

Long-term treatment with tetrahydrobiopterin increases phenylalanine tolerance in children with severe phenotype of phenylketonuria

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Abstract

Hyperphenylalaninemia caused by phenylalanine hydroxylase (PAH) deficiency requires lifelong rigorous diet starting in early infancy to prevent severe neurodevelopmental handicap. In a considerable number of children with mild hyperphenylalaninemia, long-term tetrahydrobiopterin (BH4) treatment significantly improves phenylalanine (phe) tolerance, but it has never been investigated in classic phenylketonuria (PKU). We performed a BH4-loading test in 40 consecutive infants with phe serum concentrations exceeding 240 μ M, who had been detected by newborn screening programs. Eighteen out of 40 infants were found to be BH4 responsive. Five of them, responding to the neonatal BH4-loading test, showed a phe tolerance of less than 20 mg/kg/day and a phe pre-treatment level of >1000 μ M. They were treated with BH4 (20 mg/kg/day) over a period of 24 months. All five children had a sustained response to BH4, allowing substantial easing of dietary restrictions. Before BH4 treatment daily phe tolerance was 18–19 mg/kg, increasing to 30–80 mg/kg on BH4 treatment and decreasing again to 12–17 mg/kg after termination of BH4 treatment. Mutation analysis revealed compound heterozygosity for a putative null and a variant *PAH* mutation in four patients and homozygosity for a variant *PAH* mutation in one patient. We conclude that BH4 sensitivity is not restricted to mild hyperphenylalaninemia and that long-term BH4 treatment may also improve phenylalanine tolerance in a considerable number of children with a more severe PKU phenotype.

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Keywords: Tetrahydrobiopterin; BH4; BH4-responsive; Hyperphenylalaninemia; Phenylketonuria; Classic; PKU

Introduction

Hyperphenylalaninemia is caused either by phenylalanine hydroxylase (PAH)¹ deficiency (Online Mendelian Inheritance in Man number 261600) or by a defect in the

synthesis or regeneration of its coenzyme tetrahydrobiopterin (BH4). While the latter requires supplementation of BH4 and various BH4-dependently synthesized neurotransmitters, normal psychomotor development can be achieved in PAH deficiency by early institution of phenylalanine (phe)-restricted diet. In 1999, Kure et al. reported for the first time on four infants with a novel subtype of BH4-responsive hyperphenylalaninemia. These infants showed a decrease of phe concentrations after oral administration of BH4 but displayed a normal BH4 metabolism and compound heterozygosity for

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¹ Abbreviations used: BH4, tetrahydrobiopterin; bw, body weight; MHPA, mild hyperphenylalaninemia; phe, phenylalanine; PAH, phenylalanine hydroxylase; PKU, phenylketonuria.

different *PAH* mutations [1]. Since then, several infants with BH4-responsive *PAH* deficiency have been reported [2–9]. Different mechanisms had been discussed to cause BH4-responsive *PAH* deficiency [2]. All hypotheses put forward to date assume an association with specific, mainly milder *PAH* mutations. Of the almost 500 *PAH* mutations known, more than 35 have been associated with BH4 responsiveness [3–5,10,11].

Muntau et al. [4] showed that BH4 significantly lowered serum phe concentrations in the majority of patients with mild hyperphenylalaninemia (MHPA), while no patients with classic phenylketonuria (PKU) had a response. This is in line with previous reports, none of which found BH4 responsiveness in patients with classic PKU [1,6–8]. BH4 responsiveness in infants with classic PKU has been reported only twice, for the first time by our group [12] and recently by Matalon et al. [13]. Long-term BH4 treatment has been described several times in children with MHPA [6,8,14,15], but was never proved in children with classic PKU. We now report for the first time the effect of long-term BH4 treatment in children with a severe PKU phenotype.

Methods and patients

BH4 loading test

Over a period of 5 years, between October 1999 and October 2004, we performed the BH4 loading test in all consecutive infants with phe serum concentrations exceeding 240 $\mu\text{mol/L}$, as detected by newborn screening programs ($n=40$). BH4 test was performed at the median age of 11 days of life. All infants received the fully active (6R)-BH4 (Schircks Laboratories, Jona, Switzerland; chemical purity 99.5%, application form: tablets solved in fluids) by mouth in one dose of 20 mg/kg body weight (bw) after a fasting period of 4 h. Phe serum concentrations were measured before, 4, 8, and in 32/40 children, 24 h after the application of BH4. During the BH4 test infants were still regularly breast- or bottle-fed. BH4 responsiveness was defined as a drop of serum phe levels 8–24 h after BH4 application by more than 30% from the value obtained before the administration of BH4 [4,9]. A defect of BH4 synthesis or regeneration was excluded in all 40 infants by measuring dihydropteridine reductase activity in dried blood filter card samples and by determination of pterins in urine samples collected before and during BH4 loading tests.

Classification

Adapted to previously established criteria, hyperphenylalaninemia was classified by using pretreatment phe levels [$>1200 \mu\text{M}$ = classic PKU; 900 – $1200 \mu\text{M}$ = mild PKU; $<900 \mu\text{M}$ = MHPA] and maximum daily phe

tolerance [$<20 \text{ mg/kg}$ = classic PKU; 20 – 25 mg/kg = mild PKU; $>25 \text{ mg/kg}$ = MHPA] [16,17]. Pretreatment phe levels were established at a median age of 9.5 days (4–12 days of life) before starting any diet or medication. Classic PKU was diagnosed in 23 children, mild PKU in five children, and MHPA in 12 children. Two children (IDs 1 and 2) with the clinical phenotype of classic PKU but a milder genotype carrying the variant mutation Y414C were classified as “atypical” classic PKU. Two children (IDs 4 and 5) showed phe pretreatment levels between 1000 and 1200 μM and a low phe tolerance of $<20 \text{ mg/kg/day}$. Due to their genotype they were classified as mild PKU, but—as their clinical course resembled that of children with classic PKU—they were included into the study (Tables 1 and 2).

Laboratory methods

Serum amino acids were analyzed by automated cation exchange chromatography (Biotronic/Eppendorf). Pterins in urine were analyzed by HPLC [18] and dihydropteridine reductase activity was measured in whole blood in filter cards as described previously [19]. Genomic DNA was amplified by polymerase chain reaction and sequenced as described previously [20]. Parental DNA analysis was performed to exclude monoallelic double heterozygosity.

Long-term BH4 treatment

Long-term BH4 treatment was started in all BH4-responsive PKU children with initial phe levels $>1000 \mu\text{M}$ and a phe tolerance of less than 20 mg/kg (IDs 1–5). As there was no need for strong phe-restricted diet, the remaining children with mild PKU or MHPA (IDs 6–18) did not receive BH4 treatment. Long-term BH4 treatment was started at a median age of 9.25 months (from 2 weeks to 42 months) and continued over a median time of 24 months (5.5–29 months). BH4 was given in a dose of 10 mg/kg bw twice a day. Phe and tyrosine serum concentrations were measured weekly or fortnightly. Phe tolerance was evaluated by repeated 3 day dietary protocols. Side effects and positive effects of BH4 treatment were controlled regularly by interviews.

Results

The BH4 loading test revealed a significant decline of plasma phe concentrations in 18/40 infants with hyperphenylalaninemia: in 3/23 infants with classic/atypical classic PKU, in 4/5 infants with mild PKU, and in 11/12 infants with MHPA (Table 1). In all BH4-responsive children, a median decrease of phe serum concentrations of 46% (12.3–82.6%) was achieved 8 h after BH4 application, and of 46% (31.6–59.6%) 24 h after BH4

Table 1
Children with BH4-responsive PKU, as detected by newborn screening

ID	PKU-form	Genotype	
		Allele 1	Allele 2
1	Atypical classic PKU	Y414C ^{a,b}	R252W ^c
2	Atypical classic PKU	Y414C ^{a,b}	R408W ^c
3	Classic PKU	A309V ^d	R408W ^c
4	Mild PKU ^e	R261Q ^{a,b}	R261Q ^{a,b}
5	Mild PKU ^e	D129G ^f	R408W ^c
6	Mild PKU	I306V ^{b,f}	?
7	Mild PKU	?	R408W ^c
8	MHPA	A403V ^{b,f}	R408W ^c
9	MHPA	A403V ^{b,f}	L41F ^d
10	MHPA	P211T ^d	P211T ^d
11	MHPA	A403V ^{b,f}	IVS10nt-11g> ^{a,c}
12	MHPA	E390G ^{b,f}	E390G ^{b,f}
13	MHPA	A403V ^{b,f}	K274fsdel11bp ^c
14	MHPA	S110L ^d	P281L ^c
15	MHPA	A403V ^{b,f}	R408W ^c
16	MHPA	?	R408W ^c
17	MHPA	A403V ^{b,f}	A300S ^{b,f}
18	MHPA	nd	nd

nd, not determined.
Mutations are printed in bold: newly identified mutations in BH4-sensitive PKU.

- ^a Variant mutation [10,17].
- ^b Previously described in BH4-responsive PAH deficiency [3].
- ^c Putative null mutation [10,17].
- ^d Unclassified mutation [10,17].
- ^e Phe pretreatment levels were >1000 μM, but phe tolerance was <20 mg/kg/day.
- ^f Mild mutation [10,17].

Table 2
Maximum phe pretreatment level and decrease of phe levels during initial phe loading test in children with BH4-responsive PKU and low phe tolerance (<20 mg/kg)

ID	Maximum pretreatment phe concentration (μmol/L)	Effect of BH4 on phe decrease (%)		Sex
		8 h	24 h	
1	1816	46.2	na	Female
2	1459	34.2	na	Male
3	1316	25.4	43.8	Female
4	1150	22.1	58.4	Male
5	1077	82.2	na	Male

na, not applicable.

application. Tyrosine concentrations remained unchanged in all children. Mutation analysis revealed heterozygosity for *PAH* mutations in 11 children and homozygosity in three children with BH4 responsiveness. In three of the children only one *PAH* mutation was identified, although all exons and splice sites had been sequenced. S110L, D129G, P211T, and A309V were newly identified in our patients to be BH4-responsive missense mutations (Table 1).

Long-term BH4 treatment was performed in 5/18 children with BH4-responsive PAH deficiency and a low phe tolerance of <20 mg/kg (Tables 2 and 3). All five children had a sustained response to long-term BH4

Table 3
Course of long-term BH4 therapy in BH4-responsive children with classic and mild PKU

ID	Phe tolerance (mg/kg/day)		Phe tolerance (mg/day)		Median phe concentrations (μmol/L)		Age at start of BH4 therapy (months)	Duration of BH4 therapy (months)
	Before (without) BH4 therapy	On BH4 therapy	Before (without) BH4 therapy	After (with) BH4 therapy	Before (without) BH4 therapy	After (with) BH4 therapy		
1	19	35	220	420	143 (18–557) ^a n = 65	299 (61–1065) ^a n = 78	18	24
2	19	80	100	850	77 (30–157) ^b n = 6	314 (36–726) n = 52	1.2	29
3	— ^c	40	— ^c	240	— ^c	293 (30–720) n = 49	0.5	8
4	— ^c	30	— ^c	240	— ^c	190 (30–490) n = 21	0.5	5.5
5	18	120	180	1800	208 (18–775) n = 75	249 (54–799) ^a n = 51	42	24

- ^a The slight increase of median phe serum concentrations on long-term BH4 treatment is associated with commencement of kindergarten and subsequent recurrent febrile infections.
- ^b BH4 treatment was started already at the age of 1.2 months. Therefore only few data on phe levels before BH4 treatment do occur and they cannot be compared to those on BH4 treatment.
- ^c In patients 3 and 4, BH4 treatment was started already at the age of 2 weeks. Therefore data on treatment before BH4-treatment do not exist.

treatment, allowing substantial easing of dietary restrictions. Before BH4 treatment daily median phe tolerance was 18 mg/kg, increasing to 40 mg/kg on BH4 treatment and decreasing again to 17 mg/kg after termination of BH4 treatment (Table 3). Serum phe concentrations increased immediately in children who had not received BH4 or had vomited it. Serum phe concentrations also increased in catabolic situations, such as fever or enteritis. As in children managed by diet alone, median phe serum concentrations increased slightly in all children with age, associated with commencement of kindergarten and subsequent recurrent febrile infections, independently if they were on long-term BH4 treatment or on diet alone.

No side effects were observed during BH4 short- and long-term treatment. In one boy, parents reported of hyperactivity and sleeplessness after the first dosage of BH4. These symptoms resolved on the second day of treatment and never recurred. Growth, length, and head circumference were within the percentiles for age and sex. All children showed normal mental and motor development, documented by regular neurodevelopmental testing. All parents reported of an increase of quality of life due to the stop or relaxation of protein-restricted diet.

Discussion

This is the first report of the effectiveness of long-term BH4 treatment in children with a severe PKU phenotype. In contrast to previous reports [4,21], our data show that responsiveness to BH4 is not restricted to mild PKU but may occur in severe phenotypes as well, defined as children with a low phe tolerance of <20 mg/kg. All children showed a sustained response to long-term BH4 treatment, resulting in a distinct relaxation of phe-restricted diet and hence an improvement of the quality of life. Remarkably, none of the infants with a BH4-responsive severe PKU phenotype carried two null mutations, but in spite of the severe phenotype one variant or mild *PAH* mutation in trans with a null mutation. A comparable result was reported by Matalon et al. [13] showing compound heterozygosity for one mild or variant *PAH* mutation and one classic *PAH* mutation in all children with BH4-responsive classic PKU. As shown by our data and also reported by Matalon et al. [12,13], one common variant mutation in classic BH4-responsive PKU is Y414C, which is associated with all different PKU phenotypes [17]. Also children with mild PKU and MHPA carried at least one mild or variant *PAH* mutation, with a high frequency of A403V in children with BH4-responsive MHPA. We identified four new BH4-responsive mutations: A309V in classic PKU, D129G in mild PKU, and P211T and S110L in MHPA. Except

D129G, which has been associated with a milder phenotype, these newly described BH4-responsive *PAH* mutations are still unclassified [10,17]. Considering that P211T occurred in homozygosity and that A309V, D129G, and S110L occurred in trans with a putative BH4-non-responsive null mutation, these *PAH* mutations may account for BH4 responsiveness. S110L is located in the N-terminal regulatory *PAH* domain, while D129G, P211T, and A309V are located in the catalytic *PAH* domain.

The effect of BH4 has been explained by several possible mechanisms [2]. BH4-responsive mutations may be located within or near the BH4 binding regions [22], but none of our newly identified BH4-responsive mutations are located within that region. Moreover, mutations in the catalytic domain of *PAH* may alter the tertiary structure of PAH, resulting in a K_m -variant of PAH which is activated by BH4 by stabilizing the PAH tetramer [6,7]. But as only a few of our newly identified BH4-responsive mutations are located within the catalytic *PAH* domain, further mechanisms may account for the BH4 responsiveness. Kure et al. [23] recently indicated that in vivo suboptimal physiological BH4 concentrations may occur and that wild-type and mutant residual PAH activity may be enhanced by BH4 supplementation. This mechanism may explain the effect of BH4 in children with a severe PKU phenotype. Although all children with a severe PKU phenotype and BH4 responsiveness carried at least one milder or variant *PAH* mutation, the severe PKU phenotype was evident in these patients. We speculate that an inconsistent expression of two different *PAH* alleles with a dominance of the null mutation may result in a severe PKU phenotype. After the application of BH4 the dominant-negative effect may be compensated resulting in a decrease of phe levels. As null mutations may not be stimulated by BH4 [5], BH4 responsiveness may only occur in those children with a severe PKU phenotype who carry at least one mild or variant mutation.

Presenting the data on the long-term BH4 treatment in patients with a severe PKU phenotype, we conclude that BH4 responsiveness is not only restricted to mild PKU and may improve phenylalanine tolerance in children with a severe PKU phenotype as well. These data have implications for clinical classification schemes and therapeutic issues. However, on BH4 treatment children significantly could ease their phe-restricted diet. The increased phe tolerance by BH4 eases management and improves the quality of life. Therefore, BH4 responsiveness should be considered in all infants with elevated phe, regardless of baseline concentrations and phe tolerance. Long-term BH4 treatment may be considered in all children with a positive BH4 loading test in infancy regardless of baseline phe tolerance and at least one milder or variant *PAH* mutation.

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