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Unresponsiveness to tetrahydrobiopterin of phenylalanine hydroxylase deficiency

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Abstract

Conflicting results have been reported concerning the efficacy of tetrahydrobiopterin (BH4), the cofactor of phenylalanine hydroxylase, for reducing phenylalanine (Phe) concentration in phenylketonuria (PKU). We aimed to test quantitatively the effects of BH4 in PKU patients. Seven fully characterized patients were selected among a population of 130 PKU subjects as harboring PKU mutations predicted as BH4 responsive and previously considered responsive to a cofactor challenge. They received a simple Phe (100 mg/kg) and 2 combined Phe (100 mg/kg) and BH4 (20 mg/kg) oral loading tests. Cofactor was administered either before or after the amino acid. The concentrations of Phe, tyrosine (Tyr), and biopterin were measured over 24 hours after loading. The comparative analysis of the loading tests showed that in all patients plasma Phe concentrations peaked within 3 hours, and fell within 24 hours by about 50% in benign, 20% in mild, and 15% in severe phenylalanine hydroxylase deficiency regardless of BH4 administration. A consistent or moderate increase of plasma Tyr, again independent of the cofactor challenge, was observed only in the less severe forms of PAH deficiency. Mean blood biopterin concentration increased 6 times after simple Phe and 34 to 39 times after combined loading tests. The administration of BH4 does not alter Phe and Tyr metabolism in PKU patients. The clearance of plasma Phe after oral loading and, as well as Tyr production, is not related to cofactor challenge but to patient's phenotype. The assessment of BH4 responsiveness by the methods so far used is not reliable, and the occurrence of BH4-responsive forms of PKU still has to be definitely proven.

1. Introduction

Phenylketonuria (PKU, OMIM 261600) is primarily due to more than 500 mutations in the gene for phenylalanine-4-hydroxylase (PAH, EC 1.14.16.1), leading to a defective enzyme. Because PAH is the main determinant of phenylalanine (Phe) homeostasis and disposal, the impairment of its activity results in hyperphenylalaninemia (HPA) and reduced tyrosine (Tyr) production. The biochemical phenotype of PKU is mostly complicated in severe untreated forms by permanent neurologic damage, as accumulated Phe itself is a neurotoxic molecule.

An artificial diet able to reduce the Phe dietary intake and HPA was introduced in the 1950s and still remains the cornerstone of treatment of PKU. After its inception and diffusion, refinements of diet products and supplementation with Tyr, micronutrients, and long-chain polyunsaturated fatty acids further improved compliance and effectiveness. For many years, the dietary Phe restriction was stopped at the beginning of school age, whereas in the last decades many studies showed that diet discontinuation is unavoidably followed by a decline in intellectual performance with increasing behavioral disturbances. A "diet for life" was then encouraged [1,2]. However, distortion of the normal habit and culture of nutrition, as well as unsatisfactory organoleptic characteristics of artificial foods, may lead to social isolation and make dietary treatment of PKU a difficult option in the teenage years and when diet reintroduction is necessary, like before and during pregnancies of affected women.

Alternative ways of treatment are now being explored, such as liver transplantation [3] or repopulation [4], enzyme replacement with PAH [5] or enzyme substitution with Phe

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ammonia lyase [6], and somatic gene therapy [7]; but they still lie at the experimental stage, as seldom appropriate or limited for duration or effectiveness.

Within this framework, the observation that some PKU patients could respond to the oral administration of synthetic tetrahydrobiopterin (BH4), the nonprotein cofactor of PAH, by lowering their Phe plasma level [8] encouraged a large number of studies [9-14]. Most of them claimed that BH4 responsiveness might be shared by a consistent population of PKU patients generally affected by milder forms of the disease. The rationale for this therapy, however, is scanty. Blood biopterin concentration is elevated rather than decreased in HPA genetically determined by PAH deficiency [15], and the cofactor also acts as an inhibitor of PAH activation on the increase of plasma Phe concentration [16]. Moreover, there is no consensus as to the gold standard method for the assessment of BH4 responsiveness; and cofactor effectiveness was only evaluated by means of rough clinical procedures.

We therefore elected to perform a quantitative self-controlled study by comparing Phe and Tyr metabolism after a simple Phe and 2 combined Phe and BH4 loading tests in PKU patients who harbor mutations in the PAH gene already reported as BH4 responsive and who could be considered "responsive" after a previous cofactor challenge.

2. Patients and methods

2.1. Case selection

Patient population consisted of 130 subjects affected by PAH deficiency who had a definite diagnosis after the search of PKU causal mutations and the exclusion of BH4 deficiency. As concerns the latter, all these PKU patients had been loaded with BH4 and assigned to 3 classes of PAH deficiency—severe, mild, and benign—according to the metabolic phenotype and the genotype-phenotype correlation [17]. Seven patients, representative of the 3 classes of PAH deficiency, were elected for further investigation on the basis of the following requisites: (*a*) patients' informed assent or parents' consent; (*b*) good compliance at the follow-up for type and amount of diet received, and accuracy of plasma Phe monitoring; and (*c*) prediction of BH4 responsiveness on the basis of the genotype [18] and of a plasma Phe reduction greater than 40% 24 hours after a classic (20 mg/kg) oral BH4 loading test [19].

2.2. Patients' genotyping and phenotyping

Patients' biochemical phenotype was assessed on the basis of the dietary tolerance to Phe, recorded during the first year of life. Two standards of tolerance were considered. Maximal Phe tolerance was intended as the maximal Phe daily intake allowed to maintain plasma Phe levels steadily lasting less than 600 μmol/L; and minimal Phe tolerance, as the minimal daily Phe intake sufficient to push Phe levels up to 120 to 180 μmol/L [17]. Patients' genotyping was performed on peripheral blood cells by standard methods, as earlier described [20].

2.3. Loading tests with Phe and BH4

A self-controlled study was developed by applying to each patient 3 types of oral loading tests. A simple loading with 100 mg/kg Phe (L-Phe; Merck, Darmstadt, Germany), a combined loading with 100 mg/kg Phe followed after 3 hours by 20 mg/kg BH4 (6R-L-*erythro*-5,6,7,8-tetrahydrobiopterin; Schirks Laboratories, Jona, Switzerland) administration, and a combined loading with BH4 (20 mg/kg) followed after 3 hours by Phe (100 mg/kg) administration.

The tests were performed after an overnight fast according to the procedures already reported [21], but with some modifications: (*a*) normal levels of plasma Phe were attained before the test by adjusting the diet to the minimal Phe tolerance; (*b*) the duration of the tests was extended to 24 hours after Phe administration; (*c*) during the test, all patients had a protein-free, normocaloric diet supplemented with an amino acid mixture free from Phe and Tyr (Milupa TYR 2; Nutricia, Schiphol, the Netherlands) to avoid any additional Phe and Tyr intake.

Phenylalanine and Tyr were measured in plasma by high-performance liquid chromatography before and after Phe administration at −3, 0, 1.5, 3, 6, 9, 12, and 24 hours.

Total biopterin was measured in dried blood spots according to the method of Zurflüh et al [22] in the Division of Clinical Chemistry and Biochemistry, University Children's Hospital, Zurich (courtesy of Prof Dr N Blau).

Statistical analysis was performed using the Statistical Package for Social Sciences version 13.0 (SPSS, Chicago, IL). Normality of continuous variables was checked by the Shapiro-Wilk test. In case of normal distribution, the Student *t* test for paired samples was applied. In case of nonnormal distribution, the Wilcoxon test was used.

3. Results

The genotypes and the clinical and metabolic phenotypes of the 7 PKU patients are given in Table 1, including the outcome of the neonatal BH4 loading test. Two of the patients (patients 2 and 4) had a single PAH mutant allele reported as allowing BH4 responsiveness, whereas 5 of 7 had both [18]. Patients' phenotyping confirmed that the values of minimal and maximal Phe tolerance are equivalent in severe PAH deficiency, whereas mild and benign forms show doubled or tripled values of Phe tolerance, respectively, by increasing the dietary Phe intake (Table 1) [17]. Patients belonging to the three classes of PAH deficiency showed different hydroxylating capacity after an oral load of 100 mg/kg Phe, a feature not modified by the administration of synthetic BH4. The outcomes of the simple Phe loading test and of the combined loading tests with Phe and BH4 in cases belonging to the severe, mild, and benign class of PAH deficiency are reported in Figs. 1 to 3.

Table 1
Phenotype, genotype, and outcome of a neonatal BH4 loading test in 7 patients affected by PAH deficiency

Patient no.	Age (y)	Weight (kg)	Dietary Phe tolerance (mg/d)		Mutant PAH alleles	Reduction of plasma Phe 24 h after the BH4 loading test	Class of PAH deficiency
			Minimal[a]	Maximal[b]			
1	2	12	240	<250	IVS10nt-11g>a/R158Q	42%	Severe
2	3	13	240	<250	G218V/R158Q	48%	Severe
3	21	59	300	700	L48S/S67P	70%	Mild
4	7	19	290	400	Y343C/L48S	60%	Mild
5	1.5	12	300	550	R261Q/R261Q	82%	Mild
6	13	29	390	>1000	A403V/R158Q	92%	Benign
7	1.5	12	360	>1000	R261Q/E390G	95%	Benign

[a] Minimal daily Phe intake resulting in plasma Phe concentration of 120 to 180 μmol/L.
[b] Maximal daily Phe intake allowed to keep plasma Phe concentration less than 600 μmol/L.

Plasma peak levels of Phe were attained in all patients within 3 hours after loading, irrespective of the administration of BH4 and of the impairment of PAH activity. Patients with benign PAH deficiency showed lower Phe peak levels, which decreased by about 50% within 24 hours after Phe loading, regardless of BH4 administration. Higher plasma Phe level and slower Phe clearance were observed in the severe and mild forms, with a decrease of 15% and of 20%, respectively, regardless of BH4 administration. After the simple Phe loading, a consistent increase of plasma Tyr was observed in patients with benign PAH deficiency and a moderate increase was found in patients with the mild form, whereas no increase was observed in patients with severe PAH deficiency. In each case, the administration of BH4 after or before Phe loading did not alter the time course and the levels of Tyr concentration.

Statistical analysis of variations of plasma Phe and Tyr concentrations, area under curve, and slope of the curve in the 3- to 24-hour interval after Phe administration within the same class of PAH deficiency showed no significant differences among the 3 types of loading.

The kinetics of blood biopterin during the loading tests is reported in Fig. 4. The administration of Phe alone induced in all patients a sustained rise of plasma biopterin, with peak levels about 6 times higher as a mean with respect to basal levels and still lasting after 24 hours. During the combined loading tests, a sharp increase of total biopterin concentration was observed in all patients, with peak levels 3 to 6 hours

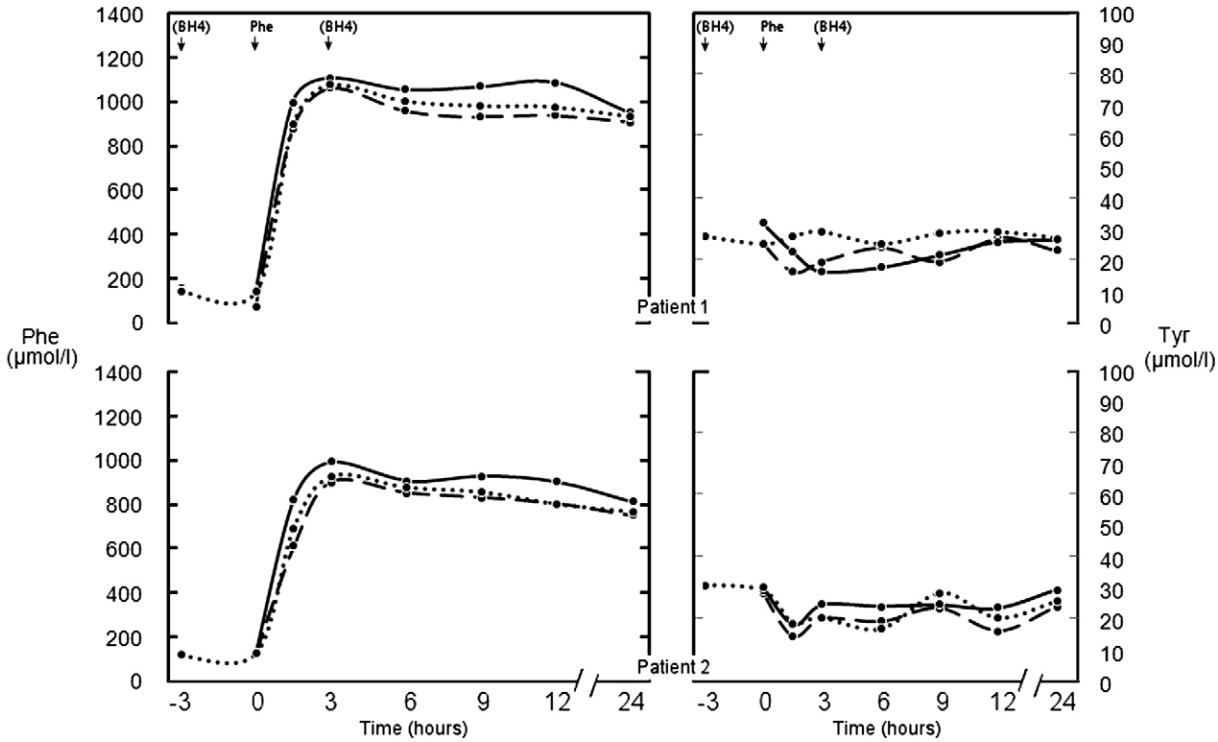

Fig. 1. Time course of plasma Phe and Tyr in patients 1 and 2 affected by severe PAH deficiency during simple Phe loading (100 mg/kg, continuous line) and combined Phe (100 mg/kg) and BH4 (20 mg/kg) loading. Tetrahydrobiopterin was administered 3 hours after (dashed line) or before (dotted line) the Phe load.

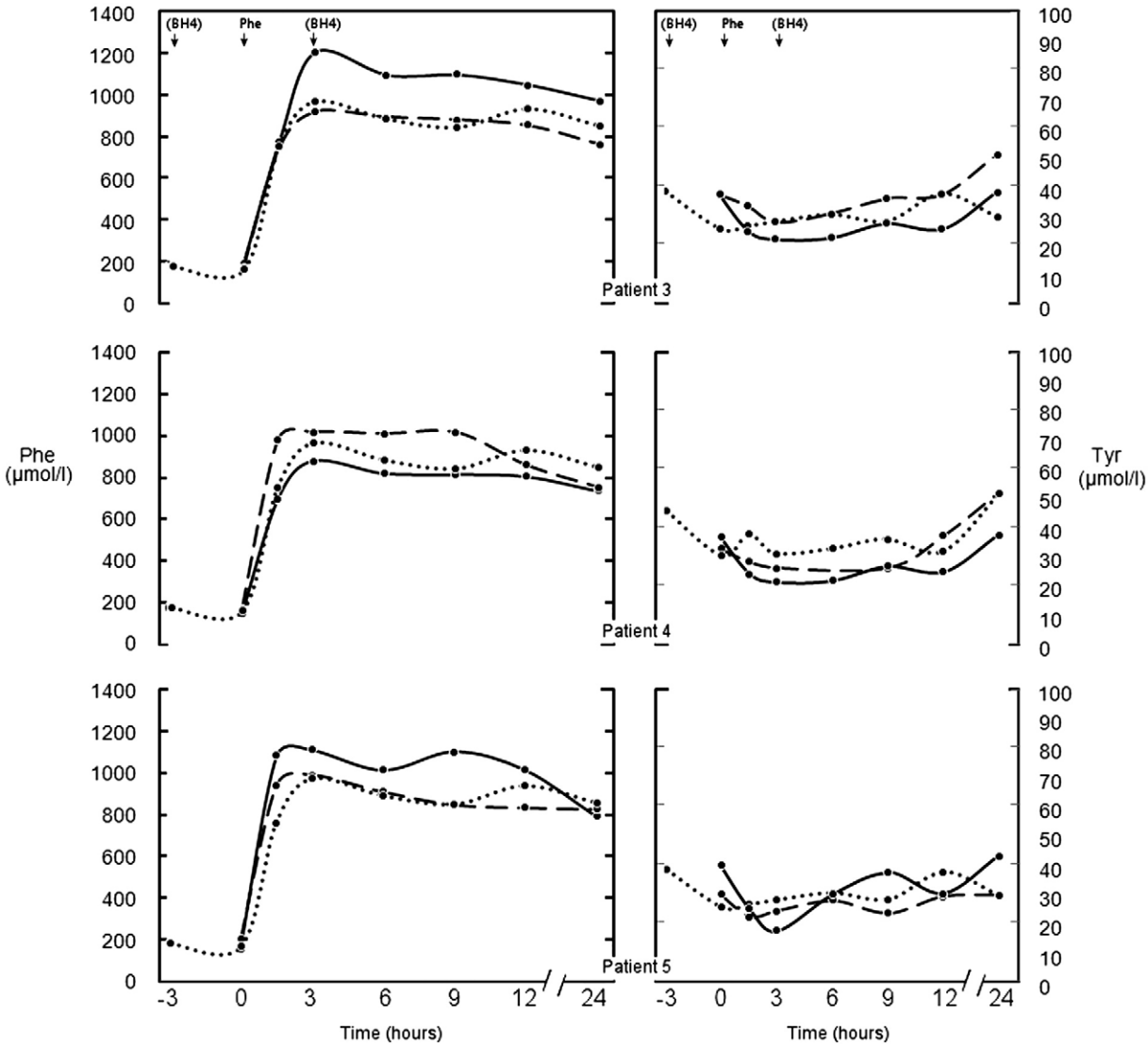

Fig. 2. Time course of plasma Phe and Tyr in patients 3, 4, and 5 affected by mild PAH deficiency during simple Phe loading (100 mg/kg, continuous line) and combined Phe (100 mg/kg) and BH4 (20 mg/kg) loading. Tetrahydrobiopterin was administered 3 hours after (dashed line) or before (dotted line) the Phe load.

after BH4 administration, followed by a rapid decrease. With respect to basal values, biopterin peak levels were 34 times higher when Phe administration preceded that of BH4 and 39 times higher when Phe administration followed that of BH4.

4. Discussion

A positive response of PKU patients to BH4 was first reported in 4 Japanese children apparently affected by nonsevere forms of the disease [8], and an increasing number of pilot studies confirmed and extended this finding in the last years [9-14]. Arbitrarily assuming that a decrease of plasma Phe concentration greater than 30% within 24 hours after BH4 administration is indicative of responsiveness to the cofactor, up to 85% of patients affected by nonsevere PKU and some affected by the classic form [9,11] were considered responsive. This peculiarity was originally suggested to be a result of a K_m mutant PAH enzyme, displaying a reduced binding affinity for BH4 [8]. This occurrence has been clinically ruled out, however, since the inception of the selective screening for BH4 deficiency [23]. Actually, only 60% of the mutations harbored by PKU patients are located in the catalytic domain of PAH; and very few are located within the cofactor binding region or show reduced binding affinity [24-28].

Other mechanisms have been postulated on the basis of present knowledge about PAH regulation by Phe or BH4. Many cases of PKU are proposed to be the result of mutations that interfere with PAH folding and oligomerization, leading to an increased rate of enzyme degradation by the ubiquitin proteasome-dependent pathway [25,29,30]. A preventive, chaperon-like chemical activity of BH4 has been investigated by in vitro experiments [26,27], as well as its

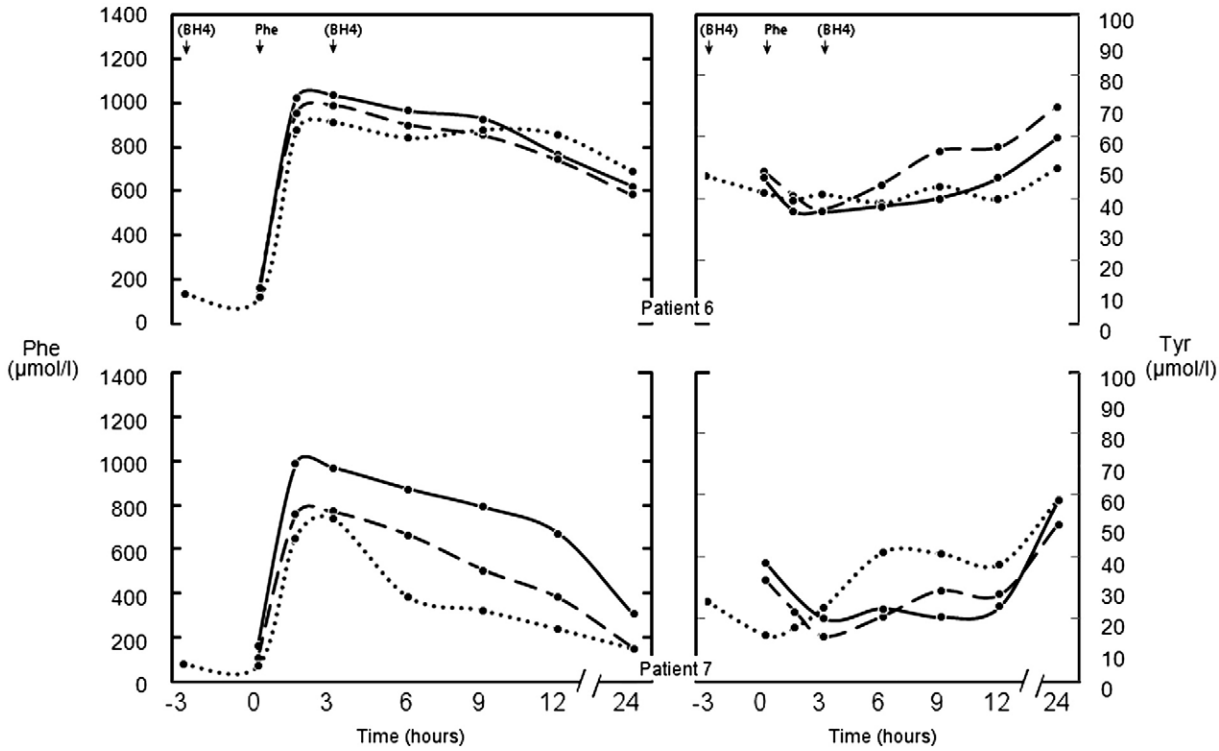

Fig. 3. Time course of plasma Phe and Tyr in patients 6 and 7 affected by benign PAH deficiency during simple Phe loading (100 mg/kg, continuous line) and combined Phe (100 mg/kg) and BH4 (20 mg/kg) loading. Tetrahydrobiopterin was administered 3 hours after (dashed line) or before (dotted line) the Phe load.

ability to prevent peroxide formation, possibly due to uncoupled reaction of Phe hydroxylation and cofactor oxidation [27,31]. None of the BH4-responsive mutations, however, showed uncoupling when kinetically characterized [27]. Animal experiments recently suggested, but did not prove, that BH4 supplementation might increase PAH activity or regulate PAH gene expression and mRNA stabilization [32,33].

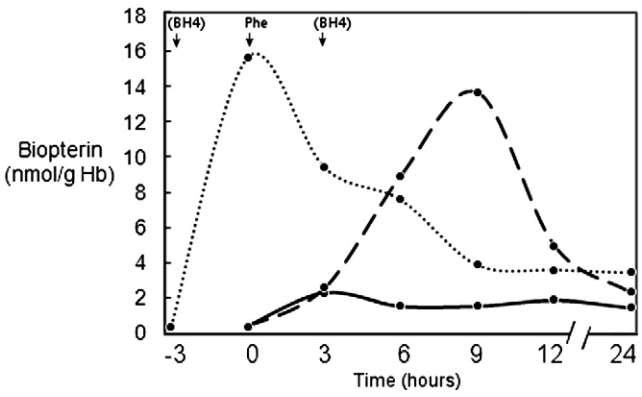

Fig. 4. Mean concentration of blood biopterin in the 7 patients affected by PAH deficiency during simple Phe loading (100 mg/kg, continuous line) and combined Phe (100 mg/dl) and BH4 (20 mg/kg) loading tests. Tetrahydrobiopterin was administered 3 hours after (dashed line) or before (dotted line) the Phe load.

Thus, despite intensive investigations, no molecular mechanism supports PKU responsiveness to cofactor, which only relies on the blood Phe variations subsequent to BH4 administration in patients with spontaneous or induced HPA [9-11]. Three larger studies on this topic have appeared in the last year [12-14]. The definition of responsiveness to cofactor and its prevalence among PKU patients was first investigated using a 20-mg/kg BH4 challenge over 24 hours. By assuming that a decrease of 30% or more in plasma Phe level 24 hours after BH4 loading was indicative of responsiveness, 135 out of 293 patients (46%) were considered responders and so candidates for cofactor treatment [12]. A phase II, open-label screening study was independently performed on 485 PKU patients who received 10 mg/kg BH4 once daily for 8 days. Ninety-six patients (20%) were considered responders to cofactor on the basis of a reduction of at least 30% in plasma Phe level from baseline to day 8. One hundred twenty-seven patients (26%), however, had increased Phe in plasma [13]. A phase III, double-blind, placebo-controlled study was finally performed on 88 of these responder patients. Forty-one received 10 mg/kg BH4 once daily for 6 weeks; but only 18 (44%), and none with basal plasma Phe greater than 1200 μmol/L, had again a plasma Phe reduction of 30% or more from baseline. A similar proportion of patients assigned to receive either cofactor or placebo (28/88, 32%), however, had increased plasma Phe. On the basis of these data, the authors concluded that some patients responsive to cofactor

are suitable for this treatment as an adjunct to a low-Phe diet and even a replacement [14].

A number of evidences, however, weaken this suggestion. The prevalence of BH4-responsive cases appears to be heavily reduced as cofactor administration is prolonged (from 50%-85% in pilot studies to 46% in the screening study, to 20% in the phase II study, and to less than 10% in the phase III study). A large proportion of patients, already identified as responsive, afterward resulted to be unresponsive; and a consistent proportion of patients showed under treatment an increase of plasma Phe.

Such erratic results appear largely dependent on the poor accuracy of the procedures so far used. Patients of phase II and III studies were selected among those who had relaxed or abandoned a strict low-Phe diet, possibly sharing residual PAH activity and so belonging to the forms of PKU more sensitive to fluctuations of Phe dietary intake. As they were simply advised to adhere to their current diet during the treatment with cofactor, the observed variations of plasma Phe, positive or negative, are within the range of diurnal variations that can occur in PKU patients under these conditions [34]. Moreover, the definition of BH4 responsiveness was arbitrarily chosen on the basis of a reduction of at least 30% in plasma Phe after treatment, irrespective of the basal level of the amino acid. It is obvious that, with regard to the amount of Phe hydroxylated, a 30% reduction from a basal level of 600 μmol/L plasma Phe in a patient approximates a 10% reduction in another patient with the same weight and with a basal level of 1800 μmol/L plasma Phe. This deceptive procedure unavoidably led to the identification of "responders" among patients with milder forms of PKU and moderate HPA. Actually, the main determinants of the plasma Phe variations in PKU are represented by the patients' residual PAH activity and Phe tolerance, body weight, dietary Phe intake, and basal Phe level, whereas the Tyr production is the major index of the Phe hydroxylation rate [35,36]. These basic aspects, however, were totally or partially disregarded in the above studies; and any comparison under the same clinical and experimental conditions between patients challenged and unchallenged with BH4 was omitted. More recently, 2 studies claimed that the treatment with BH4 may increase Phe tolerance in patients affected by milder forms of PKU [37,38]. Unfortunately, their basic Phe tolerance had not been correctly assessed before the cofactor challenge; and the values of Phe tolerance obtained after the treatment match those characteristic of these subjects [17].

To correctly evaluate this matter, we compared the outcomes of a simple Phe and of 2 combined Phe and BH4 loading tests in a self-controlled study. Similar procedures had been repeatedly recommended [25,39] but never applied. The high-dose BH4 loading test [19,40] and the combined loading test [21] were originally introduced by us to screen BH4 deficiency among neonates with HPA and were also shown to be suitable to analyze Phe and Tyr metabolism in older patients [41]. With these methods, we already stated that BH4 administration is totally ineffective in altering the time course of plasma Phe concentration. In the present study, the loading tests were made more accurate and quantitative by excluding any additional Phe and Tyr intake, and extended to 24 hours after Phe administration. Standardization of the whole procedure provides reproducible results and the comparison among patients with different phenotype or on different treatment. As the amount of administered Phe matches the daily intake of the amino acid in subjects on a free diet and the cofactor dose is within the suggested therapeutic range, the simple Phe and the combined Phe and BH4 loading tests well comply with the conditions of spontaneous HPA off and on treatment, respectively.

Seven fully characterized PKU subjects were selected among a population of 130 PKU patients as core representative of cases "responsive" to the cofactor administration because of sharing genotypes described as responsive and because of appearing to be "responders" to a simple BH4 loading test. Ineffectiveness of cofactor administration was proven by comparing the results of simple and combined loading tests, according to these lines of evidence. A 15% to 50% reduction of plasma Phe peak level was attained 12 to 24 hours after either simple Phe or combined Phe and BH4 loading test. The timing and the extent of Phe clearance were clearly related to the different severity of the clinical phenotype. After loading, a significant increase of plasma Tyr paralleled the sharp Phe decrease in the less severe forms. The clearance of plasma Phe and the rate of Tyr production were not enhanced by the administration of BH4 either after or before the Phe loading. The latter procedure also excludes any effect of BH4 on Phe absorption or PAH preincubation and stresses the physiologic role of Phe in PAH activation. The kinetics of blood biopterin after simple Phe loading, on the other hand, confirmed that in PKU patients the mechanism of guanosine triphospate cyclohydrolase I activation and the pathway of the de novo biosynthesis of BH4 are intact, resulting in high cofactor availability during spontaneous or induced HPA [15,16].

Although provocative, the conclusions of the above findings and observations are that the methods so far used to predict or to assess BH4 responsiveness in PKU can be misleading. Because neither patients' genotyping nor a decrease of at least 30% in plasma Phe after simple or repeated cofactor challenges allows the definition of BH4 responsiveness in the absence of adequate controls, we are still awaiting for the first case of PAH deficiency to be ultimately proven as responsive to BH4. We showed that the accurate patients' characterization and the use of proper methods, such as the one we developed, will help solve this question. Moreover, our method can be applied for clinical purposes even to PKU patients nongenotyped or not accurately phenotyped, a common worldwide condition appearing in recent literature. As a consequence, many concerns can be raised with the use of BH4 in the treatment of

PKU and of maternal PKU [42]. Actually, in contrast with the high rate of patients predicted as responsive on the basis of the genotype or of the response to a single cofactor administration, a small number appeared to be still responsive to continuous treatment [14,37,38,43-45]. These patients, when their genotypes and phenotypes are recorded, appear generally as affected by benign PKU variants, in whom Phe itself induces consistent activation of PAH enzyme, even making a restrictive dietary regimen unnecessary.

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