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 4)-responsive phenylalanine hydroxylase (PAH) deficiency, in which administration of BH 4 reduced elevated levels of serum phenylalanine [J. Pediatr. 135 (1999) 375–378]. Subsequent reports indicate that the prevalence of BH 4-responsive PAH deficiency is much higher than initially anticipated. Although growing attention surrounds treatment with BH 4, little is known about the mechanism of BH 4 responsiveness. An early report indicates that BH4 concentration in rat liver was 5 μM where K m for BH4 of rat PAH was estimated to be 25 μM in an oxidation experiment using a liver slice, suggesting relative insufficiency of BH 4 in liver in vivo. In the present study, we developed a breath test for mice using [1-13C]phenylalanine in order to examine the BH 4 responsiveness of normal PAH in vivo. The reliability of the test was verified using BTBR mice and its mutant strain lacking PAH activity, Pahenu2.B H 4 supplementation significantly enhanced 13CO2 production in C57BL/6 mice when phenylalanine was pre-loaded. Furthermore, BH 4 apparently activated PAH in just 5 min. These observations suggest that submaximal PAH activity occurs at the physiological concentrations of BH 4 in vivo, and that PAH activity can be rapidly enhanced by supplementation with BH 4. Thus, we propose a possible hypothesis that the responsiveness to BH 4 in patients with PAH deficiency is due to the fact that suboptimal physiological concentrations of BH 4 are normally present in hepatocytes and the enhancement of the residual activity may be associated with a wide range of mutations.

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 burden 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-RR-LL-erythro-5,6,7-tetrahydrobiopterin (BH 4) administration. These patients had mutations in the PAH gene, but none had abnormalities of BH 4 metabolism. This led to identification of a novel subset of patients with hyperphenylalaninemia (HPA),
those with BH4-responsive PAH deficiency [7]. Our observation was subsequently confirmed by other groups in 2001 [8–10]. Recent studies show that the prevalence of BH4-responsive PAH deficiency is much higher than initially anticipated. More than 70% of patients with mild HPA respond to BH4 [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 BH4 using a [1-13C]phenylalanine (13C-Phe) breath test. The PAH mutations of BH4-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 BH4, both with and without Phe-restriction [8,15–17]. More recently, studies describe favorable responses to BH4 treatment among patients with classical PKU, as well as those with mild HPA [18,19].

The mechanism underlying BH4 responsiveness remains unknown. Three possible mechanisms have been proposed [11]. First, a reduced affinity of BH4 for the PAH enzyme (Km mutant) may be overcome by high levels of BH4. Three-dimensional models of BH4-responsiveness suggest that some BH4-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 BH4-responsiveness may be caused by BH4-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 BH4 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 BH4.

Earlier kinetic studies of PAH may provide a clue. The K_m of purified PAH for BH4 was estimated to be 2 μM in vitro [23], and the concentration of BH4 in the liver was 5 μM [24–26], thus, it was initially thought that the level of hepatic BH4 was sufficient for full PAH activity. However, when a liver slice method was used to determine the apparent K_m of rat PAH for BH4 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 BH4 in hepatocytes. Thus, there may be room for increased hepatic PAH activity with BH4 supplementation. In this paper, we demonstrate enhancement of wild-type PAH activity by BH4 in vivo using a 15C-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 BH4 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-Pahenu2 [29,30], and its wild type strain mouse, BTBR, were generously donated by Dr. Alexander Shedlovsky in McArdle Laboratory for Cancer Research, University of Wisconsin, and maintained as described in a previous report [5]. Female BTBR-Pahenu2 and BTBR mice (15 weeks old), weighing 26–28 g, were used for breath testing.

[1-13C]Phenylalanine breath testing in mice

[1-13C]-L-Phenylalanine (13C-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 (BH4) was generously donated by Daiichi Suntory Pharma (Tokyo, Japan). 13C-Phe, Phe, and BH4 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 13CO2 analysis. Differences in the 13CO2 concentrations (Δ13CO2) 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 [13C]urea ([13C]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

[1-13C]Phenylalanine is converted to [1-13C]tyrosine by PAH as shown in Fig. 1A. [1-13C]Tyrosine is then broken down to yield homogentidinic acid and 13CO2 by two enzymatic reactions. To test whether the in vivo activity of mouse PAH can be evaluated by measuring the amount of 13CO2 in breath samples, we examined mice of Pahenu2 strain, a mutant strain of the BTBR mouse that lacks PAH activity [30]. Breath samples were collected from BTBR mice and Pahenu2 mice by placing them into a plastic box containing 350 ml of air 5–10,
20–25, 35–40, 50–55, and 80–85 min after injection of $^{13}$C-Phe (Fig. 1B). Preliminarily breath testing was performed using three different doses, 100, 20, and 10 mg/kg of $^{13}$C-Phe, from which it was determined that 20 mg/kg was the most appropriate (data not shown). In BTBR mice, $^{13}$CO$_2$ production peaked (33.8 ± 2.3) 10–25 min after injection of $^{13}$C-Phe (20 mg/kg). Reference breath samples were collected before injection. The solid and broken lines indicate the time course of $\Delta^{13}$CO$_2$ in BTBR and $Pah^{enu2}$ mice, respectively. Mean and SD values of $\Delta^{13}$CO$_2$ are indicated by the horizontal and vertical lines, respectively ($n = 3$).

Fig. 1. (A) Pathway of [1-$^{13}$C]-Phe oxidation and $^{13}$CO$_2$ production. The carboxyl carbon in $^{13}$C-Phe is converted to $^{13}$CO$_2$ by three reactions. (B) Time course of $^{13}$CO$_2$ production in BTBR and $Pah^{enu2}$ 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 $^{13}$C-Phe (20 mg/kg). Reference breath samples were collected before injection. The solid and broken lines indicate the time course of $\Delta^{13}$CO$_2$ in BTBR and $Pah^{enu2}$ mice, respectively. Mean and SD values of $\Delta^{13}$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 $^{13}$C-Phe (Fig. 1B). Preliminarily breath testing was performed using three different doses, 100, 20, and 10 mg/kg of $^{13}$C-Phe, from which it was determined that 20 mg/kg was the most appropriate (data not shown). In BTBR mice, $^{13}$CO$_2$ production peaked (33.8 ± 2.3) 10–25 min after injection of $^{13}$C-Phe, after which it fell to less than one-fifth of its peak value within 60 min. In contrast, $\Delta^{13}$CO$_2$ did not exceed 4% at any point during the test in $Pah^{enu2}$ 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$_2$ concentrations within the box at various time points when C57BL/6 mice were subjected to breath testing. CO$_2$ levels in the box reached 6.7 ± 0.6% at 60 min, and the mice became less active at 75 min, probably due to high CO$_2$ 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 $^{13}$CO$_2$ analysis (Fig. 2B). The BTBR and its mutant, $Pah^{enu2}$ mice were then re-exam-

Fig. 2. Sixty minutes of $^{13}$C-Phe breath test. (A) The concentration of CO$_2$ produced by C57BL/6 mice was measured. Three 9-week-old female mice were used to measure CO$_2$ concentrations at various time points. The vertical bars indicate the SD. (B) The $^{13}$CO$_2$ production of BTBR mice ($n = 6$) and $Pah^{enu2}$ mice ($n = 6$) was measured over the course of 60 min following intraperitoneal injection of $^{13}$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 $Pah^{ema2}$ ($n = 6$) mice were 25.3 $\pm$ 4.2 and 1.7 $\pm$ 0.6%$^{oo}$, 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$_4$ on the enzymatic activity of normal PAH in vivo, we administered 50mg/kg of BH$_4$ twice by intraperitoneal (i.p.) injection to C57BL/6 mice, 12 and 3h prior to the onset of breath sampling (Fig. 3A). Breath samples were then collected over the course of 60min and subjected to $^{13}\text{CO}_2$ analysis. The mean $\pm$ SD $\Delta^{13}\text{CO}_2$ value in the BH$_4$-treated group ($n = 6$) was 16.6 $\pm$ 2.7%$^{oo}$, while that of non-treated group ($n = 6$) was 15.1 $\pm$ 1.8%$^{oo}$, yielding no significant difference between the two groups ($p = 0.61$). We then administered 500mg/kg Phe 30min prior to the start of breath sampling (Fig. 3B). The group of mice that received BH$_4$ injections had a significantly greater ($p < 0.01$) mean $\Delta^{13}\text{CO}_2$ value (39.8 $\pm$ 8.2%$^{oo}$) than the group that did not (26.1 $\pm$ 7.3%$^{oo}$). On average, BH$_4$ administration caused a 1.7-fold increase in $^{13}\text{CO}_2$ production. The $\Delta^{13}\text{CO}_2$ of mice not administered BH$_4$ in Fig. 2B was significantly ($p < 0.05$) greater than that of mice not administered BH$_4$ in Fig. 2A, indicating enhancement of PAH activity by Phe pre-loading in vivo. The enhancing effect of BH$_4$ was not observed in $Pah^{ema2}$ mice (data not shown).

To examine the latency of the BH$_4$ effect, we injected BH$_4$ at the onset of breath sampling. Based on the time course shown in Fig. 1B, we collected breath samples 10–15min after $^{13}$C-Phe injections. The mice were ex-

![Fig. 3](image)

Fig. 3. (A) Effect of BH$_4$ 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$_4$ pre-loading. BH$_4$ (50mg/kg) was twice injected (i.p.) 12 and 3h prior to the onset of breath sampling. (B) The effect of BH$_4$ on PAH activity in vivo with Phe pre-loading. Phe (500mg/kg) was injected 30min prior to $^{13}$C-Phe injection (i.p.). All other experimental conditions were the same as those described in (A).

![Fig. 4](image)

Fig. 4. Effect of BH$_4$ on PAH activity in the first 5min. BH$_4$ (50mg/kg) was injected immediately prior to the onset of 5-min breath sampling, in which samples were collected in a 350ml plastic box. Unlabelled Phe (500mg/kg) and $^{13}$C-Phe (20mg/kg) were administered 30 and 10min 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$_4$ injections.
expected to produce high level of $^{13}$CO$_2$ during this period. The mean ± SD values for mice that did and did not receive BH$_4$ injections were 54.4 ± 8.7$^{\%}$ and 36.2 ± 9.3$^{\%}$, respectively (Fig. 4), indicating that BH$_4$ significantly ($p < 0.001$) enhanced PAH activity in the first 5 min.

Discussion

We developed a $^{13}$C-Phe breath test for mice in order to study the effect of BH$_4$ on PAH activity in vivo. The reliability of the test was verified by examining PKU model mice, $Pah^{hnu2}$. The PAH activity of normal mice increased 1.7-fold following 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 BH$_4$ causes a 1.6-fold increase in the conversion of Phe to tyrosine [27]. The concentration of BH$_4$ in the liver was reported to be 5$\mu$M [24–26]. The apparent $K_m$ of rat PAH for BH$_4$ was reported to be around 25$\mu$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 BH$_4$ in the liver and that its activity is enhanced by tetrahydrobioppterin 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 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 BH$_4$ supplementation is likely to be observed not only in mice, but also in rats and humans. This may explain the mechanism of BH$_4$ responsiveness in patients with BH$_4$-responsive PAH deficiency.

Our hypothesis may explain why a range of PAH mutations demonstrate responsiveness to BH$_4$, and why there is such a high prevalence of patients with 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 BH$_4$, for which pharmacological doses of BH$_4$ might be expected to restore PAH activity. However, it is unlikely that all BH$_4$-responsive mutations are $K_m$ variants for BH$_4$ because “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 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 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 BH$_4$. Recent observations indicate that patients with classical PKU, as well as patients with mild HPA, responded favorably to BH$_4$ supplementation [18,34]. Those patients may have very low but not negligible level of residual PAH activity, which is enhanced by BH$_4$.

In normal mice, pre-administration of Phe was required for augmentation of PAH activity by BH$_4$. Initially, we performed the breath test after overnight fasting based on the human protocol for $^{13}$C-Phe breath testing [13,28]. However, $^{13}$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 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 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 BH$_4$ without Phe pre-loading (Fig. 3A), which may explain why patients with the same genotype do not always respond to 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 $^{13}$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 $^{13}$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 $^{13}$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 BH$_4$ and its substrate Phe, which may suggest the pivotal roles of BH$_4$ and Phe as physiological regulators of PAH activity.

The apparent activation of PAH by BH$_4$ was observed within 5 min after administration of BH$_4$ in the present study. In contrast, serum Phe levels do not fall
until several hours after administration of BH₄ in patients with BH₄-responsive PAH deficiency [7]. This is probably because most mutant PAH enzymes have reduced Vₘₐₓ 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 BH₄ supplementation might increase PAH activity. However, these changes are unlikely to occur within 5 min of BH₄ 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 BH₄ responsiveness. Further study is necessary to clarify the mechanism of BH₄ responsiveness and to determine indications for BH₄ therapy.

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References


