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Measurement of hepatic phenylalanine metabolism in children using the [^{13}C]-phenylalanine breath test and gas chromatography–mass spectrometry

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Abstract

The essential amino acid, phenylalanine (PA), is known to be metabolized mainly in the liver of human adults. Because the liver is still in the developmental phase, the PA-related metabolic events in infants remain unsolved. In this study, evaluations of development in hepatic PA metabolism in 37 children and 16 adults were attempted using the ^{13}C-PA breath test (PBT). The subjects were categorized into four groups according to their ages in years and months: 2 years and 0 month to 3 years and 5 months (group I; $n = 12$); 3 years and 6 months to 4 years and 11 months (group II, $n = 12$); 5 years and 0 month to 6 years and 11 months (group III, $n = 13$); and healthy adults (group IV; $n = 16$). Changes in CO_2 level of exhaled gas at various time intervals after oral administration of ^{13}C-PA were monitored using gas chromatography–mass spectrometry to derive the ^{13}C excretion rate, cumulative excretion curve and time maximum ^{13}C excretion rate (T_{MAX}). In the present investigation involving children, significant increases of maximum ^{13}C excretion rate and cumulative excretion at 120 min after administration were established in group III. Furthermore, differences in PBT were not established between groups III and IV. The index for first-pass effect, T_{MAX}, did not change with time. From the above findings, the ^{13}C excretion rate increased with time although hepatic PA metabolism in infants remained underdeveloped, and children at the age of 5–7 years manifested PA metabolism similar to that of adults.

Keywords: ^{13}C-Breath test; Phenylalanine

1. Introduction

Among the various ^{13}C-exhaled gas tests used mainly in human adults [1], ^{13}C-phenacetin [2,3], ^{13}C-aminopyrine [4,5] and ^{13}C-methacetin [6] breath tests have been employed as evaluation methods for monitoring hepatic function.

However, the essential amino acid, phenylalanine (PA), is absorbed through the neutral amino acid channel of the proximal region of the small intestine by active transport before assimilation via the hepatic portal vein. The major metabolism of PA occurs in the liver, and is converted to tyrosine before following the main pathway to be eventually degraded to fumarate and acetoacetate [7,8]. As metabolism in infants is not well developed [7–9], hypometabolism obviously reflects hepatic impairment—a finding similarly observed in adults as well [10].

In recent years, L-[1-^{13}C]-PA breath test (PBT) application has been initiated as an evaluation method for hepatic function in the clinical field [11], and reports reflecting PBT in monitoring hepatic function have been demonstrated by Lara et al. [12] and Kobayashi et al. [13]. The principle basically involves evaluation of hepatic function by measuring the concentration and excretion rate of $^{13}CO_2$, which is excreted in the metabolic process of previously administered L-[1-^{13}C]-PA (^{13}C-PA) in the liver (Fig. 1). However, PBT evaluation in infants is rare as: (i) it is practically difficult to sample exhaled gas in children; and (ii) unlike adults, normal controls in children, whose hepatic function is still in the developmental stage, are difficult to establish.

Hitherto, we have demonstrated PBT studies in children where hepatic PA metabolism is not well developed [14,15], and the immature/incomplete metabolism is further exacerbated in infants with acute hepatitis [16]. However, hepatic metabolism in children has to be elucidated for PBT to serve

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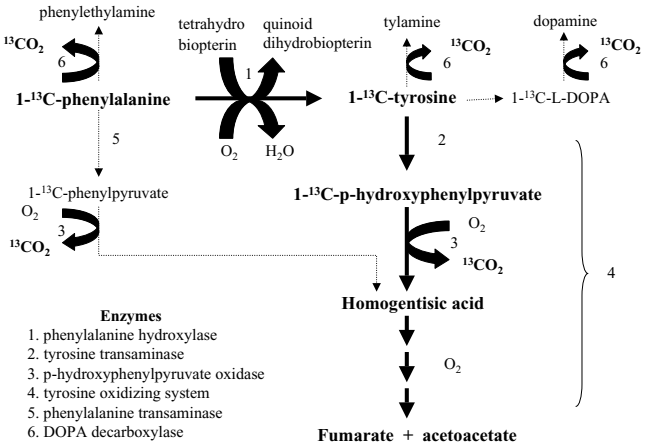

Fig. 1. Metabolic pathways of ^{13}C-phenylalanine (PA) in human liver. Two-thirds of 1-^{13}C-PA is metabolized via the main pathway (bold line), and the other side pathways are indicated as broken lines.

as an index of hepatic function. As such, PBT evaluation with age in healthy children was attempted in our present study.

2. Experimental

2.1. Subjects

Thirty-seven children of excellent physical condition born at term and without any afterbirth events or hyperphenylalaninemia [17], as well as 16 non-related healthy adults participated in the study. The subjects were categorized into four groups (Table 1) according to their ages expressed in years and months: 2 years and 0 month to 3 years and 5 months (group I, $n = 12$); 3 years and 6 months to 4 years and 11 months (group II, $n = 12$); 5 years and 0 month to 6 years and 11 months (group III, $n = 13$); and healthy adults (group IV, $n = 16$). The study was approved by the Ethical Committee of Niigata University, and subjects or their immediate next-of-kin consented to participation in the investigation.

2.2. Experimental design

^{13}C-PA (99% atom isoenrichment; Shoko, Tokyo, Japan) was administered orally at 3.5 mg/kg [3,15]. Children exceeding a body mass of 28.5 kg and the adults were treated at 100 mg per head [12,13,15]. ^{13}C-PA dissolved in 5% glucose solution was filtration-sterilized and given orally before being complemented with 50 ml distilled water. The exhaled gas was collected by blowing into an exhaled gas bag (Shiseido Fine Chemicals, Tokyo, Japan) immediately after the subject had established a stable spontaneous inhalation–exhalation cycle. Gas sampling was performed at seven time-points; before (0 min), up to 60 min (at 15 min intervals), and up to 120 min (at 30 min intervals) after PA administration. Subjects, who were tested in the morning, abstained from breakfast, and oral consumption of any kind was prohibited until completion of the investigation.

2.3. GC–MS analysis

Measurement of $^{13}CO_2$ levels in exhaled gas was conducted using gas chromatography–mass spectrometry (GC–MS; Breath MAT plus, Finnigan MAT, Bremen, Germany). The breath sample was transferred to the analyzer via a needle assembly with helium (loop volume 2 ml). The breath sample was automatically injected into the built-in gas chromatography where CO_2 was then separated from N_2, O_2 and H_2O. The conditions of GC were follows: a HayeSep D 100/120 column (10 ft × 1/8 in. SS, Alltech Associates, Deerfield, IL, USA) was used with an oven temperature of 90 °C. He was used as the carrier gas and the flow rate was 86 ml/min. The GC was connected to the mass spectrometric analyzer via a continuous flow interface which added pulses of CO_2 calibration gas under a fully computerized control system. The analyzer consisted of an electron impact ion source, a highly stable single focusing magnetic sector, and a universal triple Faraday cup collection for simultaneous measurement of masses 44 ($^{12}C^{16}O_2$), 45 ($^{13}C^{16}O_2$) and 46 ($^{12}C^{16}O^{18}O$) of CO_2. The CO_2 peak was integrated by the collectors at 88–122 s after injection of sample.

2.4. Calculation

The $^{13}CO_2/^{12}CO_2$ sample was compared with CO_2 gas (NBS no. 20; National Bureau of Standards, $^{13}CO_2/^{12}CO_2 = 0.011225$) to derive the relative concentration ($\delta^{13}C$) as

Table 1
Characteristics of 53 subjects

	Group I	Group II	Group III	Group IV
Number	12	12	13	16
Sex (male:female)	6:6	6:6	6:7	11:5
Age (mean ± S.D.)	2 years and 0 month to 3 years and 5 months (33.9 ± 6.28 months)	3 years and 6 months to 4 years and 11 months (51.9 ± 4.95 months)	5 years and 0 month to 6 years and 11 months (72.88 ± 7.82 months)	27–35 years (31.75 ± 2.57 years)
Phenylalanine (mean ± S.D., μmol/l)	72.35 ± 5.26	68.83 ± 4.72	70.55 ± 6.38	59.58 ± 4.59
Tyrosine (mean ± S.D., μmol/l)	71.44 ± 6.35	67.38 ± 5.82	69.31 ± 5.69	58.27 ± 4.13

follows:

$$\delta^{13}\mathrm{C}(‰) = \left(\frac{R_{\mathrm{sample}} - R_{\mathrm{standard}}}{R_{\mathrm{standard}}}\right) \times 10^3$$

$$R_{\mathrm{sample}} = \left(\frac{^{13}\mathrm{CO_2}}{^{12}\mathrm{CO_2}}\right)_{\mathrm{sample}}$$

$$R_{\mathrm{standard}} = \left(\frac{^{13}\mathrm{CO_2}}{^{12}\mathrm{CO_2}}\right)_{\mathrm{standard}}$$

The $^{13}CO_2$ increase (at time t in min) after ^{13}C-PA administration represented the difference from the baseline, or

$$\Delta^{13}\mathrm{C}(‰) = (\delta^{13}\mathrm{C})_t - (\delta^{13}\mathrm{C})_0 = \left(\frac{R_t - R_0}{R_{\mathrm{standard}}}\right) \times 10^3$$

The ratio of $^{13}CO_2$ per unit $^{13}CO_2$ in the next exhaled gas sample is expressed as $^{13}CO_2$ yield (% dose/mM CO_2):

$$^{13}\mathrm{CO_2}\,\%\,\mathrm{dose/mM\,CO_2} = \Delta^{13}\mathrm{CO_2} \times \left(\frac{^{13}\mathrm{CO_2}}{^{12}\mathrm{CO_2}}\right)_{\mathrm{standard}} \times \mathrm{MW} \times \frac{10}{\%\,\mathrm{excess}} \times \# \times \mathrm{dose}$$

where MW represents the molecular mass of ^{13}C-PA (i.e. 166), % excess equals ^{13}C concentration (0.99), and # indicates the number of ^{13}C per unit molecule of ^{13}C-PA (1).

By correlating the endogenous $^{13}CO_2$ production rate per unit hour in terms of body surface area at the respective time-points with $^{13}CO_2$ excretion rate (% dose/h) and integrating the plot against time, the cumulative excretion (% dose) or the metabolic rate within a fixed interval was thus derived:

$$\mathrm{surface\ area\ (m^2)} = \frac{3.2}{1000} \times M^{0.7285 - 0.0188 \log M} \times H^{0.3}$$

where M and H represent the body mass (kg) and body height (cm), respectively. Time maximum ^{13}C excretion rate (T_{MAX}), the index of first-pass effect [18,19], was derived using the measured values.

2.5. Statistical analysis

PBT-evaluated values were expressed as mean ± S.D. Differences, considered statistically significant with a risk factor of less than 5%, were compared using the Bartlet test, one-way ANOVA, Fisher's protected least significant difference and Student's t-test, accordingly.

3. Results

3.1. $^{13}CO_2$ excretion rate

After ^{13}C-PA administration, rapid increases in the ^{13}C excretion rate were observed. The maximum ^{13}C excretion rate (MAX) was established at 30 min post-administration. In children, the MAX values decreased with age, and the ^{13}C excretion rate gradually decreased 30 min after administration. In groups III and IV, differences were not significant and changes in the ^{13}C excretion rate were rather similar 30 min post-administration (Fig. 2A).

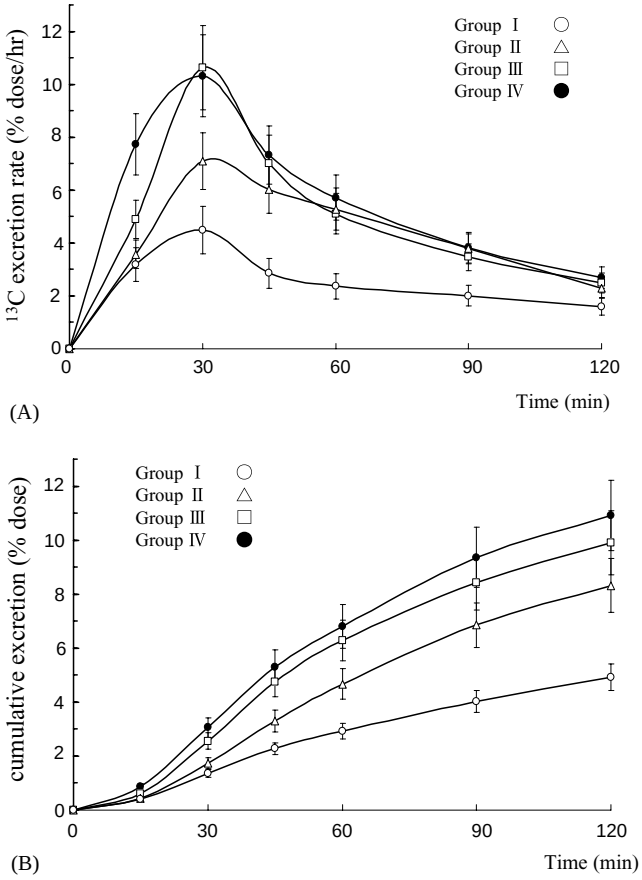

Fig. 2. ^{13}C excretion rate curves (A) and cumulative excretion curves (B) after oral administration of 3.5 mg/kg (~100 mg) L-[1-^{13}C]-phenylalanine for groups I (○, $n = 12$), II (△, $n = 12$), III (□, $n = 13$) and IV (●, $n = 16$). Data are expressed as mean ± S.D.

3.2. Cumulative excretion curves

Groups III and IV displayed a similar cumulative curve without any significant difference. In children, the cumulative curve value decreased with age in various groups; the low values were significant, especially at a time juncture 45 min after administration (Fig. 2B).

3.3. Relationship of MAX with age in children

The MAX values in groups I, II and III registered 4.50 ± 1.60, 7.10 ± 2.10 and 11.17 ± 3.97% dose/h, respectively. In children, the MAX value decreased with age in the various groups, yielding significant differences between any two groups (Fig. 3).

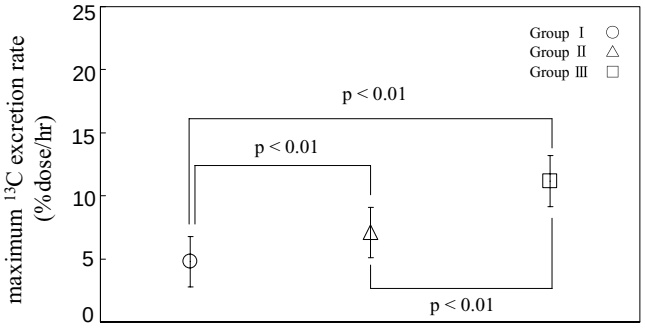

Fig. 3. Maximum ^{13}C excretion rate. Comparison between any two of the following three groups: groups I (○, $n = 12$, 4.50 ± 1.60% dose/h), II (△, $n = 12$, 7.10 ± 2.10% dose/h) and III (□, $n = 13$, 11.17 ± 3.97% dose/h). Data are expressed as mean ± S.D. Differences between any two of the three groups were significant (one-way ANOVA and Fisher's protected least significant difference).

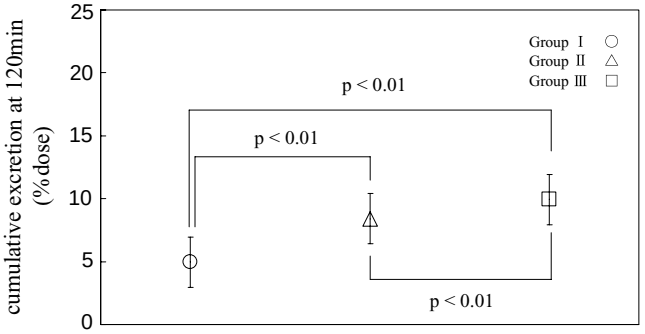

Fig. 4. Cumulative excretion at 120 min. Comparisons were performed between any two of the following three groups: groups I (○, $n = 12$, 4.92 ± 1.65% dose), II (△, $n = 12$, 8.42 ± 1.12% dose) and III (□, $n = 13$, 9.95 ± 1.82% dose). Data are expressed as mean±S.D. Differences between any two of the three groups were significant (one-way ANOVA and Fisher's protected least significant difference).

3.4. Relationship of cumulative excretion at 120 min (CE_{120}) with age in children

CE_{120} registered 4.92 ± 1.65, 8.42 ± 1.12 and 9.95 ± 1.82% doses in groups I, II and III, respectively. In children, the CE_{120} value decreased with age in the various groups, scoring significant difference between any two groups on comparison (Fig. 4).

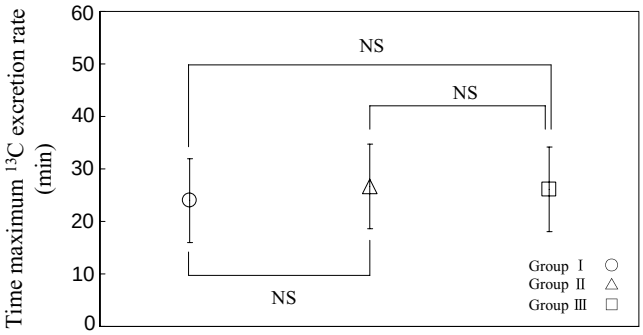

Fig. 5. Time maximum ^{13}C excretion rate. Comparisons were performed between any two of the following three groups: groups I (○, $n = 12$, 24.00 ± 7.75 min), II (△, $n = 12$, 26.67 ± 6.61 min) and III (□, $n = 13$, 26.11 ± 6.58 min). Data are expressed as mean±S.D. Differences between any two of the three groups were not significant (one-way ANOVA and Fisher's protected least significant difference).

3.5. Comparison of T_{MAX} in children

The respective T_{MAX} values registered 24.00 ± 7.75, 26.67 ± 6.61 and 26.11 ± 6.58 min in groups I, II and III, albeit differences between any two groups were not statistically significant (Fig. 5).

3.6. Comparison of PBT findings in groups III and IV

On comparison between groups III and IV, no significant differences were demonstrated in MAX, CE_{120} and T_{MAX} (Student's-test) (Table 2).

4. Discussion

Since transient hyperphenylalaninemia and hypertyrosinemia in neonates and infants have been demonstrated [20], the extensively known documented findings lead Kretchmer and Etzwiler [21] to probe into the association of enzymes in the metabolic pathways of PA and tyrosine with neonatal development, leading eventually to reduced enzymatic activation in the liver of neonates immediately after parturition [21]. In short, *p*-hydroxyphenylpyruvate oxidase activity in neonates has been found to be 30% that of adults. Although the activity of this enzyme can be enhanced with

Table 2
Comparison between groups III and IV

	Group III	Group IV	Student's *t*-test
Number	13	16	
Sex (male:female)	6:7	11:5	
Age (mean ± S.D.)	5 years and 0 month to 6 years and 11 months (72.88 ± 7.82 months)	27–35 years (31.75 ± 2.57 years)	
Maximum ^{13}C excretion rate (mean ± S.D., % dose/h)	11.17 ± 3.97	11.45 ± 3.92	NS
Cumulative excretion at 120 min (mean ± S.D., % dose)	9.95 ± 1.82	10.92 ± 1.61	NS
T_{MAX} (mean ± S.D., min)	26.11 ± 6.58	26.25 ± 8.66	NS

the addition of ascorbic acid, others remain unaffected and indicate extremely low levels of activity [21]. Moreover, tyrosine transaminase activity increases in neonatal rats from 2 h and escalates to a level higher than that of adult rats at 12 h after birth; transient two-fold increases in activity that promptly recover thereafter to the level of adult rats have been observed. Comparatively, changes in phenylalanine hydroxylase activity do not increase at a rate more rapid than that of tyrosine transaminase. Furthermore, the increases are gradual without overwhelming the activity levels of adult rats, a pattern that very much resembles that of humans [9,21].

In addition, as phenylalanine hydroxylase prevails even in the fetal liver, deficiencies of certain enzymatic systems and coenzymes of preceding pathways leading to this enzyme are the usual factors in children having PA hypometabolism [22]. As such, although dynamic changes are manifested in individual enzymes related with PA and tyrosine metabolism, current knowledge on changes in the enzymatic systems and development of metabolic pathways in living systems (in vivo) remains ambiguous.

In the present study, PBT was conducted in children. PBT involved initial oral administration of ^{13}C-PA followed by absorption in the intestines before ^{13}C-PA metabolism occurred in the liver. The metabolized $^{13}CO_2$ excreted in exhaled gas and a series of preceding events render evaluation of liver metabolic function possible. As such, elucidation of the stepwise factors influencing the PBT results, such as the first-pass effect, absorption, hepatic metabolism and CO_2 production, is warranted.

Firstly, although ^{13}C-PA is not absorbed in the stomach, it is imbibed by active transport in the proximal region of the intestines. On investigating the PBT finding by Tutekin et al. in a different approach [23], a delay of 26 min in T_{MAX} has been indicated with oral administration compared with i.v. treatment, a time delay that corresponds to the interval required for the first-pass effect and intestinal absorption to be effected. Previous findings on T_{MAX} by Braden et al. [18] and Gatti et al. [19] propose that T_{MAX} is the only single parameter in evaluating the first-pass effect by the ^{13}C-acetate breath test. Therefore, this index can thus assess the first-pass effect and absorption aspect. In our present study, comparison of T_{MAX} between any three of the four groups (I–IV) indicated no significant differences. In addition, the values measured by Tutekin et al. [23] approximate well to our present results, suggesting that the first-pass effect and absorption of ^{13}C-PA in infants and adults are similar.

Although ^{13}C-PA is metabolized mainly in the liver to *p*-hydroxyphenylpyruvic acid and homogentisic acid via tyrosine, the main pathway is responsible for their eventual conversions to $^{13}CO_2$ (Fig. 1). Although $^{13}CO_2$ is concurrently produced in the other pathways, the rates are negligible [7,8].

Moreover, Basile-Filho et al. [24] and Roberts et al. [25] have reported that absorption and metabolic enzyme activities change on loading with amino acids. As such, it is necessary to investigate plasma amino acid levels in the evaluation of hepatic PA metabolism.

Including plasma PA and tyrosine levels, the plasma amino acid levels of subjects in the present study indicated values of normal range for all age groups. As examination was conducted in subjects with excellent health in the morning without breakfast, a consistent amino acid load was therefore established.

Furthermore, Hoshi et al. [26] have demonstrated that CO_2 production in adults can be employed in infants, although correction is warranted. Moreover, as collection of exhaled gas is done by having the subject rest in a comfortable supine position without noise, the margin of error is negligible compared with CO_2 production in a mobile living system.

From the above findings, PBT results of in vivo activities of PA hydroxylase and the tyrosine oxidizing system can be tabulated. On group comparisons, PBT parameters, such as MAX and CE_{120}, manifested increases in the older children when compared with the younger infants.

As such, hepatic PA metabolism supposedly increased with age in infants. However, as significant differences were not established in any of the parameters employed in group III versus group IV comparison (Table 2), hepatic PA metabolism probably elevated gradually during infancy to eventually establish the adult level at the age of 5–7 years.

The various individual enzymes associated with PA metabolism began to elevate in nursing infancy (vide supra), and development of the systemic enzymatic activities in vivo was probably delayed as a result of deferred development of the respective enzymes. This is manifested in the case of the enzyme, PA hydroxylase, where the activity was enhanced in a very gradual manner [9]. As a result, functional development of a global enzymatic system was delayed, influencing thus lateral neuronal systems and triggering issues related with the hepatic volume. With reference to the lateral neuronal systems, ^{13}C-labeled positions of PA were changed for further investigations, and combined use of other stable isotopes such as ^{15}N might also be useful. In addition, accuracy related to hepatic function should be confirmed with imaging approaches such as ultrasonic examination and computerized tomography. In any case, with regards to development and growth aspects in infants, the age-dependent PA metabolism has to be taken into consideration when intravenous hyperalimentation and intestine-absorbed nutrient supplements are given.

5. Conclusion

The PBT approach is non-invasive, non-radioactive in nature and yields negligible after-effects. As time-related exhaled gas collection is required, diagnosis may be time-consuming. However, as reproducible real-time evaluations of in vivo functions can be established, the PBT application is potentially useful.

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