muscle intracellular phenylalanine concentration was 57 ± 3 nmol/ml before intake of the drinks. After intake of drinks, the concentration increased, and the value at 150 min (115 ± 24 nmol/ml) was significantly higher than the baseline value. At 240 min, the concentration (88 ± 14 nmol/ml) was not statistically different from the baseline value.

Phenylalanine kinetics. Enrichment of phenylalanine in blood was relatively constant throughout the experiment despite the large changes in concentration (Fig. 3). No statistical changes in enrichment were observed during the different calculation periods. This was accomplished by adding tracer to the ingested amino acids.

The rate of appearance of phenylalanine into the blood from the muscle (Rd) did not change during the first 2 h after the drink but increased significantly during the 3rd h (Fig. 4). The average rate of disappearance (Rd) of phenylalanine from the blood into the muscle (i.e., protein synthesis from plasma phenylalanine) increased significantly from the basal value during the first 2 h after intake of drink but was not different from the basal value during the 3rd h.

Ingestion of the EAA drink caused inward transport of phenylalanine from the artery to the muscle to increase (Table 3, P = 0.055). At 30 min after exercise, the total rate of intracellular phenylalanine release from protein breakdown was 43 ± 9 nmol/min · 100 ml −1 · 100 ml −1 (Table 3). There was no change in Rd during the first 2 h after the first drink, but it increased significantly during the 3rd h to 75 ± 13 nmol/min · 100 ml −1 · 100 ml −1. The rate of utilization of phenylalanine for protein synthesis was 30 ± 9 nmol/min · 100 ml −1 · 100 ml −1 at 30 min

Fig. 2. Changes in arterial phenylalanine concentration and net phenylalanine balance during the recovery period after a resistance exercise bout (means ± SE; n = 6). Essential amino acid (EAA) drink was consumed at 60 and 120 min after exercise.
after exercise, and this value increased significantly during the 1st and 2nd h after drink to $121 \pm 16$ and $139 \pm 21$ nmol·min$^{-1}$·100 ml leg$^{-1}$, respectively (Table 3). During the 3rd h after drink, the value returned to the basal level, despite the fact that the total intracellular appearance of phenylalanine (inward transport + breakdown) was elevated approximately threefold above the basal value.

**Leucine concentration and kinetics.** The pattern of response of blood concentration and kinetics of leucine was similar to that of phenylalanine. As was the case for phenylalanine, leucine tracer was added to the ingested amino acids. Therefore, enrichment of leucine in blood was essentially constant throughout the experiment despite changes in concentrations (Fig. 3).

Leucine concentration increased significantly within 10 min of ingestion of the first EAA drink, from a value of $118 \pm 4$ nmol/ml at 30 min after exercise to a peak of $407 \pm 24$ nmol/ml at 20 min after ingestion of the drink. The concentration declined before the second drink, but not to the basal level. Leucine concentration increased again after ingestion of the second drink, and although the value again began falling 30 min after ingestion of the second drink, it was still elevated above basal at 210 min after ingestion of the first drink. Muscle intracellular leucine concentration increased significantly from the basal value of $144 \pm 7$ to $307 \pm 52$ nmol/ml at 150 min. At 240 min, the concentration was not different from basal value at 30 min after exercise.

Leucine net uptake increased as a result of drink. No significant difference was found between the AUC value for net uptake during the 1st h after the first drink ($159 \pm 36$ mg/leg) and that during the 1st h after the second drink ($165 \pm 32$ mg/leg), but the area was significantly smaller by the 3rd h ($20 \pm 22$ mg/leg).

The average $R_a$ of leucine into the blood over each of the hours after intake of drink did not change, whereas the average rate of muscle protein synthesis and oxidation from plasma leucine increased significantly from basal values during the first 2 h after intake of drink but was not different from basal values during the 3rd h (Fig. 5). As a result, leucine net balance increased from a slightly negative basal value to $\sim 250–260$ nmol·min$^{-1}$·100 ml leg$^{-1}$ during the 2 h after the first drink (not significant between hours; Fig. 5). During the 3rd h, net balance decreased again.

Table 4 shows leucine kinetics. The delivery of leucine to the leg muscle increased as a result of intake of the drink. Also, the movement of leucine into the muscle increased and stayed elevated above the predrink value throughout the following 3 h. The rate of intracellular $R_a$ of leucine increased slightly after the drink, and during the 1st h the value was significantly different from the predrink value. The rate of utilization of intracellular leucine, which includes utilization for both protein synthesis and oxidation, increased significantly during the 1st and 2nd h after drink, whereas it returned to basal value during the 3rd h.

The rate of incorporation of leucine into protein can be calculated from the rate of protein synthesis as calculated with the phenylalanine tracer and the ratio between leucine and phenylalanine in mixed muscle protein. The ratio between leucine and phenylalanine in mixed muscle protein was calculated from the ratio between the rates of leucine and phenylalanine release from muscle protein breakdown at the predrink time point (30 min). The mean ratio for the group was $2.6 \pm 0.2$. When calculated accordingly, the rate of leucine incorporated into protein increased significantly during the 1st h after each drink, but during the 3rd h it decreased to a value not different from the predrink rate (Table 4). The rate of leucine oxidation was calculated from the difference between the rate of utilization of intracellular leucine and the rate at which leucine was used for protein synthesis. This calculation showed that leucine oxidation increased significantly during the 1st h after the first drink but that, during
Table 3. Calculated kinetics for phenylalanine before drink and during 1st, 2nd, and 3rd h after intake of first drink

<table>
<thead>
<tr>
<th></th>
<th>Predrink</th>
<th>1st h</th>
<th>2nd h</th>
<th>3rd h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflow</td>
<td>219 ± 27</td>
<td>514 ± 64*</td>
<td>598 ± 87*</td>
<td>547 ± 92*</td>
</tr>
<tr>
<td>Inward transport</td>
<td>201 ± 31</td>
<td>590 ± 74</td>
<td>659 ± 193</td>
<td>454 ± 105</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>30 ± 9</td>
<td>121 ± 16*</td>
<td>139 ± 21*</td>
<td>75 ± 31</td>
</tr>
<tr>
<td>Protein breakdown</td>
<td>43 ± 9</td>
<td>40 ± 6</td>
<td>55 ± 5</td>
<td>75 ± 13</td>
</tr>
</tbody>
</table>

Inflow, rate at which phenylalanine enters leg via artery; inward transport, rate of net phenylalanine movement from artery to muscle; protein synthesis, rate of phenylalanine incorporation into protein; protein breakdown, rate of intracellular release of phenylalanine from protein breakdown. Values are nmol min⁻¹ 100 ml leg⁻¹ (means ± SE; n = 6). Value vs. predrink value: *P < 0.05.

the 2nd and 3rd h, the values were not different from the predrink value.

Plasma and muscle intracellular amino acid concentrations. Intake of drinks increased the arterial plasma concentrations of total EAAs significantly. At 150 min after exercise (90 min after the first drink and 30 min after the second drink), the concentrations for most of the EAAs given in the drinks were increased by ~75–150%, except for isoleucine and leucine, which showed greater responses (317 ± 69 and 212 ± 29% increase, respectively; Fig. 6A). Arterial plasma concentrations of the amino acids not given in the drink generally showed smaller changes (Fig. 6B). Tyrosine increased significantly, whereas glycine, alanine, and tryptophan decreased during the study.

Whereas there was no change in intracellular concentration of total EAAs or in total NEAAs during the recovery period, some individual changes were observed. Threonine was not different between first and second biopsies but increased thereafter to a concentration at 150 min significantly higher than predrink concentration (741 ± 99 vs. 561 ± 59 nmol/ml H₂O). Similarly, valine, isoleucine, and leucine concentrations all increased after the first drink. The concentrations increased further at 150 min but decreased again at the end of the study at 240 min. For valine, the values at 150 and 240 min were significantly higher than the predrink value (228 ± 17 and 212 ± 16 vs. 168 ± 9 nmol/ml, respectively). For isoleucine, 90- and 150-min values were higher than the predrink value (86 ± 5 and 98 ± 6 vs. 61 ± 2 nmol/ml, respectively). For leucine, 90-, 150-, and 240-min values were higher than the predrink value (249 ± 5, 297 ± 22, and 240 ± 15 vs. 186 ± 7 nmol/ml, respectively). For the amino acids not given in the drink, a significant fall during the day was found for asparagine, serine, arginine, and alanine. The relative changes in intracellular concentrations of EAAs were small compared with the changes in arterial plasma (Fig. 6).

Urea production. Plasma urea enrichment was stable at ~4% throughout the study. Thus no change in urea production was seen over time. The value was stable at 330 µmol-kg⁻¹ h⁻¹.

Plasma glucose and insulin concentrations. Arterial glucose concentration decreased slightly during the experiment from a value of 91 ± 3 mg/dl at 30 min after exercise to 87 ± 3 mg/dl at the end of the study (P < 0.05). No change was found in net uptake of glucose during the study. Similarly, insulin concentrations did not change over time.

DISCUSSION

The principal finding of this study was that ingestion of a relatively small amount (6 g) of EAA effectively stimulated net muscle protein balance after resistance exercise. The response of net muscle protein balance could be explained largely by a change in synthesis, as the rate of breakdown was not significantly affected. The response of net balance was about twice the previous published response to 6 g of mixed amino acids (14), leading to the conclusions that there is a dose response to the amount of EAAs given and that ingestion of NEAAs is not necessary to stimulate net protein synthesis. The latter conclusion is supported by changes in NEAA concentrations after ingestion of the EAA drink. Although plasma concentrations of some individual NEAAs fell after ingestion of EAAs, intracellular concentrations of NEAAs were generally maintained, indicating that availability of NEAAs did not limit the response of muscle protein synthesis. Finally, the response of net muscle protein synthesis to the drink ingested 2 h after exercise was comparable to that of the drink ingested 1 h after exercise.

Independent effect of EAA on net protein balance. In previous studies, both intravenous infusion (5) and oral intake of amino acids (17) after resistance exercise have been shown to stimulate muscle protein synthesis. However, optimal proportions and amounts of individual amino acids to stimulate muscle protein synthesis are not known. Intake of only a small amount (6 g) of a mixture of EAA given along with 35 g of carbohydrate after resistance exercise transiently increased muscle protein synthesis 3.5-fold (14), but the independent effects of amino acids and carbohydrate were not assessed. The results of the present study show that ingestion of 6 g of EAA alone without addition of
carbohydrate effectively stimulated muscle protein synthesis after resistance exercise (Figs. 4 and 5, Tables 3 and 4).

In a recent study, Miller et al. (14) compared the independent and combined effects of a balanced mixture of amino acids (i.e., EAAs/H11001\textsuperscript{NEAAs}) and carbohydrate on muscle protein synthesis after resistance exercise. Addition of 35 g of carbohydrate to 6 g of mixed AA did not cause a greater stimulation of net muscle protein synthesis than the AAs alone. The effect of adding carbohydrate to 6 g of EAA can be seen in Fig. 7, which compares the AUC for net phenylalanine uptake for the 1st h after intake of drink (i.e., 60–120 min) in the present study with the previously published response to 35 g of carbohydrate (16). The additional carbohydrate provided no advantage to EAAs alone. From these results, it is clear that the stimulation of protein synthesis by EAAs is not a caloric effect, because ingestion of an additional 3 g of EAA (difference in EAA content between mixed AA and EAA groups) caused a much larger effect than addition of 35 g of carbohydrate to the amino acid mixture (Fig. 7), and 35 g of carbohydrate alone had a minimal effect (14). Although direct comparison with historical data may be problematic, the cited studies (14, 16) were performed in the same laboratory, approximately contemporaneously, and by use of the same general experimental protocol and techniques.

The results of the current study indicate that ~3.5 ± 1.1 g of muscle protein were synthesized during the 3 h after the first drink in one leg, or 7.0 ± 2.0 g in both legs, on the basis of irreversible loss of phenylalanine and the composition of muscle protein. This represents ~27% of ingested EAAs, because each gram of muscle protein synthesized includes both EAAs and NEAAs.

<table>
<thead>
<tr>
<th></th>
<th>Predrink</th>
<th>1st h</th>
<th>2nd h</th>
<th>3rd h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflow</td>
<td>433 ± 42</td>
<td>1228 ± 128*</td>
<td>1366 ± 143*</td>
<td>1318 ± 167*</td>
</tr>
<tr>
<td>Inward transport</td>
<td>302 ± 64</td>
<td>1008 ± 210*</td>
<td>990 ± 104*</td>
<td>718 ± 91*</td>
</tr>
<tr>
<td>Utilization</td>
<td>100 ± 16</td>
<td>458 ± 58*</td>
<td>416 ± 54*</td>
<td>176 ± 43</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>73 ± 17</td>
<td>306 ± 25*</td>
<td>360 ± 60*</td>
<td>158 ± 41</td>
</tr>
<tr>
<td>Oxidation</td>
<td>27 ± 7</td>
<td>151 ± 45*</td>
<td>56 ± 34†</td>
<td>18 ± 10</td>
</tr>
<tr>
<td>Protein breakdown</td>
<td>106 ± 14</td>
<td>195 ± 30*</td>
<td>164 ± 26</td>
<td>147 ± 30</td>
</tr>
</tbody>
</table>

Utilization, rate of utilization of intracellular leucine (i.e., protein synthesis and oxidation); oxidation, rate of leucine oxidation. Values are nmol·min\(^{-1}\)·100 ml leg\(^{-1}\) (means ± SE; n = 6). *P < 0.05, value vs. predrink value. †P < 0.05, 2nd h vs. 1st h value.

Fig. 6. Percentage changes in arterial plasma concentrations of EAAs (A), arterial plasma concentrations of non-essential amino acids (NEAAs) and tryptophan (B), intracellular concentrations of EAAs (C), and intracellular concentrations of NEAAs and tryptophan (D) from 30 min to 150 min after exercise (means ± SE; n = 6). EAA drink was consumed at 60 and 120 min after exercise.
detectable by available means to quantify changes in muscle mass, during which time all variables such as activity and other nutritional intake would have to be absolutely controlled. Thus, although the stimulation in net muscle synthesis resulting from the total of 12 g of EAA reported in this study would eventually be expected to enhance the rate of muscle gain during a resistance training program, an outcome study based on measured differences in muscle mass would have to be carefully designed and executed to demonstrate an effect of an EAA supplement.

EAA vs. NEAA. Because ingestion of 6 g of EAAs alone stimulates muscle protein synthesis after resistance exercise, NEAAs are apparently not required to stimulate protein synthesis. In a previous study, Tipton et al. (17) found no difference in net muscle protein balance response to the ingestion of 40 g of mixed AAs (roughly in proportion to their relative contributions to muscle protein) or 40 g of EAAs. These results could also be interpreted to indicate that NEAAs are not needed for stimulation of protein synthesis. However, if the NEAAs were not necessary for stimulation of synthesis, it was unclear why ingestion of the EAAs alone did not stimulate muscle protein synthesis to a greater extent than did the balanced mixture. The response in the present study was about twice that when 6 g of mixed AAs were given after a similar exercise bout (Fig. 7) (14). Thus, it seems likely that there is a dose response of muscle protein synthesis to the intake of EAAs and that the amount of EAAs in the balanced mixture (21 g) in the study by Tipton et al. was equal to or exceeded the maximal effective dose. However, it should be noted that, in this study by Tipton et al., the amino acids were ingested as small boluses over 3 h, and the different pattern of intake may also have contributed to the lack of difference between the two mixtures of amino acids.

Pattern of ingestion. No differences were found in the protein synthesis response between the first and the second dose (Tables 3 and 4, Figs. 4 and 5), and the AUC for net phenylalanine uptake was similar after the first and the second drinks. Net balance increased rapidly after intake of the drink, and the relative increase in net balance was greater than the change in arterial phenylalanine concentrations (Fig. 2). However, when arterial AA concentrations started to drop, net balance rapidly decreased to the basal level, even though the arterial AA concentrations were still elevated (Fig. 2). In fact, at 60 min after ingestion of the first drink, the phenylalanine concentration was still higher than the maximal concentration previously observed to coincide with stimulation of protein synthesis when 6 g of mixed amino acids were given (14). Thus muscle protein synthesis is apparently stimulated when there is an increase in arterial, and presumably interstitial, AA concentrations, rather than by the absolute AA concentration. This observation is consistent with our previous findings (6) that, when blood amino acid concentrations were elevated to a steady-state level about twice the basal values for 6 h, muscle protein synthesis was stimulated over the first 2 h but thereafter returned to the resting level despite the persistent elevation in blood amino acid concentrations.

The similarity of response to both boluses suggests that there is little effect of the exact time of ingestion after exercise. This is consistent with our previous work, in which we observed similar responses to single doses of amino acids and glucose given either immediately, 1 h, or 3 h after resistance exercise (16, 18). In contrast, Esmarck et al. (9) recently reported that a protein-carbohydrate-fat supplement was effective in stimulating muscle protein gain over a period of resistance training in elderly men only when ingested immediately after, as opposed to 2 h after, exercise. Differences between that study and our studies include age of subjects, ingestion of protein rather than free amino acids, and end point. Whereas we measured the acute response of muscle protein, they measured net muscle gain, which includes the response to all food intake over a period of time. Thus it is possible that ingestion of a protein-carbohydrate-fat supplement 2 h after exercise might have interfered with either the amount eaten at the next meal or the response to the next ingested meal. In another study that addressed the timing of intake on response, Levenhagen et al. (11) found a greater stimulation of net muscle protein synthesis when a protein-carbohydrate-fat supplement was given immediately after aerobic exercise than when it was given 2 h later. There likely is a difference between timing after aerobic and timing after resistance exercise, as performed in the current study. The stimulation of muscle protein synthesis is modest after aerobic exercise (7). Rather, the interaction effect of exercise and supplement ingestion may be due to increased muscle blood flow, and thus substrate delivery, immediately after exercise. In contrast, fractional synthetic rate remains elevated for ≥48 h after resistance exercise (15), so an effect on timing of supplement ingestion after resistance exercise is less likely.
Composition of drink. The composition of the mixture of EAA we have tested in this and previous studies (16, 18) was originally based on the composition of muscle protein, with the notion of increasing the availability of each EAA in proportion to its requirement for synthesis of muscle protein. The results of the current study show that isoleucine and leucine increased more than the others in the blood (Fig. 6), meaning that the goal of causing proportional increases in all EAA was not fully achieved. Different changes in concentrations of blood amino acids after ingestion of the drink may be caused by different clearance rates of individual amino acids, thereby resulting in rates of uptake that do not directly correspond to the composition of the ingested mixture. Furthermore, it is possible that the effect is largely, or even entirely, mediated by leucine alone (1). Therefore, it is possible that adjustments in the composition of the drink could further improve the response of net muscle balance.

Whereas NEAAs can be synthesized within the body at a rate generally sufficient to meet requirements, certain amino acids may be limited in the rapidity with which changes in production can occur. Thus glycine is known to be slowly transaminated (12), and this likely explains the fall in plasma glycine concentration as its utilization is increased because of the stimulated rate of protein synthesis after EAA, not only in muscle but also throughout the body. The fact that the EAA mixture was twice as effective as the same amount of the balanced mixture of AAs (14) in stimulating muscle protein synthesis indicates that glycine was probably not rate limiting, despite the decrease in plasma concentration, because the muscle concentration of free glycine was maintained. Similarly, the fall in alanine concentration in plasma probably reflected increased uptake elsewhere than in muscle for incorporation into protein, as muscle is normally highly efficient in producing alanine, and its rate of production is increased during exercise (22).

Methodological considerations. Drinks normally are ingested as boluses. However, quantifying the response to a bolus ingestion of unlabeled amino acids introduces potential methodological problems because of rapid dilution of the tracer. The resulting isotopic nonsteady state violates a fundamental assumption of the three-pool compartment model (2, 3). We therefore added tracer to the ingested amino acids so that a relatively constant enrichment in the blood was maintained, even during absorption of the bolus. Thus the calculated values for synthesis and breakdown should theoretically be accurate. Nonetheless, we used net protein balance, which is not dependent on the measurement of isotopic enrichment, as our primary end point, and the other variables were considered as secondary end points. This is not only because of methodological issues but also because, in terms of gain or loss of muscle protein, the net balance (i.e., synthesis minus breakdown) is the most relevant parameter. Furthermore, it can be determined in non-steady-state conditions. In relation to net balance, the pertinent kinetic parameters are the rate of synthesis from plasma amino acids (phenylalanine and leucine, respectively) and the rate of appearance of amino acids into plasma from protein breakdown. This is because, although the synthesis of protein from amino acids derived from protein breakdown (i.e., intracellular recycling of amino acids) is important from the standpoint of understanding the regulation of the process of synthesis, it does not represent any net gain or loss of protein. The protein synthesis from plasma amino acids and appearance in plasma from protein breakdown are calculated by the two-pool model. We also have calculated rates of synthesis and breakdown by use of the three-pool model that we have described previously, which includes synthesis from all sources and the total rate of breakdown. However, even though we avoided changes in enrichment by adding tracer to the exogenous amino acids, the calculated values of synthesis and breakdown were variable. This is because calculation of the parameters from the three-pool model requires a gradient in enrichment from blood to the intracellular phenylalanine pools, and the rapid influx of amino acids from plasma caused the gradient in enrichment between compartments to narrow to the point where accurate measurement is difficult.

The primary potential problem in the interpretation of net balance results in the nonsteady state is the possibility that amino acids entered the intracellular pool from the plasma that eventually reentered the blood at some time after the final blood sample was drawn. To the extent that this occurred in this experiment, we would have overestimated net uptake. However, the free intracellular phenylalanine concentration was not significantly elevated at the time we drew our last sample, so the magnitude of error due to this potential problem was likely not large.

Because previous studies have shown that net muscle protein balance remains slightly negative for several hours after resistance exercise in the absence of nutrient intake (4, 8), a control group was not included in this study. Furthermore, in a previous study from our laboratory, Rasmussen et al. (16) gave a placebo drink at 60 min after a similar resistance exercise bout. They found no significant change in net balance over the first 3 h after exercise. Hence, the significant positive net balance observed after drinks in the present study can be ascribed to the intake of the amino acids, rather than to changes that would have occurred anyway.

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