Assessment of tetrahydrobiopterin responsiveness in Turkish hyperphenylalaninemic patients

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Tetrahydrobiopterin (BH_4) 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 BH_4 was evaluated in 20 HPA patients (4 mild HPA, 9 mild phenylketonuria-PKU, 7 moderate PKU) by a single oral dose of BH_4. Overall, 60% of the patients responded (45% favorably, 15% partially). All of the mild HPA patients and 55% of mild PKU patients responded to BH_4 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 BH_4 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 BH_4 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 BH_4 administration is performing BH_4 loading test. Long-term BH_4 loading test should be performed in classical and moderate PKU patients to confirm that they are not responsive to BH_4.

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 (BH_4)[1]. Hyperphenylalaninemia due to a mutated enzyme protein produces a spectrum of disorders including classic phenylketonuria (PKU), moderate PKU, mild PKU and mild HPA[2]. 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 therapy[3]. 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 ongoing[4]. BH_4 therapy, which is possible in patients with certain mutations, is the latest concept put forward[5]. Several independent groups have searched for BH_4 responsive mutations and possible mechanism of BH_4 responsiveness, but since there are too many mutations and many patients are compound heterozygote for the PAH gene, BH_4 responsiveness cannot be predicted based solely on the genotype[6]. In this study, therapeutic efficacy of BH_4 was evaluated in 20 HPA patients by BH_4 loading test, and the BH_4 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 Ihsan 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[2]. 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[7].

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[8]. 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[9]. We performed Phe/BH_4 loading test in 4 patients and BH_4 loading test in 1 patient who carry this

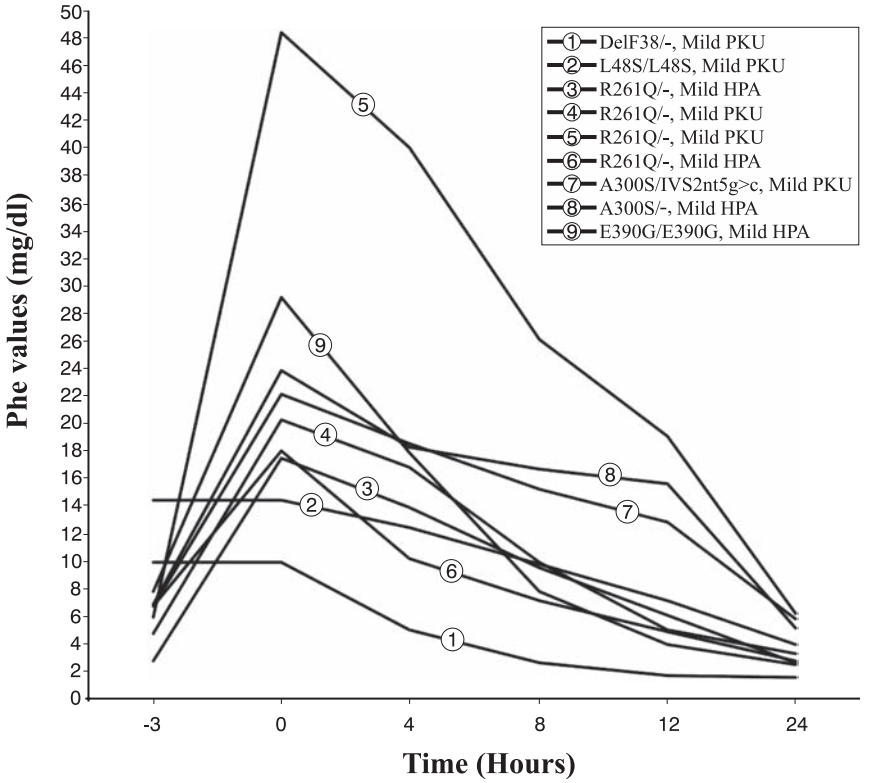

Fig. 1. Courses of plasma Phe concentrations during a challenge with BH_4 or Phe/BH_4 of nine good responder patients with indicated genotypes and phenotypes.

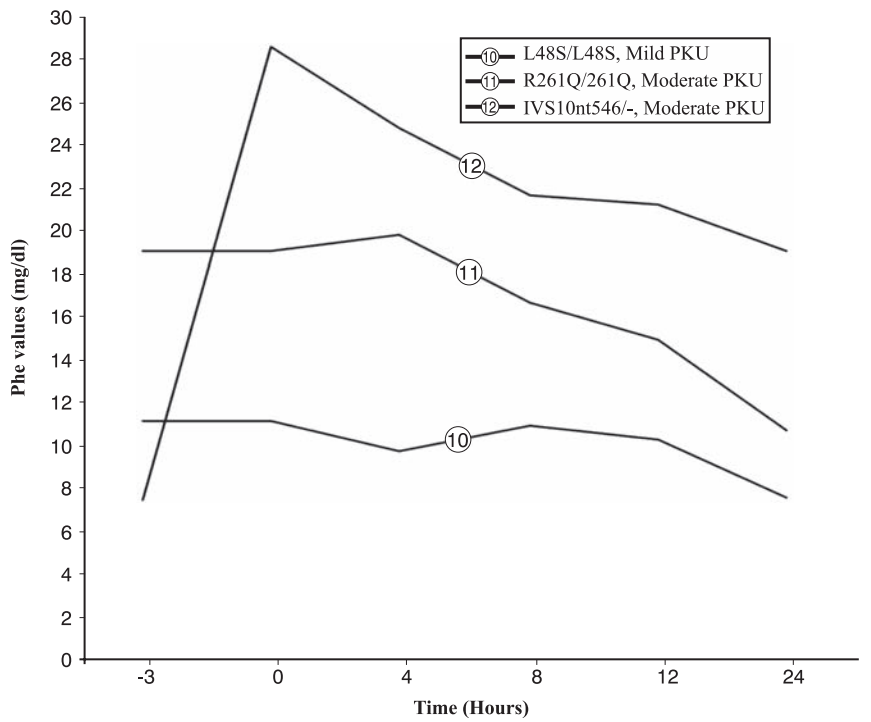

Fig. 2. Courses of plasma Phe concentrations during a challenge with BH_4 or Phe/BH_4 of three slow responder patients with indicated genotypes and phenotypes.

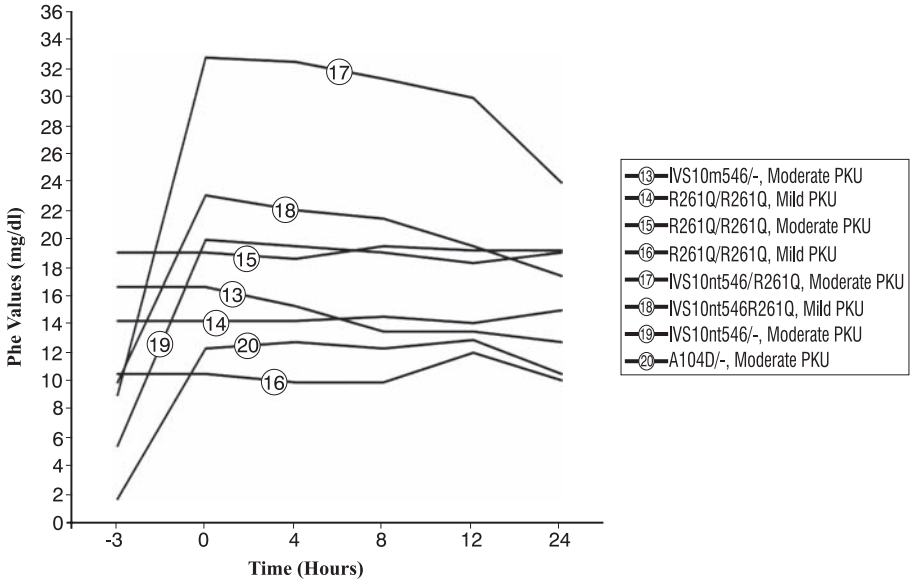

Fig. 3. Courses of plasma Phe concentrations during a challenge with BH_4 or Phe/BH_4 of nonresponders with indicated genotypes and phenotypes.

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 response 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

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[20]. We concluded that the 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[3,10,16,21] and is located in the catalytic domain of PAH[14]. Phenylalanine/BH_4 laoding 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 response[3,10,16,21].

The A104D mutation is located in the regulatory domain of PAH and causes a 26% residual enzyme activity[14]. When combined with K320 and Y414C mutations in the other allele, it is reported to be responsive to BH_4[15,22,23]. Our mild PKU patient with A104D in 1 allele did not response 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[14]. 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[21].

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[24].

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[14].

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

1. Blau N. A Differential Diagnosis and International Database of Tetrahydrobiopterin Deficiencies. In: Blau N (ed). Marburg: Tectum Verlag; 1996: 5-57.
2. Guldberg P, Rey F, Zschocke J, et al. A European multicenter study of phenylalanine hydroxylase deficiency: classification of 105 mutations and a general system for genotype-based prediction of metabolic phenotype. Am J Hum Genet 1998; 63: 71-79.
3. Muntau AC, Roschinger W, Habich M, et al. Tetrahydrobiopterin as an alternative treatment for mild phenylketonuria. N Engl J Med 2002; 347: 2122-2132.
4. Blau N, Scriver CR. New approaches to treat PKU: How far are we? Mol Genet Metab 2004; 81: 1-2.
5. Kure S, Hou DC, Ohura T, et al. Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. J Pediatr 1999; 135: 375-378.
6. Lucke T, Illsinger S, Aulehla-Scholz C, Sander J, Das AM. BH_4-sensitive hyperphenylalaninemia: new case and review of literature. Pediatr Neurol 2003; 28: 228-230.
7. 34th European Metabolic Group (EMG) Meeting Zürich, May 31st -June 2nd 2002. BH_4-Responsive HPA/PKU. Publication of Workshop Results.
8. Özgüç M, Özalp İ, Coşkun T, Yılmaz E, Erdem H, Ayter Ş. Frequency of the IVS10nt546 mutation in 44 Turkish phenylketonuria patients. Turk J Pediatr 1993; 35: 11-14.
9. Dworniczak B, Grudda K, Stumper J, Bartholome K, Aulehla-Scholz C, Horst J. Phenylalanine hydroxylase gene: novel missense mutation in exon 7 causing severe phenylketonuria. Genomics 1991; 9: 193-199.
10. Desviat LR, Perez B, Belanger-Quintana A, et al. Tetrahydrobiopterin responsiveness: results of the BH_4 loading test in 31 Spanish PKU patients and correlation with their genotype. Mol Genet Metab 2004; 83: 157-162.
11. Hyland K, Munk-Martin TL. Tetrahydrobiopterin regulates tyrosine hydroxylase and phenylalanine hydroxylase gene expression in dominantly inherited GTP cyclohydrolase deficiency. J Inherit Metab Dis 2001; 24 (Suppl 1): 30.
12. Linschied P, Schaffner A, Schoedon G. Modulation of inducible nitric oxide synthase mRNA stability by tetrahydrobiopterin in vascular smooth muscle cells. Biochem Biophys Res Commun 1998; 243: 137-141.
13. Özgüç M, Özalp İ, Coşkun T, Yılmaz E, Erdem H, Ayter Ş. Mutation analysis in Turkish phenylketonuria patients. J Med Genet 1993; 30: 129-130.
14. Blau N, Erlandsen H. The metabolic and molecular bases of tetrahydrobiopterin-responsive phenylalanine deficiency. Mol Genet Metab 2004; 82: 101-111.
15. Weglage J, Grenzebach M, Teeffelen-Heithhoff A, et al. Tetrahydrobiopterin responsiveness in a large series of phenylketonuria patients. J Inherit Metab Dis 2002; 25: 321-322.
16. Matalon R, Koch R, Michals-Matalon K, Mosley K, Stevens R. Tetrahydrobiopterin-responsive phenylalanine hydroxylase mutations. J Inherit Metab Dis 2002; 25 (Suppl): 23.
17. Doskeland AP, Flatmark T. Recombinant human phenylalanine hydroxylase is a substrate for the ubiquitin-conjugating enzyme system. Biochem J 1996; 319: 941-945.
18. International Database of Patients and Mutations causing BH4-responsive HPA/PKU (http://bh4.org/BH4DatabasesBiopku.asp).
19. Blau N, Trefz FK. Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency: possible regulation of gene expression in a patient with the homozygous L48S mutation. Mol Genet Metab 2002; 75: 186-187.
20. Lindner M, Haas D, Zschocke J, Burgard P. Tetrahydrobiopterin responsiveness in phenylketonuria differs between patients with the same genotype. Mol Genet Metab 2001; 73: 104-106.
21. Spaapen LJ, Estela Rubio-Gozalbo M. Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, state of art. Mol Genet Metab 2003; 78: 228-230.
22. Steinfeld R, Kohlschutter A, Zschocke J, Lindner M, Ullrich K, Lukacs Z. Tetrahydrobiopterin monotherapy for phenylketonuria patients with common mild mutations. Eur J Pediatr 2002; 161: 403-405.
23. Lindner M, Steinfeld R, Burgard P, Schulze A, Mayatepek E, Zschocke J. Tetrahydrobiopterin sensitivity in German patients with mild phenylalanine hydroxylase deficiency. Hum Mutat 2003; 21: 400.
24. Perez-Duenas B, Vilaseca MA, Mas A, et al. Tetrahydrobiopterin responsiveness in patients with phenylketonuria. Clin Biochem 2004; 7: 1083-1090.