

# Wild-type phenylalanine hydroxylase activity is enhanced by tetrahydrobiopterin supplementation in vivo: an implication for therapeutic basis of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency

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## Abstract

We previously proposed a novel disease entity, tetrahydrobiopterin (BH<sub>4</sub>)-responsive phenylalanine hydroxylase (PAH) deficiency, in which administration of BH<sub>4</sub> reduced elevated levels of serum phenylalanine [J. Pediatr. 135 (1999) 375–378]. Subsequent reports indicate that the prevalence of BH<sub>4</sub>-responsive PAH deficiency is much higher than initially anticipated. Although growing attention surrounds treatment with BH<sub>4</sub>, little is known about the mechanism of BH<sub>4</sub> responsiveness. An early report indicates that BH<sub>4</sub> concentration in rat liver was 5 μM where *K<sub>m</sub>* for BH<sub>4</sub> of rat PAH was estimated to be 25 μM in an oxidation experiment using a liver slice, suggesting relative insufficiency of BH<sub>4</sub> in liver in vivo. In the present study, we developed a breath test for mice using [1-<sup>13</sup>C]phenylalanine in order to examine the BH<sub>4</sub> responsiveness of normal PAH in vivo. The reliability of the test was verified using BTBR mice and its mutant strain lacking PAH activity, *Pah*<sup>enu2</sup>. BH<sub>4</sub> supplementation significantly enhanced <sup>13</sup>CO<sub>2</sub> production in C57BL/6 mice when phenylalanine was pre-loaded. Furthermore, BH<sub>4</sub> apparently activated PAH in just 5 min. These observations suggest that submaximal PAH activity occurs at the physiological concentrations of BH<sub>4</sub> in vivo, and that PAH activity can be rapidly enhanced by supplementation with BH<sub>4</sub>. Thus, we propose a possible hypothesis that the responsiveness to BH<sub>4</sub> in patients with PAH deficiency is due to the fact that suboptimal physiological concentrations of BH<sub>4</sub> are normally present in hepatocytes and the enhancement of the residual activity may be associated with a wide range of mutations.

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## Introduction

Phenylketonuria (PKU) is caused by phenylalanine hydroxylase (PAH) deficiency. [1]. A phenylalanine (Phe)-restricted diet can ameliorate the effects of high serum Phe on cognitive function [2]. However, Phe restriction, which is the life-long recommended treatment for PKU, often fails since it presents a heavy bur-

den to patients and their families. Alternative therapeutic approaches by which to maintain optimal mental functioning in patients with PKU have been explored, including gene therapy and enzyme replacement therapy [3–6]. In 1999, we described four patients with hyperphenylalaninemia whose serum Phe levels decreased in response to 6-R-L-erythro-5,6,7-tetrahydrobiopterin (BH<sub>4</sub>) administration. These patients had mutations in the PAH gene, but none had abnormalities of BH<sub>4</sub> metabolism. This led to identification of a novel subset of patients with hyperphenylalaninemia (HPA),

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those with BH<sub>4</sub>-responsive PAH deficiency [7]. Our observation was subsequently confirmed by other groups in 2001 [8–10]. Recent studies show that the prevalence of BH<sub>4</sub>-responsive PAH deficiency is much higher than initially anticipated. More than 70% of patients with mild HPA respond to BH<sub>4</sub> [11,12]. Muntau et al. [13] showed that a reduction in blood Phe in patients was caused by activation of Phe oxidation following administration of BH<sub>4</sub> using a [1-<sup>13</sup>C]phenylalanine (<sup>13</sup>C-Phe) breath test. The PAH mutations of BH<sub>4</sub>-responsive patients are not specific to a particular region of the PAH gene [11,13,14]. A number of studies report successful results of long-term treatment with BH<sub>4</sub>, both with and without Phe-restriction [8,15–17]. More recently, studies describe favorable responses to BH<sub>4</sub> treatment among patients with classical PKU, as well as those with mild HPA [18,19].

The mechanism underlying BH<sub>4</sub> responsiveness remains unknown. Three possible mechanisms have been proposed [11]. First, a reduced affinity of BH<sub>4</sub> for the PAH enzyme ( $K_m$  mutant) may be overcome by high levels of BH<sub>4</sub>. Three-dimensional models of BH<sub>4</sub>-responsiveness suggest that some BH<sub>4</sub>-sensitive mutations mapped onto the catalytic domain of the PAH gene may be located either in the cofactor binding regions or in the regions that interact with secondary structure in the protein involved in cofactor binding [20]. Alternatively, Blau and Treftz suggest that BH<sub>4</sub>-responsiveness may be caused by BH<sub>4</sub>-mediated activation of PAH gene expression in patients with a L48S mutation in the N-terminal regulatory domain of the PAH gene [21]. Another suggestion is that BH<sub>4</sub> may function as a chemical chaperone and enhance the stability of mutated PAH, particularly with missense mutations of the C-terminal tetramerization domain [22]. To date, experimental evidence to support these mechanisms is lacking and it is difficult to explain why such a broad range of mutations respond to BH<sub>4</sub>.

Earlier kinetic studies of PAH may provide a clue. The  $K_m$  of purified PAH for BH<sub>4</sub> was estimated to be 2 μM in vitro [23], and the concentration of BH<sub>4</sub> in the liver was 5 μM [24–26], thus, it was initially thought that the level of hepatic BH<sub>4</sub> was sufficient for full PAH activity. However, when a liver slice method was used to determine the apparent  $K_m$  of rat PAH for BH<sub>4</sub> in vivo, it was found to be 25 μM [27], suggesting that hepatic PAH may not be fully active in vivo due to suboptimal physiological concentrations of BH<sub>4</sub> in hepatocytes. Thus, there may be room for increased hepatic PAH activity with BH<sub>4</sub> supplementation. In this paper, we demonstrate enhancement of wild-type PAH activity by BH<sub>4</sub> in vivo using a <sup>13</sup>C-Phe breath test in mice, which was first developed to assess Phe oxidation in PKU patients in vivo [28]. Based on the results of this study, we propose a novel mechanism of BH<sub>4</sub> responsiveness.

## Materials and methods

### Mice

Female C57BL/6 mice (9 weeks of age), weighing 20–21 g, were purchased from Japan SLC (Hamamatsu, Japan) and subjected to breath testing. A PKU model mouse, BTBR-*Pah*<sup>enu2</sup> [29,30], and its wild type strain mouse, BTBR, were generously donated by Dr. Alexandra Shedlovsky in McArdle Laboratory for Cancer Research, University of Wisconsin, and maintained as described in a previous report [5]. Female BTBR-*Pah*<sup>enu2</sup> and BTBR mice (15 weeks old), weighing 26–28 g, were used for breath testing.

### [1-<sup>13</sup>C]Phenylalanine breath testing in mice

[1-<sup>13</sup>C]L-Phenylalanine (<sup>13</sup>C-Phe) with >99% purity was purchased from Cambridge Isotope Laboratories (Andover, MA), and L-phenylalanine (Phe) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Tetrahydrobiopterin (BH<sub>4</sub>) was generously donated by Daiichi Suntory Pharma (Tokyo, Japan). <sup>13</sup>C-Phe, Phe, and BH<sub>4</sub> were dissolved in saline at concentrations of 20, 50, and 5 mg/ml, respectively. The solutions were sterilized by passage through a 0.22-μm filter, Millex-GV (Millipore, Bedford, MA), immediately prior to intraperitoneal injection (i.p.). Each mouse was kept in a sealable plastic box containing 350 or 700 ml of air, which was sampled at 5 and 60 min, respectively. A total volume of 120 ml of air was collected from the plastic box using a glass syringe, after which it was transferred to a sampling bag for UBiT-IR300 (Otsuka Electronics, Osaka, Japan) and subjected to Δ<sup>13</sup>CO<sub>2</sub> analysis. Differences in the <sup>13</sup>CO<sub>2</sub> concentrations (Δ<sup>13</sup>CO<sub>2</sub>) of the reference and test samples were measured using an infrared spectrophotometer, UBiT-IR300, which was originally developed for the detection of *Helicobacter pylori* in stomach using [<sup>13</sup>C]urea ([<sup>13</sup>C]urea breath test) [31]. Differences of the means were statistically analyzed using the *t* test using SPSS software version 11.0J (SPSS Japan, Tokyo, Japan).

## Results

[1-<sup>13</sup>C]Phenylalanine is converted to [1-<sup>13</sup>C]tyrosine by PAH as shown in Fig. 1A. [1-<sup>13</sup>C]Tyrosine is then broken down to yield homogentidinic acid and <sup>13</sup>CO<sub>2</sub> by two enzymatic reactions. To test whether the in vivo activity of mouse PAH can be evaluated by measuring the amount of <sup>13</sup>CO<sub>2</sub> in breath samples, we examined mice of *Pah*<sup>enu2</sup> strain, a mutant strain of the BTBR mouse that lacks PAH activity [30]. Breath samples were collected from BTBR mice and *Pah*<sup>enu2</sup> mice by placing them into a plastic box containing 350 ml of air 5–10,

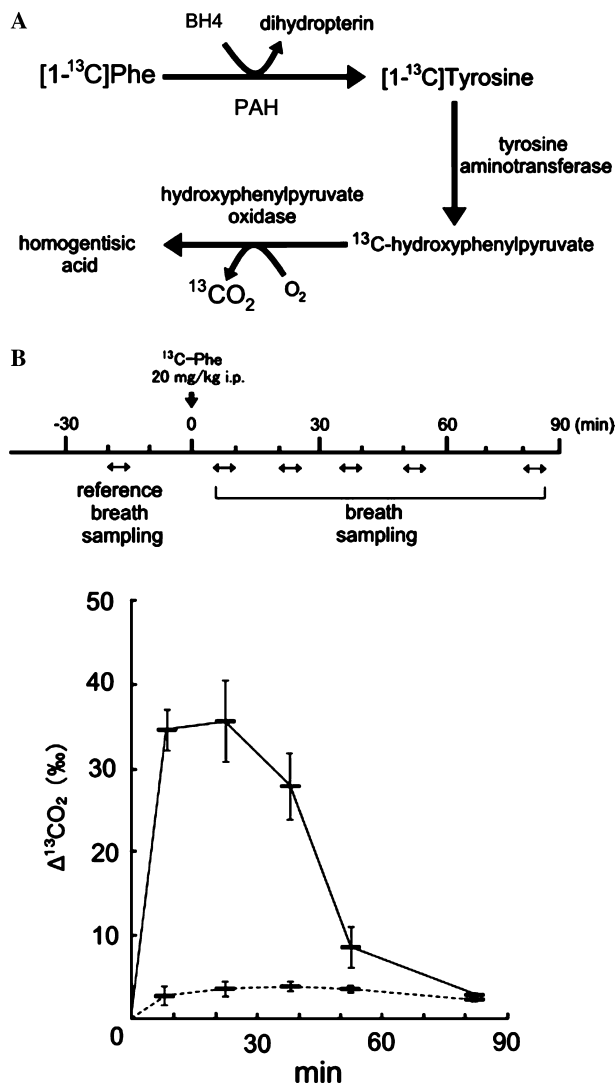


Fig. 1. (A) Pathway of [1-<sup>13</sup>C]-Phe oxidation and <sup>13</sup>CO<sub>2</sub> production. The carboxyl carbon in <sup>13</sup>C-Phe is converted to <sup>13</sup>CO<sub>2</sub> by three reactions. (B) Time course of <sup>13</sup>CO<sub>2</sub> production in BTBR and *Pah*<sup>enu2</sup> mice. Breath samples taken over 5 min were collected in a 350-ml sealable plastic box 5–10, 20–25, 35–40, 50–55, and 80–85 min after injection of <sup>13</sup>C-Phe (20 mg/kg). Reference breath samples were collected before injection. The solid and broken lines indicate the time course of  $\Delta^{13}\text{CO}_2$  in BTBR and *Pah*<sup>enu2</sup> mice, respectively. Mean and SD values of  $\Delta^{13}\text{CO}_2$  are indicated by the horizontal and vertical lines, respectively ( $n = 3$ ).

20–25, 35–40, 50–55, and 80–85 min after injection of <sup>13</sup>C-Phe (Fig. 1B). Preliminary breath testing was performed using three different doses, 100, 20, and 10 mg/kg of <sup>13</sup>C-Phe, from which it was determined that 20 mg/kg was the most appropriate (data not shown). In BTBR mice, <sup>13</sup>CO<sub>2</sub> production peaked ( $33.8 \pm 2.3\%$ ) 10–25 min after injection of <sup>13</sup>C-Phe, after which it fell to less than one-fifth of its peak value within 60 min. In contrast,  $\Delta^{13}\text{CO}_2$  did not exceed 4‰ at any point during the test in *Pah*<sup>enu2</sup> mice.

The breath test protocol in Fig. 1B requires six samplings of the mouse breath. To make the method more

easier we modified the sampling protocol. We collected breath samples over a longer period of time in a large plastic box containing 700 ml of air. Fig. 2A shows CO<sub>2</sub> concentrations within the box at various time points when C57BL/6 mice were subjected to breath testing. CO<sub>2</sub> levels in the box reached  $6.7 \pm 0.6\%$  at 60 min, and the mice became less active at 75 min, probably due to high CO<sub>2</sub> concentrations in the box. Based on the observations shown in Figs. 1B and 2A, we decided it would be best to collect breath samples over a period of 60 min for <sup>13</sup>CO<sub>2</sub> analysis (Fig. 2B). The BTBR and its mutant, *Pah*<sup>enu2</sup> mice were then re-exam-

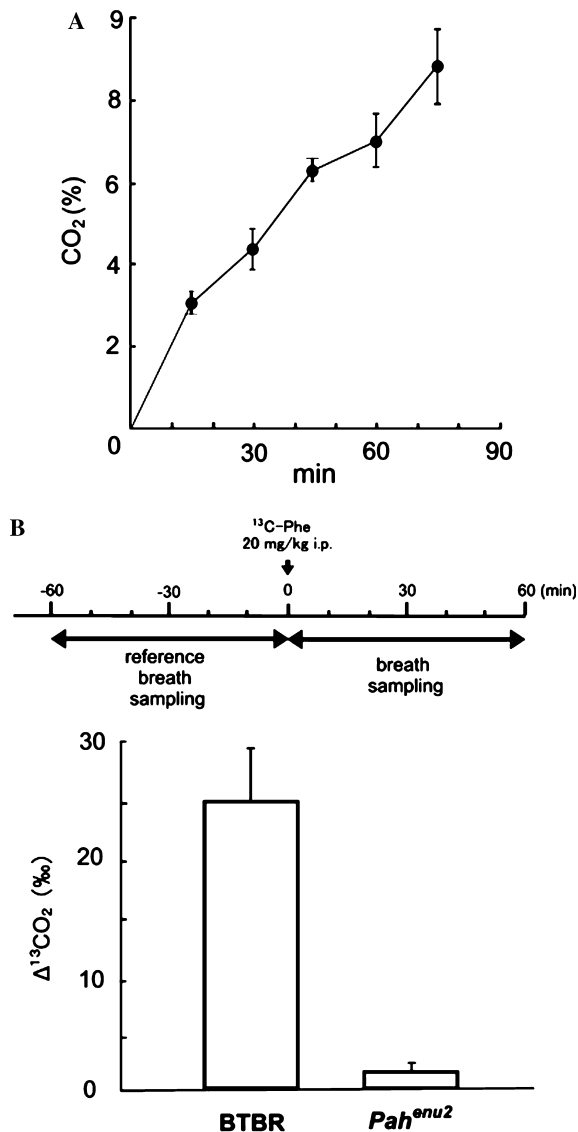


Fig. 2. Sixty minutes of <sup>13</sup>C-Phe breath test. (A) The concentration of CO<sub>2</sub> produced by C57BL/6 mice was measured. Three 9-week-old female mice were used to measure CO<sub>2</sub> concentrations at various time points. The vertical bars indicate the SD. (B) The <sup>13</sup>CO<sub>2</sub> production of BTBR mice ( $n = 6$ ) and *Pah*<sup>enu2</sup> mice ( $n = 6$ ) was measured over the course of 60 min following intraperitoneal injection of <sup>13</sup>C-Phe. The vertical bars indicate the SD.

ined using this 60-min protocol. The mean  $\Delta^{13}\text{CO}_2$  values in BTBR ( $n = 6$ ) and *Pah*<sup>enu2</sup> ( $n = 6$ ) mice were  $25.3 \pm 4.2$  and  $1.7 \pm 0.62\%$ , respectively. This breath-pooling method enables us to evaluate in vivo PAH activity more easily by sampling breath only twice.

To evaluate the effect of BH<sub>4</sub> on the enzymatic activity of normal PAH in vivo, we administered 50 mg/kg of

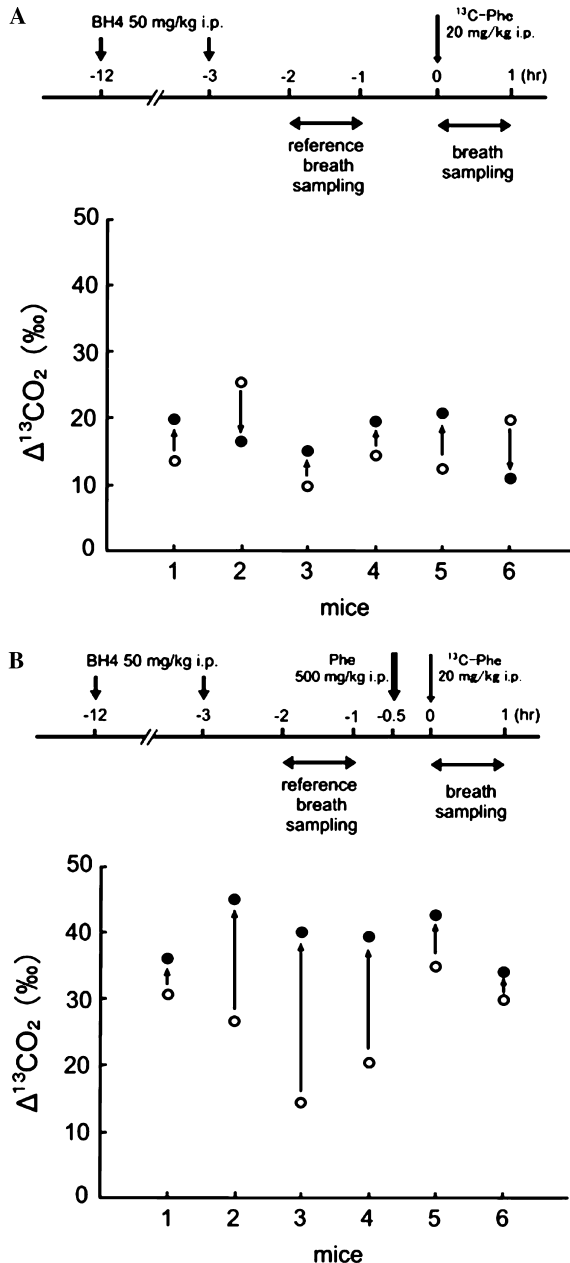


Fig. 3. (A) Effect of BH<sub>4</sub> on PAH activity in vivo without Phe pre-loading. The  $\Delta^{13}\text{CO}_2$  of C57BL/6 mice (Nos. 1–6) was measured using a 60-min sampling method with (closed circles), or without (open circles), BH<sub>4</sub> pre-loading. BH<sub>4</sub> (50 mg/kg) was twice injected (i.p.) 12 and 3 h prior to the onset of breath sampling. (B) The effect of BH<sub>4</sub> on PAH activity in vivo with Phe pre-loading. Phe (500 mg/kg) was injected 30 min prior to <sup>13</sup>C-Phe injection (i.p.). All other experimental conditions were the same as those described in (A).

BH<sub>4</sub> twice by intraperitoneal (i.p.) injection to C57BL/6 mice, 12 and 3 h prior to the onset of breath sampling (Fig. 3A). Breath samples were then collected over the course of 60 min and subjected to <sup>13</sup>CO<sub>2</sub> analysis. The mean  $\pm$  SD  $\Delta^{13}\text{CO}_2$  value in the BH<sub>4</sub>-treated group ( $n = 6$ ) was  $16.6 \pm 2.7\%$ , while that of non-treated group ( $n = 6$ ) was  $15.1 \pm 1.8\%$ , yielding no significant difference between the two groups ( $p = 0.61$ ). We then administered 500 mg/kg Phe 30 min prior to the start of breath sampling (Fig. 3B). The group of mice that received BH<sub>4</sub> injections had a significantly greater ( $p < 0.01$ ) mean  $\Delta^{13}\text{CO}_2$  value ( $39.8 \pm 8.2\%$ ) than the group that did not ( $26.1 \pm 7.3\%$ ). On average, BH<sub>4</sub> administration caused a 1.7-fold increase in <sup>13</sup>CO<sub>2</sub> production. The  $\Delta^{13}\text{CO}_2$  of mice not administered BH<sub>4</sub> in Fig. 2B was significantly ( $p < 0.05$ ) greater than that of mice not administered BH<sub>4</sub> in Fig. 2A, indicating enhancement of PAH activity by Phe pre-loading in vivo. The enhancing effect of BH<sub>4</sub> was not observed in *Pah*<sup>enu2</sup> mice (data not shown).

To examine the latency of the BH<sub>4</sub> effect, we injected BH<sub>4</sub> at the onset of breath sampling. Based on the time course shown in Fig. 1B, we collected breath samples 10–15 min after <sup>13</sup>C-Phe injections. The mice were ex-

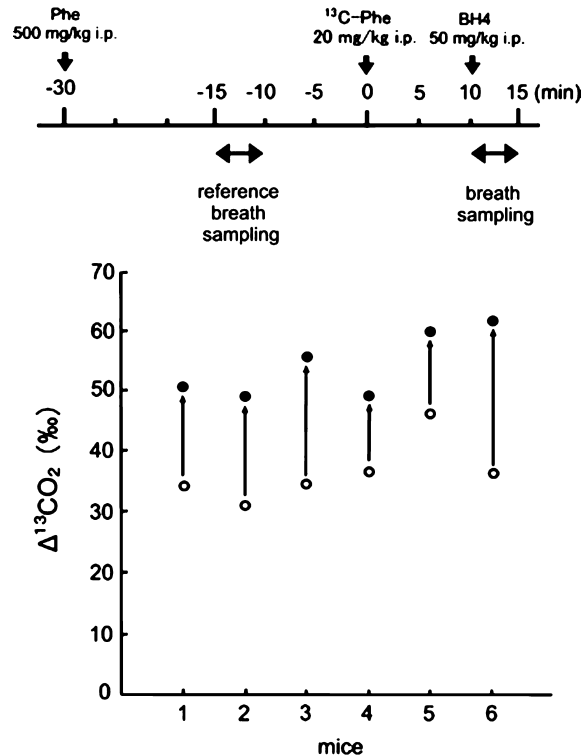


Fig. 4. Effect of BH<sub>4</sub> on PAH activity in the first 5 min. BH<sub>4</sub> (50 mg/kg) was injected immediately prior to the onset of 5-min breath sampling, in which samples were collected in a 350 ml plastic box. Unlabelled Phe (500 mg/kg) and <sup>13</sup>C-Phe (20 mg/kg) were administered 30 and 10 min prior to the onset of breath sampling, respectively.  $\Delta^{13}\text{CO}_2$  was measured in mice that received (closed circles,  $n = 6$ ), or did not receive (open circles,  $n = 6$ ), BH<sub>4</sub> injections.

pected to produce high level of  $^{13}\text{CO}_2$  during this period. The mean  $\pm$  SD values for mice that did and did not receive  $\text{BH}_4$  injections were  $54.4 \pm 8.7\%$  and  $36.2 \pm 9.3\%$ , respectively (Fig. 4), indicating that  $\text{BH}_4$  significantly ( $p < 0.001$ ) enhanced PAH activity in the first 5 min.

## Discussion

We developed a  $^{13}\text{C}$ -Phe breath test for mice in order to study the effect of  $\text{BH}_4$  on PAH activity in vivo. The reliability of the test was verified by examining PKU model mice, *Pah*<sup>enu2</sup>. The PAH activity of normal mice increased 1.7-fold following  $\text{BH}_4$  supplementation when a pre-loading dose of Phe was given. This result is in agreement with a previous report, in which a rat liver slice method was used to demonstrate that administration of  $\text{BH}_4$  causes a 1.6-fold increase in the conversion of Phe to tyrosine [27]. The concentration of  $\text{BH}_4$  in the liver was reported to be  $5\ \mu\text{M}$  [24–26]. The apparent  $K_m$  of rat PAH for  $\text{BH}_4$  was reported to be around  $25\ \mu\text{M}$  in vivo, based on results obtained using a liver slice method [27]. This suggests that normal PAH is not fully active in vivo due to a suboptimal concentration of  $\text{BH}_4$  in the liver and that its activity is enhanced by tetrahydrobiopterin supplementation. There is 97 and 92% amino acid sequence homology between mouse PAH and rat and human PAH, respectively. Moreover, purified human PAH has a similar  $K_m$  for  $\text{BH}_4$  as rat PAH [32,33], indicating that there are both structural and kinetic similarities between mouse, rat, and human PAH. Therefore, enhancement of PAH activity by  $\text{BH}_4$  supplementation is likely to be observed not only in mice, but also in rats and humans. This may explain the mechanism of  $\text{BH}_4$  responsiveness in patients with  $\text{BH}_4$ -responsive PAH deficiency.

Our hypothesis may explain why a range of PAH mutations demonstrate responsiveness to  $\text{BH}_4$ , and why there is such a high prevalence of patients with  $\text{BH}_4$ -responsive PAH deficiency. PAH mutants with amino acid substitutions in their cofactor-binding regions are likely to have elevated  $K_m$  values for  $\text{BH}_4$ , for which pharmacological doses of  $\text{BH}_4$  might be expected to restore PAH activity. However, it is unlikely that all  $\text{BH}_4$ -responsive mutations are  $K_m$  variants for  $\text{BH}_4$  because “ $\text{BH}_4$ -responsive mutations” have been dispersed over various regions of PAH enzyme, including mutations far from the cofactor-binding site. There are other vitamin responsive diseases requiring vitamin cofactor supplementation. Usually, a very limited number of mutations respond to cofactor administration. For example, in pyridoxine-responsive homocysteinuria, patients from various ethnic groups all share the same I278T missense mutation [13]. Therefore, the  $\text{BH}_4$ -responsiveness in hyperphenylalaninemic patients is unlikely to be caused by a subset of unique mutations that

alter the enzyme structure to gain specific function. Rather, enhancement of in vivo PAH activity by  $\text{BH}_4$  appears to be due to an inherent physiological characteristic of the enzyme. In this context, PKU patients who have null mutations in both PAH alleles are likely to be unresponsive to  $\text{BH}_4$ . Recent observations indicate that patients with classical PKU, as well as patients with mild HPA, responded favorably to  $\text{BH}_4$  supplementation [18,34]. Those patients may have very low but not negligible level of residual PAH activity, which is enhanced by  $\text{BH}_4$ .

In normal mice, pre-administration of Phe was required for augmentation of PAH activity by  $\text{BH}_4$ . Initially, we performed the breath test after overnight fasting based on the human protocol for  $^{13}\text{C}$ -Phe breath testing [13,28]. However,  $^{13}\text{CO}_2$  production was observed to decrease in all the mice tested (data not shown). When the mice were not fasted overnight, some were observed to respond to  $\text{BH}_4$ , as shown in Fig. 2A. The observation prompted us to pre-load the mice with Phe, the results of which are shown in Fig. 2B. In patients with untreated hyperphenylalaninemia, Phe pre-loading is not required to demonstrate  $\text{BH}_4$  responsiveness since the Phe concentration is already sufficiently high. Although all mice tested here shared the same genetic background, each mouse showed various response to  $\text{BH}_4$  without Phe pre-loading (Fig. 3A), which may explain why patients with the same genotype do not always respond to  $\text{BH}_4$  in the same manner [10]. Our data indicate that in vivo PAH activity is difficult to evaluate by breath testing when the hepatic Phe concentration is low. Pre-loading with Phe may increase the reliability and accuracy of  $^{13}\text{C}$ -Phe breath testing, particularly when evaluating patients on a Phe-restricted diet or heterozygous carriers.

It is not known why pre-loading with Phe was required for sufficient oxidation of  $^{13}\text{C}$ -Phe. Tourian [35] observed a change in the oligomeric composition of PAH following pre-incubation of PAH with Phe, causing a shift from dimer to tetramer formation and activation of PAH activity in vitro. Tipper and Kaufman [36] reported increased phosphorylation of rat PAH in the presence of Phe, concomitant with activation of PAH activity in vivo. In light of this evidence suggesting that phenylalanine can activate PAH, there might be a second activating site for Phe, distinct from the catalytic site of PAH [35]. Pre-loading with Phe may increase  $^{13}\text{CO}_2$  production by changing the phosphorylation status and/or oligomeric composition of PAH. In this study we demonstrated PAH activity can be enhanced in vivo by administration of its cofactor  $\text{BH}_4$  and its substrate Phe, which may suggest the pivotal roles of  $\text{BH}_4$  and Phe as physiological regulators of PAH activity.

The apparent activation of PAH by  $\text{BH}_4$  was observed within 5 min after administration of  $\text{BH}_4$  in the present study. In contrast, serum Phe levels do not fall

until several hours after administration of BH<sub>4</sub> in patients with BH<sub>4</sub>-responsive PAH deficiency [7]. This is probably because most mutant PAH enzymes have reduced  $V_{\max}$  values and require longer to metabolize accumulated Phe within hepatocytes and body fluids. It is also possible that altered enzyme stability or PAH gene expression resulting from BH<sub>4</sub> supplementation might increase PAH activity. However, these changes are unlikely to occur within 5 min of BH<sub>4</sub> administration and may take place in a later phase of the response. Our hypothesis remains open for experimental evidence and it is also possible that multiple mechanisms operate in BH<sub>4</sub> responsiveness. Further study is necessary to clarify the mechanism of BH<sub>4</sub> responsiveness and to determine indications for BH<sub>4</sub> therapy.

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### References

- [1] C. Scriver, S. Kaufman, Hyperphenylalaninemia: phenylalanine hydroxylase deficiency, in: C. Scriver, A. Baudet, D. Valle, W. Sly (Eds.), *The Metabolic and Molecular Bases of Inherited Diseases*, McGraw-Hill, New York, 2001, pp. 1667–1724.
- [2] H. Bickel, J. Gerrard, E.M. Hickmans, Influence of phenylalanine intake on phenylketonuria, *Lancet* 265 (1953) 812–813.
- [3] Z. Ding, C.O. Harding, B. Thony, State-of-the-art 2003 on PKU gene therapy, *Mol. Genet. Metab.* 81 (2004) 3–8.
- [4] A. Gamez, L. Wang, M. Straub, M.G. Patch, R.C. Stevens, Toward PKU enzyme replacement therapy: PEGylation with activity retention for three forms of recombinant phenylalanine hydroxylase, *Mol. Ther.* 9 (2004) 124–129.
- [5] Y. Nagasaki, Y. Matsubara, H. Takano, K. Fujii, M. Senoo, J. Akanuma, K. Takahashi, S. Kure, M. Hara, Y. Kanegae, I. Saito, K. Narisawa, Reversal of hypopigmentation in phenylketonuria mice by adenovirus-mediated gene transfer, *Pediatr. Res.* 45 (1999) 465–473.
- [6] S. Mochizuki, H. Mizukami, T. Ogura, S. Kure, A. Ichinohe, K. Kojima, Y. Matsubara, E. Kobayahi, T. Okada, A. Hoshika, K. Ozawa, A. Kume, Long-term correction of hyperphenylalaninemia by AAV-mediated gene transfer leads to behavioral recovery in phenylketonuria mice, *Gene Ther.* 11 (2004) 1081–1086.
- [7] S. Kure, D.C. Hou, T. Ohura, H. Iwamoto, S. Suzuki, N. Sugiyama, O. Sakamoto, K. Fujii, Y. Matsubara, K. Narisawa, Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, *J. Pediatr.* 135 (1999) 375–378.
- [8] F.K. Trefz, C. Aulela-Scholz, N. Blau, Successful treatment of phenylketonuria with tetrahydrobiopterin, *Eur. J. Pediatr.* 160 (2001) 315.
- [9] L.J. Spaapen, J.A. Bakker, C. Velter, W. Loots, M.E. Rubio-Gozalbo, P.P. Forget, L. Dorland, T.J. De Koning, B.T. Poll-The, H.K. Ploos van Amstel, J. Bekhof, N. Blau, M. Duran, M.E. Rubio-Gonzalbo, Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency in Dutch neonates, *J. Inherit. Metab. Dis.* 24 (2001) 352–358.
- [10] M. Lindner, D. Haas, E. Mayatepek, J. Zschocke, P. Burgard, Tetrahydrobiopterin responsiveness in phenylketonuria differs between patients with the same genotype, *Mol. Genet. Metab.* 73 (2001) 104–106.
- [11] L.J. Spaapen, M.E. Rubio-Gozalbo, Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, state of the art, *Mol. Genet. Metab.* 78 (2003) 93–99.
- [12] C. Bernegger, N. Blau, High frequency of tetrahydrobiopterin-responsiveness among hyperphenylalaninemias: a study of 1,919 patients observed from 1988 to 2002, *Mol. Genet. Metab.* 77 (2002) 304–313.
- [13] A.C. Muntau, W. Roschinger, M. Habich, H. Demmelmair, B. Hoffmann, C.P. Sommerhoff, A.A. Roscher, Tetrahydrobiopterin as an alternative treatment for mild phenylketonuria, *N. Engl. J. Med.* 347 (2002) 2122–2132.
- [14] C.R. Scriver, P.J. Waters, C. Sarkissian, S. Ryan, L. Prevost, D. Cote, J. Novak, S. Teebi, P.M. Nowacki, PAHdb: a locus-specific knowledgebase, *Hum. Mutat.* 15 (2000) 99–104.
- [15] R. Steinfeld, A. Kohlschutter, J. Zschocke, M. Lindner, K. Ullrich, Z. Lukacs, Tetrahydrobiopterin monotherapy for phenylketonuria patients with common mild mutations, *Eur. J. Pediatr.* 161 (2002) 403–405.
- [16] R. Koch, F. Guttler, N. Blau, Mental illness in mild PKU responds to biopterin, *Mol. Genet. Metab.* 75 (2002) 284–286.
- [17] H. Shintaku, S. Kure, T. Ohura, Y. Okano, M. Ohwada, N. Sugiyama, N. Sakura, I. Yoshida, M. Yoshino, Y. Matsubara, K. Suzuki, K. Aoki, T. Kitagawa, Long-term treatment and diagnosis of tetrahydrobiopterin-responsive hyperphenylalaninemia with a mutant phenylalanine hydroxylase gene, *Pediatr. Res.* 55 (2004) 425–430.
- [18] R. Matalon, R. Koch, K. Michals-Matalon, K. Moseley, S. Surendran, S. Tying, H. Erlandsen, A. Gamez, R.C. Stevens, A. Romstad, L.B. Moller, F. Guttler, Biopterin responsive phenylalanine hydroxylase deficiency, *Genet. Med.* 6 (2004) 27–32.
- [19] J. Weglage, M. Grenzebach, A. von Teeffelen-Heithoff, T. Marquardt, R. Feldmann, J. Denecke, D. Godde, H.G. Koch, Tetrahydrobiopterin responsiveness in a large series of phenylketonuria patients, *J. Inherit. Metab. Dis.* 25 (2002) 321–322.
- [20] H. Erlandsen, R.C. Stevens, A structural hypothesis for BH<sub>4</sub> responsiveness in patients with mild forms of hyperphenylalaninemia and phenylketonuria, *J. Inherit. Metab. Dis.* 24 (2001) 213–230.
- [21] N. Blau, F.K. Trefz, Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency: possible regulation of gene expression in a patient with the homozygous L48S mutation, *Mol. Genet. Metab.* 75 (2002) 186–187.
- [22] C.R. Scriver, P.J. Waters, Monogenic traits are not simple: lessons from phenylketonuria, *Trends Genet.* 15 (1999) 267–272.
- [23] S. Kaufman, The phenylalanine hydroxylating system from mammalian liver, *Adv. Enzymol. Relat. Areas Mol. Biol.* 35 (1971) 245–319.
- [24] T. Fukushima, J.C. Nixon, Analysis of reduced forms of biopterin in biological tissues and fluids, *Anal. Biochem.* 102 (1980) 176–188.
- [25] D.S. Duch, S.W. Bowers, J.H. Woolf, C.A. Nichol, Biopterin cofactor biosynthesis: GTP cyclohydrolase, neopterin and biopterin in tissues and body fluids of mammalian species, *Life Sci.* 35 (1984) 1895–1901.
- [26] C.O. Harding, M. Neff, K. Wild, K. Jones, L. Elzaouk, B. Thony, S. Milstien, The fate of intravenously administered tetrahydrobiopterin and its implications for heterologous gene therapy of phenylketonuria, *Mol. Genet. Metab.* 81 (2004) 52–57.
- [27] S. Milstien, S. Kaufman, Studies on the phenylalanine hydroxylase system in liver slices, *J. Biol. Chem.* 250 (1975) 4777–4781.
- [28] E.P. Treacy, J.J. Delente, G. Elkas, K. Carter, M. Lambert, P.J. Waters, C.R. Scriver, Analysis of phenylalanine hydroxylase genotypes and hyperphenylalaninemia phenotypes using L-

- [1-<sup>13</sup>C]phenylalanine oxidation rates in vivo: a pilot study, *Pediatr. Res.* 42 (1997) 430–435.
- [29] A. Shedlovsky, J.D. McDonald, D. Symula, W.F. Dove, Mouse models of human phenylketonuria, *Genetics* 134 (1993) 1205–1210.
- [30] J.D. McDonald, M. Andriolo, F. Cali, M. Mirisola, S. Puglisi-Allegra, V. Romano, C.N. Sarkissian, C.B. Smith, The phenylketonuria mouse model: a meeting review, *Mol. Genet. Metab.* 76 (2002) 256–261.
- [31] S. Kato, K. Ozawa, M. Konno, H. Tajiri, N. Yoshimura, T. Shimizu, T. Fujisawa, D. Abukawa, T. Minoura, K. Inuma, Diagnostic accuracy of the <sup>13</sup>C-urea breath test for childhood *Helicobacter pylori* infection: a multicenter Japanese study, *Am. J. Gastroenterol.* 97 (2002) 1668–1673.
- [32] S.C. Kwok, F.D. Ledley, A.G. DiLella, K.J. Robson, S.L. Woo, Nucleotide sequence of a full-length complementary DNA clone and amino acid sequence of human phenylalanine hydroxylase, *Biochemistry* 24 (1985) 556–561.
- [33] F.D. Ledley, H.E. Grenett, B.S. Dunbar, S.L. Woo, Mouse phenylalanine hydroxylase. Homology and divergence from human phenylalanine hydroxylase, *Biochem. J.* 267 (1990) 399–405.
- [34] J. Weglage, M. Grenzebach, A.V. Teeffelen-Heithoff, T. Marquardt, R. Feldmann, J. Denecke, D. Godde, H.G. Koch, Tetrahydrobiopterin responsiveness in a large series of phenylketonuria patients, *J. Inherit. Metab. Dis.* 25 (2002) 321–322.
- [35] A. Tourian, Activation of phenylalanine hydroxylase by phenylalanine, *Biochim. Biophys. Acta* 242 (1971) 345–354.
- [36] J. Tipper, S. Kaufman, Phenylalanine-induced phosphorylation and activation of rat hepatic phenylalanine hydroxylase in vivo, *J. Biol. Chem.* 267 (1992) 889–896.