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Tetrahydrobiopterin Loading Test in Hyperphenylalaninemia

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ABSTRACT. Some cases of primary hyperphenylalaninemia are not caused by the lack of phenylalanine hydroxylase, but by the lack of its cofactor tetrahydrobiopterin. These patients are not clinically responsive to a phenylalanine-restricted diet, but need specific substitution therapy. Thus, it became necessary to examine all newborns screened as positive with the Guthrie test for tetrahydrobiopterin deficiency. Methods based on urinary pterin or on specific enzyme activity measurements are limited in their availability, and the simplest method, based on the lowering of serum phenylalanine after loading with cofactor, was discouraged by the finding that some dihydropteridine reductase-deficient patients were unresponsive. The preliminary observation that this limitation could be overcome by increasing the dose of the administered cofactor prompted us to reevaluate the potential of the tetrahydrobiopterin loading test in hyperphenylalaninemia. Fifteen patients, eight with ultimate diagnosis of phenylketonuria, three with 6-pyruvoyl tetrahydropterin synthase-, and four with dihydropterine reductase-deficiency, have been examined by administering synthetic tetrahydrobiopterin both orally, at doses of 7.5 and 20 mg/kg, and i.v., at a dose of 2 mg/kg. All the tetrahydrobiopterin-deficient patients, unlike those with phenylketonuria, responded to the oral dose of 20 mg/kg cofactor by lowering their serum phenylalanine concentration markedly below baseline to an extent easily detectable by Guthrie cards. This method allows for a simple screening method when enzyme or pterin studies are not available. (*Pediatr Res* 30: 435–438, 1991)

Abbreviations

BH_4, tetrahydrobiopterin
CRM, cross reacting material
DHPR, dihdropteridine reductase
HPA, hyperphenylalaninemia
PH, phenylalanine hydroxylase
Phe, phenylalanine
PKU, phenylketonuria
6-PPH_4S, 6-pyruvoyl tetrahydropterin synthase
Tyr, tyrosine

The recognition that not all cases of primary HPA are caused by the lack of PH activity was first evidenced by the introduction of terms such as "atypical," "malignant," or "variant" forms of PKU to describe some cases clinically unresponsive to a Phe-restricted diet and later shown to be due to BH_4 deficiency (1–4).

By analyzing all the essential components of the complex hydroxylation system of aromatic amino acids, it became apparent that a defect in the BH_4 recycling enzyme DHPR (EC 1.66.99.7) and two defects in BH_4 synthetic pathway enzymes, guanosine triphosphate cyclohydrolase I (EC 3.5.4.16) and 6-PPH_4S, may lead to cofactor deficiency resulting in HPA and in impaired production of dopamine and serotonin (5–7). Dietary treatment in these patients does not prevent brain damage. These patients need early cofactor and/or neurotransmitter substitutive therapy. Thus, it became necessary to reexamine all the newborns having HPA for BH_4 deficiency (8–11).

A frequency of 1–3% among patients with HPA has been estimated for BH_4 deficiency in worldwide surveys (12). Further study might prove the frequency of these disorders to be higher not only in the Mediterranean area, as previously suggested (13), but also in non-Caucasian populations. The prevalence of BH_4 deficiency among all HPA is over 15% in Piedmont, Italy, appears to be even higher in infants of Turkish origin (14), and is up to 50% among Guthrie-positive patients in Taiwan (15).

Several procedures are now available for defining which component of the Phe hydroxylating system is affected (10). Ideal methods are those based on the direct measurement of specific enzyme activities. However, with the exception of DHPR assay, which is easily performed on dried blood spots (16), they are not widely used because of their complexity and because of the limited expression of PH, guanosine triphosphate cyclohydrolase I and 6-PPH_4S activity in body tissues. Indirect methods, relying on HPLC to define urinary pterin excretion, are widely used. They enable, with some minor uncertainties (17), the detection of BH_4 deficiency as well as the discrimination of the different types of deficiency. The limitation is that only a few laboratories are able to carry them out.

Historically, the first method tried (18), and predicted as the most convenient for a selected screening among HPA (19), exploited the lowering of serum Phe in BH_4-deficient patients after the administration of exogenous BH_4. An i.v. loading with 2 mg/kg BH_4 was originally proposed by Danks *et al.* (19). With the increased purity and availability of synthetic cofactor, an oral loading test was introduced by Curtius *et al.* (20) and standardized at the dose of 7.5 mg/kg in its current form by Niederwieser *et al.* (21), possibly allowing a very simple method to discriminate between hyperphenylalaninemic patients with PH and BH_4 deficiency. However, it soon became obvious that some DHPR-deficient patients could be misdiagnosed, inasmuch as their serum Phe is not lowered after the load, introducing an unacceptable limitation of this test if solely used (10).

The recent observations that BH_4 nonresponsiveness in DHPR deficiency correlated with the presence of a mutant enzyme (22) and that it could be overcome by increasing the dose of the

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administered synthetic cofactor (23) prompted us to reevaluate the potential power of different BH_4 loading tests in HPA.

PATIENTS AND METHODS

Patient selection. The study population consisted of 15 patients (seven males and eight females) positively identified through the neonatal mass screening for PKU or subsequently at an older age as having HPA at the ages of 12 d to 5 y. Differential diagnosis among HPA patients was performed by following the criteria and the procedures previously reported (10). Diagnosis of PKU was accepted after exclusion of transient or secondary forms of HPA and, after the selected screening of BH_4 deficiency through the analysis of urinary pterins (10), the measurement of specific enzyme activities (10, 16, 22, 24), in addition to the BH_4 oral loading test (10). The PKU phenotype was settled by the evaluation of the dietary Phe tolerance and the oral Phe loading test (25).

Eight patients had an ultimate diagnosis of PH deficiency with the phenotype of classical PKU (cases 1–8), three had 6-PPH_4S deficiency (cases I–III), and four had DHPR deficiency (cases a–d) (Table 1). All DHPR-deficient patients showed no enzyme activity by assay of dried blood spots (16), and an antibody study (22) demonstrated in one case (a) the absence of any enzyme protein (DHPR-CRM−) and in three cases (b, c, and d) the presence of a mutant protein lacking catalytic activity (DHPR-CRM+).

BH_4 loading tests. (6RS)-L-erthro-tetrahydrobiopterin dihydrochloride ($BH_4 \cdot 2HCl$; Dr. Schirks Laboratory, Wettswill A.A., Switzerland) was administered orally to all patients at the dose of 7.5 mg/kg before the institution of dietary restriction or cofactor therapy. BH_4 was administered after 6 h fasting and 30 min before the meal to ensure good absorption, after dissolving the tablets in 20 mL of water in dim light. Blood samples were taken immediately before and 4 and 8 h after loading. Serum Phe and Tyr were measured chromagraphically with the Kromakon 500 automatic analyzer (Kontron, Everett, MA). The three DHPR-CRM+ patients who did not respond to the load with 7.5 mg/kg BH_4 and five PKU patients were reloaded orally with 20 mg/kg BH_4, following the above procedures. At this time, fasting Phe levels were raised because of fortuitous discontinuation of diet. Two DHPR-CRM+ patients and two PKU patients were also loaded i.v. with 2 mg/kg BH_4, as originally proposed by Danks *et al.* (19). Serum Phe and Tyr concentrations were measured every 2 h after the load. All these different BH_4 loading tests were performed at basal serum Phe concentrations ranging from 331 to 2391 μmol/L. Cofactor absorption was proven in all cases by the measurement of total biopterin in urine collected 12–0 h before and 4–8 h after oral loading.

RESULTS

BH_4 orally, 7.5 mg/kg. None of the PKU patients responded to this oral loading test (Table 2). Serum Phe and Tyr concentrations showed limited and unequivocal fluctuations, possibly due to resumption of a free diet just after loading. All three 6-PPH_4S-deficient patients and only one DHPR-deficient patient (the CRM− patient) lowered their basal serum Phe concentration (Tables 3 and 4). All the BH_4-deficient patients (including the three DHPR-CRM+ patients) increased their basal serum Tyr concentration after the load. When positive, the response appeared more rapid and lowered the Phe more in 6-PPH_4S deficiency when compared with DHPR deficiency.

BH_4 orally, 20 mg/kg. When loaded with 20 mg/kg BH_4, the PKU patients again showed no significant fluctuation of serum Phe concentrations (Table 2), whereas serum Phe concentration fell in all the DHPR-CRM+ patients who previously had not responded to the oral load with 7.5 mg/kg (Table 4). The time course of serum Tyr concentration in the DHPR-deficient patients was similar to that observed with the lesser dose of BH_4 but with a more pronounced increase. Again, maximum effect was observed at 8 h. Surprisingly, serum Tyr concentration did rise in some PKU patients, with a maximal response 4 h after the load (Table 2).

BH_4 i.v., 2 mg/kg. Both in PKU patients and in DHPR-CRM+ patients, the response was similar to that obtained with the oral loading at the dose of 20 mg/kg, but the cofactor effects appeared more pronounced by i.v. loading in the first hours after the load (Tables 2 and 4). Even so, the kinetics and the extent of the response appeared markedly different between patients b and c (Table 4).

Table 1. *Diagnostic procedures in 15 patients with HPA**

Enzyme defect	Age at diagnosis (mo)	Urine			Erythrocytes		CRM¶ (DHPR)	Response to BH_4 loading (7.5 mg/kg)
		Biopterin†	Neopterin†	%B‡	6-PPH_4S activity§	DHPR activity‖		
PH^- (cases 1–8)	1	1.4–6.9	3–12.3	24–45	Not done	4.9–7.2	Not done	Negative
6-PPH_4S^-								
Case I	11	0	13.4	0	<1	1.2	Not done	Positive
Case II	1	0	27.8	0	1.8	5.0	Not done	Positive
Case III	5	0	27.1	0	<1	6.9	Not done	Positive
$DHPR^-$								
Case a (A.R.)	14	6.5	1.0	86	Not done	0	Negative	Positive
Case b (G.C.)	13	13.8	2.8	83	Not done	0	Positive	Negative
Case c (M.P.)	14	12.5	2.1	85	Not done	0	Positive	Negative
Case d (M.B.)	1	9.3	12.6	42	Not done	0	Positive	Negative
Controls	1–2 ($n = 65$)						Positive	Negative
	>2 ($n = 312$)					4.2–7.5		
	1–12**	0.5–3	1.1–4	18–63		2.5–4.1		
	Adults**				11–29.6			

* Diagnosis of PH deficiency (PH^-), 6-PPH_4S deficiency (6-PPH_4S^-) and DHPR deficiency ($DHPR^-$) was obtained by the analysis of urinary pterin excretion, the measurement of 6-PPH_4S and DHPR activity, and the evaluation of a BH_4 oral loading test.

† mmol/mol creatinine.

‡ %B = 100 × biopterin/(biopterin + neopterin).

§ μU/g Hb.

‖ nmol cytochrome *c* reduced/min/5-mm diameter filter paper disc.

¶ On cultured skin fibroblasts.

** Reference values from Dr. N. Blau, Head, Screening Laboratory, University Children's Hospital, Zurich, Switzerland.

Table 2. *BH_4 loading tests in PKU patients: time course of serum Phe and Tyr concentrations (μmol/L)*

Case	Hours after load	Dose and route of administration of BH_4: 7.5 mg/kg, orally Phe	Tyr	20 mg/kg, orally Phe	Tyr	2 mg/kg, i.v. Phe	Tyr
1	0	2391	58	1851	17	1337	22
	2					1429	48
	4	2480	60	2094	58	1401	42
	6					1359	31
	8	2723	47	1820	34	1496	50
2	0	1995	41	711	24	694	27
	2					742	49
	4	2188	41	820	84	859	42
	6					871	40
	8	2023	44	961	48	926	56
3	0	535	41	659	41		
	4	607	27	603	81		
	8	591	29	504	49		
4	0	670	79	840	20		
	4	730	74	790	34		
	8	722	64	1080	36		
5	0	443	87	1104	33		
	4	453	75	1218	28		
	8	540	62	1280	28		
6	0	1226	46				
	4	1233	45				
	8	1172	50				
7	0	614	65				
	4	585	46				
	8	516	54				
8	0	1476	57				
	4	1460	48				
	8	1476	48				

Table 3. *BH_4 loading test in patients with 6-PPH_4S deficiency: time course of serum Phe and Tyr concentrations (μmol/L)*

Case	Hours after load	Dose and route of administration of BH_4 (7.5 mg/kg, orally) Phe	Tyr
I	0	1320	37
	4	80	72
	8	53	52
II	0	331	41
	4	35	59
	8	17	55
III	0	744	52
	4	65	133
	8	32	68

DISCUSSION

Epidemiologic evidence suggests that every newborn with HPA should be evaluated for BH_4 deficiency (8–11). The nature of BH_4 deficiency justifies its inclusion in screening programs as part of the PKU mass screening program, needing additional screening in only positively identified newborns. The BH_4 loading test was recognized as most convenient for this purpose (26), depending on its reliability for the detection of DHPR deficiency (10). The data obtained from the present study confirm and extend our previous preliminary report (23) that DHPR-deficient

Table 4. *BH_4 loading tests in patients with DHPR deficiency: time course of serum Phe and Tyr concentrations (μmol/L)*

Case	Hours after load	Dose and route of administration of BH_4: 7.5 mg/kg, orally Phe	Tyr	20 mg/kg, orally Phe	Tyr	2 mg/kg, i.v. Phe	Tyr
a	0	478	41				
	4	208	47				
	8	97	64				
b	0	542	30	836	42	570	62
	2					336	153
	4	571	45	395	68	231	118
	6					224	131
	8	640	59	214	96	199	125
c	0	1458	88	583	43	1361	31
	2					1035	57
	4	1377	119	358	79	1040	45
	6					1171	35
	8	1478	153	63	102	1366	33
d	0	1939	39	816	38		
	4	1896	84	567	83		
	8	1864	56	134	108		

patients who do not respond to a load dose of 7.5 mg/kg BH_4 in fact do respond (by lowering serum Phe) to 20 mg/kg. This finding does not clarify the paradox that in DHPR deficiency BH_4 should be functioning stoichiometrically and not catalytically (27), because BH_4 should barely lower serum Phe in these cases if there were a stoichiometric conversation of Phe to Tyr (28). Serum amino acid kinetics of the load test response are quite different in 6-PPH_4S deficiency and in DHPR deficiency, allowing to some extent the discrimination of BH_4 synthesis from BH_4 regeneration defects. None of the five PKU patients tested at the higher cofactor level in this study showed a positive response, but possibly larger numbers of PKU patients have to be analyzed to exclude that some PKU variant might be BH_4-responsive. The main point regarding the diagnostic potential of the BH_4 loading test among HPA patients is the present observation that higher cofactor doses did produce a positive response in our "nonresponding" DHPR-deficient patients, allowing discrimination of all present cases of BH_4 deficiency from PKU. Yet ultimate diagnoses of subtypes will require more complex tests (10).

Since the publication of our preliminary findings (23), two DHPR-deficient patients have been described who did not respond to the higher level of BH_4 (17, 29, Kaufman S, personal communication). The lack of response in these patients might be explained by a specific mutation causing unresponsiveness, the coexistence of DHPR deficiency and PKU, or defective BH_4 absorption. Unfortunately, those patients died before any of these hypotheses could be tested, including still higher doses of BH_4, which might have elicited a Phe-lowering response.

This is a critical point, considering the clinical application of just the BH_4 loading test for the screening for BH_4 deficiency. Cofactor unresponsiveness of some very rare DHPR-deficient patients must still be regarded as a remotely possible cause of missing a diagnosis with the high BH_4 level oral loading test. Consequently, present knowledge suggests that for full reliability this simple clinic-based test should be coupled with a blood spot test (16) for DHPR activity assay.

An additional reason for performing the BH_4 loading test in HPA is that it is a functional test, and results may give some indication on the patient's treatment, outcome, and overall prognosis. Thus, in DHPR deficiency, several different BH_4 response categories can now be characterized: 7.5 mg/kg responsive, 7.5 mg/kg unresponsive but 20 mg/kg responsive, and, possibly, 20 mg/kg unresponsive. So far we have little data on

the correlation of these categories with response to therapy and prognosis. However, an earlier study (30) suggested that cases responding to lower levels of BH_4 may be less severe. These studies also indicated that nonresponsiveness to 7.5 mg/kg of BH_4 correlated with the presence of inactive enzymes (22). It is also conceivable that a cofactor-responsive form of PKU may exist and that a BH_4 loading test would detect such cases.

The outcome of a BH_4 loading test is usually judged by the presence or absence of a precipitous fall in previously elevated serum Phe concentrations, and slight falls are usually regarded as negative. This leaves open to question whether it is the percentage of the absolute fall of serum Phe level that is more relevant. If larger series of cases are studied with standardized BH_4 loading tests, it may be possible to discriminate the different types of BH_4 deficiency and indeed the different DHPR response categories. Also, the kinetics of serum Tyr response may appear to be quite different in patients with cofactor synthesis or regeneration defects, and the evaluation of the changes in the Phe/Tyr ratio at 0.4, and 8 h may help to discriminate less obvious differences.

Among the present cases studied, the oral test gave a more clear-cut response than the i.v. test in one case of DHPR deficiency. The reason for this unexpected discrepancy is unknown, but it may be that genotypic definition of underlying mutant alleles will allow us to distinguish the type of response.

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