Postprandial body protein synthesis and amino acid catabolism measured with leucine and phenylalanine-tyrosine tracers

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Submitted 26 September 2002; accepted in final form 14 January 2003

METHODS

A precise estimate of postprandial whole body protein synthesis is essential to determine the anabolic effects of meal ingestion, i.e., the key physiological stimulus of net protein deposition in body tissues (13, 14, 20, 27). By use of amino acid tracer techniques, whole body protein synthesis is commonly calculated from the portion of amino acid flux (e.g., disposal) that is not irreversibly catabolized and therefore must be incorporated into the proteins (3, 27, 31). Protein synthesis is stimulated by a meal of adequate energy and protein content (13, 18, 27). An index of such an adequacy is the stimulation of postprandial amino acid catabolism (33). Therefore, the accurate measurement of both the flux and the catabolism of indispensable amino acid(s) is essential to estimate whole body protein synthesis precisely.

Leucine and phenylalanine tracers are extensively used for this purpose (3, 13, 14, 20, 27, 31). The first step of leucine irreversible catabolism is oxidation (3, 24, 31); that of phenylalanine is hydroxylation to tyrosine (7, 21). The in vivo measurement of leucine oxidation (Leu Ox) requires analyses in both plasma and expired air, whereas phenylalanine hydroxylation (Phe Hy) is measured after the combined infusion of phenylalanine and tyrosine tracers, with analyses only in plasma (7). Therefore, the latter method is attractive because it requires a simpler sample collection.

Whether these two isotopic amino acid methods are equivalent as regards the measurements of changes in protein synthesis and amino acid catabolism after a mixed meal is unclear. In previous studies (9, 18), Leu Ox was stimulated postprandially, whereas Phe Hy was not. However, neither plasma amino acid concentrations nor meal composition was provided; therefore, the tested meal might not have been a sufficient stimulus for hydroxylation. In addition, although protein synthesis was enhanced to a similar extent with both tracers by use of adjusted intracellular precursor pools (18), the impact of the choice of the precursor pools on amino acid kinetics was not thoroughly evaluated.

Therefore, the aims of the present study were the following: 1) to measure whole body protein synthesis and amino acid catabolism in healthy volunteers by use of leucine and phenylalanine-1-tyrosine tracers simultaneously both before and after the ingestion of a mixed meal of generous energy and protein content; 2) to evaluate the impact on the kinetic measurements of the choice of the precursor pool(s); and 3) to look for the relationships between leucine and phenylalanine concentrations, catabolism, and protein synthesis.

METHODS

Subjects. Eight healthy male subjects (age 34 ± 6 yr; body mass index 23.7 ± 1.2 kg/m²), metabolically stable and adapted to a standard weight-maintaining diet, were included. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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crutied. They were informed about the aims of the study and signed their consent to it. The protocol was approved by the Ethics Committee of the Medical Faculty at the University of Padova, Italy, and it was performed according to the Helsinki Declaration (as revised in 1983) (32) as well as following the recommendations of the local Radiation Safety Officer. Whole body radiation exposure due to the [14C]leucine isotope was estimated to be 0.0009 mg (8). Isotopes. L-[1-14C]leucine ([14C]Leu; specific activity (SA) = 2 GBq/mmol) and sodium [14C]bicarbonate (SA = 2 GBq/mmol) were purchased from Amersham (Buckinghamshire, UK). L-[ring-2H3]phenylalanine ([2H3]Phe) and L-[2H2]tyrosine ([2H2]Tyr) were purchased from MassTrace (Woburn, MA). L-[ring-2H5]tyrosine ([2H5]Tyr) was obtained from Eurisotop (Gi-sur-Yvette, France). The stable isotopes were >99% mole percent enriched. All tracers were dissolved in sterile saline and proven to be sterile and pyrogen free before use.

Experimental design. The study was conducted as previously described in detail (27). Briefly, a polyethylenet catheter was placed percutaneously in retrograde fashion into a superficial vein of one arm, which was kept at +60°C in a heatingavoiding venous blood sampling. Another catheter was placed into an antecubital vein of the opposite arm for isotope infusions. At ~240 min, continuous infusions of [14C]Leu (5,841 ± 407 dpm/kg·min·1), [2H3]Phe (0.0503 ± 0.0016 mg·kg·min·1), and [2H2]Tyr (0.0214 ± 0.0009 mg·kg·min·1) were started by means of calibrated pumps. Priming doses of each isotope (equivalent to 60× continuous infusion rate/min), as well as priming doses of [2H4]Tyr (0.08 mg/kg) and of [14C]bicarbonate (3 μCi), were administered at ~240 min. Samples were frequently taken over 3 h to allow the achievement of steady state in plasma amino acid concentrations, [14C]Leu and α-[14C]ketosuccinocroato (KIC) SAs, and phenylalanine and tyrosine enrichments. Steady state was defined as absence of a slope significantly different from zero as well as of changes in concentrations, SAs, and enrichments >5%, and it was usually achieved after ~2.5 h (data not shown). Between ~60 and 0 min, four 10-mL blood samples were collected at 20-min intervals into EDTA tubes and rapidly centrifuged at +4°C. The plasma was then stored at ~20°C before assay. Samples of expired air (2-min collections) for 14CO2 measurements were taken at the same time points.

At 0 min, the administration of a mixed-liquid meal of defined composition (Nutrodrip Protein; Sandoz Nutrition, Wander Italia, Milan, Italy) was started. The meal contained 14.8 g/100 mL carbohydrates, 6.6 g/100 mL protein hydrolysate, and 4 g/100 mL lipids, minerals, and vitamins. The proteins were derived from soy and casein. The lipids were constituted by 40% linoleic acid, 6.5% medium-chain triglyceride oil, and the remainder by mono- and diglycerides. Thus ~49% of calories were represented by carbohydrates, ~22% by proteins and ~29% by lipids. The entire meal (~62 kJ/kg body wt) was given as isocaloric aliquots of 0.94 mL/kg body wt every 20 min over 4 h. Blood and expired air samples were again collected at 170, 190, 210, 230, and 250 min (i.e., every 20 min) after the administration of each meal aliquot and after the achievement of the new steady state, as reported elsewhere (27). Although this pattern of meal administration (continuous) might not be considered physiological, it allowed us to perform all kinetic measurements at near steady state, thus avoiding uncertainties due to time-dependent changes in amino acid pool sizes and SAs after a bolus meal.

Analytical measurements. Plasma leucine, phenylalanine, and KIC concentrations, and plasma leucine and KIC 14C SAs were determined by high-pressure liquid chromatography (HPLC) (17, 26). Plasma [Tyr] was determined by ion exchange chromatography using a Beckman amino acid analyzer. Plasma [3H3]Phe, [3H4]Tyr, and [3H4]Tyr mole percent enrichments were determined by gas chromatography-mass spectrometry (GC-MS) as tert-butyldimethylsilyl derivaties and electron impact ionization (23). The monitored fragments were mass-to-charge ratios (m/z) 293/294 for [3H3]Phe, 468/466 for [3H3]Tyr, and 470/466 for [3H4]Tyr, respectively. Enrichments were expressed as tracer-to-tracee ratios (TTR) (31). The 14CO2 in the expired air was determined as described previously (4, 27). Insulin and glucagon concentrations were measured by radioimmunoassay, as referenced elsewhere (27). Plasma glucose was determined using a Beckman Glucose Analyzer 2.

Calculations. The values of plasma leucine and KIC SA, and of phenylalanine and tyrosine enrichments in the two steady-state periods, i.e., in the last 60 min of the basal, postabsorptive state as well as in the last 60 min of meal administration (i.e., between 190 and 250 min), were averaged. All calculations were performed using these mean values.

Whole body leucine rate of appearance (Leu Ra) was calculated using both [14C]Leu SA (plasma data) and [14C]KIC SA (intracellular data) as precursor pools (22), i.e., by dividing the [14C]Leu infusion rate (in dpm·kg·min·1) over either [14C]Leu or [14C]KIC SA (in dpm/nmol), respectively. Leu Ox was calculated by dividing the rate of 14CO2 expiration (in dpm·kg·min·1; corrected for 76% fixation in body bicarbonate pool in the postabsorptive state and for 91% fixation in the fed state) (12) over either plasma [14C]Leu SA or [14C]KIC SA (i.e., plasma and intracellular data, respectively). The rate of nonoxidative leucine disposal (NOLD), e.g., the index of whole body protein synthesis, was calculated by subtracting Leu Ox from total Leu Rd (equal to the rate of disappearance at steady state) by use of either precursor pool.

Whole body Phe Rd and Tyr Rd were calculated at steady state using standard formulas (7, 31). Whole body phenylalanine hydroxylation to tyrosine (Phe Hy) was calculated using the following equation (4, 28)

\[
\text{Hy} = R_d, \text{Tyr} \times \frac{[3H4] \text{Tyr TTR}}{[3H3] \text{Phe TTR}}
\]

where [Rd, Tyr] is the rate of appearance of tyrosine in plasma (in μmol·kg·min·1), and [3H4]Tyr TTR and [3H3]Phe TTR are TTR of [3H4]Tyr and [3H3]Phe in plasma, respectively. The rate of nonhydroxylative phenylalanine disposal (NHPD), indicating incorporation into protein, was calculated by subtracting Hy from Phe Rd.

Phe Rd, Tyr Rd, Hy, and NHPD were also calculated using estimates of intracellular [3H3]Phe and [3H4]Tyr enrichments (18). As concerns phenylalanine in the postabsorptive state, as well as tyrosine in both the postabsorptive and the fed states, the adjusted intracellular TTRs were calculated by multiplying the plasma TTR times the ratios between plasma KIC and leucine 14C SA determined in each individual. The phenylalanine TTR measured in plasma in the fed state was not adjusted further, because the ratio between plasma and apolipoprotein (apo)B-100-bound phenylalanine enrichment was found to be 1 in the fed state (19). Plasma [3H4]Tyr TTR was not modified, as it is produced intracellularly, respectively. Extrapolation of the kinetic data from the amino acids (in μmol·kg·min·1) to the proteins (in mg body protein·kg·min·1) was performed by assuming that leucine accounts for 9% of average body proteins and phenylalanine for 4.2% (3, 16, 30).
Glucagon concentrations did not change (from increments of leucine and phenylalanine concentrations and after meal administration (from P Meal ingestion increased (Plasma data Table 2. Leu Ra, Leu Ox, and NOLD and Phe and Tyr Ra, Phe Hy, and NHPD at steady state and after meal administration (190–250 min)

<table>
<thead>
<tr>
<th>[Phe]</th>
<th>[Tyr]</th>
<th>[Leu]</th>
<th>[KIC]</th>
<th>[%H3]Phe TTR</th>
<th>[%H4]Tyr TTR</th>
<th>[%H2]Tyr TTR</th>
<th>Leu SA</th>
<th>KIC SA</th>
<th>Exp. 14CO2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>57 ± 4</td>
<td>51 ± 4</td>
<td>132 ± 6</td>
<td>30 ± 3</td>
<td>8.45 ± 0.38</td>
<td>0.73 ± 0.07</td>
<td>4.86 ± 0.49</td>
<td>3.20 ± 0.23</td>
<td>2.52 ± 0.20</td>
</tr>
<tr>
<td>Meal</td>
<td>92 ± 8</td>
<td>96 ± 6</td>
<td>229 ± 19</td>
<td>27 ± 4</td>
<td>5.51 ± 0.33</td>
<td>0.54 ± 0.04</td>
<td>3.04 ± 0.36</td>
<td>2.28 ± 0.23</td>
<td>1.80 ± 0.16</td>
</tr>
</tbody>
</table>

Values are means ± SE. Concentrations are in μmol/l; specific activities (SA) are in dpm/nmol; rate of 14CO2 expiration (exp.) is in dpm·kg⁻¹·min⁻¹. *P = 0.01 vs. basal.

**Statistical analysis.** All data are expressed as means ± SE. The comparison between the postprandial and the basal amino acid kinetic data (i.e., Leu, Phe, and Tyr Rn and Leu Ox, Phe Hy, NOLD, and NHPD) within each group was performed using the two-tailed Student's t-test for paired data. The same test was used to compare two sets of paired-related data (such as the relative changes vs. baseline, expressed as either % or Δ, of Ox vs. Hy, NOLD vs. NHPD, Leu Ra vs. Phe Ra, etc.). The one-way analysis of variance (ANOVA) was used to compare more than two sets of data (such as the relative changes vs. basal of Leu Ox and Tyr Rn). The two-way ANOVA for repeated measurements was used to analyze whole body protein synthesis, determined with the two tracers, in the fasting and postprandial states simultaneously. A P value < 0.05 was considered statistically significant.

**RESULTS**

**Substrate concentrations, SAs, and enrichments.** Meal ingestion increased (P < 0.01) plasma glucose (from 84 ± 6 to 101 ± 8 mg/dl), insulin (from 13 ± 2 to 77 ± 12 mU/l), leucine, phenylalanine, and tyrosine concentrations (Table 1). The percent postprandial increments of leucine and phenylalanine concentrations were similar (+63 ± 10% and +61 ± 3%, respectively). Glucagon concentrations did not change (from 106 ± 14 to 115 ± 16 ng/l). Plasma SAs and enrichments decreased postprandially, whereas the expired 14CO2 increased (Table 1).

**Amino acid kinetics.** Leu Ra, Phe Ra, and Tyr Ra increased postprandially (Table 2). The percent increments of the flux of each amino acid were substantially similar (~50–70%) using either plasma or intracellular precursor pools, with the exception of the increase of phenylalanine flux using the calculated intracellular precursor pool, which was lower (+23 ± 9%, P < 0.005 by ANOVA vs. the other increments; Table 2).

Both Leu Ox and Phe Hy increased postprandially (Table 2). Using plasma precursor pools, the percent increase of Phe Hy (+95 ± 25%) was similar to that of Leu Ox (+11 ± 9%), but it was lower when the intracellular precursor pool was used (Phe Hy +28 ± 14% vs. +110 ± 9% of Leu Ox, P < 0.0025 by ANOVA).

Both estimates of amino acid disposal into protein synthesis (i.e., NOLD for leucine and NHPD for phenylalanine) showed stimulation by the meal (Table 2). The percent increases of NOLD with either precursor pool, as well as that of NHPD with the intracellular pool(s), were substantially similar (~20–25%, P = not significant (NS) by ANOVA), whereas the percent increase of NHPD using the plasma precursor pool (+53 ± 8%) was greater (P < 0.005 by ANOVA vs. others).

When protein synthesis was expressed as milligrams of protein per kilogram per minute, no differences between the leucine and the phenylalanine-tyrosine data were observed in the postabsorptive state (Fig. 1, A and B) by use of each precursor pool. After the meal, no differences were again observed between the two amino acid tracers when the data were calculated using intracellular precursor pools (Figs. 1B and 2). In contrast, by use of the plasma pools, postprandial protein synthesis calculated with phenylalanine-tyrosine was significantly greater (P = 0.011 by ANOVA; Fig. 1A), and it increased more (Fig. 2) than the corresponding values derived from the leucine data.

Significant correlations were found between leucine concentrations and oxidation with use of both the plasma and intracellular TTRs, Leu and α-KIC 14C SAs, and rate of 14CO2 expiration in the basal state (from −60 to 0 min) and after meal administration (190–250 min).

Table 2. Leu Ra, Leu Ox, and NOLD and Phe and Tyr Ra, Phe Hy, and NHPD at steady state and after meal administration

<table>
<thead>
<tr>
<th>Plasma data</th>
<th>Leu Ra</th>
<th>Leu Ox</th>
<th>NOLD</th>
<th>Phe Ra</th>
<th>Tyr Ra</th>
<th>Phe Hy</th>
<th>NHPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>1.85 ± 0.11</td>
<td>0.45 ± 0.06</td>
<td>1.40 ± 0.08</td>
<td>0.60 ± 0.03</td>
<td>0.47 ± 0.04</td>
<td>0.040 ± 0.005</td>
<td>0.56 ± 0.03</td>
</tr>
<tr>
<td>Meal</td>
<td>2.68 ± 0.24</td>
<td>0.94 ± 0.1</td>
<td>1.74 ± 0.16</td>
<td>0.93 ± 0.06</td>
<td>0.77 ± 0.08</td>
<td>0.076 ± 0.011</td>
<td>0.86 ± 0.06</td>
</tr>
<tr>
<td>%Change</td>
<td>43 ± 6</td>
<td>22 ± 6</td>
<td>23 ± 6</td>
<td>55 ± 7</td>
<td>62 ± 5</td>
<td>95 ± 25</td>
<td>53 ± 8</td>
</tr>
<tr>
<td>Intracell. data</td>
<td>2.36 ± 0.13</td>
<td>0.57 ± 0.06</td>
<td>1.79 ± 0.12</td>
<td>0.78 ± 0.06</td>
<td>0.60 ± 0.05</td>
<td>0.135 ± 0.019</td>
<td>0.64 ± 0.05</td>
</tr>
<tr>
<td>Meal</td>
<td>3.35 ± 0.24</td>
<td>1.17 ± 0.11</td>
<td>2.18 ± 0.17</td>
<td>0.93 ± 0.06</td>
<td>0.96 ± 0.08</td>
<td>0.162 ± 0.020</td>
<td>0.77 ± 0.06</td>
</tr>
<tr>
<td>%Change</td>
<td>42 ± 7</td>
<td>110 ± 9</td>
<td>22 ± 6</td>
<td>23 ± 9</td>
<td>62 ± 8</td>
<td>28 ± 14</td>
<td>23 ± 9</td>
</tr>
</tbody>
</table>

Values are means ± SE, expressed in μmol·kg⁻¹·min⁻¹, all calculated using either plasma or intracellular TTRs and SAs. Ra, rate of appearance; Ox, oxidation, Hy, hydroxylation; NOLD, nonoxidative Leu disposal; NHPD, nonhydroxylative Phe disposal. *P ≤ 0.05 vs. basal.
plasma (Fig. 3A) and the intracellular pool (Fig. 3B), whereas no correlation was found between phenylalanine concentration and Phe Hy with either the plasma or the intracellular precursor pool (data not shown). In addition, Leu Ox and Phe Hy were correlated with each other with the plasma ($r = 0.83$, $P < 0.001$) but not the intracellular pools. No correlations were found between the concentrations of the two amino acids and either NOLD or NHPD, between insulin concentrations and NOLD or NHPD, between glucagon concentrations and either Leu Ox or Phe Hy, and, finally, between NOLD and NHPD.

DISCUSSION

The aim of this study was to test whether the phenylalanine-tyrosine tracer method is suitable to measure whole body protein synthesis in human subjects postprandially. The use of phenylalanine-tyrosine tracers is attractive, because the measurement of phenylalanine catabolism (hydroxylation), which is required for the calculation of whole body protein synthesis (3, 18), does not require expired-air collection. As a reference, we used the widely employed leucine tracer method. Both plasma and intracellular precursor pools were used.

The data show that, after the acute ingestion of a mixed meal of abundant energy and nitrogen content, whole body protein synthesis increased with both amino acid tracers when either plasma or intracellular calculations were used. When expressed as milligrams of newly synthesized protein, the use of intracellular precursor pool(s) resulted in similar rates of protein synthesis with the leucine and phenylalanine-tyrosine tracers, in both the postabsorptive and the postprandial states. In contrast, by use of plasma precursor pools, the phenylalanine-tyrosine approach led to an apparent overestimation (twofold; Fig. 2) of the increment of postprandial protein synthesis with respect to the leucine data.
Amino acid kinetics are calculated using SA and/or enrichments either measured in accessible pools (i.e., plasma), or estimated intracellularly, i.e., at the sites where the metabolic reactions take place. As regards leucine, either the SA or the enrichment of its deamination product KIC, measured in plasma, is commonly used as an indicator of intracellular leucine SA/enrichment (1, 22). However, no similar compounds are available for phenylalanine and tyrosine (21). Therefore, the intracellular phenylalanine enrichment in the fasting state as well as the intracellular tyrosine enrichments in both the fasted and fed states have been extrapolated by assuming a ratio between intracellular and extracellular enrichments similar to that of KIC to leucine SA measured in each subject (18). In the postprandial state, the intracellular/plasma phenylalanine enrichment ratio has been set to 1 (i.e., plasma phenylalanine enrichment has been used throughout) on the basis of the phenylalanine enrichment attained in a liver-synthesized protein, i.e., apoB-100, measured at steady state following meal ingestion (20). We acknowledge that some of these assumptions are unproven. However, the observation that by use of these adjustments protein synthesis was similar between the two tracer models in both the fasting and the fed states indirectly supports their validity.

Both phenylalanine and leucine catabolism increased using either the plasma or the intracellular precursor pool, indicating the nutritional adequacy (i.e., beyond requirement) of the meal tested. This finding is new, because in previous studies Phe Hy did not increase after a meal, at variance with the reported finding is new, because in previous studies Phe Hy did (i.e., beyond requirement) of the meal tested. This precursor pool, indicating the nutritional adequacy.

The postprandial leucine and phenylalanine concentrations achieved in this study were well below the values of the enzymes regulating the catabolic steps of these two amino acids. Indeed, the K_m of the branched-chain dehydrogenase complex is >1 mM (11), whereas that of phenylalanine hydroxylase is between 200 and 300 μM (6, 29). In dose-response studies in vivo, Leu Ox increased linearly up to ~600 μM of leucine concentration (10). Similarly, at increasing phenylalanine intakes, both phenylalanine oxidation and its conversion to tyrosine were dose dependent and did not reach a plateau (34). Thus it is unlikely that the differences between the increments of Leu Ox and Phe Hy observed postprandially were due to a saturation of the phenylalanine-catabolic step; rather, it is possible that other factors, either not considered or unmeasured in this study, played a role in the different response of the catabolism of these two essential amino acids to the meal.

The source of the meal protein used in this study was a casein hydrolysate. In casein, the leucine-to-phenylalanine ratio is 3.03 (5), i.e., greater than the commonly assumed ratio between leucine and phenylalanine in average body proteins (2.14; see Calculations (30)). This may imply that more leucine had to be oxidized (with respect to the amount of phenylalanine to be hydroxylated) to meet the body requirements for these essential amino acids. This may provide another possible explanation for the larger increase of leucine than phenylalanine catabolism observed postprandially.

No correlation was found between protein synthesis and any of the measured variables (in particular, amino acid and insulin concentrations). Thus the factors regulating the postprandial increase of whole body protein synthesis are probably more complex than is currently believed, and only partially known.

In conclusion, this study demonstrates that the leucine and phenylalanine tracer models yield similar estimates of postprandial protein synthesis in human subjects provided that adequate adjustments for intracellular specific activities or enrichments of the precursor pool are adopted. The observed differences in the relationships between leucine and phenylalanine concentrations and their catabolism might be related to the specific site of each amino acid catabolism. These limitations should be kept in mind in the choice of amino acid tracer(s) to measure amino acid catabolism and whole body protein synthesis in humans postprandially.

We thank M. Vettore for excellent technical assistance.

This study was supported by a grant from The National Research Council (CNR) of Italy (Grant no. 0704298C0704) and by the Joint Project between CNR and Regione Veneto. Study of Energy Metabolism in the Elderly.

AJP-Endocrinol Metab • VOL 284 • MAY 2003 • www.ajpendo.org
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REFERENCES


