Diagnosis of Dopa-responsive Dystonia and Other Tetrahydrobiopterin Disorders by the Study of Biopterin Metabolism in Fibroblasts

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Background: Dopa-responsive dystonia (DRD) and tetrahydrobiopterin (BH₄) defects are inherited disorders characterized by monoamine neurotransmitter deficiency with decreased activity of one of the BH₄-metabolizing enzymes. The aim of the study was to determine the utility of cultured skin fibroblasts for the diagnosis of these diseases.

Methods: Neopterin and biopterin production and GTP cyclohydrolase I (GTPCH) activity were measured in cytokine-stimulated fibroblasts; 6-pyruvoyltetrahydropterin synthase (PTPS), sepiapterin reductase (SR), and dihydropteridine reductase (DHPR) activities were measured in unstimulated fibroblasts. We examined 8 patients with DRD, 3 with autosomal recessive GTPCH deficiency, 7 with PTPS deficiency, 3 with DHPR deficiency, and 49 controls (35 fibroblast and 14 amniocyte samples).

Results: Fibroblasts from patients with DRD and autosomal recessive GTPCH deficiency showed reduced GTPCH activity (15.4% and 30.7% of normal activity, respectively) compared with controls (P < 0.001). Neopterin production was very low and biopterin production was reduced in both disorders. PTPS- and DHPR-deficient cells showed no enzyme activities; in PTPS deficiency the pattern of pterin production was typical (neopterin, 334–734 pmol/mg; controls, 18–98 pmol/mg; biopterin, 0 pmol/mg; controls, 154–303 pmol/mg). Reference values of all enzyme activities and pterin production were measured in fibroblasts and also in amniocytes for prenatal diagnosis.

Conclusions: Cultured skin fibroblasts are a useful tool in the diagnosis of BH₄ deficiencies. Intracellular neopterin and biopterin concentrations and GTPCH activity in cytokine-stimulated fibroblasts are particularly helpful in diagnosing patients with DRD.

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Tetrahydrobiopterin (BH₄)¹ is the essential cofactor for aromatic amino acid mono-oxygenases (Fig. 1) (1). Phenylalanine-3-hydroxylase is responsible for hydroxylation of phenylalanine to tyrosine in the liver. Tyrosine-4-hydroxylase and tryptophan-5-hydroxylase are the rate-limiting enzymes in the biosynthesis of the neurotransmitters dopamine and serotonin. BH₄ is also a cofactor for the different forms of nitric oxide synthase.

GTP cyclohydrolase I (GTPCH; EC 3.5.4.16) catalyzes the first and rate-limiting step in the BH₄ pathway. It is expressed in peripheral tissues as well as in the brain, and its expression can be induced at the transcriptional level in T lymphocytes, macrophages, and fibroblasts by cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (2, 3). The enzyme converts GTP to the intermediate 7,8-dihydroneopterin triphosphate (Fig. 1). 6-Pyruvoyltetrahydropterin synthase (PTPS; EC 4.6.1.10) is the second enzyme in the biosynthesis of BH₄. It is constitutively expressed in many cell types, including fibroblasts, and catalyzes the synthesis of 6-pyruvoyltetrahydropterin from 7,8-dihydroneopterin triphosphate. The last step in the biosynthesis of BH₄ is catalyzed by sepiapterin reductase (SR; EC 1.1.1.153), which reduces 6-pyruvoyltetrahydropterin to BH₄. Two enzymes are responsible for the regeneration of BH₄ following oxidation by the amino acid mono-oxygenases: pterin-4α-carbinolamine dehydratase (EC 4.2.1.96) and dihydropteridine reductase (DHPR; EC 1.6.99.7; Fig. 1). At least DHPR is

¹ Nonstandard abbreviations: BH₄, tetrahydrobiopterin; GTPCH, GTP cyclohydrolase I; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; PTPS, 6-pyruvoyltetrahydropterin synthase; SR, sepiapterin reductase; DHPR, dihydropteridine reductase; DRD, Dopa-responsive dystonia; and CSF, cerebrospinal fluid.
and many cases appear to be sporadic (15). The clinical phenotypic expression is quite diverse. Neonatal hyperphenylalaninemia, developmental delay, dystonia typically affects the legs, causing gait disturbance and postural instability. Signs and symptoms usually occur, especially in adulthood (11). Early motor development usually is normal, and hyperphenylalaninemia is absent. Recent studies have demonstrated that DRD is caused by autosomal dominant mutations in the coding region of the GCH1 gene, which encodes for GTPCH (12), but only in 40–50% of cases (13). Reduced penetrance and gender-related vulnerability are reported, with females being more affected than males (2.5:1) (14). The clinical phenotypic expression is quite diverse (15), and many cases appear to be sporadic (16).

Today, the diagnosis of BH₄ deficiencies is based on neonatal screening for phenylketonuria and urinary pterin analysis (17). DRD is diagnosed mainly by clinical signs and symptoms and by response to L-Dopa/Carbidopa treatment. The examination of pterins and neurotransmitter metabolites in cerebrospinal fluid (CSF) requires an invasive procedure, but it is the only way to make a reliable diagnosis of DRD (10,18). Definitive diagnosis of BH₄ deficiencies is achieved by enzyme activity determinations and DNA analysis (8).

PTPS and DHPR activities can be measured in liver biopsies (19,20), erythrocytes (21,22), and fibroblasts (23,24), whereas GTPCH is measurable in liver biopsies (25). In mononuclear blood cells, GTPCH can be induced by phytohemagglutinin (26), and reference values have been reported recently by Hibiya et al. (27). However, this test is quite complex and must be performed within 20 h after sample collection (27). In previous studies, fibroblasts were used for indirect measurement of pterin metabolism (28), based on the induction of GTPCH expression with cytokines, but no data are available about nonpathological and pathological values. The possibility of measuring GTPCH activity in cytokine-stimulated fibroblasts was shown by Werner et al. (29). In the present study, we used a similar stimulation procedure for measurement of both pterins and GTPCH activity. To our knowledge, fibroblasts have not been systematically tested for the other BH₄-metabolizing enzymes. Cultured skin fibroblasts are easily used and practical for the diagnosis of many metabolic disorders. The samples can be transported at room temperature and stored for long periods after early passages for later analysis.

The aim of the present study was to demonstrate the utility of cultured skin fibroblasts as a diagnostic tool for DRD and BH₄ deficiencies. Particularly for DRD, the enzyme activity measurement is essential for final diagnosis. BH₄ metabolism was studied in fibroblasts from a large number of controls and in patients with different forms of BH₄ deficiency.

Materials and Methods

Subjects

Primary skin fibroblasts were obtained from 21 patients and 35 controls. Eight patients (four males, four females), 10–45 years of age at the time of the skin biopsy, were affected by DRD (McKusick 128230). Thirteen patients, 2 months to 11 years of age at the time of the skin biopsy, were affected by BH₄ deficiencies. Of these, three patients (two males, one female) had an autosomal recessive GTPCH deficiency (McKusick 233910), seven (five males, two females) had a PTPS deficiency (McKusick 261640), and three (all males) had a DHPR deficiency (McKusick 261630). The diagnosis was confirmed by mutation analysis in all patients.

Control value biopsies were obtained from 17 subjects (10 males, 7 females) between birth and 1 year of age and 18 subjects (11 males, 7 females) between 1 and 45 years of age. No neurological abnormalities were detected in any of the controls. To establish reference values for prenatal diagnosis, 14 amniocyte samples collected between 15 and
18 weeks of gestational age were also studied. The activities of all of the BH₄-metabolizing enzymes, as well as neopterin and biopoterin production after stimulation with cytokines, were measured in all samples.

The procedures used were in accordance with the current revision of the Helsinki Declaration of 1975.

**MATERIALS**

Sephadex G-50 Nick spin columns were from Pharmacia. DMEM, AmnioMax C-100 medium, trypsin/EDTA solution, antibiotics, and fetal calf serum were purchased from Life Technologies. GTP lithium salt, recombinant human IFN-γ, and TNF-α were from Sigma. Calf intestine alkaline phosphatase, NADH, NADPH, DHPR, peroxidase, leupeptin, and pepstatin were from Roche. Falcon cell culture plasticware was from Becton-Dickinson. γ-Globulin and the protein assay dye reagent were from Bio-Rad. Ultrafree-MC filters with PL10 membrane were from Millipore.

Pteridine derivatives (6,7-dimethyltetrahydropterin and sepiapterin) were from Schircks Laboratories. Dihydroneopterin triphosphate was prepared enzymatically as described previously (30), but using recombinant GTPCH (Thöny B, Blau N. Enzymatic reactor for synthesis of dihydronopterin triphosphate. Manuscript in preparation). Recombinant SR was purified in our laboratory (Thöny B, unpublished work). All other chemicals were from Fluka.

**CELL CULTURE AND TREATMENT WITH CYTOKINES**

Fibroblasts were cultured in DMEM containing 100 mL/L fetal bovine serum, 50 kilounits/L penicillin, and 50 mg/L streptomycin. Amniocytes were cultured in AmnioMax C-100 medium. Cells were passaged by trypsinization and studied at low passage numbers (3–12). All cells were *Mycoplasma* negative, as checked by the Hoechst staining method (31). Cultures were kept at 37 °C in humidified air containing 5% CO₂.

To induce the expression of GTPCH, confluent cell monolayers in 78-cm² plates were stimulated with recombinant human IFN-γ and TNF-α at concentrations of 250 and 100 kilounits/L, respectively, in fresh medium. After incubation for 24 h, cells were harvested by trypsinization, washed with phosphate-buffered saline, and immediately lysed for neopterin and biopoterin measurement and for GTPCH activity assay. PTPS, DHPR, and SR are constitutively expressed in fibroblasts; therefore, their activities were tested in unstimulated cells. Confluent cells in 78-cm² plates were harvested, washed with phosphate-buffered saline, and kept at −80 °C until analyzed for PTPS, DHPR and SR.

**MEASUREMENT OF NEOPTERIN AND BIOPTERIN PRODUCTION IN CELL EXTRACTS**

Cytokine-stimulated cells from one confluent 78-cm² plate were lysed in 150 μL of 50 mmol/L Tris-HCl (pH 7.4) containing 1 mmol/L EDTA and 1 mmol/L dithioerythritol by freezing and thawing six times. Lysate was centrifuged at 15 000g, and 100 μL of the supernatant was oxidized with 6 μL of 1 mol/L HCl and 20 μL of 10 g/L iodine solution for 15 min at room temperature in the dark. Oxidation was stopped by the addition of 20 μL of 10 g/L ascorbic acid (freshly prepared). Ten microliters of buffer (1 mol/L Tris-HCl, pH 9.6, containing 80 mmol/L MgCl₂) was added to the sample to adjust to pH 8.0–9.0. Dephosphorylation of neopterin triphosphate was achieved by hydrolysis with alkaline phosphatase (16.8 U) for 60 min at 37 °C. The reaction was stopped by acidification to pH 2.0 with 7 μL of 2 mol/L HCl. The sample (final volume, 175 μL) was subsequently deproteinized through an Ultrafree-MC filter and analyzed by HPLC (32). The intracellular concentrations of neopterin and biopoterin after 24-h stimulation with cytokines are expressed as pmol per mg of protein.

**GTPCH ASSAY**

The assay was performed as described previously by Hatakeyama and Yoneyama (33), with some modifications. The assay monitors the conversion of the substrate GTP under saturating conditions to neopterin triphosphate, which is detected as neopterin, the oxidized and dephosphorylated product (Fig. 1).

Fibroblasts were analyzed immediately after 24-h incubation with cytokines. Cells from one confluent 78-cm² plate were lysed in 200 μL of freshly prepared homogenization buffer (50 mmol/L Tris·HCl, pH 7.5, containing 0.1 mol/L KCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 0.2 mmol/L phenylmethylsulfonyl fluoride, 1 μmol/L leupeptin, and 1 μmol/L pepstatin) by freezing and thawing six times and were subsequently centrifuged at 15 000g for 5 min. A 150-μL aliquot of the supernatant was desalted on a spin column (Nick spin column, Sephadex G50; Pharmacia Biotech), and 50 μL of the filtrate was added to 148 μL of reaction buffer (50 mmol/L Tris·HCl, pH 7.5, containing 0.1 mol/L KCl and 1 mmol/L EDTA) and 2 μL of 100 mmol/L GTP. The mixture was divided into two 100-μL portions; one was incubated for 60 min at 37 °C, whereas the other was immediately oxidized (blank with cell extract). A blank without cell extract (25 μL of homogenization buffer added to 1 μL of 100 mmol/L GTP and to 74 μL of reaction mixture) was incubated together with the sample. The reaction was stopped by cooling the sample on ice and adding 10 μL of oxidizing solution (5 g/L iodine and 10 g/L potassium iodide in 1 mol/L HCl). After oxidation in the dark for 60 min, the reaction was stopped by adding 10 μL of 20 g/L ascorbic acid (freshly prepared). The mixture was adjusted to pH 8.5 by adding 14 μL of 1 mol/L NaOH, and the sample was incubated with 20 μL of alkaline phosphatase solution (300 KU/L calf intestine alkaline phosphatase in 0.1 mol/L Tris·HCl, pH 8.0, containing 1 mmol/L MgCl₂ and 0.1 mmol/L ZnCl₂) for 60 min at 37 °C. Neopterin was measured, after deproteinization through an Ultrafree-MC filter, by HPLC.
A blank without cell extract, containing 50 \( \mu \)mol phosphate buffer, pH 6.4, 125 \( \mu \)mol of BH4 is then necessary to stabilize the formation of BH4. BH4 is then samples on ice and adding 15 \( \mu \)mol of BH4 for 120 min at 37 °C; another 50 \( \mu \)mol of BH4 is then used as a blank. BH4 is then measured as the oxidized product, biopterin.

Unstimulated cells from one confluent 78-cm² plate were suspended in 150 \( \mu \)L of lysis buffer (10 mmol/L Tris-HCl, pH 7.4, containing 10 mL/L Triton X-100) and lysed by freezing and thawing six times. After centrifugation at 15 000g for 5 min, 50 \( \mu \)L of the supernatant was added to 60 \( \mu \)L of reaction mixture containing 100 mmol/L Tris-HCl, pH 7.4, 10 mmol/L MgCl₂, 1 mmol/L NADPH, 1 mmol/L NADH, 3 mM of SR, 220 mM of DHPR, and 60 \( \mu \)mol/L dihydroneopterin triphosphate in a final volume of 110 \( \mu \)L. A 50-\( \mu \)L aliquot was incubated for 120 min at 37 °C; another 50 \( \mu \)L was used as a blank. A blank without cell extract, containing 50 \( \mu \)L of lysis buffer and 60 \( \mu \)L of reaction mixture, was incubated in the same run. The reaction was stopped by cooling the samples on ice and adding 15 \( \mu \)L of 300 g/L trichloroacetic acid for protein precipitation, followed by centrifugation (5 min at 15 000g) and oxidation. Oxidation was performed by incubation of 50 \( \mu \)L of the supernatant with 10 \( \mu \)L of 10 g/L iodine for 60 min at room temperature in the dark. The same procedure was used for the blanks. After oxidation, 15 \( \mu \)L of 10 g/L ascorbic acid was added to destroy excess iodine. Samples were diluted (1:4 by volume) with water and heated at 60 °C for 2 h before being deproteinized through an Ultrafree-MC filter and analyzed by HPLC (32). One unit of PTPS produces 1 \( \mu \)mol of biopterin per minute at 37 °C.

SR ASSAY
The activity was assayed according to the method described by Ferre and Naylor (34) with some modifications. The assay monitors the conversion of sepiapterin to BH₂, which is then measured as the oxidized product, biopterin.

Unstimulated cells from one confluent 78-cm² plate were suspended in 1 mL of lysis buffer (0.1 mol/L potassium phosphate buffer, pH 6.4, containing 0.15 mol/L KCl, 0.68 mol/L glycerol, and 2.5 mmol/L EDTA), lysed by freezing and thawing six times, and centrifuged for 5 min at 15 000g. A 20-\( \mu \)L portion of the supernatant was incubated in a freshly prepared reaction mixture (final volume, 50 \( \mu \)L) containing 0.1 mol/L potassium phosphate buffer, pH 6.4, 125 \( \mu \)mol/L sepiapterin, and 250 \( \mu \)mol/L NADPH at 37 °C for 30 min in the dark. A blank with cell extract was immediately oxidized, and a blank without cell extract (20 \( \mu \)L of lysis buffer in 50 \( \mu \)L of reaction mixture) was incubated with the samples. The reaction was stopped by adding 10 \( \mu \)L of oxidizing solution (5 g/L iodine and 10 g/L potassium iodide in 1 mol/L HCl) and maintained at room temperature in the dark for 30 min. Excess iodine was removed by adding 10 \( \mu \)L of 10 g/L ascorbic acid solution (freshly prepared). The sample was then deproteinized through an Ultrafree-MC filter and analyzed by HPLC (32). One unit of enzyme activity produces 1 \( \mu \)mol of biopterin per minute at 37 °C.

DHPR ASSAY
The activity of this enzyme was assayed, as described previously (24), by monitoring the oxidation of NADH during the reduction of 6,7-dimethyldihydropterin (quinonoid isomer) to 6,7-dimethyltetrahydropterin, catalyzed by DHPR. The pterin derivative 6,7-dimethyltetrahydropterin was oxidized in situ by peroxidase in the presence of H₂O₂ to q-6,7-dimethyldihydropterin and thus was recycled for the DHPR reaction.

Unstimulated fibroblasts from three confluent 78-cm² plates were suspended in 450 \( \mu \)L of lysis buffer (5 mmol/L Tris-HCl, pH 7.4, containing 0.1 mol/L KCl), lysed by freezing and thawing six times, and centrifuged at 15 000g for 20 min. The supernatant was then analyzed for protein concentration, and an amount corresponding to 120 \( \mu \)g of protein was incubated in the assay. The assay mixture contained 8.8 mmol/L H₂O₂, 1 mmol/L NADH, 25 kU/L peroxidase, and reaction buffer (50 mmol/L Tris-HCl, pH 7.0) in a final volume of 1 mL. After a 5-min incubation at 25 °C and before the substrate was added, the absorbance at 340 nm of the samples and blanks (without cell extract) was measured. Subsequently, 15 \( \mu \)L of substrate (1 mmol/L 6,7-dimethyltetrahydropterin) was added and mixed, and the absorbance was measured at the same wavelength (340 nm). The consumption of NADH was measured against corresponding blanks at 340 nm for 5 min. One unit of DHPR oxidizes 1 \( \mu \)mol of NADH per minute at 25 °C.

PROTEIN MEASUREMENT
Protein concentrations in cell lysates were determined by the Bradford spectrophotometric method (35) using γ-globulin as a calibrator. The activities of the various enzymes are expressed as units per mg of protein.

STATISTICAL ANALYSIS
Normal values of neopterin, biopterin, and enzyme activities for controls were expressed as 5th, 50th, and 95th percentiles (Table 1). Medians and minimum and maximum concentrations of the same analytes were used for pathological values because of the limited number of samples (Table 2). Statistical differences between multiple groups (neopterin and biopterin concentrations in controls and patients with different disorders; GTPCH activity in controls and patients with DRD and GTPCH defi-
creased in fibroblasts collected after birth (GTPCH activity was very low in amniocytes but in fibroblasts from males (n = 5) and females, fibroblasts 0–1 year vs fibroblasts >1 year) and biopterin (males, 189–303 pmol/mg) were found between 17–99 pmol/mg) and neopterin (males, 30–94 pmol/mg; females, 0.4–1.8 pmol/mg) showed no significant differences. SR activity was similar in cells collected before and after birth, as well as in males and females (males, 96–202 mU/mg; females, 120–155 mU/mg) and also in different age groups. DHPR activity was also similar in amniocytes and fibroblasts, as well as in males and females (males, 5–7.8 mU/mg; females, 4.2–8.9 mU/mg). A decrease in activity was observed after 1 year of age (0–1 year, 5.4–8.9 mU/mg; >1 year, 4.2–7.0 mU/mg; P = 0.03). No differences were observed in other age groups after 1 year. There were no significant increases in PTPS, SR, and DHPR activities after stimulation with cytokines (data not shown).

MEASUREMENT OF NEOPTERIN AND BIOPTERIN PRODUCTION AND ENZYME ACTIVITIES IN FIBROBLASTS FROM PATIENTS

Neopterin and biopterin concentrations were significantly different between controls and patients (P <0.001; Table 2). Cytokine-stimulated GTPCH-deficient cells (autosomal recessive form, three cases) showed extremely low

Table 1. Reference values for neopterin and biopterin production and GTPCH activity in cytokine-stimulated control cells, and PTPS, SR, and DHPR activities in unstimulated control cells.

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Amniocytes (n = 14)</th>
<th>Fibroblasts (n = 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neopterin, pmol/mg</td>
<td>5       0–18</td>
<td>50       4–43</td>
</tr>
<tr>
<td></td>
<td>95       14–98</td>
<td>95       154</td>
</tr>
<tr>
<td>GTPCH activity, μU/mg</td>
<td>5       0.04–1.4</td>
<td>50       0.2–2.6</td>
</tr>
<tr>
<td></td>
<td>95       1.8–6.5</td>
<td>95       3.0–0.7</td>
</tr>
<tr>
<td>PTPS activity, μU/mg</td>
<td>5       1.0–0.4</td>
<td>50       3.0–0.7</td>
</tr>
<tr>
<td></td>
<td>95       3.4–1.6</td>
<td>95       3.4–1.6</td>
</tr>
<tr>
<td>SR activity, μU/mg</td>
<td>5       89–99</td>
<td>50       143–138</td>
</tr>
<tr>
<td></td>
<td>95       313–185</td>
<td>95       313–185</td>
</tr>
<tr>
<td>DHPR activity, μU/mg</td>
<td>5       5.6–4.5</td>
<td>50       7.5–6.7</td>
</tr>
<tr>
<td></td>
<td>95       9.6–8.3</td>
<td>95       9.6–8.3</td>
</tr>
</tbody>
</table>

* Activity measured in unstimulated cells.

Table 2. Neopterin and biopterin production and enzyme activity in fibroblasts of patients with DRD and autosomal recessive BH4 deficiencies.

<table>
<thead>
<tr>
<th>BH4 Deficiency</th>
<th>Neopterin, pmol/mg</th>
<th>Biopterin, pmol/mg</th>
<th>Enzyme activity, μU/mg (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRD (n = 8)</td>
<td>Median 0</td>
<td>84</td>
<td>0.4 (15.4)</td>
</tr>
<tr>
<td></td>
<td>Range 0–27</td>
<td>0–139</td>
<td>0.1–0.6 (3.8–23)</td>
</tr>
<tr>
<td>GTPCH deficiency (n = 3)</td>
<td>Median 0</td>
<td>24</td>
<td>0.8 (30.7)</td>
</tr>
<tr>
<td></td>
<td>Range 0–26</td>
<td>0–74</td>
<td>0.7–1.0 (26.9–38.5)</td>
</tr>
<tr>
<td>PTPS deficiency (n = 7)</td>
<td>Median 475</td>
<td>0</td>
<td>&lt;0.05 (&lt;7)b</td>
</tr>
<tr>
<td></td>
<td>Range 334–734</td>
<td>0</td>
<td>&lt;0.05 (&lt;7)b</td>
</tr>
<tr>
<td>DHPR deficiency (n = 3)</td>
<td>Median 49</td>
<td>179</td>
<td>&lt;0.3 (&lt;5)c</td>
</tr>
<tr>
<td></td>
<td>Range 29–81</td>
<td>98–181</td>
<td>&lt;0.3 (&lt;5)c</td>
</tr>
<tr>
<td>Controls (n = 35)</td>
<td>43–232</td>
<td>(18–98)</td>
<td>(154–303)</td>
</tr>
</tbody>
</table>

* Neopterin and biopterin concentrations (in all patients) and GTPCH activity (in patients with DRD and autosomal recessive GTPCH deficiency) were measured in cytokine-stimulated fibroblasts. PTPS and DHPR activities (in PTPS- and DHPR-deficient patients, respectively) were measured in unstimulated fibroblasts.

b Activity measured in unstimulated fibroblasts.

Results

REFERENCE VALUES FOR NEOPTERIN AND BIOPTERIN PRODUCTION AND GTPCH ACTIVITY IN STIMULATED CELLS

After stimulation for 24 h with cytokines, the concentrations of neopterin and biopterin in amniocytes were significantly lower (P <0.001) than in skin fibroblasts collected after birth (Table 1). There were no differences in the production of neopterin (males, 30–94 pmol/mg; females, 17–99 pmol/mg) and biopterin (males, 189–301 pmol/mg; females, 153–327 pmol/mg) were found between fibroblasts from males (n = 21) and females (n = 14). GTPCH activity was very low in amniocytes but increased in fibroblasts collected after birth (P <0.01). Fibroblast GTPCH activity was higher in females than in males (males, 1.4–7.3 μU/mg; females, 1.4–3.9 μU/mg; P = 0.016). No significant differences was observed at different ages (0–1 year, 1.7–4.9 μU/mg; >1 year, 1.3–7.3 μU/mg; P = 0.97; activity 0–10 years vs activity >10 years, P = 0.74).
concentrations of neopterin and biopterin (Fig. 2) and a median enzyme activity of 0.8 μU/mg of protein (30.7% of the 50th percentile for normal activity). Cells from DRD patients (eight cases) also showed reduced neopterin and biopterin concentrations compared with controls (Fig. 2). In both diseases, GTPCH activity was lower than in controls (P <0.001). In DRD patients, the median enzyme activity was 0.4 μU/mg of protein (15.4% of the 50th percentile for normal activity), ranging between 0.1 (3.8%) and 0.6 μU/mg (23%). GTPCH activity in the autosomal recessive form was higher than in the autosomal dominant form (GTPCH deficiency vs DRD, P <0.01). PTPS-deficient cells (seven cases) contained extremely high concentrations of neopterin (median, 475 pmol/mg; range, 29–81 pmol/mg; for biopterin, median, 179 pmol/mg; range, 99–181 pmol/mg; Fig. 2), with biopterin concentrations significantly lower than in controls (P <0.001). Enzyme activity was not detectable in unstimulated fibroblasts from the same patients (<0.3 mU/mg, 5% of 50th percentile for controls).

**Discussion**

Autosomal recessive BH4 deficiencies are characterized by the presence of neonatal hyperphenylalaninemia with abnormal urinary pterin excretion. The differential diagnosis between phenylketonuria and BH4 deficiencies is extremely important because BH4-deficient patients do not respond to low phenylalanine dietary treatment. However, they benefit from early substitution therapy with BH4 and neurotransmitter precursors (1-Dopa and 5-hydroxytryptophan) (36, 37).

DRD is diagnosed mainly by the typical clinical signs and symptoms and by the responsiveness to therapy with 1-Dopa/Carbidopa. Hyperphenylalaninemia usually is not present in these patients; however, the oral phenylalanine loading test is consistent with a partial deficiency of BH4 in the liver. Plasma phenylalanine/tyrosine ratios increase significantly 4–6 h after the challenge (38). Unfortunately, false-positive results with this test are possible; in fact, heterozygous phenylketonuric subjects show the same abnormal phenylalanine/tyrosine profiles (39), whereas a small number of genetically confirmed DRD subjects showed no abnormalities with this test (40). A lumbar puncture for CSF examination is invasive but most informative for the diagnosis of both recessive BH4 deficiencies (41) and DRD (10). Low CSF concentrations of homovanillic acid and 5-hydroxyindoleacetic acid, metabolites of dopamine and serotonin, respectively, are found in all variants of BH4 deficiencies. Furthermore, both neopterin and biopterin are very low in GTPCH deficiency, neopterin is high and biopterin low in PTPS deficiency, and biopterin concentrations are very high in DHPR deficiency (8, 41). CSF examinations in DRD patients revealed low concentrations of homovanillic acid, indicating a lack of dopamine synthesis, and low neopterin and biopterin concentrations are consistent with a GTPCH deficiency (10).

The final diagnosis of BH4 deficiencies is achieved by enzyme activity determinations and, with some limitations, by detection of mutations. PTPS and DHPR activities are measurable in many different tissues and cells such as erythrocytes (21, 22), fibroblasts (23, 24), and liver (19, 20). Dried blood spots are routinely used for DHPR deficiency screening among hyperphenylalaninemic newborns (22). Measurement of GTPCH activity is rather difficult because this enzyme is not expressed in blood cells and fibroblasts. Blau et al. (26) described an assay in phytohemagglutinin-stimulated mononuclear blood cells that was used to measure reduced activity in heterozygous and homozygous GTPCH subjects, and subsequently in DRD patients (12). The test must be performed.

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**Figure 2.** Neopterin (A) and biopterin (B) concentrations [median (•) and range (bars)] in cytokine-stimulated fibroblasts from controls (n = 35), DRD patients (n = 8), autosomal recessive GTPCH-deficient patients (n = 3), PTPS-deficient patients (n = 7), and DHPR-deficient patients (n = 3).
with fresh blood samples, within 20 h after sample collection (27). Bezin et al. (42) suggested that the stimulation of lymphoblasts could lead to incorrect determinations of the actual activity of GTPCH in DRD patients.

Because not all GTPCH-deficient DRD patients have GCH1 mutations in the coding region or in the splice sites, which are detectable by the current genomic DNA sequencing of GCH1 (43), and because of the high occurrence of sporadic mutations in this gene (14), DNA testing for the autosomal dominant DRD is not suitable for routine clinical practice. Suzuki et al. (44) reported that in 40% of DRD patients no mutation was found in the GCH1 gene but that some of them showed reduced GTPCH activity in phytohemagglutinin-stimulated mononuclear blood cells. Therefore, measurement of enzyme activity is extremely important for the diagnosis of DRD. Recent studies demonstrated that cytokine-stimulated fibroblasts may be used for investigating BH4 pathway integrity (28), by measuring intracellular neopterin and biopterin production patterns, and for GTPCH activity measurement (29). Here, we adapted these methods to investigate in cytokine-stimulated fibroblasts both pterin production patterns and GTPCH activity, with a diagnostic purpose. We first determined the reference values of intracellular neopterin and biopterin in fibroblasts and amniocytes after stimulation with cytokines. Under these conditions, amniocytes appeared to produce rather low amounts of pterins, consistent with their low GTPCH activity (Table 1). Therefore, these cells are not suitable for prenatal diagnosis of GTPCH deficiency. On the other hand, prenatal diagnosis of PTPS and DHPR deficiency should be possible because the activities of these two enzymes are well detectable in amniocytes (Table 1).

We observed that cells collected in the first year of life showed higher PTPS and DHPR activities than cells collected at a later age. GTPCH activity was slightly but not significantly higher when cells were collected during the first year of life. Because Hibiya et al. (27) reported a higher GTPCH activity in mononuclear blood cells in the first 10 years of life, we compared fibroblasts of these two control groups and found no significant difference. Only GTPCH activity was different between males and females, with higher activities in females. In contrast, lower activities in females were reported by Ichinose et al. (12), and no difference was found by Hibiya et al. (27) in mononuclear blood cells. The analysis of a larger collection of controls is probably needed to better define these differences in fibroblasts; however, this is not of diagnostic importance.

The study of pathological samples allowed us to define the utility of cultured skin fibroblasts in the diagnosis of BH4 defects and DRD. Except for SR, for which no patients have as yet been reported, all the studied enzymes exhibited very low activities in patient samples compared with controls (Table 2). Indeed, PTPS and DHPR activities in unstimulated fibroblasts from the corresponding patients were below the detection limit (<7% and <5% of the 50th percentile of normal activity, respectively). PTPS deficiency was characterized by a typical pattern of pterin production in stimulated fibroblasts, with high neopterin concentrations and no measurable biopterin. The same pattern was observed in urine and CSF (8). DHPR-deficient patients showed normal intracellular neopterin concentrations after stimulation, whereas biopterin concentrations were lower than in controls. Milstien et al. (28) suggested that the possible pattern of pterin production in these cells could be characterized by increased neopterin because of the lack of feedback inhibition of GTPCH by BH4, and reduced biopterin because of the fast transport rates of the oxidized forms of pterins out of the cells. This hypothesis would explain the differences of pterin patterns between the cell extracts and the extracellular fluids (plasma, urine, CSF), where increased biopterin is characteristic of the disease (41).

GTPCH activity was lower in stimulated fibroblasts from patients with the autosomal dominant form than in those from patients with the autosomal recessive form. Previous measurements in phytohemagglutinin-stimulated mononuclear blood cells showed a reduction of GTPCH activity in DRD patients to <20% (12), which was comparable to our finding, and a reduction of activity to <3% in autosomal recessive GTPCH deficiency (26), in contrast to our results in stimulated fibroblasts (Table 2). A dominant-negative effect of the mutated subunit on the activity of the wild-type subunit in DRD was suggested by Hirano and Ueno (45). Indeed, cotransfection experiments with two mutant GTPCH alleles, identified in DRD patients, demonstrated that mutant proteins inhibit the activity of the wild-type enzyme. In contrast, cotransfection of the mutant allele found in a patient with autosomal recessive GTPCH deficiency failed to inhibit the wild-type activity, producing much higher GTPCH activity (46). The molecular mechanism of this dominant-negative effect and the different phenotype, which is more severe in recessive GTPCH deficiency despite higher enzyme activity in vitro, remain to be explained. Recently, Suzuki et al. (44) reported that a reduction in the amount of enzymatic protein could be responsible for the lower GTPCH activity in DRD compared with the autosomal recessive GTPCH deficiency. A dominant-negative effect might still be acting at an earlier stage, at either the translational or the transcriptional level. Regardless, GTPCH activity measurement in cytokine-stimulated fibroblasts was diagnostic for both disorders.

In conclusion, skin cultured fibroblast assays are confirmatory for all suspected BH4 deficiencies. Our studies now provide for the first time reference values for direct and indirect (intracellular neopterin and biopterin) estimation of enzyme activity. The study of BH4 metabolism is particularly useful in DRD patients because it allows an etiologic diagnosis when other tests are inconclusive.
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