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# Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency in Dutch neonates

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**Summary** Four neonates with a positive phenylalanine screening test (Phe concentrations between 258 and 1250 $\mu$mol/L) were investigated further to differentiate between phenylalanine hydroxylase (PAH) deficiency and variant hyperphenylalaninaemia (HPA) forms. In patients 1 and 2 a tetrahydrobiopterin ($BH_4$) load caused a significant decrease of the plasma Phe levels. A combined phenylalanine/$BH_4$ loading test was performed in patients 2, 3 and 4. In the latter two patients, plasma Phe concentrations completely normalized within 8 h after the $BH_4$ load (20 mg/kg). Basal urinary pterins were normal in all four patients. The activity of dihydropteridine reductase (DHPR) was normal in patients 1, 2 and 3 and 50% of control values in patient 4 (not in the range of DHPR-deficient patients). In patient 3 a subsequent phenylalanine loading test with concomitant analysis of plasma biopterins revealed a normal increase of biopterin, excluding a $BH_4$ biosynthesis defect. Pterins and neurotransmitter metabolites in CSF of patients 1, 3 and 4 were normal. DNA mutations detected in the PAH gene of patients 1–4 were A313T, and L367fsinsC; V190A and R243X; A300S and A403V; R241C and A403V. The results are suggestive for mutant PAH enzymes with decreased affinity for the cofactor $BH_4$.

Hyperphenylalaninaemia (HPA) is a disorder caused by a deficient or a decreased activity of phenylalanine-4-hydroxylase (PAH, EC 1.14.16.1) due either to a

mutated enzyme protein or to a deficiency of its obligatory cofactor tetrahydrobiopterin ($BH_4$). The latter group comprises defects in the biosynthesis and in the regeneration of $BH_4$ (Scriver et al 1995). Detection of HPA is included in the newborn mass screening programme. Differential diagnostic investigations are necessary, however to detect $BH_4$ deficiencies even if phenylalanine (Phe) concentrations are only slightly elevated (Ponzone et al 1993). Screening for $BH_4$ deficiency is performed by analysis of pterins in urine and measurement of dihydropteridine reductase (DHPR) activity in erythrocytes or skin fibroblasts (Blau and Blaskoviks 1996). In the Netherlands as well as in several other countries, a $BH_4$ loading test is included in the screening protocol of HPA. However, if the initial Phe concentration is below 400 $\mu$mol/L, a combined Phe/$BH_4$ loading is performed (Ponzone et al 1993). Recently, Kure and colleagues (1999) reported that serum Phe concentrations in four patients with mild HPA decreased after a $BH_4$ challenge. Urinary pterins in their patients and DHPR activities in blood appeared to be normal. In addition, mutations were detected in the PAH genes of those patients.

In the follow-up of a positive newborn PKU-screening test we found four children to be responsive after either a $BH_4$ challenge or a combined Phe/$BH_4$ loading test. DHPR deficiency was excluded and urinary pterins appeared to be normal. Mutations were found in the PAH genes of all these patients; thus this group of patients belongs to a new variant of $BH_4$-responsive PAH deficiency.

## PATIENTS AND METHODS

Patient 1, a girl, was born prematurely after a pregnancy of 28 weeks because of maternal complications. Her birth weight was 750 g, she was dysmature and suffered from severe neonatal complications. Patients 2, 3 and 4 were born at term after uncomplicated pregnancies. They were admitted to the academic hospital for evaluation of a positive newborn PKU screening test. Combined Phe/$BH_4$ loading tests were performed according to Ponzone and colleagues (1993).

Amino acids were analysed by means of automated ion-exchange chromatography with postcolumn ninhydrin derivatization (Biochrom 20, Amersham Pharmacia Biotech). DHPR activity in erythrocytes was measured as described previously (Surplice et al 1990) or in cultured skin fibroblasts according to Bonafé and colleagues (2000). Urinary pterins were analysed by a HPLC procedure adapted from Fukushima and Nixon (1980) and Nixon and colleagues (1980). Neurotransmitter metabolites were analysed in CSF of patients 1, 3 and 4 as described (Blau et al 1999). Mutations in the PAH gene were detected by means of single-strand conformational analysis and subsequent sequence analysis (van der Sijs-Bos et al 1996).

## RESULTS AND DISCUSSION

The positive PKU screening test in patient 1 was followed by a $BH_4$ load of 20 mg/kg body weight. Figure 1 shows the response of plasma Phe to the $BH_4$ load. Because of the rapid decrease of the plasma Phe concentration, treatment with $BH_4$ (5 mg/kg per day) was continued during the next 8 days. From day 9 the treatment was stopped

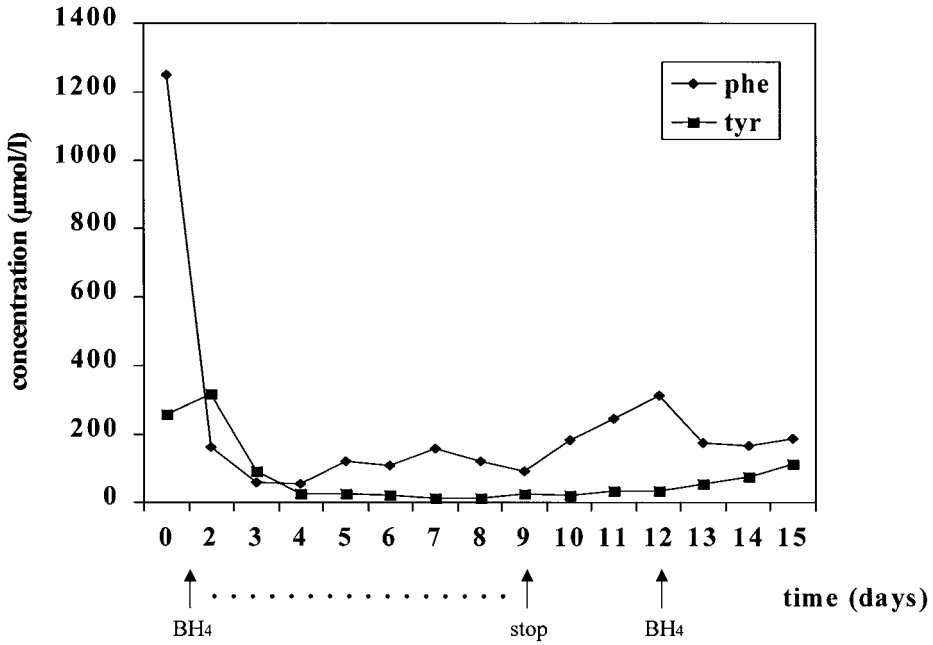


**Figure 1** Course of plasma phenylalanine and tyrosine concentrations in patient 1 after $BH_4$ supplementation: day 1, 20 mg/kg; days 2–9, 5 mg/kg per day; days 9–12 stop supplementation; from day 12 on continuation of treatment with $BH_4$ 5 mg/kg per day

for 3 days, resulting in an increase of the Phe level. Subsequently, the treatment was instituted again. The initial Phe concentration in patient 2 was 450 $\mu$mol/L. A single $BH_4$ load of 20 mg/kg resulted in a decrease of Phe from 445 $\mu$mol/L before load to 251 $\mu$mol/L and 26 $\mu$mol/L, 8 and 33 h following load, respectively.

Because of the moderately increased Phe concentrations in patients 3 and 4, combined Phe/$BH_4$ loading tests were performed. Patient 2 was retested in the same way. Figure 2 shows the courses of the plasma Phe concentrations during the tests. Urinary pterins were normal in all four patients. DHPR activity in cultured fibroblasts of patient 1 and in erythrocytes of patients 2 and 3 were found normal, whereas in patient 4 DHPR activity was 46% of control (data not shown). Phenylalanine-induced biopterin synthesis was studied in patient 3 following an oral Phe load (100 mg/kg). The normal increase of biopterin (Figure 3) excluded a $BH_4$ biosynthesis defect. Analysis of neurotransmitter metabolites in CSF of patients 1, 3 and 4 did not reveal deficiencies of neurotransmitters (Table 1); CSF of patient 2 was not analysed. In addition, CSF pterins were found normal (data not shown). These normal findings prompted us to analyse the PAH gene. Mutations in both alleles of the PAH gene were identified in all four patients (Table 2). Patients 3 and 4 share the same mutation (A403V) although they are not related. They showed a similar rapid response on $BH_4$ loading, resulting in normalization of plasma Phe

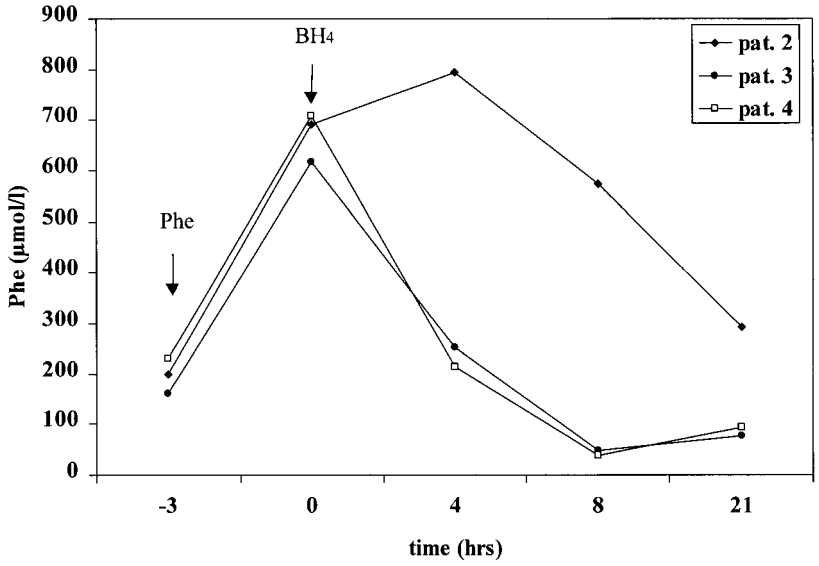


**Figure 2** Course of plasma phenylalanine concentrations during a combined Phe/$BH_4$ loading test in patients 2, 3 and 4

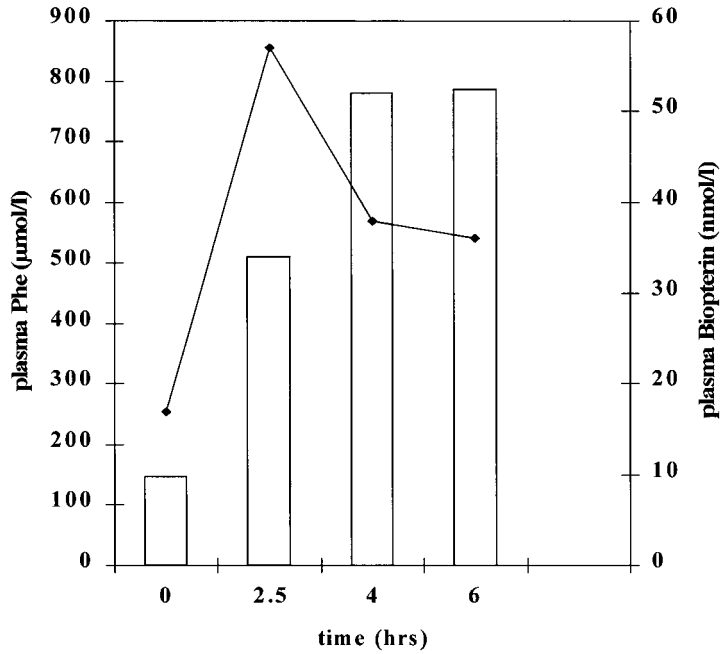


**Figure 3** Course of plasma biopterin (—◆—) and phenylalanine (bars) concentrations in patient 3 after a phenylalanine load (100 mg/kg)

**Table 1 Biogenic amines in CSF (nmol/L)**

| | *5-HIAA* | *HVA* | *HVA/5-HIAA* | *DOPAC* | *MHPG* | *3-OMD* | *5-OH-Trp* | *L-Dopa* |
|---|---|---|---|---|---|---|---|---|
| Patient 1 | 697 | 758 | 1.1 | 16.3 | | 335 | 17 | 12.5 |
| Patient 3 | 212 | 563 | 2.7 | | | 75.0 | 8.7 | 12.3 |
| Patient 4 | 316 | 523 | 1.7 | 29.9 | 67.2 | 71.3 | <5 | 38.5 |
| Controls[a] | 150–800 | 310–1100 | 1.5–3.5 | 8–18 | 98–168 | <300 | <10 | <25 |

[a] Age range: 0–0.5 years

5-HIAA, 5-hydroxyindoleacetic acid; HVA, homovanillic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; MPHG, 3-methoxy-4-hydroxyphenylglycol; 3-OMD: 3-*O*-methyl-dopa; 5-OH-Trp, 5-hydroxytryptophan

**Table 2 Mutations in the PAH gene**

| | *Allele 1* | *Allele 2* |
|---|---|---|
| Patient 1 | A313T ex 9 | L367fsinsC ex 11 |
| Patient 2 | V190A ex 6 | R243X ex 7 |
| Patient 3 | A300S ex 8 | A403V ex 12 |
| Patient 4 | R241C ex 7 | A403V ex 12 |

within 8 h (Figure 2). The R241C mutation found in patient 4 was detected earlier in two other $BH_4$-responsive HPA patients. In contrast to our patient 4, plasma Phe concentrations in those two patients apparently did not decrease to normal values within 24 h after the $BH_4$ load. However, they received a lower dose of 10 mg/kg $BH_4$ (Kure et al 1999). The A300S and A403V mutations have been found in non-PKU HPA, though no $BH_4$-responsiveness was reported (Mallolas et al 1999). The A313T mutation due to a 937G>A transition, detected in patient 1, has not been reported before.

Normal human PAH protein is present as homopolymer produced by a single genetic locus (Scriver et al 1995). The HPA patients described in this study all bear two different allele mutations, leading to the presence of various heteropolymeric and homopolymeric PAH proteins. Increasing intracellular $BH_4$ concentrations by oral supplementation apparently increases residual PAH activity, possibly as result of a decreased affinity of the enzyme for this cofactor. In the patients who showed a fast normalization of plasma Phe within 8 h after $BH_4$ load, both heteropolymeric and homopolymeric PAH subunits may be involved in the restoration of enzymic activity. In patients who responded partially to $BH_4$ supplementation, only homopolymeric PAH molecules bearing one of the mutations may be activated by the cofactor, leading to a partially restored enzyme activity. So far, PAH mutations specifically affecting the $BH_4$ binding site of the enzyme are unknown.

The patients presented above belong to a group with a new variant of PAH deficiency, a $BH_4$-responsive one. Patient 1 had Phe and Tyr concentrations in the 120–150 $\mu$mol/L range on $BH_4$ monotherapy. In patients 2 and 4, plasma Phe levels remain well controlled (<350 $\mu$mol/L) on protein restriction and a PKU formula. Without protein restriction, Phe concentrations in patient 3 remain mostly below 250 $\mu$mol/L. In principle this form of PAH deficiency is treatable with $BH_4$. Institution of $BH_4$ supplementation in our patients is a point at issue. These novel $BH_4$-responsive subtypes of PAH deficiency can only be detected if a $BH_4$ loading test is included in the differential diagnostic investigations of HPA patients.

## ACKNOWLEDGEMENTS

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