

# Assessment of Tetrahydrobiopterin (BH<sub>4</sub>) Responsiveness in Phenylketonuria

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**Objective** To determine the prevalence of and identify subjects with phenylketonuria (PKU; phenylalanine hydroxylase deficiency) responsive to 6R-tetrahydrobiopterin (BH<sub>4</sub>) and to establish selection criteria for potential treatment with BH<sub>4</sub>.

**Study design** Blood phenylalanine levels from 557 newborns and children with various degrees of PKU (blood phenylalanine, 301 to 4743  $\mu\text{mol/L}$ ) challenged with BH<sub>4</sub> (20 mg/kg of body weight) were analyzed at 8 and 24 hours after BH<sub>4</sub> administration. The 2 modalities were compared in terms of phenylalanine reduction.

**Results** The overall prevalence of BH<sub>4</sub> responsiveness within patients with PKU for blood phenylalanine reductions of 20%, 30%, 40%, and 50% was 48%, 38%, 31%, and 24%, respectively, using the 8-hour modus and 55%, 46%, 41%, and 33%, respectively, using the 24-hour modus. Using the 30% cutoff, BH<sub>4</sub> responsiveness was similar regardless of the modality in patients with mild hyperphenylalaninemia (79% to 83% responders), mild PKU (49% to 60% responders), and classical PKU (7% to 10% responders).

**Conclusions** BH<sub>4</sub> responsiveness is more prevalent than was previously assumed, particularly in patients with mild hyperphenylalaninemia and mild PKU. Depending on the severity of hyperphenylalaninemia, selection criteria for the potential treatment with BH<sub>4</sub> may range from 20% to 40% blood phenylalanine reduction after 24 hours. (*J Pediatr* 2007;150:627-30)

Phenylketonuria (PKU), the most common inborn error in amino acid metabolism, is caused by mutations in the phenylalanine hydroxylase (PAH) gene.<sup>1</sup> Blood phenylalanine concentration during childhood is the major determinant of cognitive outcome in these patients. Particularly, adolescents and young adults generally do not comply with recommendations for the monitoring and control of phenylalanine concentrations,<sup>2</sup> and 2/3 of pregnant women with PKU in the United States do not follow the diet before becoming pregnant.<sup>3</sup> Recently, it was shown that some patients with PKU respond to the 6R-tetrahydrobiopterin (BH<sub>4</sub>)-loading test with decreased plasma phenylalanine concentrations, and that these patients can be treated with BH<sub>4</sub>.<sup>4</sup> We found that >60% of patients with plasma phenylalanine concentrations between 400 and 800  $\mu\text{mol/L}$  responded to a BH<sub>4</sub> challenge with decreased blood phenylalanine levels by 30% 8 hours after administration, and that the responsiveness was dose-dependent.<sup>5</sup> Keep in mind, however, that this study used data obtained with both the old 33% less active formulation of BH<sub>4</sub> and the new fully active compound.

Various authors have suggested different criteria for the definition of BH<sub>4</sub> responsiveness using 10 mg BH<sub>4</sub>/kg,<sup>6-8</sup> 20 mg BH<sub>4</sub>/kg,<sup>9-15</sup> or a combined phenylalanine (100 mg/kg) and BH<sub>4</sub> (20 mg/kg)<sup>16-19</sup> challenge over 24 hours. Numerous patients with PKU have currently been on BH<sub>4</sub> therapy for more than 3 years, and BH<sub>4</sub> administration has been shown to increase phenylalanine tolerance in both mild and moderate phenotypes.<sup>7,16,20-25</sup> Thus, replacement of the low-phenylalanine diet with the commercially available BH<sub>4</sub> may significantly improve compliance in patients with PKU and may be an option for better control of blood phenylalanine levels in pregnant women with PKU.

Although most laboratories use a 30% phenylalanine reduction cutoff after a 24-hour challenge as a definition of responsiveness, some clinicians suggest lower cutoffs to assess additional patients. The aim of the present study was to compare 2 loading test modalities (8 hours and 24 hours) to assess the best criteria for the selection of BH<sub>4</sub>-responsive patients, based on the BH<sub>4</sub> loading tests (20 mg/kg) performed in 557 newborns and infants with PKU.

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BH <sub>4</sub>	Tetrahydrobiopterin	PAH	Phenylalanine hydroxylase
HPA	Hyperphenylalaninemia	PKU	Phenylketonuria

**Table. Summary of the BH<sub>4</sub> loading test (20 mg/kg) in patients with PKU**

Phe* ( $\mu\text{mol/L}$ )	8-hour loading test					24-hour loading test				
	n	Phe reduction after 8 hours				n	Phe reduction after 24 hours			
		>50%	>40%	>30%	>20%		>50%	>40%	>30%	>20%
301 to 4743	557	24	31	38	48	293	33	41	46	55
300 to 400	59	58	68	76	83	35	60	71	89	91
400 to 500	54	63	70	81	89	29	72	79	79	93
500 to 700	65	55	68	75	78	29	62	69	76	76
700 to 900	64	27	38	45	70	35	40	60	69	74
900 to 1100	48	10	25	42	56	31	32	42	55	77
1100 to 1300	44	9	14	27	30	24	21	29	29	42
1300 to 1500	44	7	7	11	16	28	4	7	7	29
1500 to 1800	56	2	5	5	11	27	15	15	15	15
1800 to 2100	50	0	2	4	10	22	0	1	1	2
>2100	73	1	1	5	19	33	3	6	12	21

\*Blood phenylalanine concentrations before BH<sub>4</sub> administration.

## METHODS

### Patients

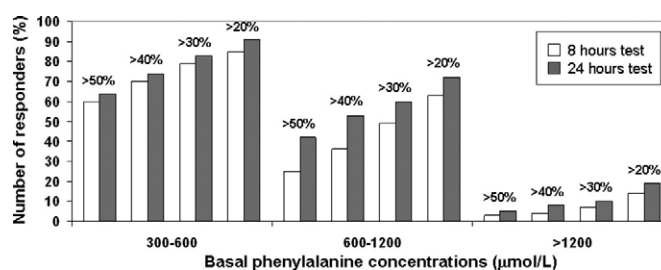
A total of 557 patients from Switzerland, Germany, Italy, Austria, Slovenia, Hungary, Turkey, and Israel (age range, 1 week to 7 years; median, 2 weeks) diagnosed with hyperphenylalaninemia (HPA) (blood phenylalanine 301 to 4743  $\mu\text{mol/L}$ ) between 2000 and 2006 were investigated for possible BH<sub>4</sub> deficiency. There was an almost equal distribution between females (43%) and males (57%). BH<sub>4</sub> deficiency was excluded in all patients by measuring urinary pterins and dried blood dihydropteridine reductase activity. In all 557 patients, a loading test with 6R-BH<sub>4</sub> (20 mg/kg) was performed over a period of 8 hours. In 293 of the 557 patients, the same loading test was extended to 24 hours. Patients were divided into 3 phenotype groups (mild HPA with phenylalanine levels <600  $\mu\text{mol/L}$ , mild PKU with phenylalanine levels 600 to 1200  $\mu\text{mol/L}$ , and classical PKU with blood phenylalanine levels >1200  $\mu\text{mol/L}$ ). This classification is based on phenylalanine levels before the test and may differ from the actual phenotype. PAH gene mutation analysis was done in only a few patients and was not included in this study. All tests were performed as a part of routine investigations at the time of diagnosis and before introducing the diet.

### BH<sub>4</sub>-Loading Test

Plasma or blood phenylalanine was measured before and 4, 8, and 24 hours after oral administration of 20 mg/kg BH<sub>4</sub> (6R-BH<sub>4</sub>; Schircks Laboratories, Jona, Switzerland) as described previously.<sup>26</sup> Responsiveness to BH<sub>4</sub> was calculated as a percentage of blood phenylalanine reduction 8 or 24 hours after administration. Phenylalanine concentrations were measured using an amino acid analyzer or tandem-mass spectrometry.

### Statistical Analyses

WinSTAT for Excel, version 2003.1 (R. Fitch Software, Bad Krozingen, Germany) was used for descriptive statistics.



**Figure.** Responsiveness to BH<sub>4</sub> in patients with mild HPA (phenylalanine 300 to 600  $\mu\text{mol/L}$ ), mild PKU (phenylalanine 600 to 1200  $\mu\text{mol/L}$ ), and classical PKU (phenylalanine >1200  $\mu\text{mol/L}$ ) according to different cutoffs (20% to 50% phenylalanine reduction) calculated 8 hours (white bars) and 24 hours (dark bars) after BH<sub>4</sub> administration (20 mg/kg).

## RESULTS

The outcome of the 8- and 24-hour loading tests with 20 mg/kg BH<sub>4</sub> in patients with PKU is summarized in the Table (according to initial blood phenylalanine levels) and the Figure (according to phenotype groups). Of 557 patients with various degrees of PKU (301 to 4743  $\mu\text{mol/L}$ ), 48% responded to BH<sub>4</sub> administration (20 mg/kg) with at least a 20% decrease in their initial blood phenylalanine levels after 8 hours. Increasing the cutoff for phenylalanine reduction to 30%, 40%, and 50% reduced the sensitivity of the 8-hour loading test to 38%, 31%, and 24%, respectively, in the overall population with PKU.

In 293 of the total of 557 patients, the loading test was performed over 24 hours; the overall responsiveness was higher than in the 8-hour test (55%, 46%, 41%, and 33% of patients with 20%, 30%, 40%, and 50% reduction, respectively). The patients with mild HPA (phenylalanine <600  $\mu\text{mol/L}$ ) responded better than those with mild PKU (phenylalanine 600 to 1200  $\mu\text{mol/L}$ ) or classical PKU (phenylalanine >1200  $\mu\text{mol/L}$ ), regardless of the selected cutoff (20% to 50%) or test modality (8 or 24 hours) (Figure).

Using the currently accepted cutoff of 30% phenylalanine reduction in the 24-hour test, the highest incidence of responders was in patients with blood phenylalanine levels between 301 and 1100  $\mu\text{mol/L}$  (55% to 89%) (Table). About 1/3 of patients with blood phenylalanine levels between 1100 and 1300  $\mu\text{mol/L}$  responded to the same protocol, and only 1% to 15% of patients with blood phenylalanine levels  $>1300$   $\mu\text{mol/L}$  responded to  $\text{BH}_4$  administration according to the same protocol. Of the 27 patients with blood phenylalanine levels between 1500 and 1800  $\mu\text{mol/L}$ , 4 patients responded to  $\text{BH}_4$  administration with a 50% decrease in their initial phenylalanine levels.

Although there was an overall consistency in responsiveness between the 8-hour and 24-hour loading test, 8 of the 295 patients in whom the loading test was performed over 24 hours were defined as responders only at 8 hours. In addition, 26 of the 295 patients were responders at 24 hours, but not at 8 hours. All other patients were either responders at both 8 and 24 hours or nonresponders (data not shown). In about 15% of all patients who responded to  $\text{BH}_4$  with a 20% decrease in initial blood phenylalanine level, the blood phenylalanine level increased by at least 10% from 8 to 24 hours.

## DISCUSSION

Between 2000 and 2006, a total of 557 patients with PKU were challenged with  $\text{BH}_4$  (20 mg/kg) and evaluated for possible  $\text{BH}_4$  deficiency. Although  $\text{BH}_4$  deficiency was excluded in all of these patients, 38% of the patients responded to  $\text{BH}_4$  administration with at least a 30% decrease in their initial blood phenylalanine levels after 8 hours, regardless of the phenotype. This prevalence was even higher (46%) when using a 24-hour loading test. Patients with a milder phenotype (phenylalanine levels  $<1200$   $\mu\text{mol/L}$ ) were the best responders (49% to 79%). These figures are much higher than previously reported,<sup>5</sup> and reducing the cutoff for responsiveness at 24 hours from 30% to 20% produced 93% of responders within the subgroup of patients with mild HPA (phenylalanine 400 to 500  $\mu\text{mol/L}$ ). Although recommendations for treating HPA and PKU differ among countries,<sup>27,28</sup> patients with mild HPA are not a main target for  $\text{BH}_4$  treatment.

There remains no consensus as to the gold standard protocol for the diagnosis of  $\text{BH}_4$ -responsive HPA/PKU and the point at which a patient should be defined as a  $\text{BH}_4$  responder. A 24-hour protocol with 20 mg/kg  $\text{BH}_4$  is the most commonly used method,<sup>11,13-15,26,29,30</sup> and multiple administrations of  $\text{BH}_4$  and extension of the test to up to 1 week may detect additional "slow" responders.<sup>7,31</sup> Results from different studies are difficult to compare because some used lower doses of  $\text{BH}_4$  (10 mg/kg)<sup>7,8</sup> or a combined phenylalanine (100 mg/kg) and  $\text{BH}_4$  (20 mg/kg) challenge.<sup>16,17,19</sup> Some authors use a 20% cutoff,<sup>7</sup> some a 30% cutoff,<sup>5,6,8,13-16</sup> and some a 50% cutoff<sup>32</sup> at 8, 15, or 24 hours after a single administration of  $\text{BH}_4$ .

Several factors hamper the interpretation of published reports. The first such factor concerns the use of different methods for determining the phenylalanine reduction. Mun-

tau et al<sup>16</sup> and Okano et al<sup>18</sup> investigated the therapeutic efficacy of  $\text{BH}_4$  by measuring in vivo rates of [ $^{13}\text{C}$ ] phenylalanine oxidation. Leuzzi et al<sup>15</sup> considered daily fluctuations of basal phenylalanine levels and defined  $\text{BH}_4$  responsiveness as variations exceeding the individual variability of blood phenylalanine levels. Second, although under normal conditions and in most cases newborns are tested under a normal protein intake, some authors tested patients who were already on a low-phenylalanine diet with<sup>8,17-19</sup> or without additional phenylalanine supplementation.<sup>8,15</sup> The total amount of phenylalanine intake and the resulting blood phenylalanine concentrations seem to be determining factors for  $\text{BH}_4$  responsiveness. Pay and Martinez<sup>33</sup> demonstrated in an in vitro system that a 5-fold increase in phenylalanine concentrations doubles the  $K_m$  values for  $\text{BH}_4$  in the PAH assay. Thus, the higher the blood phenylalanine level, the more  $\text{BH}_4$  is needed to activate the mutated protein.

Our results demonstrate that a considerable number of patients with classical PKU are responsive to the  $\text{BH}_4$  loading test, particularly if the cutoff is reduced to 20% (see the Table and Figure). Other authors excluded classical PKU from responsiveness to oral  $\text{BH}_4$  administration when different criteria were applied.<sup>14,16</sup> Thus, selection criteria for the phenotype classification may be responsible for the relatively high number of classical PKU responders. Most authors use newborn screening phenylalanine values or fasting phenylalanine concentrations in basal conditions (before the loading test) for the phenotype classification. Tolerance to dietary phenylalanine intake and genotype analysis might be better methods for phenotype classification than blood phenylalanine levels, but even then there is not always a good correlation among these 3 variables.<sup>14</sup>

Evaluation of responsiveness at 8 and 24 hours after  $\text{BH}_4$  administration revealed a greater number of responders at 24 hours than at 8 hours (see the Table and Figure); for example, 8.9% of patients were responsive at 24 hours but not at 8 hours after  $\text{BH}_4$  administration. Extension of the  $\text{BH}_4$  loading test up to 24 hours seems to be fundamental for detecting possible  $\text{BH}_4$ -responsive patients. Nevertheless, some patients (2.7%) were responsive at 8 hours but not at 24 hours after  $\text{BH}_4$  administration. Based on the present knowledge of  $\text{BH}_4$  pharmacokinetics<sup>34,35</sup> a second administration of  $\text{BH}_4$  12 hours after the first might further improve the sensitivity of the test and detect slow responders. Although most patients on  $\text{BH}_4$  therapy receive doses between 5 and 20 mg/kg,<sup>7,16,20-25</sup> and 20 mg is given almost exclusively during the  $\text{BH}_4$  loading test, another rather high dose of  $\text{BH}_4$  may enhance enzyme catalytic activity. Accordingly, we suggest modifying the  $\text{BH}_4$ -loading test to include a second administration of 10 to 20 mg/kg  $\text{BH}_4$  at 12 hours, with assessment of plasma phenylalanine levels at 0, 8, 12, and 24 hours after  $\text{BH}_4$  administration.

In summary, the cutoff level for  $\text{BH}_4$  responsiveness depends on the patient's clinical phenotype, being lower for patients with classical PKU ( $>20\%$  phenylalanine reduction) and higher for those with mild or moderate PKU ( $>30\%$

phenylalanine reduction). Patients with mild PKU may replace a phenylalanine-restricted diet with BH<sub>4</sub> monotherapy, whereas those with moderate or classical PKU may combine BH<sub>4</sub> with a less-strict diet regimen. A 20% to 30% reduction in plasma phenylalanine can be significant in a patient with classical PKU, because it may allow the patient to relax his or her strict dietetic regimen in association with BH<sub>4</sub> therapy. Well-designed long-term studies are needed to compare short-term (BH<sub>4</sub> loading test) and long-term (BH<sub>4</sub> therapy) responsiveness.

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