

The spectrum of phenylalanine variations under tetrahydrobiopterin load in subjects affected by phenylalanine hydroxylase deficiency

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Summary A fall in blood phenylalanine (Phe) after tetrahydrobiopterin (BH₄) administration is a common trait in phenylalanine hydroxylase (PAH, EC 1.14.16.1) deficiency (McKusick 261600). To explore the extent and biological correlates of this phenomenon we studied: (a) the spectrum of BH₄ response in patients with PAH deficiency; (b) the variability of BH₄ response according to the severity of the biochemical phenotype; and (c) the variability of the response to BH₄ in subjects with the same genotype. Fifty PAH-deficient subjects (age 1 month–35 years) were enrolled for the study (5 with mild hyperphenylalaninaemia (MHPHE), 15 with mild phenylketonuria (MPKU) and 30 with classic phenylketonuria (CPKU) and underwent an identical schedule of blood samplings 24 h before and after oral BH₄ challenge (6(*R*)-BH₄, 20 mg/kg per day), leaving Phe intake unchanged. The effect of BH₄ on blood Phe concentration was evaluated according to the percent decrease of Phe during the 24 h following the challenge (criterion *a*), and as variation exceeding the individual variability of blood Phe (criterion *b*). The number of BH₄-responders according to

criterion *b* was 31 (including all the 14 detected by criterion *a*): 17 out of 30 CPKU (57%), 9 out of 15 MPKU (60%), and all the MHPHE subjects ($\chi^2 = 3.45$, $df = 2$, $p = 0.178$). The effect of BH₄ showed a large interindividual variability unrelated to diagnostic classification, basal value of blood Phe, maximum percentage of Phe reduction, Phe intake, and genotype. Some inconsistencies were found in patients with identical genotype. The first responsive case homozygous for the severe R408W mutation was found. Two new mutations, Y387X and G352C, were identified (the former was BH₄-responsive), and the responsiveness of three already reported mutations (R261Q, D338Y, T92I) was substantiated.

Phenylalanine hydroxylase (PAH, EC 1.14.16.1), the rate-controlling enzyme of phenylalanine (Phe) homeostasis converting phenylalanine to tyrosine, requires 6(*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH₄) as a cofactor. PAH deficiency (McKusick 261600) leads to different degrees of hyperphenylalaninaemia, whose clinical consequences can be prevented by early dietary treatment: the only therapy whose efficacy has so far been demonstrated (Scriver and Kaufman 2001). After about 40 years of undisputed supremacy of diet therapy, the cases reported by Kure and collaborators (Kure et al 1999) have opened the way to a possible alternative treatment of (some) phenylketonuric patients, pointing out a new subtype of PAH deficiency, i.e. the 'BH₄-responsive' form. BH₄-responsiveness was subsequently shown as a frequent trait of subjects with the milder forms of PAH deficiency (Bardelli et al 2002; Bernegger and Blau 2002; Blau and Trefz 2002; Lindner et al 2003; Matalon et al 2002; Muntau et al 2002; Spaapen et al 2001; Steinfeld et al 2003; Weglage et al 2002).

To evaluate the response of PAH-deficient patients to BH₄, a diagnostic loading procedure was adopted, giving 20 mg/kg BH₄ by mouth and assuming a decline of blood

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Phe greater than 30% after 4–8 h as a conventional threshold for BH₄-responsiveness (Bernegger and Blau 2002). This approach is in fact designed to identify levels of BH₄-responsiveness potentially relevant for therapeutic purposes and does not take into account smaller but significant Phe variations following BH₄ administration. Consequently, the labelling of a given patient as ‘nonresponsive’ might not be entirely correct from a physiological standpoint. This is relevant concerning the biological mechanisms of PAH BH₄-responsiveness. Another intrinsic limit of the current loading procedure is the fact that it does not take into account the daily variation in blood Phe concentration—assuming that blood Phe value is stable throughout the day and is reliably expressed by basal Phe (the value of Phe after an overnight fast). Contrary to this, MacDonald and colleagues (1998) showed wide variability in Phe concentration in a 24 h period in children with phenylketonuria (PKU), notwithstanding strict control of Phe intake.

The aim of the present study was to investigate the response to BH₄ in PAH-deficient patients, considering: (a) the spectrum of BH₄ response detectable, taking into account the individual daily variation of blood Phe; (b) the relationship between BH₄ response and severity of the biochemical phenotype; and (c) the relationship between the response to BH₄ and the genotype, focusing on the variability of the response in subjects with the same genotype.

Patients and methods

Patients: The study was conducted from January to December 2003. Written informed consent was obtained from each family or patient enrolled in the study. Fifty subjects (30 female and 20 male, age range 1 month–35 years) were classified according to plasma Phe concentration before treatment or under free diet (normal 30–120 μmol/L): 5 patients (age 8 months–5 years) had mild hyperphenylalaninaemia (MHPHE) (Phe < 600 μmol/L); 15 (age 4 months–24 years) had mild phenylketonuria (MPKU) (Phe 600–1200 μmol/L); and 30 (age 1 month–35 years) had classic phenylketonuria (CPKU) (Phe > 1200 μmol/L). The tolerance to Phe (Phe intake compatible with blood Phe below 400 μmol/L) was less than 450 mg/day for CPKU (range 120–450), between 600 and 1000 mg/day for MPKU, and near the normal Phe intake for MHPHE subjects. Twenty-nine patients were on diet (mean age 16.6 years, range 4 months–24 years) and 21 (mean age 15.60 years, range 1 month–35 years) were off diet when the study was performed. This group included all MHPHE subjects. Eight affected siblings from four families were included (patients 19 and 20, 22 and 23, 26 and 27, 34 and 35) (see Table 1). A defect in the synthesis or recycling of tetrahydrobiopterin was excluded by analysis of urinary pterins and dihydropteridine reductase activity in erythrocytes.

PAH genotype: To characterize the genotype of each subject, DNA was extracted from leukocytes according to standard phenol–chloroform plus SDS–proteinase K protocol. Except for exon 1 and the 5′ UTR region, which were directly sequenced, mutation analysis was performed by an exon scanning method (DGGE) (Guldberg et al 1993). The variations detected by this method were then characterized by direct sequencing, performed using Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) and analysed by an automated genetic analyser ABI PRISM 310 (Applied Biosystems). All the mutations identified were confirmed by analysing parental DNA, which also enabled us to follow the segregation of the mutations. Table 1 shows the genotypes of all the patients, classified according to the severity of their biochemical phenotype.

BH₄ loading procedure: Tetrahydrobiopterin, 6(*R*)-BH₄ (BH₄), was obtained from Schircks Laboratories (Jona, Switzerland). The conventional protocol of BH₄ loading (Bernegger and Blau 2002), which schedules blood Phe assessments before and 4, 8, 12, and 24 h (here denoted T0, T4, T8, T12, T24, respectively) after oral administration of BH₄ (20 mg/kg per day), was modified by performing an identical schedule of blood samplings in each patient the day before the BH₄ challenge (here named T00, T04, T08, T012, T024, respectively). To evaluate the possible bias arising from the lack of Phe determination between 12 and 24 h, we assessed blood Phe 12, 18 and 24 h after the basal sample in a subgroup of 8 PKU subjects: Phe value at the 18th hour did not substantially influence the trend of Phe depicted according to the values obtained at the 12th and 24th hours, or the area under the curve used to quantify the effect of BH₄ treatment (see Data Analysis for more details). We therefore decided that this sample was not necessary for the ensuing study.

In each subject, blood was collected before the corresponding meal and Phe intake was not modified during the 48 h test period. Blood amino acid concentrations were determined by high-performance liquid chromatography (HPLC) in dried capillary blood spots (Moretti et al 1990). Pterin assessment in urine was performed in samples collected before and 4–8 h after BH₄ administration using an HPLC method (Antonozzi et al 1998).

Data analysis: Quantitative data are presented as means ± standard deviations and ranges. In addition, the coefficient of variation (CV) is used to evaluate the within-day variability of blood Phe before and after BH₄ loading in each patient. Qualitative variables are presented as absolute and percentage frequencies.

Comparisons between groups (based on diagnosis, dietary control, sex, responsiveness to BH₄ challenge, etc.) were performed using Student’s *t*-test (in the case of two groups) or analysis of variance for completely randomized design

Table 1 Biochemical phenotype and genotype of the patients (left columns). BH₄-responsiveness according to the criteria (a and b) used in the present work (right columns)

Patient group (n)	Patient no.	Genotype ^a		Responders (a) ^b		Responders (b) (area) ^d
		Allele 1	Allele 2	Blood Phe (μmol/L) and decline (%)	Latency ^c	
CPKU ^e (30)						
	1	IVS10–11G>A	IVS10–11G>A			
	2 ^f	IVS10–11G>A	R176X			
	3 ^f	R261Q	R261X			
	4	R281L	R158Q			114.34
	5	R261Q	ND ^g			
	7 ^f	IVS10–11G>A	IVS10–11G>A			
	8	R243X	ND			
	10	IVS7+1A>A	IVS7+1G>A			113.72
	11 ^f	P281L	P281L			
	15	R261Q	IVS4+5G>T			2460.02
	17 ^f	L48S	L48S	401 → 226 (44)	24	502.65
	18 ^f	IVS12+1G>A	Y277C+V245A			718.87
	19 ^f	R408W	R408W			
	20 ^f	R408W	R408W	1080 → 715 (34)	24	718.87
	21	L48S	ND			
	22 ^f	R408W	R408W			6.53
	23 ^f	R408W	R408W			
	24 ^f	R261X	I65T			419.17
	25 ^f	R261Q	R243X			
	26 ^f	R158Q	IVS4+5G>T			317.22
	27 ^f	R158Q	IVS4+5G>T			1504.93
	29	R261Q	IVS10–11G>T			224.79
	31 ^f	R261Q	R261Q	857 → 561 (35)	24	1519.31
	32 ^f	R408W	IVS4+5G>T			361.53
	38	R158Q	IVS10–11G>A			604.75
	40	P281L	Y387X	2035 → 1130 (45)	24	8252.89
	42 ^f	R111X	delF39			500.84
	43	P281L	P281L			
	45 ^f	R252W	R252W			423.21
	50 ^f	IVS7+1G>A	P281L			
MPKU ^e (15)						
	6 ^f	R261Q	R158Q	528 → 340 (35.6)	4	84.41
	9 ^f	G352C	IVS10–11G>A			
	12 ^f	R261Q	R408Q			
	13 ^f	R261Q	IVS8–7A>G			
	28 ^f	L194P	R261X			
	33 ^f	IVS10–11G>A	R261Q			
	34	L48S	L48S	753 → 300 (60)	24	1197.12
	35	L48S	L48S			2602.56
	36 ^f	Y414C	IVS10–11G>A	753 → 300 (60)	12	229.20
	37 ^f	P281L	D338Y	307 → 186 (39.5)	12	1753.95
	39	R261Q	L48S	1183 → 820 (31)	8	585.11
	41	D338Y	R261X			
	43	R158Q	L48S	1242 → 857 (31)	8	68.77
	46 ^f	R261Q	R261Q	687 → 416 (39.5)	12	615.61
	47 ^f	L48S	F55fs	446 → 216 (52)	24	1287.77

(Continued on next page)

Table 1 (Continued)

Patient group (<i>n</i>)	Patient no.	Genotype ^a		Responders (a) ^b		Responders (b) (area) ^d
		Allele 1	Allele 2	Blood Phe (μmol/L) and decline (%)	Latency ^c	
MHPHE ^e (5)						
	14	A300S	R408W	258 → 128 (50)	24	386.86
	16	F55fs	IVS4+5G>T	303 → 128 (58)	4	507.18
	30	A403V	R216Q	256 → 46 (82)	8	2393.97
	48	T92I	R261Q	355 → 80 (68)	12	2512.97
	49	R408Q	Y414C	341 → 47 (76)	8	2526.12

^aMutations not so far associated with BH₄-responsiveness are shown in italic type

^bBH₄-responsiveness according to the 30% cut-off criterion (criterion *a*; see Data Analysis section for more details)

^cTime lag (hours) between BH₄ challenge and lowest concentration of blood PKU

^dCriterion *b*; see Data Analysis section for details about the computation of the area

^eCPKU, classical phenylketonuria; MPKU, mild phenylketonuria; MHPHE, mild hyperphenylalaninaemia

^fPatients on diet when the study was performed

^gND, not detected

(ANOVA, in the case of more than two groups). A mixed-model ANOVA was used to compare the Phe profile before BH₄ loading among the different subgroups of subjects. In case of nonnormally distributed matched data, the Wilcoxon test was used.

Correlation between quantitative variables was estimated using the Pearson correlation coefficient, while association between qualitative variables was tested by the chi-square test. Yates correction was applied when appropriate. To evaluate the effect of BH₄ treatment, two different criteria were applied, both using each patient as his or her own control. Criterion *a*: Each patient was classified as a responder when at least one measurement of blood phenylalanine during the 24 h following the challenge showed a 30% decline with respect to the basal value (usual criterion). Criterion *b*: The effect of BH₄ was evaluated taking into account the within-day variability of blood Phe. For each patient a reference range of 'basal' blood Phe values was built, based on the patient's basal Phe T0 and on the standard deviation (SD) of the Phe values detected in that patient before BH₄ loading on the first day of observation (that is, the SD of T00, T04, T08, T012, T024). Thus, for each patient the 95% reference range, based on the hypothesis of normal distribution of Phe values (which was verified), was T0 - 1.96SD to T0 + 1.96SD. The lower limit of such a reference range (i.e. T0 - 1.96SD) was then used as a threshold for assessing the patient's responsiveness to BH₄. Therefore, the patient was classified as responsive when at least one of the concentrations of blood Phe after BH₄ loading declined below the threshold. Finally, a quantitative estimate of BH₄ effect was obtained by evaluating for each BH₄-responsive patient the total area between the threshold (T0-1.96SD) and the portion of the curve (plotting Phe values T0 to T24) below the threshold (Fig. 1).

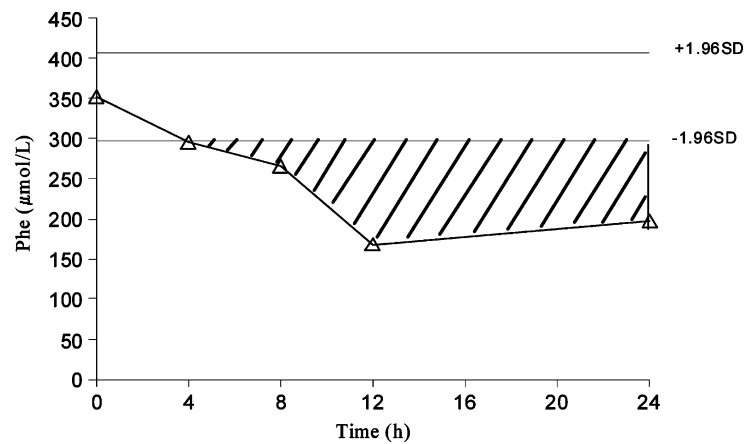
Results

Table 1 reports for each responsive subject (classified according to the usual criterion for BH₄ responsiveness—criterion *a*) the ranges of Phe reduction, the percentage of Phe decline from basal value, and the time lag to reach the lowest value of Phe after the loading. The different basal concentrations depend on the fact that some patients were on diet while others were off diet when the study was performed. Four out of 30 patients affected by CPKU (13.3%) proved to be responsive, reaching the lowest value of Phe 24 h after BH₄ loading. In the responsive MPKU subjects (8 out of 15; 53.3%), the maximum Phe reduction occurred 4–24 h after the loading. Finally, all 5 patients affected by MHPHE (100%) were responsive to BH₄ from 4 to 24 h after the challenge. The difference between diagnostic groups for the percentage of responders was statistically significant ($\chi^2 = 17.91$, *df* = 2, *p* < 0.001).

Under BH₄, the concentrations of Phe became normal or nearly normal in 6 subjects—one MPKU subject (case no. 36) and all MHPHE subject (cases 14, 16, 30, 48, 49). In the other responders, Phe remained well above the normal range.

The effect of BH₄ was also evaluated taking into account the basal daily variation of blood Phe, which was assessed during the 24 h period before BH₄ loading (criterion *b*). As a general remark, a relevant change of the CV of Phe concentrations was found after BH₄ administration (T00–T024: mean 11, SD 5, range 3–25; T0–T24: mean 17, SD 15, range 5–84), denoting an effect of the drug as well as the presence of different clusters (responders and nonresponders) inside the sample. Although there was some daily variation of blood Phe concentrations in the basal condition, no significant differences were detected when the concentrations

Fig. 1 Graphic representation illustrating the method used to measure the effect of BH₄, which was computed as the area bounded by the threshold for BH₄-responsiveness (T0-1.96SD of blood Phe concentrations detected in basal condition) and the curve of Phe values T0 to T24 (hatched area)



observed at each time point from T00 to T012 were compared between and within diagnostic groups (main effect of time: $F(3, 141) = 1.88$, $p = 0.149$; interaction diagnostic group \times time: $F(6, 141) = 0.04$, $p = 0.999$), indicating the lack of a circadian rhythm. Therefore, subjects were classified as responsive when the concentration of blood Phe after BH₄ loading declined over the individual cut-off determined according to the second criterion (see Data Analysis section). After BH₄ administration, 31 out of 50 subjects (62%) in the whole group showed a reduction of Phe above their own individual threshold at least in one of the four subsequent assessments. In addition to the 17 subjects detected as responders by the 30% criterion (Table 1), 14 further patients were classified as responders by this approach: 13 CPKU, and 1 MPKU (Table 1). The two classification criteria were in accordance (Yates corrected $\chi^2 = 13.44$, $df = 1$, $p < 0.001$), but showed different sensitivity. On the whole, once the daily oscillations of Phe in basal conditions were taken into account, BH₄ administration resulted in a relevant variation of Phe concentrations in 17 out of 30 CPKU (57%), 9 out of 15 MPKU (60%), and in all the MHPHE subjects (100%). This last group turned out to be responsive whatever the detection criterion. The difference between diagnostic groups was not significant ($\chi^2 = 3.45$, $df = 2$, $p = 0.178$). Sex and age did not influence the effect of BH₄.

The quantitative measures of BH₄ effect (computed as shown in Fig. 1) are summarized in Table 1. A wide interindividual variability characterized the area under the threshold, whose values ranged from 6.53 (case 6) to 8252.89 (case 40). No significant differences were found between the three different diagnostic groups (1207.39 ± 1970.98 , 936.06 ± 854.48 and 1665.42 ± 1114.25 for CPKU, MPKU and MHPHE, respectively; $F(2, 28) = 0.33$, $p = 0.723$) or between on-diet and off-diet subjects (1234.06 ± 1932.11 and 1164.16 ± 1070.87 , respectively; $t(29) = 0.12$, $p = 0.905$). Moreover, there was no correlation between basal value of Phe (T0) and the area ($r = 0.2767$, $p = 0.132$) when the sample of responsive subjects was taken as a whole, while

a weak correlation was found for responsive subjects with CPKU ($r = 0.5174$, $p = 0.033$). The correlation between the area and the maximum percentage reduction of Phe values after BH₄ loading (taken with its sign) was weak and not significant ($r = -0.336$, $p = 0.065$). In any case, the negative value of the coefficient denotes that larger areas correspond to larger maximum percentage reductions. Finally, no difference was found concerning this area when all responsive subjects were split in two groups (responders and nonresponders) according to the criterion $a(1582.63 \pm 1937.03$ and 740.89 ± 841.29 , for responders and nonresponders, respectively; $t(29) = 1.51$, $p = 0.142$). The widest area under the line of -1.96 SD (8252.89) was detected in case 40, a CPKU who underwent the loading test before starting the diet and responded to BH₄ administration with a decline of over 50% in blood Phe.

Following the administration of BH₄, a marked increase in urinary excretion of pterins was found in all the subjects. The mean concentration of total biopterin in urine was 2.5 mmol/mol creatinine (SD 1.2, range 0.5–6.5) (normal 0.4–2.8) before and 9.0 mmol/mol creatinine after BH₄ (SD 8.7; range 1.55–49) (Wilcoxon's paired test $p < 0.001$). The median values were 2.17 and 6.4, respectively. No significant difference was found in the excretion of biopterin either between different diagnostic groups or between responders and nonresponders.

The biochemical phenotype was consistent with genotype in most of the patients. The few inconsistencies concerned R261Q (CPKU in patient 31 and MPKU in patient 46), L48S (MPKU in patients 34 and 35 and CPKU in patient 17), and R158Q/IVS4+5G>T (CPKU in patient 29 and MPKU in patient 33) (Table 1). Data concerning patient 20, homozygous for the R408W mutation, are reported below. Patient 18, a compound heterozygote carrying two mutations in *cis* (Y277C+V245A), associated with the severe IVS12+1G>A, responded with a maximum decline of Phe (26%) 12 h after the loading (area 718.87). Other responsive mutations were Y387X (a new mutation we found

associated with P281L in patient 40: maximum Phe reduction 45%, area 8252.89), and T92I (associated with R261Q in patient 48: maximum Phe reduction 68%, area 2512.97), while the D338Y mutation acted as responsive when associated with P281L (patient 37: maximum Phe decline 39.5%, area 1753.95) but not with R261X (patient 41). Another new PAH mutation, namely G352C located in the catalytic region (exon 10), was found in a nonresponsive MPKU subject (patient 9), also carrying the severe IVS10–11G>A mutation.

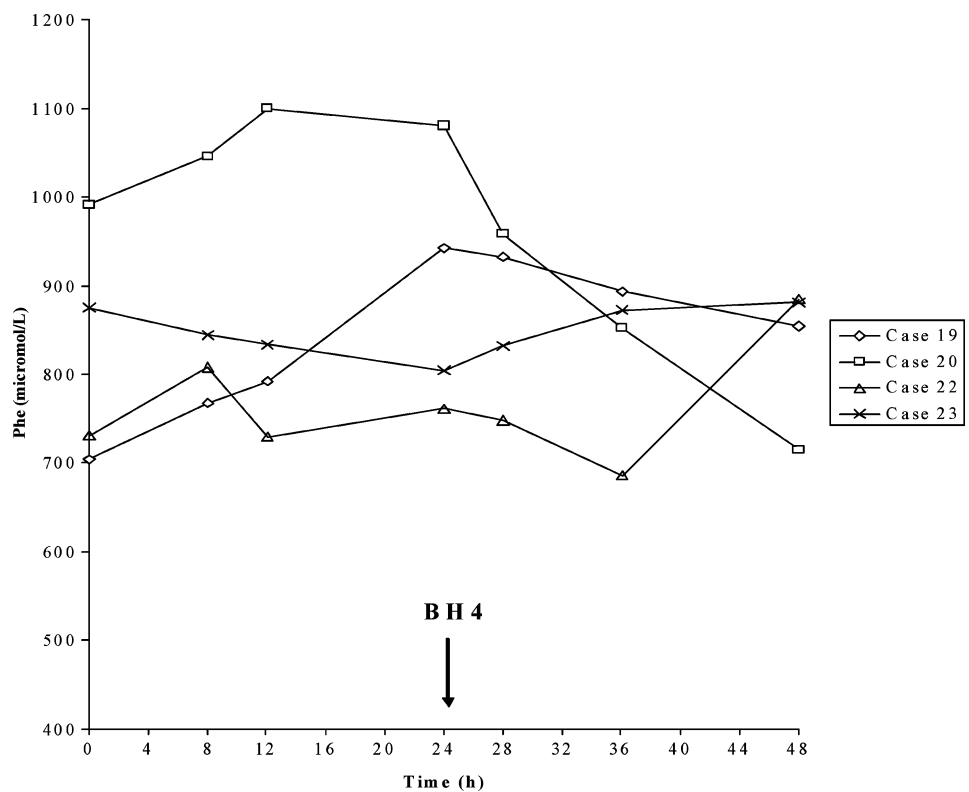
As a final point, we analysed the interindividual variability of the response in PKU subjects with the same genotype. Figure 2 shows the time course of Phe before and after BH₄ challenge in two couples of siblings homozygous for R408W mutations (patients 19 and 20, 22 and 23). Patient 20, an 8-month-old girl, was affected by a severe form of PKU (pretreatment blood Phe > 2500 µmol/L, Phe tolerance less than 20 mg/kg bw per day), that required several months of strict treatment before blood Phe was under control. BH₄ supplementation resulted in a decrease of Phe concentration, reaching the lowest level 24 h after the loading (34% reduction; area 2480.86). A similar phenotype, but not the BH₄-responsiveness, was found in her older brother as well as in the other R408W siblings (although patient 22 was marginally responsive according to criterion *b*, area 6.53). All three subjects carrying the L48S mutation in homozygous form were responsive to BH₄. Two of these (patients 17 and 34) also fitted the 30% cut-off criterion 24 h after the loading. In the two brothers (patients 34 and 35) Phe declined

60% and 24.64%, respectively, while the corresponding areas were 1197.12 and 2602.56, respectively. The incongruence between percentages of Phe decline and areas arose from the different stability of Phe values in basal conditions in these two subjects, notwithstanding a similar Phe intake. Patients 31 and 46, homozygous for the R261Q mutation, were both responsive to BH₄ (Phe decline 35% and 39.5%; areas 1519.31 and 615.61, respectively). Two sisters carrying R158Q/IVS4+5G>T genotype (patients 26 and 27) were both responsive according to criterion *b*, while a discordance was found in two patients with R261Q/IVS10–11G>A genotype: only patient 29 responded to BH₄ (maximum Phe decline 16.68%, area 224.79). Finally, in two patients homozygous for the severe IVS10–11G>A mutation, the trend of Phe was not influenced at all by the administration of BH₄.

Discussion

Loading procedure and BH₄ responsiveness: After the report by Kure and colleagues (Kure et al 1999), a number of retrospective papers, small series and single case reports focused on the effect of BH₄ in PHA-deficient subject (Lindner et al 2003; Matalon et al 2004; Spaapen et al 2001). In the largest series published so far (Bernegger and Blau 2002), among 278 subjects loaded with 6(*R*)-BH₄, a decline of Phe greater than 30% (4 and 8 h after the loading) was detected in 65%, 74% and 33% of patients with initial Phe levels of

Fig. 2 Results of BH₄ loading in two couples of siblings homozygous for the severe R408W mutations, affected by classical PKU. In patient 20, BH₄ supplementation results in decrease of Phe levels, which reaches its lowest point (about 40%) 24 h after the loading



120–400, 400–800 and 800–1200 $\mu\text{mol/L}$, respectively. The percentage of responders dropped as basal plasma Phe exceeded 1200 $\mu\text{mol/L}$. A prospective study was carried out by Muntau and colleagues (2002), who explored the efficacy of BH_4 by performing a combined Phe (100 mg/kg bw)/6(R)- BH_4 (20 mg/kg bw) loading test and analysing the *in vivo* rates of [^{13}C]Phe oxidation in 38 children with PAH deficiency, classified according to the same criterion as used in the present work. A decline of more than 30% in Phe (assessed before and 4, 8 and 15 h after BH_4 challenge) was found in all of 10 MHPHE patients, in 17 of 21 MPKU patients, and in none of 7 CPKU patients. Accordingly, the cumulative recovery of labelled carbon dioxide increased significantly in 23 out of 31 BH_4 -responsive subjects and in none of 7 CPKU patients. This paper confirmed BH_4 responsiveness as a common trait of the mildest forms of PAH defect, and, importantly, proved that the beneficial effect of *in vivo* BH_4 is the result of increased oxidation of Phe.

Under the same conditions of loading, a possible bias in the interpretation of BH_4 responsiveness (resulting in a higher percentage of responsive subjects among CPKU subjects) could arise from the classification of the biochemical phenotype. Recently, Desviat and colleagues (2004) reported the response to BH_4 in 31 PAH-deficient patients who were classified according to their tolerance to Phe. Classical and moderate PKU patients (Phe tolerance < 250 and 350–550 mg/day, respectively) had diagnostic blood Phe higher than 1200 $\mu\text{mol/L}$ (as our CPKU patients) and showed a similar response to BH_4 loading test. Moreover, the diagnosis performed according to the tolerance to Phe was generally congruent with that based on the basal concentrations of Phe (grouping together classical and moderate PKU, only one patient was differently classified by the two criteria). Therefore, the low frequency of BH_4 -responders among CPKUs observed by these authors and the high frequency that we (by criterion *a*) and other (Matalon et al 2004) have found, seem to be an inherent characteristic of the diverse samples rather than the consequences of diagnostic bias.

In all of the aforementioned studies, the trend of blood Phe under BH_4 loading was assessed with reference to basal value of Phe, i.e. the value of blood Phe after an overnight fast (challenge with BH_4), or 3 h after Phe loading (combined Phe/ BH_4 loading), and the response was evaluated according to a conventional 30% cut-off. The reliability of the cut-off and the solidity of the basal value have not been assessed so far. Although advantageous on clinical grounds, this approach gives only limited physiopathological information on the effect of BH_4 , since a significant amount of information could be lost using a cut-off point set to increase the specificity of the test. Moreover, the wide diurnal variability in blood phenylalanine concentrations found in children with PKU (MacDonald et al 1998) could affect the interpretation of the loading test, making it sometimes difficult to distin-

guish between the spontaneous and induced decline of Phe. Finally, expressing the effect of BH_4 in terms of the percentage of Phe reduction is misleading as far as the actual enzymatic activity is concerned. The hydroxylation of 400 or of 40 $\mu\text{mol/L}$ of Phe implies different activity of PAH on the substrate, even if both result in a 20% decline in blood Phe when the basal values of the amino acid are 2000 and 200, respectively.

To ascertain the extent of BH_4 response in PAH-deficient patients, we tested the response to BH_4 in the framework of the individual daily variation of blood Phe, leaving the Phe intake unchanged during the test (criterion *b*). According to this approach 56% of CPKU, 60% of MPKU and all MHPHE patients showed a detectable response to BH_4 , including all the patients whose response fitted with the 30% criterion and 14 additional subjects (13 CPKU and 1 MPKU). Moreover, once the effect of BH_4 was evaluating using a parameter relating the degree and the duration of the response, no clear difference was found between responsive subjects belonging to the three different diagnostic categories. This implies that in some PAH-deficient subjects, whatever the severity of the enzymatic defect (and the resultant biochemical phenotype), a residual capacity for Phe removal is preserved, and, through unknown mechanisms, activated by BH_4 . These results suggest that the severity of PAH deficiency is only one of the determinants of the response to BH_4 .

As regards the loading procedures, a combined Phe/ BH_4 test was suggested in patients with blood Phe values lower than 400 $\mu\text{mol/L}$ (Bernegger and Blau 2002). We did not find any correlation between basal values of Phe and response to BH_4 . Moreover, in our on-diet patients as well as in MHPHE patients, the low basal value did not affect the possibility of detecting the responsiveness to BH_4 .

Genotype, biochemical phenotype and BH_4 -responsiveness: A number of molecular mechanisms have been postulated to explain the response to BH_4 in PHA-deficient subjects (Blau and Erlandsen 2004; Blau and Trefz 2002; Erlandsen and Stevens 2001; Erlandsen et al 2004; Kure et al 1999; Matalon et al 2004; Spaapen and Rubio Gozalbo 2003): some residual PAH activity has been considered as a common prerequisite for BH_4 -responsiveness (Blau and Erlandsen 2004; Erlandsen et al 2004; Muntau et al 2002). Although the present work was not aimed at focusing on the mechanism of BH_4 effect, our results make the picture more complex.

The majority of responsive patients carried a genotype compatible with the presence of residual enzymatic activity. Focusing on conditions that had not been reported so far, we demonstrated the responsiveness of the R261Q mutation in two homozygous patients (all R261Q BH_4 -responsive subjects so far reported were compound heterozygous (Bardelli et al 2002; Desviat et al 2004; Lässker et al 2002; Spaapen and Rubio-Gozalbo 2003). In patient 18 we found two

mutations in *cis* (Y277C+V245A) and a severe mutation IVS12+1G>A on the other allele. The Y277C mutation has not so far been associated with BH₄-responsiveness, while V245A has been found in MHPHE BH₄-responsive patients (Lässker et al 2002; Muntau et al 2002; Spaapen and Rubio-Gozalbo 2003). In accordance with theoretical expectations, our patients exhibited a 26% decline of blood Phe, much less than that reported in subjects carrying the sole V245A mutation (Muntau et al 2002; Spaapen and Rubio-Gozalbo 2003). We detected a new mutation (Y387X) in a responsive subject affected by CPKU. Considering that the associated P281L is a severe mutation (www.pahdb.mcgill.ca), we ascribed BH₄-responsiveness to Y387X, which causes the lack of the C-terminal part of the protein, disturbing the assembly of the tetramer (Chehin et al 1998). We also detected the responsiveness of the T92I mutation (regulatory domain) (www.pahdb.mcgill.ca).

In some compound heterozygotes, the detection of the inactivation of one of the two mutations simplified the interpretation of their biochemical phenotype. In patients 16 (F55fs/IVS4+5G>T) and 24 (R261X/I65T) the response to BH₄ was influenced by I65T and IVS4+5G>T mutations, respectively, since the transcript arising from the second allele (R261X and F55fs, respectively) underwent to nonsense-mediated decay (Dr Carla Carducci, personal communication).

Finally, a number of observations in our study remain to be explained. D338Y, a mutation localized in the catalytic site (exon 10) never reported as BH₄-responsive, acted as responsive when associated with the severe P281L mutation and as nonresponsive when coupled with R261X. We report the first responsive patient carrying the R408W mutation in homozygous form. This severe mutation, located at the boundary between the catalytic and the tetramerization domains, results in less than 1% of residual enzymatic activity (www.pahdb.mcgill.ca). With the only exception of the case reported by Steinfeld and colleagues (2003), where R408W was coupled with IVS10–11G>A, in BH₄-responsive subjects the R408W mutation has always been associated with less severe mutations (Lindner et al 2003; Muntau et al 2002; Spaapen and Rubio-Gozalbo 2003). What is more, when our patient's brother and a different couple of siblings were tested, they were all nonresponsive to BH₄. As a last point, the analysis of the response of BH₄ in a number of subjects with the same genotype, both related and unrelated (present study; Bardelli et al 2002; Blau and Trefz 2002; Hennermann et al 2002; Koch et al 2002; Kure et al 1999; Lässker et al 2002; Lindner et al 2001, 2003; Muntau et al 2002; Spaapen and Rubio-Gozalbo 2003; Spaapen et al 2001; Trefz et al 2001), shows that even if most of the patients behave in the same way under BH₄ challenge (or therapy), a few notable exceptions remain to be explained: R261Q/R158Q (present study and Spaapen

and Rubio-Gozalbo 2003), R261Q/R243X (present study and Spaapen and Rubio-Gozalbo 2003), and R408W/R408W (present study) genotypes. The reasons for this interindividual variability of the response are unknown. Some recent papers have advocated a possible interindividual variability in BH₄ absorption and pharmacokinetics (Dhondt et al 2003; Fiege et al 2003), while a similar pattern of BH₄ bioavailability in all the patients has been found by others (Desviat et al 2004). Although this is an important issue, which deserves to be explored further, it cannot provide an explanation for those conditions in which a response to BH₄ should not be found but in fact is found (as for null or severe mutations associated with CPKU) (present data and Matalon et al 2004).

In conclusion, the response to BH₄ is a very common trait in subjects with PAH defect and involves all forms of PAH deficiency, but not all subjects in each form. Moreover, the drug results in a decline of blood Phe that (a) does not always respect the residual enzymatic activity as inferred on the base of the genotype and (b) shows a certain inconsistency in subjects with the same genotype. These findings cannot be completely explained in the present framework of the physiopathology of PAH and require further study.

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