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Long-Term Treatment and Diagnosis of Tetrahydrobiopterin-Responsive Hyperphenylalaninemia with a Mutant Phenylalanine Hydroxylase Gene

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ABSTRACT

A novel therapeutic strategy for phenylketonuria (PKU) has been initiated in Japan. A total of 12 patients who met the criteria for tetrahydrobiopterin (BH_4)-responsive hyperphenylalaninemia (HPA) with a mutant phenylalanine hydroxylase (PAH) (EC 1.14.16.1) gene were recruited at 12 medical centers in Japan between June 1995 and July 2001. Therapeutic efficacy of BH_4 was evaluated in single-dose, four-dose, and 1-wk BH_4 loading tests followed by long-term BH_4 treatment, and also examined in relation to the PAH gene mutations. The endpoints were determined as the percentage decline in serum phenylalanine from initial values after single-dose (>20%), four-dose (>30%), and 1-wk BH_4 (>50%) loading tests. Patients with mild PKU exhibiting decreases in blood phenylalanine concentrations of >20% in the single-dose test also demonstrated decreases of >30% in the four-dose test. The 1-wk test elicited BH_4 responsiveness even in patients with poor responses in the shorter tests. Patients with mild HPA, many of whom carry the R241C allele, responded to BH_4 administration. No clear correlation was noted between the degree of decrease in serum phenylalanine concentrations in the single- or four-dose tests and specific PAH mutations. The 1-wk test (20 mg/kg of BH_4 per day) is the most sensitive test for the diagnosis of BH_4-responsive PAH deficiency. Responsiveness apparently depends on mutations in the PAH gene causing mild PKU, such as R241C. BH_4 proved to be an effective therapy that may be able to replace or liberalize the phenylalanine-restricted diets for a considerable number of patients with mild PKU. **(*Pediatr Res* 55: 425–430, 2004)**

Abbreviations

PKU, phenylketonuria
BH_4, tetrahydrobiopterin
PHA, hyperphenylalaninemia
PAH, phenylalanine hydroxylase

HPA results from the deficiency of PAH enzyme activity or its cofactor, BH_4. In 1999, Kure *et al.* (1) reported four patients with PAH deficiency who showed a decrease in blood phenylalanine elevations after BH_4 loading. BH_4 is known to normalize blood phenylalanine in BH_4 deficiency, but not in PKU. However, Shintaku *et al.* (2) found that 5 patients of 15 with mild HPA (serum phenylalanine <20 mg/dL) showed a gradual decrease of serum phenylalanine at 24 h with BH_4 loading, although no patient with classical PKU (serum phenylalanine ≥20 mg/dL) responded to BH_4. We examined 12 patients with BH_4-responsive PAH deficiency discovered by PKU screening and evaluated the responses in the BH_4 loading tests in terms of specific PAH mutations. The results have important implications for diagnosis and treatment of patients with BH_4-responsive PAH deficiency.

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PATIENTS AND METHODS

The 12 HPA patients detected by neonatal PKU screening (Table 1) had normal amount of pteridine in urine or serum before BH_4 loading, and also had normal dihydropteridine reductase activity according to the test using the Guthrie card. Blood phenylalanine concentrations at screening were in the range of 2.0–12.0 mg/dL. All patients were diagnosed with mild PKU, and were given the phenylalanine-restricted diets. The criterion standard was administered between June 1995 and July 2001 at 12 medical centers in Japan.

An oral BH_4 (Suntory, Tokyo, Japan) loading test was performed after demonstrating serum phenylalanine concentrations >6 mg/dL upon instituting a normal diet, which was maintained during loading tests. In the single-dose BH_4 loading test, BH_4 (10 mg/kg) was administered before breakfast; blood samples were collected at 0, 4, 8, and 24 h after loading. In the four-dose BH_4 loading test, BH_4 was administered at doses of 10, 10, 5, and 5 mg/kg at 0, 24, 36, and 48 h, respectively. Blood samples were obtained at 0, 4, 8, 24, and 52 h after loading. In the 1-wk BH_4 loading test, BH_4 was administered for 1 wk at 20 mg/kg in 3 p.o. daily. Blood samples were obtained before loading and after 4 and 7 d. Long-term BH_4 administration was started at 10–20 mg/kg into three doses daily; the dose was adjusted with the goal of maintaining serum phenylalanine concentrations between 2 and 4 mg/dL with a normal diet. Blood samples were obtained before administration, then weekly during the first month, and subsequently every 2 wk or month. All BH_4 loading tests and long-term BH_4 administration were performed after informed consent and approval of the institutional review board.

Serum phenylalanine concentrations were determined by using an automated amino acid analyzer (L-8800; Hitachi, Tokyo, Japan). Serum pteridine was measured by HPLC (LC-10; Shimazu, Kyoto, Japan) after iodine oxidation. Dihydropteridine reductase activity was measured in Guthrie card specimens as described previously (3).

Genomic DNA was prepared from white blood cells using a phenol/chloroform extraction method. Each exon and its flanking intron region were amplified with a pair of human PAH-specific oligonucleotide primers (one primer being biotinylated) using a PCR. The amplified products were purified to single-stranded DNA using streptavidin-coated M280 magnetic beads (Dynal, Oslo, Norway). The purified single-stranded DNA was sequenced by the dye terminator method using an ABI PRISM 310 genetic analyzer (PerkinElmer Instruments, Norwalk, CT, U.S.A.).

RESULTS

Single-dose BH_4 loading test. In eight patients (cases 1, 2, 5–8, 10, and 11) with initial serum phenylalanine concentrations of at least 9.84 mg/dL, serum phenylalanine decreased gradually by 20–60% of their initial values between 4 and 24 h after BH_4 loading (Tables 2 and 3). Among cases 3, 4, and 9, with initial values of approximately 5 mg/dL, cases 3 and 9 showed the above pattern, decreasing by more than 50% of the initial phenylalanine values. Case 4 failed to show a decrease in serum phenylalanine during the loading test. Thus, the degrees of decline of serum phenylalanine in a single-dose BH_4 loading test were greater than 20% in 10 of 11 patients. This test was not performed in case 12.

Four-dose BH_4 loading test. Case 4 showed a gradual decrease after 8 h, with a 37.4% decline in serum phenylalanine in 52 h (Tables 2 and 3). Case 5 showed a similar response to the single-dose case. The four doses were no more effective than one in this case. The decline in serum phenylalanine induced by the four-dose BH_4 loading test was >30% in seven (cases 1–4, 6, 8, and 12) of eight patients, ranging from 35% to 59%. This test was omitted in some patients.

One-week BH_4 administration test. In six patients (cases 1–5 and 8) BH_4 was administered at 20 mg/kg/d for 1 wk (Tables 2 and 3). In cases 2 and 4, the 1-wk BH_4 administration was initiated immediately after the four-dose BH_4 loading test

Table 1. *Blood phenylalanine (Phe) in neonatal mass-screening, and preloading blood phenylalanine and serum and urine pteridines*

				Urine (mmol/mol creat)			Serum (nM)		
Case no.	B-Phe* (mg/dL)	Age (d)	Phe (mg/dL)	Neopterin (N)	Biopterin (B)	N/B	N	B	N/B
1	6.0	14	9.8	5.7	1.8	3.2	108.2†	45.4†	2.4†
2	10.0	31	10.5	6.0	9.2	0.7	121.7	75.0	1.6
3	5.9	44	5.8	5.1	2.5	2.0	97.9	139.7	0.7
4	2.0	50	4.5	4.1	5.2	0.8	50.1	40.8	1.2
5	6.2	23	10.1	7.3	1.5	4.8	29.0†	66.5†	0.4†
6	16.0	27	11.2	3.2	2.9	1.1	—	—	—
7	8.1	30	11	36%	45%	0.8	—	—	—
8	4.0–6.0	21	19.5	11.1	11.4	1.0	270.7	197.1	1.4
9	4.0	30<	6.8	—	—	—	—	—	—
10	8.0–10.0	25	13.2	3.3	3.7	0.9	—	—	—
11	8.3	23	11.5	4.6	0.6	7.7	—	—	—
12	10–12	14.3y†	9.6‡	—	—	—	—	—	—
Control	<2	30	1.01 (0.23)§	2.09 (0.52)§	1.08 (0.36)§	2.2 (1.0)§	33.8 (4.9)§	15.0 (1.6)§	2.5 (1.0)§

* Blood Phe value by mass-screening.
† Data from four-dose BH_4 loading test.
‡ Data from four-dose BH_4 loading test with Phe-restricted diet (42 mg/kg/d).
§ Mean (SD).

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Table 2. *Blood phenylalanine concentrations (mg/dL) in single-dose, four-dose, and 1-wk BH_4 loading tests*

Case no.	Single-dose BH_4 loading		Four-dose BH_4 loading		1-wk BH_4 loading	
	Before	After 4–24 h	Before	After 52 h	Before	After 4–7 d
1	9.8	6.5	8.6	3.8	8.5	4.7
2	10.5	4.9	4.4*	4.0	4.0†	2.1
3	5.8	2.8	5.0	3.3	7.1	2.8
4	4.5	4.5	5.9	3.7	3.9‡	3.0
5	10.1	8.0	7.0	5.9	12.0	3.0
6	11.2	4.6	7.9	4.3	—	—
7	11.0	6.2	—	—	—	—
8	19.5	11.9	14.0	5.7	0.6§	4.1
9	6.8	2.8	—	—	—	—
10	13.2	7.7	—	—	—	—
11	11.5	4.4	—	—	—	—
12	—	—	9.6	4.2	—	—

* Continued immediately after the single-dose BH_4 loading test.
† Continued immediately after single-dose and four-dose BH_4 loading tests.
‡ Continued immediately after the four-dose BH_4 loading test.
§ Continued with phenylalanine-restricted diet.

Table 3. *Serum phenylalanine (Phe) decline in BH_4 loading tests, PKU genotype, and BH_4 treatment*

	Serum Phe decline (%) in BH_4 loading test			PKU genotype allele		Treatment and clinical variables				
Case no.	Single-dose	Four-dose	1-wk	1	2	Duration* (mo)	Age (mo)	Dose† (mg/kg)	S-Phe (mg/ dL)	Low-Phe diet
1	34	56	45	R241C	T278I	9	4	12.2	3.7	Combined‡
2	54	62	80	P407S	R158W	11	23	16.4	3.4	No
3	51	35	60	R413P	A132V	7	6	7.9	3.4	No
4	0	37	50	R241C	R241C	7	29	13.2	5.3	No
5	21	16	75	R241C	P281A	3	27	17.0	3.4	No
6	59	46	—	P407S	R252W	6	63	6.6	9.6	No
7	43	—	—	R241C	R413P	19	2	1.7	3.6	Combined‡
8	39	59	71	R241C	R111X	—	—	—	—	Yes
9	59	—	—	IVS4 −1g>a	A373T	56	3	8.6	5.0	No
10	42	—	—	R413P	R241C	28	2	7.3	3.1	Combined‡
11	62	—	—	R241C	R241C	—	—	—	—	Yes
12	—	56	—	R413P	R241C	—	—	—	—	Yes

* Between the first day of treatment and the last day of observation.
† The dose of last observation day.
‡ Low-Phe diet and BH_4 treatment.

without waiting for an increase in serum phenylalanine. Values from the single-dose BH_4 loading test in case 2 and the four-dose BH_4 loading test in case 4 therefore were used as the baseline for calculating degree of decline with the 1-wk administration in these cases. In case 8, the 1-wk BH_4 administration was carried out immediately by returning the patient to a normal diet, because low-phenylalanine diet therapy had been maintained until that time. Taking the value for the four-dose BH_4 loading as a baseline, the degree of further decline was calculated. As a result, serum phenylalanine concentrations in cases 3 and 5 decreased by more than 50%. The serum phenylalanine decline in case 1 was the largest observed, being >44.7% at 15 mg/kg of BH_4/d for 1 wk. Serum phenylalanine levels decreased to 3 mg/dL or less in cases 2 and 4, with degree of reduction representing more than 50% compared with the preloading values in the single-dose or the four-dose BH_4 loading test. In case 8, the serum phenylalanine level before the 1-wk BH_4 loading test was 0.56 mg/dL within the normal range, however, during this test, serum phenylalanine kept the level as almost stable as 4 mg/dL. This level did not increase under the free diet and the degree of reduction showed more than 50% compared with the preloading values in the four-dose BH_4 loading test.

Long-term BH_4 therapy. In case 1, BH_4administration at 12.2 mg/kg/d kept serum phenylalanine at <4 mg/dL with a normal diet modified only by use of phenylalanine-free milk (Table 3). Subsequently, case 1 developed normally. Cases 2 and 3 had serum phenylalanine levels below 4 mg/dL on a normal diet under BH_4 administration at 16.4 and 7.9 mg/kg per day, respectively. They had started the BH_4 treatment early, and developed normally. Cases 4 and 5 had started the BH_4 treatment at 2 y of age and kept their serum phenylalanine levels below 4 mg/dL on a normal diet by BH_4 mono-therapy, and showed normal mental development. In case 4, compliance with the low phenylalanine diet resulted in a reduced caloric intake and stunted body development. After the introduction of BH_4 treatment, the caloric intake recovered and the body development resumed normally.

In case 6, the compliance with the low phenylalanine diet had been poor, resulting in serum phenylalanine above 10

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mg/dL. BH_4 treatment was initiated at 5 mg/kg per day and then increased gradually, based on serum phenylalanine concentrations.

Cases 7 and 9 had received BH_4 treatment from the beginning in the neonatal period, and both showed a good response in the single-dose BH_4 loading test. Blood phenylalanine values were controlled by BH_4 either given alone or combined with a restricted-phenylalanine diet. BH_4 monotherapy has been pursued in case 9 for more than 4.5 y, resulting in normal physical and mental development. In case 10, BH_4 administration was discontinued for 1 y due to parental constraints, leading to high serum phenylalanine concentrations. BH_4 treatment was resumed after this hiatus, but unfortunately this treatment was stopped after 11 mo, again by the parental request. Cases 8 and 12 were treated with a low-phenylalanine diet without BH_4 administration. The way of treatment in case 11 after BH_4 loading test was unknown. Notably, no side effects were identified in the BH_4-treated cases.

Among 12 patients with BH_4-responsive PAH deficiency, 8 patients achieved their target serum phenylalanine values with BH_4 alone or in combination with a phenylalanine-restricted diet, resulting in normal development. In addition, BH_4 greatly improved patients' compliance and reduced the mental and physical burden upon both patients and families. The effectiveness, safety, and advantages of BH_4 thus were confirmed in treatment of BH_4-responsive PAH deficiency.

Mutations in the PAH gene. The results of gene mutation analysis are listed in Table 3. All but two patients were heterozygous, and two different mutation types were identified. The R241C mutation was detected in 8 (cases 1, 4, 5, 7, 8, 10, 11, and 12) of 12 patients. Interestingly, two patients (cases 4 and 11) were homozygous for this mutation. The PAH R241C mutant has been reported showing 25% of the activity of wild-type PAH in a COS cell expression analysis (4).

Among the six compound heterozygotes, T278I, P281A, R111X, and R413P were detected in the other alleles. These four mutations are detected in classical PKU and result in nonfunctional PAH alleles (5, 6). Nevertheless, eight cases with the R241C allele showed low blood phenylalanine concentrations (7.0 mg/dL on average) in neonatal mass-screening. In cases 2 and 6, P407S was detected in one allele and R158W and R252W in the other allele. Case 9 had A373T and IVS4–1g>a alleles. Mutant PAH molecules with P407S and A373T possess residual activity, like R241C (1). However, PAH alleles with R252W, R158W, and IVS4–1g>a have no activity, thus resembling the R413P allele. Two patients with the P407S allele (cases 2 and 6) showed essentially high blood phenylalanine concentrations (10–16 mg/dL) in neonatal mass-screening.

No clear correlation was evident between the PAH mutations and the degree of decline of serum phenylalanine in the single- or four-dose BH_4 loading test. In all cases, blood phenylalanine concentrations decreased gradually after BH_4 administration. However, based on the results above, R241C, P407S, A132V, and A373T alleles represented the causes of mild HPA, whereas R413P, R252W, R158W, and IVS4–1g>a alleles resulted in severe HPA. Patients with mild HPA, many of whom had the R241C allele, responded to BH_4 administration.

DISCUSSION

Among patients with mild HPA, characterized by serum phenylalanine concentrations below 20 mg/dL, a subset has shown a gradual decrease in serum phenylalanine concentrations over 1 d after BH_4 administration (1, 2, 7–15). These patients showed no abnormalities in BH_4 metabolism but had mutations in the PAH gene. Thus, a likely mechanism for BH_4 responsiveness would involve the mutant PAH molecules with a high Michaelis-Menten constant (Km) for BH_4, requiring a higher BH_4 concentration (1, 2, 7–15). Furthermore, BH_4 might stabilize the mutant PAH molecules, considering that some of the missense mutations rendered the PAH molecule unstable, leading to a shorter half-life; this could account for the gradual nature of the effect. In either case, PAH activity should increase in response to exogenous BH_4.

Accordingly, the four-dose BH_4 loading test and the 1-wk trial administration were performed in patients showing decreases in blood phenylalanine in the single-dose BH_4 loading test. The four-dose test was used to confirm a decline of phenylalanine in serum. The high-dose, 1-wk trial administration (20 mg/kg/d) was intended to increase the diagnostic accuracy and predict the effectiveness of the long-term treatment.

We found that patients with a decrease >20% in serum phenylalanine levels in the single-dose BH_4 loading test showed a similar decrease in the four-dose test. The mean degree of lowering in the single-dose test was 40% in Japan, about half of the reduction seen in Europe; the standard dose of BH_4 is 10 mg/kg in Japan, half of the 20 mg/kg dose used in Europe. These results indicated that the response to a BH_4 load was dose dependent. Although case 5 showed no enhancement of effect by the four-dose BH_4 administration, it showed a significant decrease in serum phenylalanine levels in the 1-wk BH_4 loading test. The 1-wk trial administration confirmed BH_4 effects in responsive patients, but also showed an effect in patients without any clear responsiveness in single- or four-dose BH_4 loading tests. These results indicated that BH_4 responsiveness becomes evident slowly, and in some cases it must be taken several days to lower blood phenylalanine. Lässker *et al.* (7) reported that two patients with no response to BH_4 (7.5 mg/kg) in a loading test showed a marked decrease in plasma phenylalanine after 5 d of BH_4 administration (10 mg/kg). Moreover, although the BH_4 dose (20 mg/kg) in the 1-wk trial administration was higher than the usual therapeutic dose used in BH_4 deficiency in Japan, it may represent the optimal dose in BH_4-responsive PAH deficiency, considering that 20 mg/kg is used in European countries with only minimal side effects (diarrhea and vomiting). Information from our 1-wk trial BH_4 administration is helpful in dose-setting for the long-term treatment. More generally, the 1-wk BH_4 administration is highly important in the differential diagnosis and treatment planning in BH_4-responsive HPA.

Our genetic analyses and those reported by others indicated that BH_4 responsiveness is greatly determined by mutations in

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the PAH gene, such as R241C. Spaapen *et al.* (8) reported four patients with BH_4-responsive PAH deficiency; one patient with R241C/A403V mutations showed a rapid response in a combined phenylalanine and BH_4 loading test, resulting in normalization of plasma phenylalanine within 8 h. Mutant PAH molecules with R241C and A403V mutations were found to have respective residual activities of 25% and 32% of wild-type PAH activity. However, Trefz *et al.* (9) reported one mild PKU patient with E390G/IVS10–11g>a—a missense mutation permitting some enzyme activity and another mutation precluding activity—who showed BH_4 responsiveness. They concluded that a patient with mild PKU with one mutation, resulting in limited activity, may show the BH_4 reactivity even if the other mutation abolishes activity. Mutant PAH molecules with Y414C, the most common mild PKU mutation in Northern and Western Europe (4), have residual activity resembling that with R241C. Steinfeld *et al.* (10) reported that a patient homozygous for Y414C showed a 72% decline in serum phenylalanine in a single-dose BH_4 loading test (20 mg/kg). Nuoffer *et al.* (11) described a patient with Y414C/del194 mutations and a similar response to a BH_4 load of 20 mg/kg. On the other hand, Lindner and co-workers (12) found that three patients with the same genotype (Y414C/R408W) showed differing responses in a single-dose BH_4 loading test (20 mg/kg). Blood phenylalanine levels decreased to the normal range in one patient, but not in the other two patients, despite normal BH_4 absorption; the authors concluded that BH_4 responsiveness in PAH deficiency is at least partly independent of PAH genotype. Although the first mutation, Y414C, has some residual activity (4), R408W is a known null mutation that completely abolishes enzyme activity on the affected allele and is associated with only minimal amounts of PAH-immunoreactive protein according to *in vitro* expression studies (16). Therefore, the mutant PAH molecule with Y414C/R408W catalyzed the phenylalanine-hydroxylation reaction at a lower rate than that seen in a Y414C homozygote, rendering BH_4 responsiveness in patients with Y414C/R408W more variable than in Y414C homozygote. Although no clear correlation was seen between the degrees of lowering of the serum phenylalanine in the BH_4 loading test and PAH mutations, at least one mild PKU mutation or missense mutation was found in patients with BH_4 responsiveness. Erlandsen and Stevens (13) indicated that these mutations can be located in the cofactor-binding regions or in those that closely interact with the cofactor-binding regions. However Blau and Trefz (14) reported that a patient with BH_4 responsiveness had the homozygous L48S mutation in the PAH gene that has located in the regulatory domain (exon 1–5). Muntau *et al.* (15) classified seven mutations (P314S, Y417H, V177M, V245A, A300S, E390G, and IVS4–5C->G) as probably associated with responsiveness to BH_4, six mutations (A403V, F39L, D415N, S310Y, R158Q, and I65T) as potentially associated, and four mutations (Y414C, L48S, R261Q, and I65V) as inconsistently associated with this phenotype, and stated that mutations connected to BH_4 responsiveness were predominantly in the catalytic domain of the protein and were not directly involved in cofactor binding. Therefore, the mechanism underlying BH_4 responsiveness in PAH deficiency is still incompletely understood.

Discovery of BH_4-responsive PAH deficiency has significant clinical implications. Sufficient amounts of BH_4 easily could be given to treat this disorder, inasmuch as serum phenylalanine concentrations were controlled to within 4 mg/dL by BH_4 monotherapy or by BH_4 combined with relatively mild dietary phenylalanine restriction. A rigorous restricted-phenylalanine diet involves a great deal of long-term effort for patients and their families. BH_4 treatment of responsive patients can eliminate or reduce the need for phenylalanine-restriction. Accurate diagnosis of BH_4 responsiveness therefore is very important. In this study, BH_4 responsiveness appeared to be regulated by mild PKU mutations in PAH gene, and was affected by the dose and administration period of BH_4. Based on our genetic analysis, the rate of R241C or P407S mutations among BH_4-responsive PAH deficiency is 83% in Japan. Thus, gene analysis should be performed on all HPA patients. One-week BH_4 administration at 20 mg/kg/d was the most sensitive test for diagnosis of BH_4-responsive PAH deficiency, and this additional test should be performed in all PKU patients who show more than a 20% decrease of blood phenylalanine in a single-dose BH_4 loading test, or who have R241C or P407S mutations.

Treatment with BH_4 (5–20 mg/kg/d) is a new and effective pharmacotherapy that can replace a restricted-phenylalanine diet in some mild PKU patients. BH_4 has already been marketed as an approved drug for BH_4 deficiency. The rate of BH_4-responsive PAH deficiency among PKU is about 10% in Japan, and is apparently higher than that of BH_4 deficiency. Nevertheless, BH_4 is not yet approved for BH_4-responsive PAH deficiency, and effects of high doses of BH_4 have not been examined in large numbers of patients. Caution and careful observation of clinical changes will be necessary to successfully bring BH_4 treatment to the clinic for this indication.

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