

# Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency

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Serum phenylalanine concentrations decreased in 4 patients with hyperphenylalaninemia after loading with tetrahydrobiopterin. There were no abnormalities in urinary pteridine excretion or in dihydropteridine reductase activity. However, mutations were detected in the phenylalanine hydroxylase gene, suggesting a novel subtype of phenylalanine hydroxylase deficiency that may respond to treatment with cofactor supplementation. (J Pediatr 1999;135:375-8)

Hyperphenylalaninemia is caused by a deficiency of either phenylalanine hydroxylase or its cofactor, tetrahydrobiopterin.<sup>1</sup> Accurate differentiation is required because patients with the latter disorder must be treated with the cofactor and/or neurotransmitter as early as possible to minimize irreversible brain damage. BH<sub>4</sub> and com-

bined phenylalanine and BH<sub>4</sub> loading tests are currently used for differentiation, because the serum Phe concentration decreases after BH<sub>4</sub> administration in BH<sub>4</sub> deficiency, but not in PAH deficiency.<sup>2-4</sup>

Recently, we encountered patients with mild HPA whose elevated serum Phe concentrations gradually decreased after oral administration of BH<sub>4</sub>. Urinary pteridines and dihydropteridine reductase activities were normal, suggesting that the patients were deficient in PAH rather than BH<sub>4</sub>. Because our results differed from published reports showing that patients with PAH deficiency do not respond to BH<sub>4</sub>, the nature of the metabolic defect was not clear. Molecular analysis of the *PAH* gene showed that mutations were present in all the alleles.

## METHODS

### Patients

At neonatal screening the serum Phe concentrations of patients 1 to 5 were 16, 4, 10, 12, and 16 mg/dL, respectively. All patients were treated with a low-

Phe diet (Phe, 50-60 mg/kg/d) to maintain a serum Phe level below 4 mg/dL. All were considered to have a mild form of HPA because their serum Phe concentrations never exceeded 20 mg/dL, even when they were not on the Phe-restricted diet.

BH <sub>4</sub>	Tetrahydrobiopterin
DHPR	Dihydropteridine reductase
HPA	Hyperphenylalaninemia
PAH	Phenylalanine hydroxylase
Phe	Phenylalanine
PKU	Phenylketonuria

## Biochemical Analyses

Urinary pteridine compounds were analyzed by high-performance liquid chromatography.<sup>5</sup> DHPR activity was measured in Guthrie card specimens as described.<sup>6</sup>

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

Patients received non-Phe-restricted meals 2 days before initiating the BH<sub>4</sub> loading test and during the test. The estimated Phe intake during the test was 80 to 90 mg/kg per day. BH<sub>4</sub> (Suntory, Tokyo, Japan) was administered orally in a loading test at a dose of 5 or 10 mg/kg, and blood samples were obtained at least 2 hours after each meal. Serum Phe concentrations were determined by using an automated amino acid analyzer (L-8500; Hitachi, Hitachi, Japan).

## Mutational Analysis of PAH Gene

Seven mutations prevalent in Asian patients with classical phenylketonuria

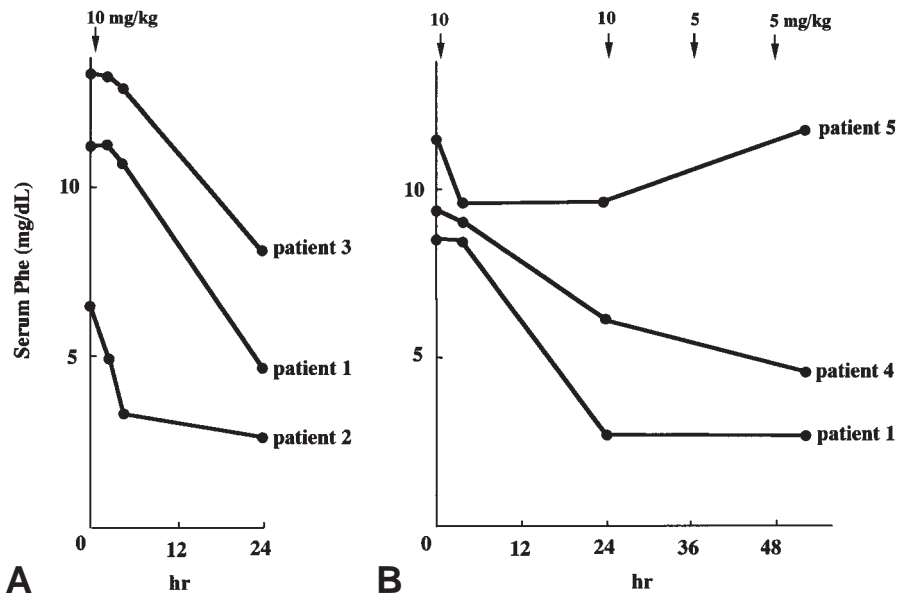
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Supported by grants from the Ministry of Education, Science, Sports and Culture and the Ministry of Health and Public Welfare, Japan.

Submitted for publication July 21, 1998; revisions received Dec 9, 1998, and Mar 26, 1999; accepted May 7, 1999.

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0022-3476/99/\$8.00 + 0 9/22/99987



**Figure.** BH<sub>4</sub> loading test in patients with PAH deficiency. **A**, Conventional protocol. Arrow indicates time of oral BH<sub>4</sub> administration (10 mg/kg body weight). Blood samples were collected to determine serum Phe concentrations at 0, 2, 4, and 24 hours after initiation of loading. Results are for patients 1, 2, and 3 during infancy. **B**, Modified protocol. BH<sub>4</sub> was administered orally 4 times. Arrows indicate times of BH<sub>4</sub> administration at 0 (10 mg/kg), 24 (10 mg/kg), 36 (5 mg/kg), and 48 hours (5 mg/kg). Blood samples were obtained at 0, 4, 24, and 52 hours. These tests were performed in patients 1, 4, and 5 at 1.3, 13, and 8 years of age, respectively.

were screened by allele-specific amplification. These mutations included R111X, IVS4-1G→A, Y204C, R241C, R243Q, Y356X, and R413P. Other mutations were identified by analyzing a minute amount of ectopically expressed PAH messenger RNA in lymphoblasts established from our patients. Briefly, the entire coding region of PAH complementary DNA was amplified by reverse-transcription-mediated nested polymerase chain reaction. Nucleotide sequences of the amplified cDNA fragments were directly determined by using a ThermoSequenase cycle sequencing kit (Amersham, Little Chalfont, England) and the A.L.F. automated DNA sequencer (Pharmacia, Uppsala, Sweden).

## RESULTS

Serum Phe concentrations were analyzed after oral administration of BH<sub>4</sub>, 10 mg/kg body weight, to 3 patients with HPA (Figure, A). Serum Phe concentrations gradually decreased, never

falling to the normal range for Phe (1 to 2 mg/dL). This type of response contrasted sharply with findings from patients with a deficiency in the BH<sub>4</sub> synthesizing system (such as that of 6-pyruvoyltetrahydropterin synthetase), in which the serum Phe concentration is typically normalized within 2 to 4 hours of administration.<sup>8</sup>

The decrease of serum Phe concentrations in our patients apparently did not reach the lowest level at 24 hours (Figure, A). To evaluate the extent to which the serum Phe concentration decreased, we modified the BH<sub>4</sub> loading protocol. The monitoring period was extended from 24 to 52 hours. Because the half-life of orally administered BH<sub>4</sub> in serum was 1.1 and 3.5 hours in rats<sup>9</sup> and humans (Suntory Co Ltd, unpublished data), respectively, BH<sub>4</sub> was administered again at 24 hours (10 mg/kg body weight) and at 36 and 48 hours (5 mg/kg body weight) to maintain high plasma BH<sub>4</sub> levels during the loading test. Patient 1 was re-evaluated according to this modified protocol at the age of 1.3 years. We were unable

to obtain parental consent to perform the modified loading test on patients 2 and 3. The serum Phe concentration gradually decreased and remained low for 24 hours (Figure, B). These findings indicate that the response of patient 1 to BH<sub>4</sub> was reproducible and excluded the possibility of a transient neonatal form of BH<sub>4</sub> deficiency.<sup>10</sup>

A gradual response has also been found in some patients with a DHPR deficiency when a low dose of BH<sub>4</sub> was used in the loading test.<sup>8</sup> Urinary pteridine analysis disclosed neopterin/total pterin ratios of 48%, 53%, and 50% in patients 1, 2, and 3, respectively (normal, 27% to 62%). DHPR activities in Guthrie card specimens from patients 1, 2, and 3 were 0.89, 0.81, and 0.94 nmol of cytochrome *c* reduced per minute per 3-mm diameter filter disk, respectively (normal, 0.8 to 1.2). These 3 patients were therefore unlikely to have biochemical abnormalities in the BH<sub>4</sub> metabolic pathway.

Patients with a PAH deficiency that responds to BH<sub>4</sub> have not been described. We therefore analyzed our patients further by mutational analysis of the *PAH* gene (Table). Five mutations (R252W<sup>1</sup>, IVS4-1G→A<sup>1</sup>, R413P<sup>1</sup>, R241C<sup>11</sup>, and P407S<sup>12</sup>) have been found in patients with classical PKU. The A373T mutation has not been described. Identification of a mutation in each *PAH* gene allele indicated that patients 1 to 3 had a PAH deficiency. Homozygotes of R252W, IVS4-1G→A, and R413P presented with clinical symptoms typical of classical PKU. Therefore these mutations appear to abolish PAH function. In contrast, the serum Phe concentrations of our patients never exceeded 20 mg/dL, even when they were not on a Phe-restricted diet, suggesting that mutant PAH molecules with P407S, A373T, and R241C have residual enzymatic activities.

If responsiveness to BH<sub>4</sub> is determined by the nature of mutations, patients who share identical mutations should respond similarly to BH<sub>4</sub>. To test this notion, we performed a BH<sub>4</sub>

**Table.** Mutations in the *PAH* gene identified in patients with HPA

Patient No.	Allele	Mutation code	Nucleotide change*	Effect on coding
1	1	P407S	C1441T in exon 12	Pro→Ser at codon 407
	2	R252W	C976T in exon 7	Arg→Trp at codon 252
2	1	IVS4-1G→A	gt→at, splicing donor site in intron 4	Splicing defect
	2	A373T <sup>†</sup>	G1339A in exon 11	Ala→Thr at codon 373
3	1	R413P	G1460C in exon 12	Arg→Pro at codon 413
	2	R241C	C943T in exon 7	Arg→Cys at codon 241
4	1	R413P	G1460C in exon 12	Arg→Pro at codon 413
	2	R241C	C943T in exon 7	Arg→Cys at codon 241
5	1	P407S	C1441T in exon 12	Pro→Ser at codon 407
	2	R111X	C553T in exon 3	Premature termination at codon 111

*Pro*, Proline; *Ser*, serine; *Arg*, arginine; *Trp*, tryptophan; *Ala*, alanine; *Tbr*, threonine; *Cys*, cysteine.

\*Numbered according to Kwok et al (1985).<sup>15</sup>

<sup>†</sup>Novel mutation identified in this study.

loading test in patient 4, whose mutations (R241C and R413P) were identical to those in patient 3 (Table). Patient 4 was an 8-year-old boy with no known relationship to patient 3. The serum Phe concentration in patient 4 decreased in response to BH4 in a manner similar to that in patient 3 (Figure, B). We then examined the response to BH4 of patient 5, a 13-year-old girl with HPA. She shared the P407S mutation with patient 1 but had a nonsense mutation, R111X, instead of the R413P mutation (Table). Her HPA was refractory to BH4 supplementation; the serum Phe concentration decreased only slightly, returning within 36 hours to the level before loading (Figure, B).

## DISCUSSION

The mechanism of BH4 responsiveness may be explained by distinct mutations in the *PAH* gene. Normal human PAH is present as a homotrimer or a homodimer.<sup>13</sup> In patient 1, who responded well to BH4 therapy, P407S and R252W subunits should associate to form various P407S/R252W heteropolymers in addition to P407S and R252W homopolymers. Patient 5 shared only one mutant allele (*P407S*) with patient 1 and responded poorly to

BH4. The other *R111X* allele is supposed to generate a truncated PAH subunit that is unlikely to associate with the P407S subunit. These observations suggested that the composition of the PAH subunits may be important for BH4 responsiveness. Namely, P407S/R252W heteropolymers or R252W homopolymers, but not P407S homopolymers, probably form mutant PAH with a high Michaelis-Menten constant  $K_m$  for BH4. It is likely that BH4 supplementation increased the intracellular BH4 concentration to restore residual PAH activity and/or to stabilize the mutant PAH molecules. Likewise, R413P/R241C heteropolymers may constitute BH4-responsive PAH subunits in patients 3 and 4.

Our results identified a novel subtype of PAH deficiency and suggest the therapeutic potential of BH4. Clinical outcomes in HPA are sometimes unsatisfactory because of limited compliance with a strict Phe-restricted diet. To date, no supportive therapy has been established to allow a diet less restrictive in Phe. Micro-encapsulated Phe lyase<sup>14</sup> and gene therapy are currently under development, but neither is presently available for clinical use. Although BH4 therapy is not always effective in PAH deficiency, it may be beneficial to a subgroup of patients with specific PAH mutations. Indeed,

the oral administration of BH4, maintained sufficiently, allowed low serum Phe concentrations in patient 1 for over 24 hours without a Phe-restricted diet (Figure, B). All patients studied here had mild HPA, representing non-PKU HPA. However, some individuals with PKU might be partially responsive to BH4. Further studies of patients with HPA associated with various mutations are necessary to evaluate the therapeutic potential of BH4 in PAH deficiency.

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