Relationships Between Phenylalanine Hydroxylation and Plasma Aromatic Amino Acid Concentrations in Humans

R. Barazzoni, M. Zanetti, M. Vettore, and P. Tessari

We investigated the relationships between phenylalanine hydroxylation (Phe Hy) and plasma concentrations of phenylalanine, tyrosine, and glucagon in healthy male volunteers (N = 13; age, 29 ± 3 years). Phe Hy, as well as the Phe and Tyr rate of appearance (Ra), were measured during L-[$^2H_5$]-Phe and L-[$^2H_2$]-Tyr continuous intravenous (IV) infusions both under basal postabsorptive conditions (N = 13) and following divergent changes of plasma aromatic amino acids (AAA) concentrations. Namely, AAA were increased by administration of a balanced synthetic mixed meal (n = 6) or selectively decreased by IV infusion of insulin along with a Phe-deficient, Tyr and tryptophan–deprived amino acid mixture ([IAA] n = 7). Following the meal, plasma Phe (54 ± 3 to 81 ± 12 μmol/L), plasma Tyr (54 ± 4 to 91 ± 7), Phe Hy (0.09 ± 0.01 to 0.15 ± 0.02 μmol/kg · min), Phe Ra (0.65 ± 0.04 to 0.96 ± 0.07), and Tyr Ra (0.51 ± 0.03 to 0.93 ± 0.11) all significantly increased ($P \leq .05$ *v* basal). IAA infusion significantly decreased plasma Phe (to 47 ± 3 μmol/L), plasma Tyr (to 25 ± 4), Phe Hy (to 0.07 ± 0.004 μmol/kg · min), and Tyr Ra (to 0.29 ± 0.02; all $P \leq .05$ *v* basal), while Phe Ra did not change (0.64 ± 0.04, NS). Plasma glucagon did not change in the three experimental periods (basal, 85 ± 7; meal, 72 ± 10; IAA, 92 ± 14 pg/mL; NS). Using linear regression analysis, plasma Phe was positively related to both Phe Hy ($R^2 = .76$, $P < .001$) and plasma Tyr ($R^2 = .80$, $P < .001$); Phe Hy and plasma Tyr were also significantly correlated ($R^2 = .60$, $P < .001$). No correlation was found between Phe Hy and basal plasma glucagon ($R^2 = .04$, NS). Using multiple regression analysis with plasma Tyr as the dependent variable, plasma Phe was still correlation with plasma Tyr ($t = 4.29$, $P = .0002$), while the relationship between Phe Hy and plasma Tyr was no longer significant ($t = 0.69$, $P = .49$). These data indicate that plasma Phe is closely associated with its own hydroxylative disposal in humans, and confirm that Phe conversion to Tyr may play a physiological role in maintaining balanced plasma phenylalanine and tyrosine concentrations.

PHENYLALANINE HYDROXYLATION (Phe Hy) to tyrosine, catalyzed by phenylalanine hydroxylase, is the first step of phenylalanine catabolism, as well as a source of tyrosine for both hepatic and whole-body protein metabolism.[1,2] The reaction is considered to be mainly located in the liver in humans.[1]

The rate of Phe Hy has been determined in vivo with phenylalanine and tyrosine isotopic intravenous (IV) infusions by measuring the ratio between plasma phenylalanine and phenylalanine-derived tyrosine enrichment.[2,3] Using such a model, most groups reported that hydroxylation accounts for about 15% of phenylalanine postabsorptive flux, as well as 15% to 20% of the tyrosine plasma rate of appearance (Ra).[2-6]

In vitro data have shown that phenylalanine itself is both the substrate and an essential activating factor of the enzyme,[7] representing a typical example of feed-forward regulation of substrate catabolism. Also in humans, the existing data suggest that phenylalanine catabolism may change in response to changes in its own exogenous supply, as demonstrated also with regard to other essential amino acids.[8] Indeed, both hydroxylation and decarboxylation, a subsequent catabolic step of phenylalanine metabolism, were acutely inhibited following a decrease of aromatic amino acid (AAA) levels in vivo.[9] However, only phenylalanine decarboxylation, not the indirectly determined hydroxylation, increased following acute stepwise increments of phenylalanine oral loads.[10] Furthermore, in adult humans, adaptation to different levels of dietary phenylalanine and tyrosine intake did not significantly modify postabsorptive hydroxylation rates, while hydroxylation increased in the fed state only at the highest amino acid supply.[5,6] Therefore, some uncertainties remain as to the possible relationships between AAA availability and Phe Hy in vivo in humans.

In particular, although a positive correlation between the plasma phenylalanine concentration and Phe Hy has been recently reported in newborns,[11,12] the relationship between hydroxylation and plasma AAA concentrations has not been systematically investigated in adult humans, especially following acute diverging modifications of plasma phenylalanine and tyrosine levels.

To address this issue, we studied a group of adult healthy male volunteers both under postabsorptive conditions and following either a balanced synthetic mixed meal or IV infusion of insulin along with a phenylalanine-deficient, tyrosine-deprived amino acid mixture, aimed at selectively decreasing plasma phenylalanine and tyrosine concentrations. Since glucagon has been shown to stimulate phenylalanine hydroxylase in vitro,[13] as well as in vivo,[14] glucagon levels were measured and a relationship between basal plasma glucagon and hydroxylation was also sought.

SUBJECTS AND METHODS

Subjects

We studied 13 healthy male volunteers (age, 29 ± 3 years; range, 22 to 55). All subjects were instructed to follow a balanced, weight-maintaining diet containing 50% carbohydrate, 32% lipid, and 18% protein with about 1.2 g protein/kg · d for at least 1 month before the study. In the absence of a local Ethical Committee at the time studies were performed, the experimental protocol was submitted to and approved by the Dean of the Faculty of Medicine and the Head of the Department. All subjects were informed about the aims and potential risks of the study, and they provided consent to participate before it was performed.

From the Department of Metabolic Diseases, University of Padova, Padova, Italy.

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Address reprint requests to P. Tessari, MD, Cattedra di Malattie del Metabolismo, Dipartimento di Medicina Clinica e Sperimentale, Policlinico, Via Giustiniani 2, 35128 Padova, Italy.

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Isotopes

L-[ring-$^2H_5$]-phenylalanine (D_5-phenylalanine) and L-[3,3-$^2H_2$]-tyrosine (D_2-tyrosine) were purchased from Tracer Technologies (Somerville, MA). L-[ring-$^2H_4$]-tyrosine (D_4-tyrosine) was purchased from Euriso-Top (Gif-Sur-Yvette, France). All isotopes were proven to be sterile and pyrogen-free before use.

Experimental Design

At 7:30 AM on the morning of study after an overnight fast, each subject was admitted to the Metabolic Unit of the Department of Metabolic Diseases at Padua University Hospital. A polyethylene catheter was placed in an antecubital vein of the right arm and used for basal blood sampling and subsequent isotope infusion. Another catheter was inserted into the contralateral brachial artery and used for blood sampling throughout the study.

At 8:00 AM, a primed-continuous infusion of D_5-phenylalanine (≈0.05 μmol/kg · min) and D_2-tyrosine (≈0.02 μmol/kg · min) was started. Priming doses of both isotopes were about 60 times the continuous rate of infusion per minute. A bolus dose of D_4-tyrosine (≈0.4 μmol/kg) was also administered to prime the phenylalanine-derived plasma tyrosine pool. After about 3 hours to allow for isotope equilibration, four samples were drawn over 30 to 60 minutes for measurement of substrate and hormone concentrations and plasma isotope enrichment under basal steady-state conditions.

Thereafter, the subjects were divided into two experimental groups.

Meal administration. In six subjects, a synthetic mixed meal (Nutrodrip Protein; Wander Sandoz Nutrition, Milan, Italy) was administered in isocaloric aliquots every 20 minutes for 4 hours for a total about 15 kcal/kg. The composition of the meal was as follows (in grams per 100 mL): carbohydrate 14.8, fat 4, and protein (N × 6.38) 6.6. The protein component consisted mainly of casein together with soy protein hydrolysates. Blood samples were collected every 20 minutes from 190 to 250 minutes for turnover calculations under the new isotopic steady state, which was effectively achieved in the last hour of this experimental period as previously shown in similar experimental conditions.[15]

Insulin and amino acid infusion. In seven subjects, insulin 0.05 U/m^2 · min (Actrapid; Novo, Bagswaert, Denmark) was infused to decrease protein breakdown and endogenous amino acid release. Plasma amino acid concentrations were selectively modified by simultaneous infusion of an L-amino acid mixture (L-Amino Acidi Selettivi; Boehringer Mannheim Italia, Milan, Italy) containing low amounts of phenylalanine and tryptophan and no tyrosine. The composition of the infused mixture was the same as reported elsewhere.[9] In particular, the phenylalanine content was 6.1 g/L, resulting in an infusion rate of about 0.13 μmol/kg · min. The rate of total amino acid infusion was approximately 1 mmol/min. Euglycemia was maintained by variable glucose infusion based on frequent blood glucose monitoring. After about 3 hours, four blood samples were again taken under isotopic steady-state conditions for kinetic calculations.

Analytical Methods

D_5-phenylalanine, D_2-tyrosine, and D_4-tyrosine plasma isotopic mole percent enrichment (MPE) was determined as *tert*-butyl-dimethyl-silyl derivatives as previously described.[16] The monitored masses were *m/z* 239/234 for M+5 (ie, deuterated) and M+0 (ie, natural) phenylalanine respectively, and *m/z* 470/468/466 for deuterated M+4 and M+2 and for M+0 tyrosine.

Amino acid concentrations were measured by ion-exchange liquid chromatography using a Beckman Amino Acid Analyzer (Beckman Instruments, Palo Alto, CA). Blood glucose was monitored using a Beckman Glucose Analyzer 2. Glucagon and insulin concentrations were measured by radioimmunoassay as previously described.[17]

Calculations

Phenylalanine and tyrosine total plasma Ra (micromoles per kilogram per minute) was conventionally calculated by dividing the infusion rate (F) of each isotope by the corresponding plasma MPE, and subsequently subtracting F.[18]

Endogenous Phe and Tyr Ra, corresponding to total Ra in the basal period, were calculated in the insulin and amino acid (IAA) period by subtracting their exogenous fixed rate of infusion from the total Ra.

Phe Hy was calculated as previously described,[3] according to the following equation:

$$\text{Phe Hy} = \text{Tyr Ra} \times \frac{\text{D}_4\text{-Tyr MPE}}{\text{D}_5\text{-Phe MPE}} \times \frac{\text{Phe Ra}}{(\text{D}_5\text{-Phe F} + \text{Phe Ra})}$$

Statistical Analysis

Comparisons between the basal period and each subsequent experimental period within each subgroup were performed using the two-tailed Student's *t* test for paired data. The basal data of both groups were pooled and are presented together in the results.

The correlation between two sets of data was determined using linear regression analysis. Multiple regression analysis was then performed among significantly related variables.

A P value of .05 or less was considered statistically significant. All data are expressed as the mean ± SE.

RESULTS

Substrates and Hormones

Meal absorption caused a significant increase of phenylalanine, tyrosine, and insulin concentrations, whereas glucagon levels did not change (Table 1). Following IAA infusion, there was an expected increase of insulin levels, whereas phenylalanine and tyrosine concentrations significantly decreased and glucagon was unaffected (Table 1).

Plasma glucose significantly increased following the meal (from 4.9 ± 0.2 to 5.7 ± 0.6 mmol/L, P = .02), whereas it did not change from basal during the IAA and the euglycemic clamp, as expected.

Phenylalanine and Tyrosine Kinetics

D_5-phenylalanine MPE significantly decreased following meal absorption, whereas it did not change during the IAA infusion (Table 2). D_2-tyrosine and D_4-tyrosine MPEs decreased following the meal, whereas they sharply increased during the IAA infusion (Table 2).

Phenylalanine and tyrosine kinetics are shown in Table 3. Both Phe Ra and Tyr Ra increased following the meal. In contrast, Phe Ra was unchanged and Tyr Ra, corresponding to endogenous Ra since no IV Tyr was infused, significantly decreased following the IAA infusion (Table 3). Endogenous

Table 1. Plasma Concentrations of Phenylalanine (μmol/L), Tyrosine (μmol/L), Glucagon (pg/mL), and Insulin (μU/mL) in the Postabsorptive (basal), Meal, and IAA Periods

Parameter	Basal	Meal	IAA
Phe	54 ± 2	78 ± 5*	47 ± 3*
Tyr	54 ± 4	91 ± 7*	25 ± 4*
Glucagon	85 ± 7	92 ± 14	72 ± 10
Insulin	10 ± 1	75 ± 14*	111 ± 5*

NOTE. Data are the mean ± SE.
*P < .05 *v* basal.

Table 2. Plasma MPE of D_5-Phenylalanine, D_4-Tyrosine, and D_2-Tyrosine in the Three Experimental Periods

Parameter	Basal	Meal	IAA
D_5-Phe	7.31 ± 0.41	5.08 ± 0.46*	7.23 ± 0.45
D_4-Tyr	1.35 ± 0.08	0.83 ± 0.08*	1.96 ± 0.19*
D_2-Tyr	4.19 ± 0.24	2.38 ± 0.26*	7.29 ± 0.53*

NOTE. Data are the mean ± SE.
*$P < .05$ *v* basal.

Phe Ra was also significantly suppressed in the IAA period (to 0.52 ± 0.04 μmol/kg · min, $P = .002$ *v* basal).

Hydroxylation significantly increased following meal ingestion (0.09 ± 0.01 to 0.15 ± 0.01 μmol/kg · min, $P < .01$), whereas it decreased following the IAA infusion period (0.09 ± 0.01 to 0.07 ± 0.01, $P < .02$; Table 3).

Correlations

Considering all data together (ie, basal, meal, and IAA periods), Phe Hy was significantly correlated with the plasma phenylalanine concentration ($R^2 = .76$, $P = .0001$; Fig 1). The correlation was statistically significant also for the basal (n = 13) and meal (n = 6) periods considered separately, although not for the IAA (n = 7) period alone (Table 4).

Ph Hy was also correlated with the plasma tyrosine concentration ($R^2 = .60$, $P = .0001$; Fig 2). The correlation was of borderline statistical significance in the basal and meal periods considered separately, but again was not significant in the IAA group alone (Table 4).

Phenylalanine and tyrosine concentrations were significantly and strongly related in both the basal and the meal periods (Table 4), with a tight overall positive correlation ($R^2 = .80$, $P = .0001$; Fig 3).

Using multiple regression analysis among hydroxylation and phenylalanine and tyrosine concentrations in the three experimental periods together considering tyrosine as the dependent variable, a strong correlation was still observed between plasma phenylalanine and tyrosine ($t = 4.29$, $P = .0002$; Table 5), whereas the correlation between hydroxylation and plasma tyrosine was no longer significant ($t = 0.69$, $P = .49$; Table 5). No correlation was found between basal plasma glucagon and hydroxylation ($R^2 = .04$, NS).

Since hydroxylation and phenylalanine levels were significantly related, we expressed the former as a function of the phenylalanine concentration, ie, by dividing Phe Hy (micromoles per kilogram per minute) by plasma Phe (micromolars). The resulting phenylalanine hydroxylative clearance (milliliters per kilogram per minute) may represent an index of phenylalanine hydroxylase activity independent of circulating phenylalanine. The hydroxylative clearance did not change in the IAA period with respect to basal, but it increased significantly during the meal (0.16 ± 0.02 to 0.19 ± 0.02 mL/kg · min, $P = .04$; Fig 4).

Table 3. Phenylalanine and Tyrosine Ra (μmol/kg · min) and Phe Hy to Tyrosine (μmol/kg · min) in the Three Experimental Periods

Parameter	Basal	Meal	IAA
Phe Ra	0.65 ± 0.04	0.96 ± 0.07*	0.64 ± 0.04
Phe Hy	0.09 ± 0.01	0.15 ± 0.02*	0.07 ± 0.01*
Tyr Ra	0.51 ± 0.03	0.93 ± 0.11*	0.29 ± 0.02*

NOTE. Data are the mean ± SE.
*$P < .05$ *v* basal.

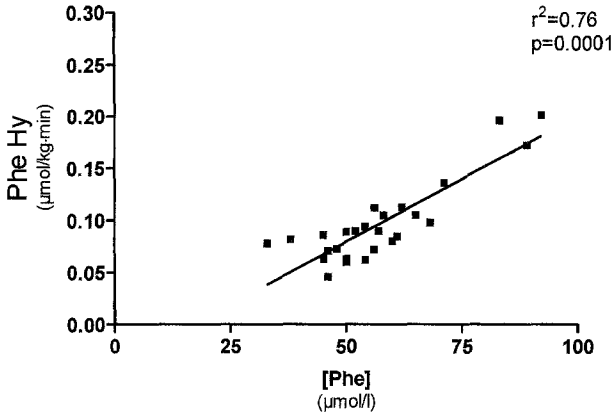

Fig 1. Correlation between Phe Hy and Phe plasma concentration [Phe].

DISCUSSION

The aim of this study was to investigate the relationships between Phe Hy to tyrosine and plasma phenylalanine, tyrosine, and glucagon concentrations in humans at different plasma AAA levels. With respect to phenylalanine and tyrosine plasma concentrations, classic data from patients with phenylketonuria show that a lack of phenylalanine hydroxylase activity leads to abnormally high phenylalanine and low tyrosine circulating levels.[19] Indeed, these data indicate that phenylalanine conversion to tyrosine is linked to the maintainance of balanced plasma AAA concentrations. However, the relationships between these variables had not been extensively investigated in adult healthy humans.

Our data show that under these experimental conditions, a strong positive correlation exists between the plasma phenylalanine concentration and its hydroxylation rate, as well as with the tyrosine concentration. A direct linear correlation was also found between hydroxylation and plasma tyrosine. In contrast, hydroxylation was not related to plasma glucagon in the basal period.

The strong correlation between the phenylalanine concentration and Phe Hy indicates that plasma phenylalanine may directly influence its own hydroxylative disposal in vivo in adult men, probably due, at least in part, to a substrate-induced activation of the enzymatic reaction.[7]

The positive overall correlation between the phenylalanine whole-body Ra, which equals disposal at steady state, and both the concentration ($R^2 = .56$, $P = .0001$) and hydroxylation ($R^2 = .43$, $P = .0002$) also suggests that phenylalanine catabolism is linked to its turnover.

Table 4. Correlations Between Phe Hy, Phenylalanine, and Tyrosine Plasma Concentrations in Each Experimental Period

Parameter	Basal	Meal	IAA
Phe Hy-Phe	$R^2 = .32$, $P = .04$	$R^2 = .88$, $P = .006$	NS
Phe Hy-Tyr	$R^2 = .28$, $P = .06$	$R^2 = .60$, $P = .07$	NS
Phe-Tyr	$R^2 = .69$, $P = .0005$	$R^2 = .78$, $P = .02$	NS

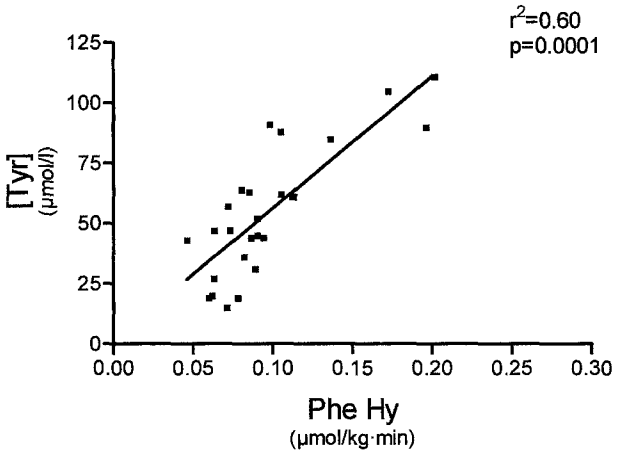

Fig 2. Correlation between Phe Hy and tyrosine plasma concentration [Tyr].

In a study focused on dietary phenylalanine requirements, stepwise increments of phenylalanine oral loads stimulated phenylalanine oxidation but not the estimated rate of Phe Hy, despite parallel increments of the plasma amino acid concentration.[10] This negative finding was presumably influenced by both the indirect method for estimation of the hydroxylation rate and the very high tyrosine dietary intake used in the study protocol. Indeed, the high tyrosine levels could have shifted intracellular phenylalanine-derived tyrosine directly toward oxidation without equilibrating with the plasma pool,[20] as actually discussed by the investigators themselves.[10] In other nutritional investigations, adaptation to different levels of dietary nitrogen and/or AAA intake did not significantly modify basal Phe Hy, whereas postmeal Phe Hy increased with respect to basal values only in the presence of an adequate dietary supply.[5,6] However, plasma

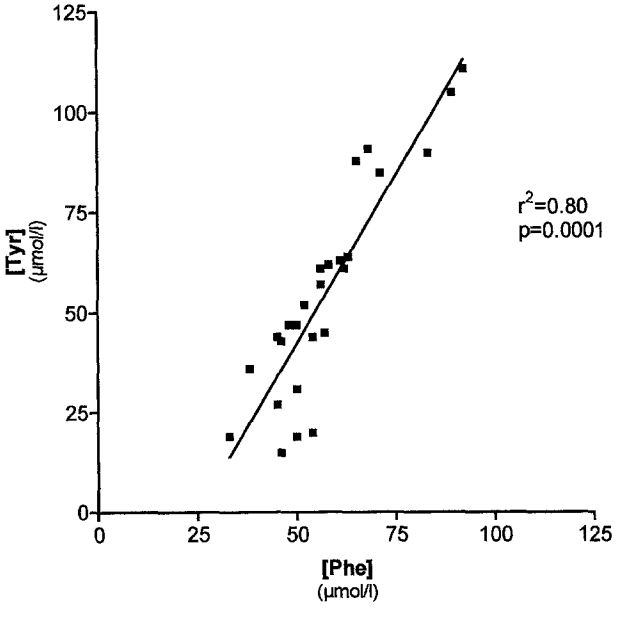

Fig 3. Correlation between phenylalanine [Phe] and tyrosine [Tyr] plasma concentrations.

Table 5. Multiple Regression Analysis Among Plasma Phenylalanine Concentration, Phe Hy, and Tyrosine Concentration

Dependent Variable	Phe	Phe Hy
Tyr	$t = 4.29$, $P = .0002$	$t = 0.69$, $P = .49$

amino acid concentrations were not reported in one study.[6] In the other investigation,[5] the near-normal basal AAA concentrations following chronic dietary restriction might explain the lack of significant differences in hydroxylation, in agreement with our finding that phenylalanine hydroxylase activity is strictly related to the plasma phenylalanine level.

In the present study, when each experimental period was considered separately, hydroxylation was still correlated with the phenylalanine concentration both under basal conditions and following meal administration (Table 4). Therefore, phenylalanine levels may affect hydroxylation also within the narrow range of physiologic postabsorptive concentrations, as well as at the higher postmeal levels. In contrast, the correlation was no longer evident at the lower phenylalanine levels achieved following the IAA infusion. Such a negative finding might be explained by the presence of a threshold level of phenylalanine to activate hydroxylation or, more likely, by a blunting effect of the fixed rates of exogenous insulin and amino acid infusions on this potential relationship, which might instead be present also at spontaneously low phenylalanine concentrations.

Phenylalanine hydroxylative clearance is an index of phenylalanine hydroxylase activity normalized for the plasma phenylalanine concentration, ie, possibly due to different activating factors. Therefore, the slight but significant increase of hydroxylative clearance following meal ingestion may suggest that the corresponding increment of Phe Hy was partly due to factors other than circulating phenylalanine, such as acute changes in splanchnic blood flow, intracellular coenzyme activity, or others. Clearly, this hypothesis is entirely speculative and should be confirmed by further investigations.

The direct correlation between Phe Hy and the tyrosine concentration also suggests that, as expected, the plasma tyrosine level is partly determined by its de novo hepatic synthesis. This relationship was somewhat weaker than the previous one, especially when each experimental period was

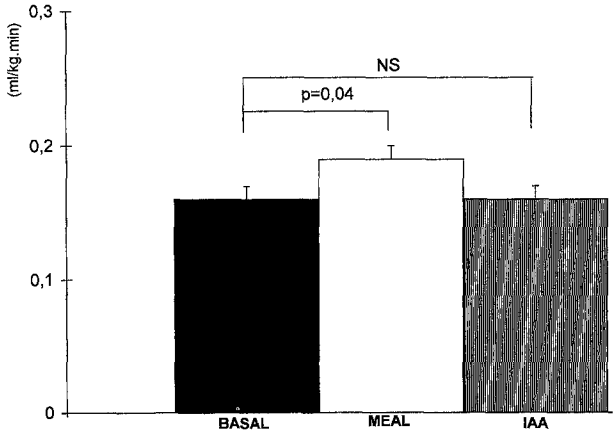

Fig 4. Phe hydroxylative clearance in the 3 experimental periods.

considered separately, probably also because only about 20% of tyrosine flux is accounted for by newly synthesized tyrosine. Again, the lack of a significant correlation in the IAA infusion period might be due, at least in part, to the peculiar experimental conditions we used to selectively decrease AAA levels. Interestingly, the squared regression coefficient in the basal period was in fair agreement with the expected impact of hydroxylation on plasma tyrosine, and it increased following meal ingestion (Table 4), suggesting an increased contribution from de novo synthesis at a time when endogenous proteolysis, ie, the main source of circulating amino acids under postabsorptive conditions, was most likely suppressed.[21]

There was a strong positive correlation between phenylalanine and tyrosine concentrations using both linear and multiple regression analysis including hydroxylation. The significant relationship in the basal and meal periods considered separately indicates that the overall correlation was not dependent on the experimental manipulations. Our finding is thus in agreement with the balanced entry of both amino acids into the plasma pool from whole-body proteolysis,[22] as well as from the casein- and soy-derived amino acids during meal absorption. However, the results of multiple regression analysis also indicate that the relationship between hydroxylation and plasma tyrosine may be explained by the influence of plasma phenylalanine on hydroxylase activity.

Therefore, the plasma phenylalanine concentration determined, at least in part, the circulating tyrosine level, thus confirming the important physiological role of phenylalanine conversion to tyrosine in maintaining balanced levels of both amino acids. Indeed, as already mentioned, plasma AAA profiles in patients with phenylketonuria, ie, lacking phenylalanine hydroxylase activity, also provide strong indirect evidence of such a regulatory role.[19]

The lack of a significant correlation between basal plasma glucagon and Phe Hy would not confirm the in vitro and in vivo reports of a glucagon-induced stimulation of Phe Hy.[13,14] On the other hand, the present data agree with our recent finding of increased phenylalanine oxidation with unchanged hydroxylation following acute isolated hyperglucagonemia at basal insulin and growth hormone levels in humans.[23] However, if glucagon stimulated both Phe Hy and oxidation, it is possible that the newly synthesized tyrosine did not equilibrate with its plasma pool, being instead driven toward oxidation inside the hepatocyte. Therefore, a possible link between glucagon and Phe Hy might be underestimated when the present isotopic model is used, since it mainly accounts for phenylalanine-derived tyrosine reaching the systemic circulation.

In conclusion, we report a direct correlation between plasma phenylalanine and both Phe Hy and plasma tyrosine in adult men in the postabsorptive state, as well as following acute diverging modifications of plasma AAA levels. The present data suggest that the plasma phenylalanine concentration is closely associated with its own hydroxylative disposal in humans. Furthermore, they confirm in a physiological setting that Phe Hy plays an important role in maintaining balanced plasma phenylalanine and tyrosine levels.

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