In vivo regulation of phenylalanine hydroxylation to tyrosine, studied using enrichment in apoB-100

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Rafii M, McKenzie JM, Roberts SA, Steiner G, Ball RO, Pencharz PB. In vivo regulation of phenylalanine hydroxylation to tyrosine, studied using enrichment in apoB-100. Am J Physiol Endocrinol Metab 294: E475–E479, 2008. First published November 27, 2007; doi:10.1152/ajpendo.00604.2007.—Phenylalanine hydroxylation is necessary for the conversion of phenylalanine to tyrosine and disposal of excess phenylalanine. Studies of in vivo regulation of phenylalanine hydroxylation suffer from the lack of a method to determine intrahepatocyte enrichment of phenylalanine and tyrosine. apoB-100, a hepatic export protein, is synthesized from intrahepatocyte amino acids. We designed an in vivo multi-isotope study, [15N]phenylalanine and [2H2]tyrosine to determine rates of phenylalanine hydroxylation from plasma enrichments in free amino acids and apoB-100. For independent verification of apoB-100 as a reflection of enrichment in the intrahepatocyte pool, [1-13C]lysine was used as an indicator amino acid (IAA) to measure in vivo changes in protein synthesis in response to tyrosine supplementation. Adult men (n = 6) were fed an amino acid-based diet with low phenylalanine (9 mg·kg⁻¹·day⁻¹, 4.54 μmol·kg⁻¹·h⁻¹) and seven graded intakes of tyrosine from 2.5 (deficient) to 12.5 (excess) mg·kg⁻¹·day⁻¹. Gas chromatography-quadrupole mass spectrometry did not detect any tracer in apoB-100 tyrosine. A new and more sensitive method to measure label enrichment in proteins using isotope ratio mass spectrometry demonstrated that phenylalanine hydroxylation measured in apoB-100 decreased linearly in response to increasing tyrosine intake and reached a break point at 6.8 mg·kg⁻¹·day⁻¹. IAA oxidation decreased with increased tyrosine intake and reached a break point at 6.0 mg·kg⁻¹·day⁻¹. We conclude: apoB-100 is an accurate and useful measure of changes in phenylalanine hydroxylation; the synthesis of tyrosine via phenylalanine hydroxylation is regulated to meet the needs for protein synthesis; and that plasma phenylalanine does not reflect changes in protein synthesis.

The hydroxylation of phenylalanine to tyrosine is considered to be the most important determinant of phenylalanine homeostasis because it is the rate-limiting step in the oxidation of phenylalanine to CO₂ and water (23). Phenylalanine hydroxylase has been shown to occur in both the liver and kidney, and in humans the liver enzyme is predominant, with an average activity level four to five times that in the kidney in vitro (1). There are contradictory reports in vivo in humans. Moller et al. (18), using measurement of phenylalanine uptake and tyrosine release across the kidney and splanchnic bed, concluded that the kidney is an important site for phenylalanine-to-tyrosine production. Conversely, Jones et al. (12), using [1-14C]phenylalanine, observed a mild impairment in the hydroxylation of phenylalanine that did not produce marked changes in plasma or urinary metabolites of phenylalanine in uremic adults.

Clarke and Bier (5) developed an in vivo model to estimate the rate of phenylalanine hydroxylation in fasted humans by use of stable isotope tracers. This model used the ratio of the plasma enrichment of tyrosine to phenylalanine, representing the product and precursor pools of hydroxylation, respectively, and tyrosine flux to calculate the rate of phenylalanine hydroxylation (5). Although originally developed for use in the fasted state, the Clarke and Bier model, and its modified version (19), were subsequently applied to studies in the fed state in parenterally fed neonates using intravenously administered isotope infusions (2, 4, 7, 15, 26, 28). The results of these studies indicated that parenterally fed neonates have the ability to hydroxylate phenylalanine. However, a follow-up appraisal by our group (10), using data from a study of parenterally fed neonatal piglets, questioned the quantitative validity of this measure of phenylalanine hydroxylation under certain circumstances. The results indicated that, at low tyrosine and high phenylalanine intakes, the estimation of rate of hydroxylation exceeded the intake of phenylalanine, indicating net tissue protein breakdown. However, those piglets were shown to be actively growing and depositing body protein. It was therefore concluded that, by use of this model at intakes of tyrosine below the mean requirement as estimated by House et al. (9), and at high intakes of phenylalanine, measurements of phenylalanine hydroxylation were overestimated. Subsequent examination of results from previous studies of phenylalanine metabolism in TPN-fed neonates (2, 15, 26) also indicates that rates of hydroxylation appeared to be overestimated. Similarly, we showed in adult humans that rates of phenylalanine hydroxylation are overestimated (27) at low tyrosine intakes. Indeed, it was not until the tyrosine intake reached 10.5 mg·kg⁻¹·day⁻¹ (r = 8.10 − 0.34x, R² = 0.21) that the rate of hydroxylation fell below the phenylalanine

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intake of 4.54 μmol·kg⁻¹·h⁻¹ (9.1 mg·kg⁻¹·day⁻¹) (see Fig. 4). Therefore, we believe that all the previous in vivo estimates of phenylalanine hydroxylation may be erroneous.

A potential source of error in the calculation of phenylalanine hydroxylation with the current model involves the site of sampling of amino acid enrichments. The issue of what is the most appropriate free amino acid pool to sample in isotope studies of amino acid metabolism has been well recognized (3, 11, 21). Most studies of amino acid metabolism in humans have traditionally been conducted using plasma amino acid enrichments, since it was suggested to be the best representation of whole body amino acid kinetics (30), and also due to lack of a suitable marker of intracellular enrichment. The notable exception is the use of the enrichment of ketoisocaproate (KIC) as a reflection of intracellular leucine enrichment (17).

apoB-100 has been shown to represent the amino acid enrichments of the hepatic intracellular pool (21): the site of phenylalanine hydroxylation. Because very-low-density lipoprotein (VLDL) particles are rapidly processed to higher-density particles, the apoB-100 contained within the VLDL fraction has a very high rate of turnover. It is sufficiently rapid that, during the course of a tracer amino acid infusion of less than 12 h, the apoB-100 enrichment reaches isotopic equilibrium (21). By definition, once this status has been achieved the isotopic enrichment of the tracer amino acid in the VLDL apoB-100 is a direct measurement of the enrichment of the apoB-100 standard curve obtained from human LDL (21).

In the present study, the main objective was to compare estimates of phenylalanine hydroxylation across a range of tyrosine intakes, determined using plasma and apoB-100 phenylalanine and tyrosine enrichments, with a measurement of in vivo change in protein synthesis. At fixed and deficient intakes of tryptophan and tyrosine enrichments, with a measurement of in vivo amino acid oxidation (IAAO) measurements were made simultaneously using L-[1-¹³C]lysine. All isotopes were given orally at hourly intervals so as to simulate a constant infusion for a total of 5 h. The study periods were separated by at least 1 wk, and all studies were carried out within 3 mo (22, 27).

Sample collection and analysis. Detailed steps for plasma and breath collections and analyses are outlined in our previous papers (22, 27).

Arterialized venous blood samples (15 ml) were also collected into tubes containing 1.5 ml of 15% Na₂EDTA-H₂O for the analysis of amino acid enrichments from apoB-100. Arterialized venous blood was obtained in a similar method to that mentioned earlier. Plasma was separated for the analysis of amino acid enrichments in apoB-100 by ultracentrifugation (Optima LE-80K Ultracentrifuge; Beckman Coulter, Fullerton, CA) at 4°C at 39,000 rpm for 16 h. The top VLDL fraction was aspirated into a separate tube and delipidated. The apolipoproteins B-48 and B-100 from the VLDL fraction were then separated for the analysis of amino acid enrichments in apoB-100 by ultracentrifugation. Stable isotope-labeled amino acids were added to the samples, which were then hydrolyzed in 300 μl of 6.0 N HCl plus 0.5% phenol at 110°C in vacuum reaction vessels placed in a heated work station (Eldex Hydrolysis and Derivatization Station; Eldex Laboratories, Napa, CA) for 24 h. The hydrolyzed samples were cooled to room temperature and frozen at −20°C for 20 min. This was followed by centrifugation at 3,000 rpm for 10 min. The supernatant fractions were dried under Speed Vac (Labconco Freeze Dry System). The dried samples were reconstituted in 50 μl of 0.1% formic acid (FA). Phenylalanine and tyrosine fractions were separated from each sample by a fraction collector (Fraction Collector Frac-100; Pharmacia, Uppsala, Sweden) attached to an HPLC ( Dionex, Sunnyvale, CA). The amino acids were separated by a Dionex Acclaim 120 Å PA (Polar Advantage), C₁₆, 150×4.6-mm, 5-μm column. An isocratic gradient at 0.75 ml/min of 0.1% FA at an oven temperature of 37°C was used. The phenylalanine and tyrosine fractions were freeze-dried overnight for enrichment analysis by continuous-flow isotope ratio mass spectrometer (CFIRMS; 20/20, ANCA GSL; Europa Scientific, Crewe, UK). The freeze-dried samples were reconstituted in 50 μl of deionized water and transferred to 6×4-mm tin capsules. The samples were evaporated to dryness on a heating block at 70°C. The dried samples in the tin capsules were squelched and combusted in the elemental analyzer (ANCA) unit attached to the CFIRMS. The samples were run against urea working standards that were calibrated against international standards [Urea Standard Reference Material 2141 Batch no. 580609; National Institute of Standards and Technology (NIST), Maryland] to calculate the APE of [¹⁵N]phenylalanine and [¹⁵N]tyrosine. Since the amount of phenylalanine and tyrosine extracted from the VLDL samples ranged from 2 and 1 μg, we determined enrichment at both 1 and 2 μg of nitrogen. Both showed very similar results, and the [¹⁵N] enrichment curves for 1 μg of nitrogen is shown (Fig. 1).

Estimation of isotope kinetics. Phenylalanine kinetics were calculated according to the stochastic model of Matthews et al. (16), as we have previously employed (31). Briefly, stable isotope-labeled amino acid is given as a continuous infusion, and the enrichment of the pool increases until a plateau is reached. The initial increase in the enrichment of the pool is relative to the pool size and turnover rate. The enrichment of the plateau represents the rate of turnover of the pool, and this relationship is used to calculate the flux of the free amino acid pool: Q = i(E/E), where, i = rate of infusion of the

### MATERIALS AND METHODS

**Subjects.** Six healthy adult male volunteers participated in the study on an outpatient basis in the Clinical Investigation Unit at the Hospital for Sick Children (HSC), Toronto, Canada. Subject characteristics are detailed in Table 1. Each subject received all diet treatments. More details on the subjects and their diet and energy intakes can be found in our previous, corollary papers (22, 27).

**Tracer protocol and experimental design.** Subjects were randomly allocated to receive each of the seven different dietary intakes of 3, 4.5, 6.0, 7.5, 9.0, 10.5, and 12.0 mg·kg⁻¹·day⁻¹. Each study consisted of 2 days on a controlled protein intake of 1 g·kg⁻¹·day⁻¹ followed by a single study day for the measurement of phenylalanine hydroxylation. On the study day, phenylalanine hydroxylation was measured using 1-[¹⁵N]phenylalanine and 1-[3,3-²H₂]tyrosine at a protein intake of 1 g·kg⁻¹·day⁻¹. Indicator amino acid oxidation (IAAO) measurements were made simultaneously using 1-[¹³C]lysine. All isotopes were given orally at hourly intervals so as to simulate a constant infusion for a total of 5 h. The study periods were separated by at least 1 wk, and all studies were carried out within 3 mo (22, 27).

**Table 1. Subject characteristics**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age, yr</th>
<th>Height, m</th>
<th>Weight, kg</th>
<th>Energy Intake, MJ/day*</th>
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<td>35</td>
<td>1.81</td>
<td>95.0</td>
<td>13.8</td>
</tr>
</tbody>
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Mean ± SD /H₁₁₀₂₁

*Calculated from the 1985 FAO/WHO/UN predictive equations, multiplied by an activity factor of 1.7 (8).

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isoacetate from tyrosine intakes of 3.0 to 6.8 mg shown in Fig. 2. In general, phenylalanine hydroxylation de-
in the apoB-100 protein to variations in tyrosine intake is

Values of enrichment are expressed in atoms percent excess (APE).

from [3,3-2H2]tyrosine, and Et/Ep is the ratio of the enrichments of

infused into the plasma metabolic pool. Tyrosine flux was estimated

ratios of the enriched peaks (the points making up the plateau (H

/\)


tyrosine to [15N]phenylalanine in either plasma or in apoB-100.

Oxidation of 13C lysine was calculated from the appearance of

isotope, Ei = enrichment of the labeled amino acid, and E =

enrichment of the pool at plateau. This equation can be modified to

account for the difference of the isotope infusion on the flux rate (17):

\[ Q = \left( \frac{E_t}{E_p} - 1 \right) \]

Isotopic steady state in the metabolic pool was

represented by plateaus in free [15N]phenylalanine, [15N]tyrosine, and

[3,3-2H2]tyrosine in plasma. The occurrence of a plateau was defined

by visual inspection, the absence of a significant slope, assessed by

linear regression analysis, and by the coefficient of variation of the

points making up the plateau (n = 4) being less than 5%. The mean

ratios of the enriched peaks (m + 1 and m + 2) to the unenriched (m)

for each amino acid at both the baseline and plateau samples were

used to calculate molecules percent excess (MPE).

Phenylalanine flux (\( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \)) was measured during isoto-

pic steady state from the dilution of the l-[1-15N]phenylalanine

infused into the plasma metabolic pool. Tyrosine flux was estimated

from the dilution of the [3,3-2H2]tyrosine infused.

Phenylalanine hydroxylation in both plasma and the apoB-100 samples were calculated from the conversion of the [15N]phenylala-

nine to [15N]tyrosine and from the independent measurement of tyrosine flux according to the model of Clarke and Bier (5):

\[ \frac{Q_{pt}}{Q_{pi}} = \frac{E_t}{E_p} \]

\( pt \) is the rate of phenylalanine hydroxylation

(\( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \)), \( pi \) is the tyrosine flux (\( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \)) estimated from [3,3-2H2]tyrosine, and

\( E/Ep \) is the ratio of the enrichments of [15N]tyrosine to [15N]phenylalanine in either plasma or in apoB-100.

Oxidation of 13C lysine was calculated from the appearance of

\( ^{13}\text{CO}_2 \) in breath (22).

Data analysis. Repeated-measures analysis of variance was per-

formed to assess the effect of tyrosine intake on phenylalanine hydroxylation, phenylalanine flux, tyrosine flux and lysine oxidation.

Where an effect was identified, the data were analyzed using a two-phase linear crossover model (29) (Proc Mixed, SAS Institute Cary, NC) to assign a break point to the response. The variance around the break point (95% confidence interval) was determined using Feiller’s theorem (24). Results were considered to be statistically significant at \( P < 0.05 \).

RESULTS

The mean (\( \pm \text{SE} \)) response of phenylalanine hydroxylation in the apoB-100 protein to variations in tyrosine intake is shown in Fig. 2. In general, phenylalanine hydroxylation decreased from tyrosine intakes of 3.0 to 6.8 mg·kg\(^{-1}\)·day\(^{-1}\), after which point there was no change in slope with further increase in tyrosine intake levels. Two-phase linear regression crossover model was the best-fit model for our data, and the break point estimate was found to be 6.8 mg·kg\(^{-1}\)·day\(^{-1}\).

Figure 3 shows the production of \( ^{13}\text{CO}_2 \) (V\(^{13}\text{CO}_2 \)) mean (\( \pm \text{SE} \)) from the oxidation of l-[1-13C]lysine at graded tyrosine intakes

using IAAO (22). The break point estimate using IAAO methodology was found to be 6.0 mg tyrosine·kg\(^{-1}\)·day\(^{-1}\).

Phenylalanine hydroxylation to tyrosine measured by plasma enrichments is shown in Fig. 4 (mean \( \pm \text{SE} \), modified from Ref. 27). In the plasma, no identifiable break point was seen in phenylalanine hydroxylation in response to tyrosine intake levels, using a two-phase linear regression crossover model. Conversely, linear regression analysis showed a significant effect of tyrosine intake on phenylalanine hydroxylation (\( P = 0.002 \)), such that the rate of hydroxylation was higher following a lower intake of tyrosine. The relationship is de-

fined by the equation, \( y = 8.10 - 0.34x \) (\( R^2 = 0.21 \), SE = 2.02). Dietary phenylalanine intake was 4.54 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \). It was not until a tyrosine intake of 10.5 mg·kg\(^{-1}\)·day\(^{-1}\) that estimated phenylalanine hydroxylation equaled phenylalanine intake. This contrasts with the phenylalanine hydroxylation estimates that were derived from apoB-100 enrichment where even at the lowest tyrosine intake phenylalanine hydroxylation was less than phenylalanine intake.

\[ \text{Fig. 1. One-microgram } ^{15}\text{N enrichment curve derived by combustion isotope ratio mass spectrometry measurements of a [15N]glycine enriched standard. Values of enrichment are expressed in atoms percent excess (APE).} \]

\[ \text{Fig. 2. Phenylalanine hydroxylation to tyrosine measured by apoB-100. Mean ( \pm \text{SE} \) phenylalanine hydroxylation at graded tyrosine intakes. Dotted vertical lines represent 95% confidence limits of break point (6.8 mg·kg\(^{-1}\)·day\(^{-1}\)) estimate.} \]

\[ \text{Fig. 3. } ^{13}\text{CO}_2 \text{ production (V}\(^{13}\text{CO}_2 \)) from the oxidation of l-[1-13C]lysine at graded tyrosine intakes (23), reproduced with permission from The American Journal of Clinical Nutrition. Mean \( \pm \text{SE} \) V\(^{13}\text{CO}_2 \) from the oxidation of l-[1-13C]lysine at graded tyrosine intakes. Dotted vertical lines represent 95% confidence limits of break point (6.0 mg·kg\(^{-1}\)·day\(^{-1}\)) estimate.} \]
We chose to do so using graded intakes of the product, tyrosine, since earlier work had suggested that tyrosine might inhibit phenylalanine hydroxylation to tyrosine (6, 25). In addition, in light of the uncertainty regarding the importance (predominance) of hepatic phenylalanine metabolism, we chose to use an independent marker of whole body protein metabolism, IAAO. The basis of the technique is that the indicator (in this case lysine) is partitioned either to incorporation into protein (protein synthesis) or to oxidation (20). Hence, in Fig. 3, as tyrosine intake increases from lower intakes and the rate of lysine oxidation decreases, so protein synthesis also increases until the break point at a tyrosine intake of 6.0 mg·kg⁻¹·day⁻¹. After the break point, increases in tyrosine intake have no effect on lysine oxidation and, hence, on whole body protein synthesis.

The most likely source of error in the present methods for determination of phenylalanine hydroxylation involves the site of sampling of amino acid enrichments. Hepatic export proteins such as apoB-100 (21) may provide more accurate estimates of liver intracellular amino acid enrichment, because the hydroxylation of phenylalanine occurs primarily in the cytosol of the hepatocytes. Analysis for apoB-100 in the present experiment showed that with increasing tyrosine intake the rate of phenylalanine hydroxylation decreased linearly from 3.0 to 6.8 mg·kg⁻¹·day⁻¹, after which point there was no further decrease (slope not significantly different from zero) with further increases in tyrosine intake (Fig. 2). At low tyrosine intake, phenylalanine was hydroxylated to provide tyrosine to support protein synthesis. Once sufficient dietary tyrosine was provided to optimize protein synthesis, there was a constant rate of phenylalanine hydroxylation. The plateau level of phenylalanine hydroxylation, after the break point shown in Fig. 2, represents the first direct in vivo estimate of the minimal “obligatory” rate of phenylalanine hydroxylation in human adults during feeding. The hydroxylation estimates obtained from apoB-100 are more rational than previous estimates based on plasma enrichment (Fig. 4), since they do not exceed dietary phenylalanine intake levels.

There was very close correspondence between the pattern of the apoB-100-derived estimates of phenylalanine hydroxylation and the pattern of (indicator) lysine oxidation. The very similar break points in hydroxylation (6.8 mg·kg⁻¹·day⁻¹) and indicator oxidation (6.0 mg·kg⁻¹·day⁻¹) (protein synthesis) clearly demonstrate for the first time in vivo that dietary tyrosine regulates phenylalanine hydroxylation to optimize whole body protein synthesis. This is also the first clear demonstration that enrichment in apoB-100 gives results that agree with in vivo changes in protein synthesis.

The present data also support the concept that, in vivo in the fed state, the liver plays a major role in the hydroxylation of phenylalanine to tyrosine. The observations of Moller et al. (18) are in the fasted state. It is currently unclear whether the feeding state is a key determinant. However, the studies of Jones et al. (12) were extended to measure cumulative oxidation of phenylalanine over 24 h, hence covering fed and fasted states and also showed a minor role for the kidney.

In conclusion, analysis of the enrichment in apoB-100 protein is a new and highly improved in vivo isotope model of hepatic intracellular enrichment of amino acids. This model can be used in the study of in vivo metabolism of most amino acids via protein synthesis. Using this new apoB-100 model,
we have shown that dietary tyrosine regulates hepatic phenylalanine hydroxylation to provide tyrosine for whole body protein synthesis.

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