Evaluation of Phenylalanine and Tyrosine Metabolism in Late Human Pregnancy

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The [$^2H_5$]-phenylalanine method for measurement of protein metabolism requires the phenylalanine hydroxylation to tyrosine to be calculated from the tyrosine flux. Although this can be estimated, for pregnancy, we made a direct measurement of the molar ratio of the fluxes of tyrosine and phenylalanine from protein breakdown (Pt/Pp) using [$^2H_2$]-tyrosine infusion. Six normal pregnant women were studied at 37 weeks' gestation. While fasting, they were administered a 3-hour primed-constant infusion with [^{13}C]-leucine, [$^2H_5$]-phenylalanine, and [$^2H_2$]-tyrosine. Leucine (α-ketoisocaproic acid [KIC]) flux was 136.2 ± 15.1 μmol/kg/h (mean ± SD), phenylalanine flux 41.2 ± 5.6, and tyrosine flux 25.0 ± 6.0, and phenylalanine hydroxylation was 3.3 ± 2.1 μmol/kg/h. The mean tyrosine to phenylalanine molar flux ratio (Pt/Pp) was 0.52 ± 0.10, lower than the ratio of 0.65 to 0.85 reported in normal nonpregnant subjects and 0.73 estimated from animal studies. We studied protein metabolism in six additional pregnant women and six nonpregnant women using [^{13}C]-leucine and [$^2H_5$]-phenylalanine infusions only and applied the lower Pt/Pp ratio to the former group. Tyrosine flux (42.0 ± 7.2 μmol/kg/h) and phenylalanine hydroxylation (9.2 ± 4.2 μmol/kg/h) were significantly higher in nonpregnant subjects than in both groups of pregnant subjects. The percent contribution of phenylalanine hydroxylation to total tyrosine flux was reduced from 20% to 14%. When using [$^2H_5$]-phenylalanine to study whole-body protein metabolism in pregnancy and tyrosine flux is not measured directly by infusion of [$^2H_2$]-tyrosine, the lower Pt/Pp ratio is required. The phenylalanine model shows that tyrosine flux derived from protein breakdown and phenylalanine hydroxylation are both reduced in pregnancy.

PREGNANCY IS A TIME of significant physiological changes during which the pregnant woman must adapt her metabolism of substrates and endogenous stores to accommodate a growing fetus as well as her own needs. Protein metabolism plays an important role in fetal growth,[1] and reports of whole-body protein metabolism in human pregnancy evaluated by the primed-constant infusion of stable isotopes[2-6] have shown data for [^{13}C]-leucine. The concomitant use of other tracers such as phenylalanine would provide useful insights, but the [$^2H_5$]-phenylalanine model for whole-body protein turnover has not been fully evaluated for late pregnancy. The calculation of phenylalanine hydroxylation is dependent on knowledge of the tyrosine flux as well. Tyrosine flux can be measured by a primed-constant infusion of [$^2H_2$]-tyrosine,[7] but the infusion is made awkward by the limited solubility of [$^2H_2$]-tyrosine. In the model described by Thompson et al,[7] phenylalanine hydroxylation could also be calculated without measuring tyrosine flux independently if the molar ratio for the fluxes of tyrosine and phenylalanine from protein breakdown (Pt/Pp) was known. In animal studies, Pt/Pp was estimated to be 0.73.[8] Direct measurement of tyrosine flux obtained a figure of 0.76[7] in normal human subjects and 0.65[9] in insulin-dependent diabetic subjects.

Since the Pt/Pp ratio may be modified under different physiological conditions, we evaluated the [$^2H_5$]-phenylalanine model in late pregnancy by direct measurement of tyrosine flux to determine the Pt/Pp ratio. We compared whole-body protein metabolism for the phenylalanine and leucine models and assessed the effect of pregnancy on phenylalanine metabolism.

SUBJECTS AND METHODS

Isotopes

L-[1-^{13}C]-leucine (99% ^{13}C), L-[ring-$^2H_5$]-phenylalanine (98% ^{2}H), L-[ring-$^2H_4$]-tyrosine (98% ^{2}H), L-[ring-3,5-$^2H_2$]-tyrosine (98% ^{2}H), and sodium [^{13}C]-bicarbonate (99% ^{13}C) were purchased from Cambridge Isotopes Laboratories (Woburn, MA). They were dissolved in normal saline and tested to be sterile and pyrogen-free by Northwick Park Hospital Pharmacy (Harrow, UK).

Subjects

Twelve healthy normal women were recruited from the antenatal clinics at the Royal Victoria Infirmary, Newcastle-upon-Tyne, UK (Table 1). They were not on any medication and had no obstetric problems. Six healthy nonpregnant women were recruited from staff at the Medical School, Newcastle. Informed consent was obtained from all of the women, and ethical approval was given by the Joint Ethics Committee of Newcastle Health Authority and University of Newcastle.

Protocol

Six pregnant women were studied at 37 weeks' gestation (group 1). All subjects maintained a protein diet of 65 g/d for 3 days before the study. The subjects reported to the research unit between 8:30 and 9:00 AM, having fasted overnight from 10:00 PM. Each subject voided the bladder and had height and weight measured. This was followed by cannulation of both hands, one for infusion of the stable isotopes and the other for sampling arterialized superficial venous blood by the hot-box technique.[10] The subjects received priming doses of L-[1-^{13}C]-leucine (0.5 mg/kg), L-[ring-$^2H_5$]-phenylalanine (0.5 mg/kg), L-[ring-$^2H_4$]-tyrosine (0.08 mg/kg), L-[ring-3,5-$^2H_2$]-tyrosine (0.25 mg/kg), and sodium [^{13}C]-bicarbonate (0.08 mg/kg) followed immediately by continuous infusions of L-[^{13}C]-leucine (0.5 mg/kg/h), L-[ring-$^2H_5$]-phenylalanine (0.5 mg/kg/h), and L-[ring-3,5-$^2H_2$]-tyrosine (0.25 mg/kg/h) for 3 hours. Blood and expired-breath samples were collected at −30, −15, and 0 minutes before the start of the infusions and thereafter at 90, 120, 140, 150, 160, 170, and 180 minutes. Indirect calorimetry was performed for 30 minutes with a Deltatrac (Datex, Helsinki, Finland) metabolic monitor before the start and again in the last half-hour of the infusions.

Six pregnant women (group 2) and six nonpregnant women (group 3) were studied with this protocol without the L-[ring-3,5-$^2H_2$]-tyrosine prime and infusion.

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Table 1. Clinical Details of the Subjects

Parameter	Group 1	Group 2	Group 3
Age (yr)	32 ± 2	30 ± 3	28 ± 5
Gestation (wk)	37 ± 1	34 ± 2	—
Weight (kg)	78.2 ± 9.5	78.0 ± 9.3	58.8 ± 4.8
Body mass index (kg/m²)	31.7 ± 4.8	27.0 ± 2.3	21.0 ± 1.3
No. of subjects	6	6	6

Assays

All samples were centrifuged at 4°C immediately after collection, and plasma was stored at −70°C until analysis. The plasma samples were derivatized using a combination of methods,[11,12] and α-ketoisocaproic acid (KIC) was derivatized to its quinoxalinol-*t*-butyldimethylsilyl derivative. Phenylalanine and tyrosine were derivatized to their *t*-butyldimethylsilyl derivatives. Electron ionization gas chromatography/mass spectrometry (GC/MS) analysis was performed on the derivatives separately using a Finnegan (Hemel Hempstead, UK) 1020 GC/MS to determine ^{13}C or deuterium enrichments using selected ion monitoring. Phenylalanine was measured at *m/z* 336 and 441. Tyrosine was measured at *m/z* 446, 468, and 470. Samples were measured in duplicate on two separate occasions. Appropriate corrections were made for all samples using the calibration equations derived from standards for each amino acid. An additional correction was made for the contribution of [$^2H_2$]-tyrosine to the [$^2H_4$]-tyrosine peak at *m/z* 470 during GC/MS analysis.[13] When only [$^2H_4$]-tyrosine was infused, enrichment of [$^2H_2$]-tyrosine remained at basal levels, confirming nondegradation of label during analysis.[7] Breath $^{13}CO_2$ enrichment was measured by standard isotope-ratio mass spectrometry.

Comparisons of protein metabolism data were made using ANOVA, paired *t* tests, or group *t* tests as appropriate, with a *P* level less than .05 taken as significant. Results are the mean ± SD.

Phenylalanine Model for Whole-Body Protein Turnover

This has been described by Thompson et al.[7] Like the leucine model, it assumes that phenylalanine, as an essential amino acid, is not synthesized in man and enters the plasma pool only from protein breakdown. It exits the pool only via protein synthesis or by hydroxylation to tyrosine, an irreversible step. The calculation of amino acid flux (Q) uses the standard equation,

$$Q = i[(E_i/E_p) - 1], \quad \text{Eq 1}$$

where i is the tracer infusion rate in micromoles per kilogram per hour and E_i/E_p is the ratio of isotopic enrichment of the infusate (E_i) and plasma amino acid (E_p).

The calculation of phenylalanine hydroxylation (Q_{pt}) is complicated by the fact that the tyrosine pool flux itself will influence the enrichment of tyrosine from phenylalanine degradation, and is given by the equation,

$$Q_{pt} = Q_t[E_t/E_p] \times [Q_p/(i_p + Q_p)], \quad \text{Eq 2}$$

where Q_t is the total tyrosine flux as measured directly by the primed-continuous infusion of [$^2H_2$]-tyrosine, Q_p is phenylalanine flux, E_t is plasma isotope enrichment of [$^2H_4$]-tyrosine, E_p is plasma isotope enrichment of [$^2H_5$]-phenylalanine, and i_p is the infusion rate of [$^2H_5$]-phenylalanine. The expression $Q_p/(i_p + Q_p)$ corrects for the contribution of the phenylalanine infusion to Q_{pt}.

The rate of movement of different amino acids into protein synthesis and breakdown is assumed to be in a constant ratio that reflects the amino acid composition of the proteins,[14] so that

$$Q_t = Q_p[P_t/P_p] + Q_{pt}[(i_p + Q_p)/Q_p], \quad \text{Eq 3}$$

where P_t/P_p is the molar ratio of the fluxes of tyrosine and phenylalanine arising from protein breakdown.

By rearranging equations 2 and 3, Q_{pt} can also be calculated without measuring the tyrosine flux directly:

$$Q_{pt} = [P_t/P_p] \times \frac{Q_p^2}{[(E_p/E_t) - 1] \times [i_p + Q_p]}. \quad \text{Eq 4}$$

Our calculations for group 2 (pregnant subjects) used the P_t/P_p of 0.52 established in group 1. For group 3 subjects, we used the ratio of 0.76 derived in nonpregnant subjects.[7]

For the postabsorptive steady-state phenylalanine flux,

$$Q_p = S_p + Q_{pt} = B_p, \quad \text{Eq 5}$$

where S_p represents phenylalanine loss from the free amino acid pool to protein synthesis and B_p is the rate of phenylalanine entry into the pool from protein breakdown. Whole-body turnover in grams per kilogram per day was calculated by assuming the phenylalanine content of whole-body protein to be 280.3 μmol/g.[8]

Leucine Model

Leucine flux was determined by a well-established model measuring the ^{13}C enrichment of KIC.[15] Leucine oxidation was calculated from breath $^{13}CO_2$ and plasma [^{13}C]-KIC enrichment. We used a value of 0.86 for bicarbonate recovery based on previous studies on bicarbonate recovery in pregnant women in our unit.[16,17] The leucine content of whole-body protein was assumed to be 629 μmol/g.[8]

RESULTS

Steady-state levels of all three infused stable isotopes were reached by 120 minutes. The arterialized isotopic enrichment (moles percent excess) at plateau was 2.72 ± 0.31 for [^{13}C]-KIC, 6.81 ± 0.91 for [$^2H_5$]-phenylalanine, 0.97 ± 0.70 for [$^2H_4$]-tyrosine, and 5.39 ± 1.44 for [$^2H_2$]-tyrosine for group 1, and these were not different for groups 2 and 3. The mean (across subjects) coefficient of variation (within subjects) for isotopic enrichment at plateau for [^{13}C]-KIC was 4.6% ± 2.3%, for [$^2H_5$]-phenylalanine 5.5% ± 1.6%, for [$^2H_4$]-tyrosine 7.5% ± 2.3%, and for [$^2H_2$]-tyrosine 6.3% ± 1.4%.

Table 2 shows that for group 1, leucine (KIC) flux was 136.2 ± 15.1, phenylalanine flux 41.2 ± 5.6, and tyrosine flux 25.0 ± 6.0 μmol/kg/h. Leucine oxidation was 15.0 ± 3.0 and phenylalanine hydroxylation was 3.3 ± 2.1 μmol/kg/h. Amino acid kinetics for group 2 were very similar to those for group 1, while the kinetics for group 3 (nonpregnant) showed that only tyrosine flux (42.0 ± 7.2) and phenylalanine hydroxylation (9.2 ± 4.2) were significantly affected by pregnancy ($P = 0.0009$ and .0001, respectively). If we had used our P_t/P_p ratio of 0.52 for group 3 (established in our pregnant group 1), tyrosine flux (30.0 ± 5.2) and phenylalanine hydroxylation (6.7 ± 3.1) still would have been significantly different from the values in the pregnant subjects ($P = .005$ and .04, respectively), suggesting that the differences are indeed physiological rather than due to derived ratios. Plasma tyrosine levels were 42 ± 8 and 44 ± 13 and phenylalanine levels were 52 ± 11 and 52 ± 10 (groups 2 and 3, respectively).

Table 3 shows that the mean phenylalanine/leucine flux ratio (measured by [^{13}C]-KIC enrichment) was 0.30 to 0.37 for the three groups. The mean total tyrosine/phenylalanine flux ratio (Q_t/Q_p) was 0.61 ± 0.14 for group 1, and the measured mean tyrosine/phenylalanine molar flux ratio from protein breakdown (P_t/P_p) was 0.52 ± 0.10. The mean contribution to total tyrosine flux from protein breakdown in group 1 was 86.3% ± 7.9% and from phenylalanine hydroxylation 13.7% ± 7.9%.

Table 2. Leucine, Phenylalanine, and Tyrosine Kinetics (μmol/kg/h)

Amino Acid	Group 1 (pregnant)	Group 2 (pregnant)	Group 3 (nonpregnant)
Leucine			
Flux	136.2 ± 15.1	112.5 ± 15.4	123.2 ± 18.3
Oxidation	15.0 ± 3.0	17.3 ± 4.4	18.3 ± 5.5
Phenylalanine			
Flux	41.2 ± 5.6	41.7 ± 7.7	44.9 ± 5.9
Hydroxylation	3.3 ± 2.1	1.8 ± 1.4	9.2 ± 4.2*
Tyrosine			
Flux	25.0 ± 6.0	23.5 ± 4.3	42.0 ± 7.2†

NOTE. Phenylalanine hydroxylation and tyrosine flux were calculated using the P_t/P_p ratio of 0.52 (from group 1) for group 2 and 0.76[7] for group 3.

*$P = .0009$, †$P = .0001$ *v* groups 1 and 2.

Whole-body protein metabolism rates in grams per kilogram per day by the leucine and phenylalanine methods are compared in Table 4. The phenylalanine model gave lower mean values for whole-body protein breakdown, synthesis, and oxidation compared with the leucine model (all $P < .01$). Phenylalanine oxidation was the only parameter significantly affected by pregnancy. The use of a correction factor[18] (see Discussion) gave phenylalanine-derived mean values for whole-body protein breakdown, synthesis, and oxidation as 5.17 ± 0.69, 4.23 ± 0.80, and 0.93 ± 0.33 g/kg/d for group 1.

DISCUSSION

Our findings of reduced tyrosine flux in late pregnancy are corroborated by another recent study[19] that reported almost the identical tyrosine flux data from which we derived the Q_t/Q_p of 0.63. Studies of nonpregnant subjects report a Q_t/Q_p between 0.8 and 1.0.[7,13,20] Moreover, in our pregnant subjects, the mean tyrosine/phenylalanine molar flux ratio from protein breakdown (P_t/P_p) was 0.52, and we can derive the same figure from the pregnancy data of Zimmer et al.[19] These P_t/P_p ratios for pregnancy were consistently lower than for (predominantly male) subjects in the literature (0.65 to 0.85).[7,9,20-22] The molar ratio of the contents of tyrosine/phenylalanine in animal protein has been estimated to be 0.73.[8] However, this figure represents "average" protein. One possible explanation for a lower P_t/P_p ratio may be that during pregnancy there is selective breakdown, especially in the liver, of proteins that are low in tyrosine content, producing a reduced tyrosine flux. Interestingly, although not directly related to reduced flux, plasma levels of tyrosine have also been shown to be significantly lower in pregnancy compared with the nonpregnant state, while phenylalanine was unchanged.[23-25] Although there is increased urinary loss of tyrosine in pregnancy, this was not thought to be responsible for the reduced plasma levels, since there was no consistent relation between the urinary excretion rate and the reduction in the plasma level.[23] In late pregnancy (group 1), the contribution of phenylalanine hydroxylation to total tyrosine flux (14%) was lower than the 22% (range, 25% to 19%) in the fasting state in normal nonpregnant subjects.[7,9,21,22] In our pregnant subjects, the proportion of phenylalanine hydroxylation to phenylalanine flux (8%) was similar to the 10% reported (without comment) by Zimmer et al,[19] lower than the value for our nonpregnant subjects (20%), and half the value (16% to 20%) previously reported for nonpregnant subjects.[7,9,21] The reduced phenylalanine hydroxylation in pregnancy was a greater proportional shift than the change in P_t/P_p, and indicated that this additional mechanism for alterations in tyrosine metabolism was an exception to the findings of Barazzoni et al,[22] who suggested that plasma phenylalanine levels determined the rate of phenylalanine hydroxylation. It is also clear from our data that the ratio of leucine oxidation to flux (15%) was unaffected by pregnancy, probably due to its derivation from muscle metabolism rather than liver. Evidence from studies on rats[26] suggested that tyrosine flux was elevated in the later half of pregnancy, but it was contradicted by the conclusion that liver protein breakdown was reduced. Data are lacking from large mammal studies and, indeed, an analysis of compartmentalization between mother, placenta, and fetus is required to understand the significance of altered tyrosine metabolism.

Table 3. Amino Acid Flux Ratios

Amino Acid	Group 1 (pregnant)	Group 2 (pregnant)	Group 3 (nonpregnant)
Phenylalanine:leucine total flux	0.30 ± 0.03	0.37 ± 0.07	0.37 ± 0.08
Tyrosine:phenylalanine total flux (Q_t/Q_p)	0.61 ± 0.14	0.56 ± 0.03	0.93 ± 0.09
Tyrosine:phenylalanine flux from protein breakdown (P_t/P_p)	0.52 ± 0.10	—	—

Table 4. Whole-Body Protein Metabolism From the Leucine and Phenylalanine Models (g/kg/d)

Tracer	Group 1 (pregnant)	Group 2 (pregnant)	Group 3 (nonpregnant)
Leucine			
Breakdown	5.20 ± 0.58	4.29 ± 0.59	4.70 ± 0.70
Synthesis	4.62 ± 0.49	3.63 ± 0.47	4.00 ± 0.73
Oxidation	0.58 ± 0.10	0.66 ± 0.17	0.70 ± 0.21
Phenylalanine			
Breakdown	3.53 ± 0.48	3.57 ± 0.66	3.84 ± 0.50
Synthesis	3.25 ± 0.54	3.42 ± 0.66	3.05 ± 0.47
Oxidation	0.28 ± 0.18	0.15 ± 0.12	0.79 ± 0.36*

*$P = .0009$ *v* groups 1 and 2.

Whole-body protein turnover parameters (Table 4) from the leucine model were similar to other published results in normal pregnant women.[3-5] The mean whole-body protein turnover parameters obtained by the phenylalanine model were lower than those obtained by the leucine model. The mean phenylalanine flux expressed as a percentage of the mean leucine flux (30% to 37%) was similar to the values of 0.32 to 0.35 previously obtained,[7,9,13,19] although the values in all studies were lower than the expected 45%,[8] the estimated molar ratio of phenylalanine to leucine in body protein. It is likely that the lower values obtained by the phenylalanine method are more model-related than physiological. Arterialized plasma KIC enrichment is used as a reflection of intracellular leucine enrichment in the leucine model, with the KIC to leucine ratio estimated to be 0.8.[15] There is no analogous plasma measurement for intracellular phenylalanine enrichment, so phenylalanine and [$^2H_2$]-tyrosine flux may be underestimated due to the inability to measure the true intracellular enrichment of phenylalanine and [$^2H_2$]-tyrosine, which would be lower than the level in plasma. Millward et al[18] suggested correction factors in the fasting state of 0.7 and 0.65 for phenylalanine and [$^2H_2$]-

tyrosine, respectively. When this correction was applied to our measurements, our phenylalanine flux (group 1) was 44% of the flux of leucine and whole-body breakdown and synthesis values were similar to those from the leucine model.

The debate over measurement of phenylalanine hydroxylation has not been fully resolved. Firstly, phenylalanine hydroxylation may be underestimated due to the inability to measure the true intracellular enrichment of phenylalanine or [$^2H_2$]-tyrosine, and the suggested correction factors approximately double the measured rate of hydroxylation. Secondly, it has been suggested that using L-[1-^{13}C]-phenylalanine as a tracer for in vivo studies underestimates "true" phenylalanine hydroxylation rates by only 30%, which was less than with L-[ring-$^2H_5$]-phenylalanine. [20] The measured phenylalanine flux in that study was unaffected by using a different label, but for this study, we could not use L-[1-^{13}C]-phenylalanine (requiring $^{13}CO_2$ breath collection) because we were already infusing our subjects with L-[1-^{13}C]-leucine at the same time. The use of two amino acids is important for comparison, since phenylalanine hydroxylation occurs principally in the liver[27] while leucine oxidation occurs predominantly in muscle and other peripheral tissues.[28] However, using uncorrected figures, we found that oxidation/hydroxylation as a proportion of total flux (group 1) was similar for [$^2H_5$]-phenylalanine and [^{13}C]-leucine (0.08 ± 0.06 *v* 0.11 ± 0.01, respectively). Previous studies suggested (uncorrected) ratios for both amino acids of 0.10 and 0.14 in pregnancy[19] and 0.16 to 0.22 in males.[7,9,21] Despite these uncertainties, the observed lower P_t/P_p ratio in pregnancy remains valid. Applying the suggested correction factors[13,18] would further decrease our P_t/P_p ratio from 0.52 to 0.42.

In conclusion, if the phenylalanine model is used to study whole-body protein metabolism in pregnancy and tyrosine flux is not measured directly with a continuous infusion of [$^2H_2$]-tyrosine, the lower P_t/P_p ratio should be used. The phenylalanine model in pregnancy shows that tyrosine flux derived from protein breakdown and phenylalanine hydroxylation are both reduced in pregnancy.

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