

# Biopterin responsive phenylalanine hydroxylase deficiency

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**Purpose:** Phenylketonuria (PKU) is an autosomal recessive disorder caused by mutations in the phenylalanine hydroxylase (PAH) gene. There have been more than 400 mutations identified in the PAH gene leading to variable degrees of deficiency in PAH activity, and consequently a wide spectrum of clinical severity. A pilot study was undertaken to examine the response to 6-R-L-erythro-5,6,7,8-tetrahydrobiopterin (BH<sub>4</sub>) in patients with atypical and classical PKU. **Methods:** PAH gene mutation analysis was performed using denaturing gradient gel electrophoresis and gene sequencing. Patients with classical, atypical, or mild PKU were orally given BH<sub>4</sub> 10 mg/kg. Blood phenylalanine and tyrosine levels were determined using tandem MS/MS at 0 hours, 4 hours, 8 hours, and 24 hours intervals. **Results:** Thirty-six patients were given a single oral dose of 10 mg/kg of BH<sub>4</sub>. Twenty one patients (58.33%) responded with a decrease in blood phenylalanine level. Of the patients that responded, 12 were classical, 7 atypical, and 2 mild. The mean decline in blood phenylalanine at 24 hours was > 30% of baseline. There were 15 patients who did not respond to the BH<sub>4</sub> challenge, 14 of those had classical and one had atypical PKU. Mapping the mutations that responded to BH<sub>4</sub> on the PAH enzyme showed that mutations were in the catalytic, regulatory, oligomerization, and BH<sub>4</sub> binding domains. Five patients responding to BH<sub>4</sub> had mutations not previously identified. **Conclusion:** The data presented suggest higher than anticipated number of PKU mutations respond to BH<sub>4</sub>, and such mutations are on all the domains of PAH. *Genet Med* 2004;6(1):27–32.

**Key Words:** phenylketonuria, PKU, tetrahydrobiopterin, BH<sub>4</sub>, phenylalanine,

Phenylketonuria (PKU) is a disorder caused by deficiency of the enzyme phenylalanine hydroxylase (PAH).<sup>1</sup> The gene for PAH has been cloned and over 400 mutations have been identified.<sup>2,3</sup> The findings of Bickel et al.,<sup>4</sup> that a phenylalanine (Phe)-restricted diet can ameliorate the effects of high blood Phe on cognitive function, has resulted in newborn screening programs for PKU.<sup>5</sup> Early results of treatment were very encouraging. However, treatment has to be continued “for life,” otherwise high blood Phe levels lead to functional deficits.<sup>6</sup> Present treatment goals for PKU include maintaining blood Phe levels between 120 and 360 μmol/L. It has been difficult to comply with this treatment.<sup>7–17</sup> In order to maintain optimal mental functioning in patients with PKU, a search for better methods of treatment has been on going. These methods in-

clude enzyme therapy, competition with transport of Phe to the brain, and potentially gene therapy.<sup>18–22</sup>

A new development in the treatment of PKU was reported by Kure et al.<sup>23</sup> in four patients with mild PKU who responded to an oral load of the cofactor for PAH, 6-R-1-erythro-5,6,7,8-tetrahydrobiopterin (BH<sub>4</sub>). These patients had normal levels and production of BH<sub>4</sub>. After this report, other cases from Europe have been reported.<sup>24–30</sup> These reports emphasize the potential response to BH<sub>4</sub> of patients with atypical or mild PKU. Recently Matalon et al.<sup>31</sup> presented favorable response to BH<sub>4</sub> in a small number of patients with classical PKU.

This pilot study was undertaken in two clinics in the United States to find out the extent of response to BH<sub>4</sub> among patients with PKU. The study included patients with classical, atypical, and mild PKU.

## MATERIALS AND METHODS

The study was performed at two sites, the Children’s Hospital of Los Angeles and University of Texas Medical Branch at Galveston. Informed consent was obtained from all participants or their parents. The study included 16 males and 20 females with PKU. There were 26 subjects with “classical” PKU, based on several quantitative determination of blood Phe levels > 1200 μmol/L after positive newborn screening for

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PKU, and also according to daily Phe intake of 150 to 250 mg. In the 8 patients with “atypical” PKU, blood Phe was 360 to 1200  $\mu\text{mol/L}$ , and daily Phe tolerance was 350 to 450 mg. In the two patients with “mild” PKU, blood Phe was 120 to 360  $\mu\text{mol/L}$ . The mean age of the subjects was 16.6 years with a range from 6 months to 43 years. Twenty-seven of the 36 patients were taking low Phe diet, with various degrees of success in attaining blood Phe levels between 120 and 360  $\mu\text{mol/L}$ . Only seven patients had blood Phe levels in the treatment range of 120 to 360  $\mu\text{mol/L}$ , whereas the other 20 patients had blood Phe levels above 360  $\mu\text{mol/L}$ . Six patients were not following a low Phe diet: one with classical PKU, four atypical, and one mild.

All of the patients had a normal urine pterin profile and blood dihydropteridine reductase (DHPR) activity. Urine pterin profile and blood DHPR were determined by Neogen (Bridgeville, PA). Mutation analysis on all patients was determined by Dr. Guttler using Denaturing Gradient Gel Electrophoresis (DGGE) of the coding sequence exons 1–12; and by sequencing of the exons with the mutations.<sup>32,33</sup>

Tetrahydrobiopterin, 6R-BH<sub>4</sub>, was obtained from Schircks Laboratories, Jona, Switzerland. Baseline blood Phe and tyrosine were drawn after an overnight fast and then the subjects were given BH<sub>4</sub> 10 mg/kg orally. Data from a single patient given 20 mg/kg BH<sub>4</sub> were contributed by Dr. C. Scriver. Patients were instructed to continue the same dietary practice during the day after oral BH<sub>4</sub> challenge. Blood Phe and tyrosine were taken at 4, 8, and 24 hours after an overnight fast. Blood analyses were determined using tandem MS/MS (Neogen, Bridgeville, PA). Analysis of variance (ANOVA) was used for statistical analysis.

### Expression analysis

*Escherichia coli* expression and activity assessment experiments were performed on mutant AH protein expressed both with and without GroES and GroEL chaperonin coexpression (pGroESL) by the method followed earlier.<sup>34</sup>

For mammalian expression, wild-type and mutant PAH proteins were expressed using transient mammalian expression in COS cells and using an in vitro transcription-translation system (TnT-T7). Transient expression analysis in COS cells were performed using PAH cDNA cloned in the rRC/CMV expression vector (Invitrogen, CA) as followed earlier.<sup>35</sup>

## RESULTS

Twenty one subjects (58.33%) responded with a decline in blood Phe compared to the baseline levels. The mean blood Phe level before BH<sub>4</sub> was 720.23  $\mu\text{mol/L}$ , and after 24 hours BH<sub>4</sub> loading, the mean level dropped to 423.33  $\mu\text{mol/L}$  as shown in Table 1 ( $P = 0.0006$ ). The mean decline at 24 hours in blood Phe was 58.77%. The maximum decline in blood Phe was at 24 hours in all patients except one who had maximum decline at 8 hours after BH<sub>4</sub>. The genotype and individual blood Phe response are shown in Table 1. Mean blood tyrosine levels before and 24 hours after the BH<sub>4</sub> load are also shown in

Table 1. Whereas tyrosine increased in some patients, the overall increase was not statistically significant.

Blood Phe and tyrosine before and after BH<sub>4</sub> load in patients that did not respond are shown in Table 1. Two individuals who had either the R261Q or Y414C allele (IVS12nt1g>a/R261Q and Y414C/IVS12nt1g>a) failed to respond to BH<sub>4</sub>. These mutations have been reported to cause favorable response to BH<sub>4</sub>. Two patients had a rise in blood tyrosine (IVS12nt1g>a/E280K and IVS12nt1g>a/R261Q), but overall there was no statistical significance in tyrosine response to BH<sub>4</sub>. The two patients with the R261Q allele were challenged again with 20 mg/kg BH<sub>4</sub> and showed less than a 10% drop in blood Phe level.

The mutations in patients with favorable response to BH<sub>4</sub> in this study are shown in Figure 1 mapped onto the structure of a composite of the monomer model of PAH. Five of these mutations have not previously been reported to respond to BH<sub>4</sub>: F39L/F55fdelT, R68S/R408W, H170D/IVS1nt5g>a, E178G/IVS10nt-11g>a, and L308F/R261Q.

*E. coli* expression of mutation A313T, Y414C, and I65T showed 45%, 50%, and 91% residual activity, respectively.

## DISCUSSION

Treatment of PKU has been undergoing reevaluation since the mid seventies. There have been an increased number of reports of intellectual decline, and white matter changes, in patients who stopped the dietary restriction of Phe.<sup>8,14</sup>

Neuropsychological and neuroimaging improvement have been reported when low Phe diet was resumed. At the same period of time, the problem of maternal PKU was looming, and the practice of diet discontinuation was questioned. A strong move toward “diet for life” has emerged. This change in treatment policy has been difficult to translate to the reality of lower blood Phe levels, especially when subjects with PKU enter the early teens.<sup>6</sup>

In an attempt to improve dietary compliance, medical foods for the treatment of PKU underwent many changes to improve taste and acceptability. New low protein foods have been developed, to be used as part of the low Phe diet plan. Such changes are still continuing. The “magic” formula and foods that a patient with PKU will readily take “for life” have yet to be found. Therefore, different approaches have been explored. Such ideas include the use of an enzyme, phenylalanine ammonia lyase (PAL), that breaks down Phe in the gastrointestinal tract.<sup>18–20</sup> Another approach is to prevent the influx of Phe to the brain by the addition of large neutral amino acids (LNAA) to the diet as suggested by Kaufman.<sup>21</sup> The LNAA include Phe, leucine, isoleucine, valine, tryptophan, tyrosine, methionine, threonine, and histidine. Studies using tyrosine, tryptophan, or branched chain amino acids and other short term trials with LNAA have been reported with no alternative treatment to Phe-restricted formulas.<sup>36–44</sup>

Treatment of PKU with BH<sub>4</sub> is a novel approach started with the report of Kure et al. in 1999.<sup>23</sup> Subsequently reports of challenging patients with PKU with BH<sub>4</sub> have dealt with atyp-

**Table 1**  
Mutations in BH<sub>4</sub> responsive PAH deficiency

Sex	Age	Mutation name	Systematic name	Phenotype	Phe level (μmol/L)				Tyr level (μmol/L)			
					0 h	4 h	8 h	24 h	0 h	4 h	8 h	24 h
F	12y	R408W/F39L	c.1222C>T/c.117C>G	classic	1008	847	792	561	23.1	20.9	20.9	51.15
M	18y	R270K/delI94	c.809G>A/c.283-285del ATC	classic	1224	1386	1374	672	48.95	59.95	45.65	69.85
M	19y	Y414C/R408W	c.1241A>G/c.1222C>T	classic	792	636	489.5	630	60.5	55.0	60.5	66.0
F	35y	Y414C/R408W	c.1241A>G/c.1222C>T	classic	1228	1109	1029	905	43	54	55	46
M	43y	delI94/?	c.283-285delATC/?	classic	1530	1230	852	816	73.15	89.65	53.9	127.05
M	6m	R408W/R68S	c.1222C>t/c.204A>T	classic	870	ND	594	516	42.35	ND	69.3	59.95
M	5y	F39L/F55delT	c.117C>G/c.165delT	classic	948	ND	900	606	44	ND	38.5	60.5
M	6m	F39L/F55delT	c.117C>G/c.165delT	classic	450	ND	246	132	24.75	ND	60.5	71.5
F	20y	R408W/R261Q	c.1222C>T/c.782G>A	classic	900	834	798	744	115.5	71.5	82.5	93.5
F	30y	IVS7nt5g>a/Y414C	c.842+5g>a/c.1241A>G	classic	1086	990	852.5	759	47.85	44.55	60.5	45.65
M	28y	I65T/R68S	c.194T>C/c.204A>T	classic	1110	996	930	852	45.6	58.3	96.8	104.5
M	2y	R261Q/L308F	c.782G>A/c.922C>T	classic	342 <sup>a</sup>	ND	256.2	96	33.55	ND	53.9	38.5
M	4y	R408W/R68S	c.1222C>T/c.204A>T	Atyp	282	ND	102	114	33.0	ND	52.25	50.6
M	14y	E390G/IVS12nt1g>a	c.1169A>G/c.1315+1g>a	Atyp	624	ND	ND	186	ND			
M	16y	IVS1nt5g>a/H170D	c.60+5g>a/c.508C>G	Atyp	510	366	270	162	79.75	154	126.5	121
F	4y	I65T/R408W	c.194T>C/c.1222C>T	Atyp	226.2 <sup>b</sup>	ND	127.8	139.8	32.45	ND	89.65	26.95
F	6y	I65T/R408W	c.194T>C/c.1222C>T	Atyp	389.4	ND	211.8	162	64.9	ND	74.2	29.7
M	9y	P407S/R408W	c.1219C>T/c.1222C>T	Atyp	504	ND	468	276	61.05	ND	60.5	55.0
F	8y	IVS10nt-11g>a/E178G	c.1066-11g>a/c.533A>G	Atyp	576	ND	384	312	50.05	ND	57.75	54.45
F	16y	E280K/I269L	c.838G>A/c.805A>C	mild	163.2	ND	100.2	100.8	ND			
F	6y	A300S/R408W	c.898G>T/c.1222C>T	mild	363	ND	240	150	41.8	ND	88	44

<sup>a</sup>Classical patients had blood Phe above 1200 μmol/L and <sup>b</sup>atypical patients had blood Phe 360–1200 μmol/L. These individuals have been on diet. For the challenge with BH<sub>4</sub> their dietary practice was not altered and therefore some have low levels during 0 h.

ical or mild PKU.<sup>23–29,45</sup> Our report is the first study showing a large number of patients with classical as well as atypical PKU responding favorably to a BH<sub>4</sub> challenge by a drop in their blood Phe levels.<sup>31</sup> Although “classical PKU” has been used to denote levels of blood Phe above 1200 μmol/L, it has become important to determine the genotype of patients with PKU, so that severity of the metabolic defect can be better correlated with the mutation and PAH residual activity. Of 12 subjects considered “classical” PKU, three had blood Phe levels > 1200 μmol/L and responded to BH<sub>4</sub>. The other patients had lower blood Phe levels because they were on some dietary restriction. In young patients who were on diet with good control of blood Phe, the drop of blood Phe level after BH<sub>4</sub> load may not be as dramatic, but still was discernible.

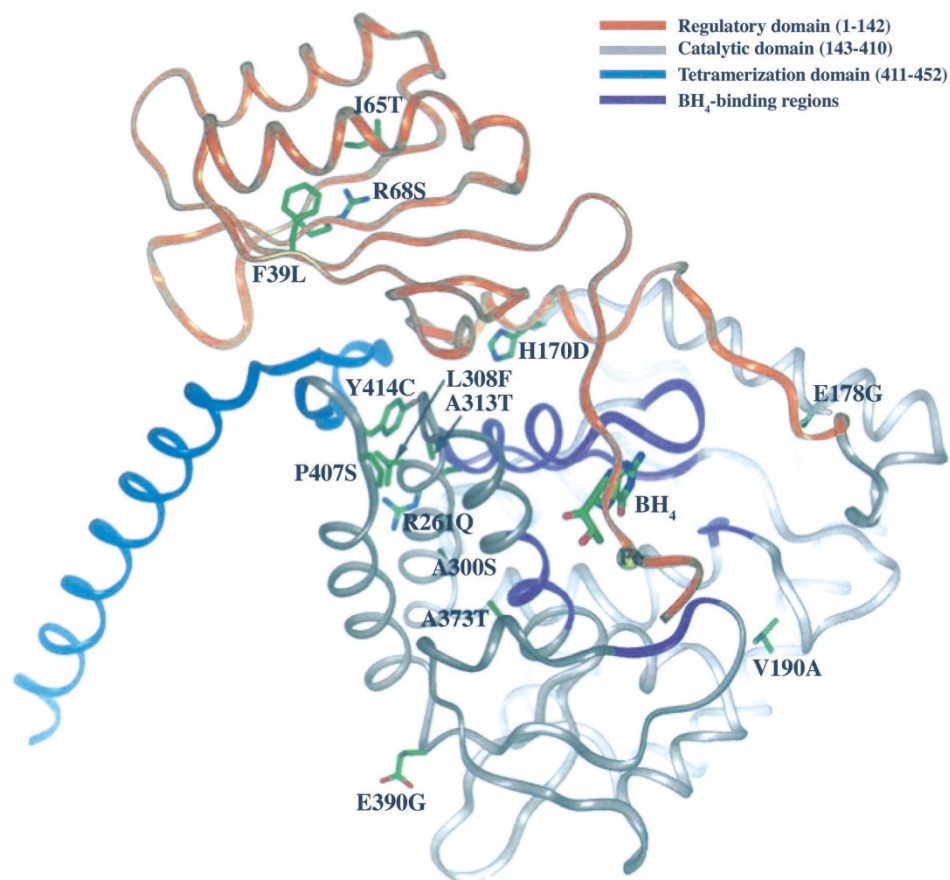
A trial with BH<sub>4</sub> supplementation on six patients with PKU showed improved tolerance to dietary Phe, suggesting the possibility of diet relaxation.<sup>46</sup> Results of treatment of 38 PKU patients with BH<sub>4</sub> showed that all the responders had mild PKU, and the six patients with classical PKU did not respond to BH<sub>4</sub>.<sup>47</sup>

Although the phenotype-genotype correlation in PKU is rather complex, the genotype of PKU patients who respond to

BH<sub>4</sub> may be predictive, although individual response may be different. Blood Phe and tyrosine have diurnal variation, so blood drawing needs to be done at similar times.<sup>48</sup>

Most of the mutations tested in this study have been reported in earlier BH<sub>4</sub> trials.<sup>31,49–50</sup> However, five previously unreported mutations have been found to be BH<sub>4</sub> responsive. The BH<sub>4</sub>-responsive mutations F39L and R68S are located in the regulatory domain of PAH,<sup>51</sup> whereas mutations H170D, E178G, and L308F are in the catalytic domain.

Mutation H170D is a newly discovered PKU mutation that is found in one of the patients of the study, together with IVS 1nt5g→a. Histidine 170 is located on the surface of the catalytic domain, close to the tetramerization domain and the regulatory domain. Substitution into aspartic acid may disrupt a hydrogen bond to Arg241 at the start of beta strand Cβ1.<sup>49</sup> Mutation L308F is another newly discovered mutation found to be BH<sub>4</sub> responsive in a patient along with the R261Q mutation on the second allele. Leucine 308 is close to the tetramerization domain residues valine 412 and tyrosine 414, and the catalytic domain residues alanine 259 and glutamic acid 305. When leucine is substituted with phenylalanine (L308F), there is no space for the phenylalanine side chain to be inserted, as



**Fig. 1** C-Alpha trace of a monomer of the composite model of phenylalanine hydroxylase.<sup>51</sup> Regulatory domain is colored in orange (residues 19–142), the catalytic domain in gray (residues 143–410), and the tetramerization domain (residues 411–452) in blue. Regions responsible for binding the BH<sub>4</sub> cofactor are shown in purple. BH<sub>4</sub> cofactor is shown together with the iron at the active site. Side chain of the residues found to be BH<sub>4</sub> responsive are shown mapped onto the structure.

would be the case in L308F. Substitution into a phenylalanine from a smaller leucine could potentially push the tetramerization domain away from the catalytic domain, and interfere with dimer/tetramer formation. Based on the BH<sub>4</sub>-responsiveness both the H170D and the L308F mutations may prove to be mild mutations with close to wild-type residual activity, but the mutations remain to be studied biochemically.

Based on the emerging large number of PKU mutations that are BH<sub>4</sub> responsive, there does seem to be a few basic points that govern the BH<sub>4</sub> response. The combination of alleles found in the PKU patient's genotype seems to initially determine whether or not the patient shows BH<sub>4</sub> responsiveness. If one of the patient's alleles contains a mild mutation that does not completely abolish activity but imposes changes in cofactor binding (i.e., a  $K_m$  mutant), this may be a sign of BH<sub>4</sub> responsiveness. If both alleles contain severe mutations, it is unlikely that the patient will be BH<sub>4</sub> responsive. A second option to explain the BH<sub>4</sub> responsiveness could be the idea of a chemical chaperone affecting the function and stability of enzymes as suggested by Scriver and Waters.<sup>52</sup> The BH<sub>4</sub> cofactor may function as a chemical chaperone by rescuing mutant PAH enzyme from host cell proteases (increasing the protein half-life), and making the enzyme able to perform its enzy-

matic functions for a longer period of time before being degraded.<sup>53</sup> We have crystallized PAH with mutation A313T, and superimposed it on the wild-type PAH. Minor differences were noted in the crystal structure of BH<sub>4</sub> binding domain. It has been proposed that BH<sub>4</sub> responsiveness resulting from BH<sub>4</sub> induced stabilization of mutant PAH dimers.<sup>54</sup> The prerequisite for BH<sub>4</sub> to function as a chemical chaperone there has to be residual activity in vivo, with enzymatic activity in expression system. A third option for BH<sub>4</sub> response is that BH<sub>4</sub> up-regulates PAH gene expression,<sup>31,55</sup> perhaps similarly to what is seen in the related and homologous enzyme tyrosine hydroxylase.<sup>56</sup>

Because PAH is a tetramer, the association of different monomers into a tetramer may vary depending on the mutations found in PAH, thus leading to functional hemizygoty that further leads to variable phenotype and variable response to BH<sub>4</sub>. More research is needed to clarify which mutations lead to better response to BH<sub>4</sub>. There is a need for more information from a new emerging discipline of genomic interaction and the effect on the proteome and the metabolome. Because the experiments presented in this study were short-term and only given at one time, it is evident that longer trial periods and double blind placebo control studies are needed in order to evaluate the effectiveness of BH<sub>4</sub> in the treatment of PKU. As to

management of PKU, BH<sub>4</sub> loading is encouraged and long-term monitoring of response is needed. It seems that treatment with BH<sub>4</sub> for all PKU patients may emerge if indeed tolerance to dietary Phe increases and blood tyrosine levels increase. Success of such a treatment should introduce a dramatic change in the way we manage PKU.

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