Assessment of tetrahydrobiopterin responsiveness in Turkish hyperphenylalaninemic patients

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Tetrahydrobiopterin (BH₄) therapy is the latest alternative approach in phenylalanine hydroxylase (PAH) deficiency, and is suggested for a number of hyperphenylalaninemic (HPA) patients with certain mutations. In our unit, therapeutic efficacy of BH₄ was evaluated in 20 HPA patients (4 mild HPA, 9 mild phenylketonuria-PKU, 7 moderate PKU) by a single oral dose of BH₄. Overall, 60% of the patients responded (45% favorably, 15% partially). All of the mild HPA patients and 55% of mild PKU patients responded to BH₄ favorably and an additional 11% of mild PKU patients responded partially. Of 7 moderate PKU patients, 2 responded partially (28%). The genotypes of the patients who responded to BH₄ favorably were: DelF39/-, L48S/L48S, R261Q/- (4 patients), A300S/IVS2nt5g>c, A300S/-, E390G/E390G. The genotypes of the patients who exhibited a partial response were: L48S/L48S, R261Q/R261Q, IVS10nt546/-. We concluded that since there are too many mutations and many patients are compound heterozygote, it is difficult to predict BH₄ responsiveness based solely on genotype, especially for the mutations which show inconsistent phenotypes. The best way to identify the patients who are more likely to benefit from BH₄ administration is performing BH₄ loading test. Long-term BH₄ loading test should be performed in classical and moderate PKU patients to confirm that they are not responsive to BH₄.

Key words: tetrahydrobiopterin responsiveness, hyperphenylalaninemic patients.

Hyperphenylalaninemia (HPA) is a disorder caused by a deficient or a decreased activity of phenylalanine-4-hydroxylase (PAH, EC 1.14.16.1) due to either a mutated enzyme protein or a deficiency of its cofactor tetrahydrobiopterin (BH₄)¹. Hyperphenylalaninemia due to a mutated enzyme protein produces a spectrum of disorders including classic phenylketonuria (PKU), moderate PKU, mild PKU and mild HPA². Treatment of classic, moderate and mild PKU involves a phenylalanine (Phe)-restricted diet, supplemented by a Phe-free amino acid formula, while mild HPA patients do not require therapy³. In almost all clinics, the principle of “diet therapy for life” is accepted, but patient compliance is difficult. Therefore, search for alternative therapeutic approaches has been ongoing⁴. BH₄ therapy, which is possible in patients with certain mutations, is the latest concept put forward⁵. Several independent groups have searched for BH₄ responsive mutations and possible mechanism of BH₄ responsiveness, but since there are too many mutations and many patients are compound heterozygote for the PAH gene, BH₄ responsiveness cannot be predicted based solely on the genotype⁶. In this study, therapeutic efficacy of BH₄ was evaluated in 20 HPA patients by BH₄ loading test, and the BH₄ responsiveness of common mutations seen in the Turkish population was investigated.

Material and Methods
The study group was formed by the 20 patients who were under the care of the Metabolic Unit of Hacettepe University İhsan Doğramacı Children’s Hospital (age range: 2 years 6 months-12 years). Inclusion criteria for the patients were as follows: (a) known mutations
in the PAH gene, (b) normal pterin profile and dihydropteridine reductase activity, (c) normal nutriture and no other additional chronic disease, and (d) patient or parental acceptance of the loading test.

The Phe levels at diagnosis of the patients included in the study ranged from 3.5 to 18.6 mg/dl. Sixteen of 20 patients were taking low Phe diet, with various degrees of success in attaining blood Phe levels between 3 and 6 mg/dl. Four patients were not following a low Phe diet as they were mild HPA. For diagnostic and therapeutic purposes, patients were classified according to the metabolic phenotype as Guldberg proposed in 1998. Therefore, 4 patients were defined as mild HPA (Phe levels on unrestricted nutrition under 10 mg/dl and tolerance above 600 mg Phe/day), 9 patients as mild PKU (Phe levels on unrestricted nutrition between 6 and 10 mg/dl and tolerance: 400-600 mg Phe/day) and 7 patients as moderate PKU (Phe levels on unrestricted nutrition between 10 and 20 mg/dl and tolerance: 350-400 mg Phe/day).

Parents of all children in this study signed an informed consent agreement in accord with the Helsinki Declarations of 1964, revised in Edinburgh in 2000. Our hospital Ethics Committee approved the study.

6R-BH$_4$ (Schirck’s Laboratories, Switzerland), the biologically active form, was used for all tests. Patients were instructed to continue the same dietary practice during the test. Basal blood samples were taken from all patients. Patients with basal Phe levels >10 mg/dl (8 patients) were given 20 mg/kg BH$_4$ after 3 hours of fasting and 30 minutes before the meal to ensure good absorption. For patients who could not swallow the tablets, tablets were dissolved in 20 ml water in dim light and the suspension was administered in 30 minutes. Blood sampling was then done at 4, 8, 12 and 24 hours. Patients with basal Phe levels <10 mg/dl (12 patients) were given 100 mg/kg Phe, and plasma Phe levels were confirmed to peak 3 hours after the challenge. The remainder of the test was the same as described above. Plasma Phe levels were determined using high performance liquid chromatography (HPLC) analysis.

The BH$_4$ loading test is considered positive when initial plasma Phe concentrations decrease by at least 30% after 8 hours. If plasma Phe values decreased >30% after 12-16 hours, patients were considered as partially responsive or slow responders.

Results

In our PKU patients, 9 out of 20 (45%; 4 mild HPA, 5 mild PKU) responded with a decrease greater than 30% in plasma Phe concentrations 8 hours after a single dose of BH$_4$, and in 4 of these 9 responder patients, the decrease was at least 50%. In all of the responder patients, the decrease was greater than 70% in the 24th hour. Phe levels during the test and metabolic phenotype and genotype of BH$_4$ responder patients are shown in Figure 1.

Three additional patients (1 mild PKU, 2 moderate PKU) may be considered as slow responders, as a >30% reduction was reached only 24 hours postloading. Phe levels during the test and metabolic phenotype and genotype of these slow responders are shown in Figure 2.

Eight of the 20 patients did not respond to BH$_4$. Phe levels during the test and metabolic phenotype and genotype of these patients are shown in Figure 3.

In our study, all of mild HPA and 55% of mild PKU patients responded to BH$_4$ favorably. One additional mild PKU patient (genotype: L48S/L48S) exhibited a partial response (11%) while the other mild PKU patient with the same mutation favorably responded to BH$_4$. Patients with moderate PKU did not have a favorable response to BH$_4$ loading; only 2 of them (genotype: R261Q/R261Q and IVS10nt546/-) exhibited a partial response (28%).

Discussion

Our aim in this study was to evaluate BH$_4$ responsiveness of common mutations seen in the Turkish population and to search for the optimal candidates for BH$_4$ therapy.

The most common mutation seen in the Turkish population is IVS10nt546. It is known as a null mutation which completely abolishes PAH enzyme activity on the affected allele and is associated with only minimal amounts of PAH-immunoreactive protein according to in vitro expression studies. We performed Phe/BH$_4$ loading test in 4 patients and BH$_4$ loading test in 1 patient who carry this
Fig. 1. Courses of plasma Phe concentrations during a challenge with BH₄ or Phe/BH₄ of nine good responder patients with indicated genotypes and phenotypes.

Fig. 2. Courses of plasma Phe concentrations during a challenge with BH₄ or Phe/BH₄ of three slow responder patients with indicated genotypes and phenotypes.
mutation in 1 allele. Four of them (genotype: IVS10nt546/R261Q, IVS10nt546/R261Q, IVS10nt546/-, IVS10nt546/-) did not respond to BH$_4$ while 1 (IVS10nt546/-) showed a 33% decline in Phe levels after 24 hours (a partial response). This is the second report of a splicing mutation potentially associated with BH$_4$ responsiveness$^{10}$. As previously suggested, this response can be explained assuming that BH$_4$ exerts some effect on PAH gene transcription or PAH mRNA stabilization, which would increase the levels of normally spliced transcript and thus residual PAH protein and activity$^{11,12}$.

The second common mutation in the Turkish population is R261Q$^{13}$. It causes a 30% residual enzyme activity in cell lysates and lies very close to one of the residues (H264) shown to interact directly with BH$_4$ analogue in the Phe crystal structure. It is conceivable this mutation will alter the tertiary structure of the catalytic domain. This may change the affinity of PAH for its cofactor, resulting in a higher Km for BH$_4$$^{14}$. Different study groups reported that this mutation along with other mutations in the other allele is associated with BH$_4$ responsiveness$^{15,16,17}$. Thus far, there is only one study in which responsiveness of the R261Q mutation is investigated in homoallelic state, and this patient exhibited a partial response$^{18}$. In this study, a single dose BH$_4$ loading test was performed in 4 patients (2 mild, 2 moderate PKU) who were homozygote for the R261Q mutation. Three of 4 patients did not respond to BH$_4$ while 1 moderate PKU patient showed a partial response. Phe/BH$_4$ loading test was performed to 4 other patients (2 mild PKU, 2 mild HPA) who carried this mutation in 1 allele. The mutation in the other allele was not known. All responded to BH$_4$ favorably. Two other patients with R261Q mutation in 1 allele and IVS10nt546 in the other did not respond to BH$_4$. We concluded that patients who carry the R261Q mutation in homoallelic state could be slow responders and that long-term BH$_4$ loading test should be performed in these patients. The response of the R261Q mutation to BH$_4$ depends on allelic combination.

The L48S is another common mutation seen in the Turkish population$^{13}$. It causes a variable metabolic phenotype and a 39% residual enzyme activity in cell lysates. It is located in the regulatory domain and associated with BH$_4$ response$^{14}$. Although the exact mechanism of BH$_4$ response is not known, it is proposed that the mutation causes a Km variant of the enzyme or that BH$_4$ regulates PAH gene expression in patients with this mutation$^{19}$. BH$_4$ response of 2 mild PKU patients was investigated in this study. Both were following a protein-restricted diet with poor compliance. No high Phe level (>11.39 mg/dl) was ever determined in one of

Fig. 3. Courses of plasma Phe concentrations during a challenge with BH$_4$ or Phe/BH$_4$ of nonresponders with indicated genotypes and phenotypes.
the patients, in spite of his poor compliance, while the other patient’s Phe levels reached 14.48 mg/dl, indicating their Phe tolerances were different. The patient with better tolerance responded to BH$_4$ favorably while the other patient responded partially. One can speculate that BH$_4$ responsiveness of mutations with variable metabolic phenotype may differ from patient to patient even if the same mutation is carried. This is the second report in which BH$_4$ responsiveness in PKU differs between patients with the same genotype. We concluded that L48S is associated with BH$_4$ response as in other studies and the response of the patient is related to Phe tolerance, which indicates residual enzyme activity.

The A300S is another mutation associated with BH$_4$ response and is located in the catalytic domain of PAH. Phenylalanine/BH$_4$ loading test was performed in 2 mild PKU patients with A300S in 1 allele (A300S/-, A300S/IVS2nt5g>c), both of whom responded to BH$_4$ favorably. This study supports that A300S and IVS2nt5g>c are mild mutations associated with BH$_4$ responsiveness.

The A104D mutation is located in the regulatory domain of PAH and causes a 26% residual enzyme activity. When combined with K320 and Y414C mutations in the other allele, it is reported to be responsive to BH$_4$. Our mild PKU patient with A104D in 1 allele did not respond to BH$_4$. We do not know if he carried an unknown null mutation in the other allele. We concluded that to confirm the relationship of the A104D mutation with BH$_4$ responsiveness, a BH$_4$ loading test should be performed to a patient homozygote for this mutation.

The E390G mutation is located in the catalytic domain of PAH and causes a 70% residual enzyme activity. It is known to be a mild mutation associated with BH$_4$ responsiveness. Our mild HPA patient homozygote for E390G responded to BH$_4$. However, when residual enzyme activity is high, some of the decline in the Phe concentrations after BH$_4$ may be spontaneous as previously suggested. A Phe loading test should be performed in these patients with a BH$_4$ loading test to see if the decline is more rapid.

Our mild PKU patient with delF39 in 1 allele exhibited an excellent response to BH$_4$, and BH$_4$ responsiveness of this mutation is supported.

To date, no BH$_4$ loading tests have been performed in PAH-deficient patients from Turkey. We have observed BH$_4$ responsiveness in common mutations seen in the Turkish population. All our mild HPA patients and 55% of our mild PKU patients responded to BH$_4$ favorably. An additional 11% of mild PKU patients responded partially. Our moderate PKU patients did not show a full response; 28% responded partially.

Our study was limited to loading tests performed within 24 hours after BH$_4$ administration and it was obvious that some patients responded very slowly. We concluded that long-term BH$_4$ loading test should be done in patients who have moderate or classical PKU to confirm that they are not responsive to BH$_4$. As previously reported, milder cases of PKU are more likely to be responsive to BH$_4$ treatment, since lower Phe levels and higher amounts of residual PAH can be expected.

The other point to be considered is the possibility of spontaneous Phe elimination in patients in whom Phe/BH$_4$ loading test is performed. To investigate this point, the Phe loading test should be performed in the patients who underwent Phe/BH$_4$ loading test. Nevertheless, we believe that starting daily BH$_4$ therapy in responsive patients with this information would be practical and that long-term therapy would confirm the BH$_4$ responsiveness of these patients.

Since there are too many mutations and too many patients are compound heterozygote, it is difficult to predict BH$_4$ response based solely on genotype, especially for inconsistent phenotypes. Thus, we conclude that the best means of selecting patients who are more likely to benefit from BH$_4$ administration is by performing the BH$_4$ loading test and if the patient’s metabolic phenotype is moderate or classic, long-term BH$_4$ administration would be more appropriate.

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REFERENCES


7. 34th European Metabolic Group (EMG) Meeting Zürich, May 31st -June 2nd 2002. BH4-Responsive HPA/PKU. Publication of Workshop Results.


