

## Tetrahydrobiopterin responsiveness in phenylketonuria. Two new cases and a review of molecular genetic findings

U. LÄSSKER<sup>1\*</sup>, J. ZSCHOCKE<sup>2</sup>, N. BLAU<sup>3</sup> and R. SANTER<sup>1</sup>

<sup>1</sup> Department of General Paediatrics, University of Kiel; <sup>2</sup> Institute of Human Genetics and Department of General Paediatrics, University of Heidelberg, Germany; <sup>3</sup> Division of Clinical Chemistry and Biochemistry, University Children's Hospital Zurich, Switzerland

\*Correspondence: University Children's Hospital, Schwanenweg 20, D-24105 Kiel, Germany. E-mail: laessker@pediatrics.uni-kiel.de

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**Summary:** We report two new patients with tetrahydrobiopterin (BH<sub>4</sub>)-responsive phenylketonuria and compare their phenylalanine hydroxylase (PAH) genotypes (A395P/IVS12+1g>a and R261Q/I65T, respectively) to those of previous cases from the literature. These case observations confirm earlier reports stating that BH<sub>4</sub>-responsive patients are frequently carriers of a missense mutation within the DNA region coding for the catalytic domain of the enzyme. Interestingly, many of the PAH gene mutations detected in BH<sub>4</sub>-responsive patients have been associated with an inconsistent phenotype in the past. Our case reports confirm that it is necessary to thoroughly examine individuals with increased phenylalanine levels, not only to detect BH<sub>4</sub> deficiency, but also to identify patients with PAH deficiency who may benefit from BH<sub>4</sub> treatment. In both of our patients, however, an effect of BH<sub>4</sub> (7.5 mg/kg) on plasma phenylalanine levels was not seen in the newborn period. We therefore conclude that a normal neonatal BH<sub>4</sub> test does not necessarily exclude BH<sub>4</sub> responsiveness in all such patients.

Hyperphenylalaninaemia can be caused by phenylalanine hydroxylase (PAH, EC 1.14.16.1) deficiency or by defects in the synthesis of its coenzyme, tetrahydrobiopterin (BH<sub>4</sub>). Patients whose blood phenylalanine concentration decreases upon treatment with BH<sub>4</sub> typically belong to the latter group (Blau et al 2001). Recently, however, several patients with a BH<sub>4</sub>-responsive type of PAH deficiency (hyperphenylalaninaemia, phenylketonuria (PKU), McKusick 261600) have been described (Kure et al 1999; Lindner et al 2001; Spaapen et al 2000; Steinfeld et al 2001; Trefz et al 2001). Here we report another two patients with

BH<sub>4</sub>-responsive PAH deficiency and discuss the molecular genetic basis of such cases. Furthermore, we present a novel aspect, age-dependency of BH<sub>4</sub> responsiveness, suggesting that neonatal testing is not optimal to detect all cases.

## PATIENTS

Two patients with mild PKU, a girl and a boy of northern German descent, were investigated. Phenylalanine concentrations in the neonatal screening samples were relatively low (360  $\mu\text{mol/L}$  in patient 1, 605  $\mu\text{mol/L}$  in patient 2). In both patients a defect of BH<sub>4</sub> metabolism was excluded during the neonatal period by pterin analysis in urine and by determination of dihydropteridine reductase activity in erythrocytes. Plasma phenylalanine concentrations measured by ion exchange chromatography were determined during an oral BH<sub>4</sub> loading test (Schircks Laboratories, Jona, Switzerland; 7.5 mg/kg as a single dose). These tests were performed during the second week of life and no change of plasma phenylalanine levels was observed (405  $\mu\text{mol/L}$  (0 h) and 460  $\mu\text{mol/L}$  (8 h) in patient 1, and 859  $\mu\text{mol/L}$  (0 h) and 938  $\mu\text{mol/L}$  (8 h) in patient 2).

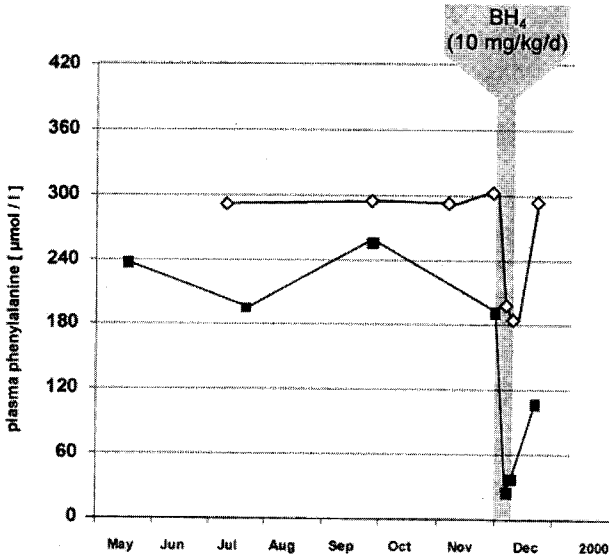
During the following years, both had very stable plasma phenylalanine concentrations on a phenylalanine-restricted diet and, at the time of this investigation at ages 5 and 9 years, respectively, phenylalanine tolerance was relatively high (46 mg/kg body weight per day in patient 1, and 20 mg/kg per day in patient 2).

At that stage, BH<sub>4</sub> responsiveness was re-evaluated in both patients under outpatient conditions but with meticulous documentation of constant phenylalanine intake. Therefore, BH<sub>4</sub> (10 mg/kg per day) divided into two oral doses was administered for 5 days. Both patients reacted with a marked decrease of their plasma phenylalanine concentration down to 62% and 14% of their starting value, respectively (Figure 1). While a significant increase of plasma BH<sub>4</sub> from 6.0 to a maximum of 22.5 nmol/L (normal 4–18) was found in patient 2 after the initiation of treatment, plasma concentrations ranged between 7.7 and 12.3 nmol/L in patient 1 before and after treatment. Similar results were found when urinary BH<sub>4</sub> excretion was measured: five days after BH<sub>4</sub> treatment was started, urinary excretion increased from 1.4 to 3.3 mmol/mol creatinine (normal 0.5–2.7) in patient 2, while no change was found in patient 1 (2.0 and 2.0 mmol/mol creatinine).

Molecular genetic analyses revealed that both patients are compound heterozygous for mutations in the *PAH* gene. In patient 1 the missense mutation A395P in exon 11 and the common splice site mutation at the donor site of intron 12 (IVS12+1g>a) was found. In patient 2 the missense mutations I65T and R261Q in exons 3 and 7, respectively, were detected.

## DISCUSSION

In this paper, two further patients with mild PKU and BH<sub>4</sub> responsiveness are reported. Although longer-term trials of BH<sub>4</sub> supplementation will be necessary to demonstrate a sustained increase in phenylalanine tolerance, this observation confirms that residual PAH activity (underlying the relatively high phenylalanine



**Figure 1.** Effect of BH<sub>4</sub> loading on plasma phenylalanine concentration in patient 1 (◇) and patient 2 (■). BH<sub>4</sub> (10 mg/kg body weight per day divided into two doses) was administered for 5 consecutive days

tolerance in our patients) can be increased in certain cases by pharmacological doses of BH<sub>4</sub>. The mechanism for this has not been fully elucidated. It has recently been suggested that patients with BH<sub>4</sub> responsiveness are carriers of at least one missense mutation of the *PAH* gene and that these mutations cluster in the region of exon 6 to the first nucleotides of exon 12 of the *PAH* gene (Erlandsen and Stevens 2001). This DNA region has been shown to encode the catalytic domain, while exons 1–5 and the second part of exon 12 together with exon 13 code for the regulatory and the tetramerization domains of the enzyme, respectively (Erlandsen et al 1997). The molecular genetic findings in our two patients are in accordance with this model, which proposes that some *PAH* mutations result in  $K_m$  variants of the enzyme. Interestingly, the R261Q mutation detected in patient 2 lies very close to one of the residues (H264) shown to interact directly with a BH<sub>4</sub> analogue in the phenylalanine crystal structure (Erlandsen et al 2000) and it is conceivable that this and other mutations will alter the tertiary structure of the catalytic domain. This may change the affinity of phenylalanine hydroxylase for its cofactor, resulting in a higher  $K_m$  for BH<sub>4</sub>.

Other genetic or nongenetic factors unrelated to the *PAH* gene may play an additional role, as recently suggested for three patients with an identical *PAH* genotype but a different response to BH<sub>4</sub> (Lindner et al 2001). In a recent meta-analysis, *PAH* mutations were classified according to their *in vivo* phenotypes (Kayaalp et al 1997) and, while the majority of missense mutations confer a consistent phenotype, some missense mutations were associated with more than one

Table 1 PAH genotype in BH<sub>4</sub>-responsive cases

Allele 1			Allele 2			Reference
Exon	Associated phenotypes <sup>a</sup>	Exon	Associated phenotypes <sup>a</sup>	Exon	Associated phenotypes <sup>a</sup>	
1	R241C	(7)	?	R111X	(11)	Kure et al 1999
2	P407S	(12)	?	R252W	(7)	Kure et al 1999
3	IVS 4-1 g>a			A373T	(11)	Kure et al 1999
4	R413P	(12)	c	R241C	(7)	Kure et al 1999
5	IVS 10-11 g>a			E390 G	(11)	Trefz et al 2001
6	A313T	(9)	?	1099 <i>ins</i> C		Spaapen et al 2000
7	V190A	(6)	?	R243X	(7)	Spaapen et al 2000
8	A300S	(8)	c   n	A403V	(12)	Spaapen et al 2000
9	R241C	(7)	?	A403V	(12)	Spaapen et al 2000
10	Y414C	(12)	c v n	Y414C	(12)	Lindner et al 2001
11	Y414C	(12)	c v n	R408W	(12)	Steinfeld et al 2001
12	K320N	(9)	?	A104D	(3)	Steinfeld et al 2001
13	A395P	(11)	v	IVS 12+1 g>a		<b>This study (patient 1)</b>
14	R261Q	(7)	c v	I65T	(3)	<b>This study (patient 2)</b>

Missense mutations considered to be responsible for the residual PAH activity in patients are underlined. This conclusion was drawn when the second mutation was a nonsense or a frameshift mutation, or in cases when the second mutation was demonstrated to result in a residual enzymic activity of < 1%.

<sup>a</sup>Associated phenotypes denotes which clinical phenotypes have been observed in patients with homozygosity or 'functional hemizyosity' for a given missense mutation (modified from Kayaalp et al 1997): c, classical PKU; v, variant PKU; n, non-PKU hyperphenylalaninaemia

phenotype and resulted in classical PKU, variant PKU or non-PKU hyperphenylalaninaemia. Interestingly, many of the mutations detected in BH<sub>4</sub>-responsive patients have been associated with an inconsistent phenotype (Table 1). Among these *PAH* mutations, two (I65T and Y414C) appeared in all three classes of phenotypes and, with the description of the I65T mutation in patient 2, both of them have now been detected in BH<sub>4</sub>-responsive patients.

This observation points to the possibility that variations in BH<sub>4</sub> metabolism among individuals with identical *PAH* mutations are responsible for the different phenotypes. For a better interpretation, one would like to know BH<sub>4</sub> tissue or plasma concentrations in individuals carrying *PAH* mutations associated with an inconsistent phenotype and/or BH<sub>4</sub> responsiveness. Differences in intestinal absorption and cellular transport, and minor alterations in metabolism of BH<sub>4</sub> could influence the results of BH<sub>4</sub> loading tests. We measured plasma concentrations and urinary excretion of BH<sub>4</sub> before and after the 5 days of our therapeutic trial, and we were surprised how these concentrations differed in our two patients despite the fact that both patients had received an identical weight-adjusted dosage of BH<sub>4</sub>.

Owing to the increasing number of BH<sub>4</sub>-responsive PKU patients, it has been suggested to test all hyperphenylalaninaemic patients by BH<sub>4</sub> loading in the newborn period (Trefz et al 2001). In many screening programmes this is already a routine practice to detect patients with congenital defects of BH<sub>4</sub> metabolism. None the less, only a relatively small number of BH<sub>4</sub>-responsive PKU patients have yet been detected. Our case report demonstrates that a normal BH<sub>4</sub> test in the neonatal period, at least with a test dose of 7.5 mg/kg, does not ultimately exclude a BH<sub>4</sub>-responsive type of PKU. It could be that other age-dependent factors play a role or that higher concentrations of BH<sub>4</sub> (e.g. 20 mg/kg) are needed for a positive loading test, as observed in patients with dihydropteridine reductase deficiency (Blau et al 2001).

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