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SEPIAPTERIN REDUCTASE DEFICIENCY: MOLECULAR ANALYSIS IN A NEW CASE PRESENTING WITH NEUROTRANSMITTER DEFICIENCY WITHOUT HYPERPHENYLALANINEMIA

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ABSTRACT

A 27 year-old woman presenting with the clinical picture characterized by hypersomnolence, mild psychomotor retardation, dystonia, oculomotor apraxia, weakness, and striking diurnal variations of symptoms was found to be sepiapterin reductase (SR)-deficient by investigations of pterins in cytokine stimulated fibroblasts. There was no detectable SR activity in the non-stimulated fibroblasts and mutation analysis revealed a homozygous Arg to Gly exchange at codon 150 (R150G). This is the third case found with SR deficiency characterized by a severe monoamine neurotransmitters deficiency without hyperphenylalaninemia.

INTRODUCTION

Tetrahydrobiopterin (BH_4) is a natural cofactor of aromatic amino acid hydroxylases and nitric oxide synthase (1). It is synthesized from GTP by the enzymes GTP cyclohydrolase I (GTPCH), 6-pyruvoyl-tetrahydropterin synthase (PTPS), and sepiapterin reductase (SR) (Figure 1).

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The last two-step reduction of 6-pyruvoyl-tetrahydropterin (PTP) to BH_4 can be alternatively catalyzed by the enzymes aldose reductase (AR) and carbonyl reductase (CR). During hydroxylation of phenylalanine to tyrosine by phenylalanine-4-hydroxylase, tyrosine to L-Dopa by tyrosine-3-hydroxylase, and tryptophan to 5-hydroxytryptophan by tryptophan-5-hydroxylase, BH_4 is oxidized to carbinolamine-4a-tetrahydrobiopterin which is subsequently reduced back to BH_4 by the enzymes pterin-4a-carbinolamine dehydratase and dihydropteridine reductase (not shown).

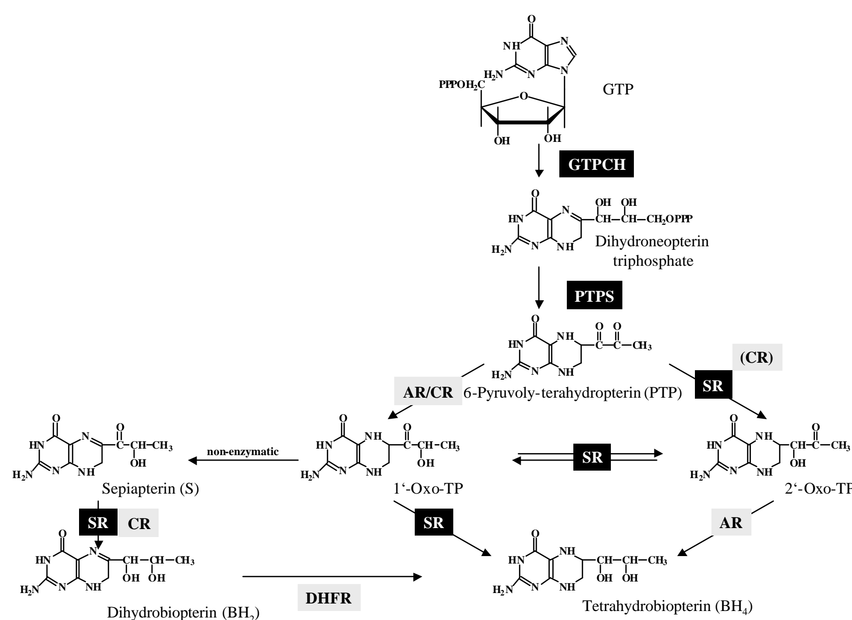

Figure 1. De novo biosynthesis of BH_4 from GTP and the proposed salvage pathway. Under normal physiological conditions PTP is reduced to BH_4 via 2'-oxo-TP and 1'-oxo-TP by SR. In the absence of SR 1'-oxo-TP is oxidized non-enzymatically to sepiapterin which is reduced to BH_4 by CR and dihydrofolate reductase (DHFR) (salvage pathway).

The classical forms of BH_4 deficiencies are characterized by severe monoamine neurotransmitters deficiency accompanied by hyperphenylalaninemia (2). They are caused by autosomal recessive mutations in genes encoding for the enzymes involved in the BH_4 biosynthesis or regeneration; e.g. GTPCH, PTPS, and DHPR (3). Pterin-4a-carbinolamine dehydratase deficiency is a benign form of BH_4 deficiency characterized by transient hyperphenylalaninemia and normal brain neurotransmitters. Autosomal dominant GTPCH deficiency (Dopa-responsive dystonia, DRD) (4) and the recently discovered autosomal

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recessive SR deficiency (5) present phenotypically without hyperphenylalaninemia. In contrast to classical forms of BH_4 deficiency, SR deficiency and DRD can not be detected by the neonatal screening for phenylketonuria (PKU). However, these patients can be detected by measurement of the neurotransmitter metabolites, 5-hydroxyindoleacetic acid (5HIAA) and homovanillic acid (HVA), and pterins in CSF or by the investigation of cytokine-stimulated fibroblasts (6). Measurement of neopterin and biopterin in cytokine-stimulated fibroblasts and SR activity in non-stimulated fibroblasts was used for the diagnosis of SR deficiency in a woman with severe monoamine neurotransmitters deficiency.

METHODS

Patient

A 27 year-old woman of Hispanic origin was initially diagnosed as DHPR-deficient (central form) (7) because of the high biopterin and dihydrobiopterin levels in CSF. Laboratory data are listed in the BIODEF database (ID# 373; www.bh4.org/biomdb1.html). Detailed clinical data will be published elsewhere (Friedman et al., in preparation).

Fibroblasts culture and treatment with cytokines

Fibroblasts were cultured in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal bovine serum, 50 units of penicillin/mL and 50 µg of streptomycin/mL. Cells were passaged by trypsinization and studied at low passage numbers. All cells were *Mycoplasma* negative, checked by the Hoechst staining method. Cultures were kept at 37°C in humidified air containing 5% CO_2.

In order to induce the expression of GTPCH, confluent cell monolayers in 78 cm^2 plates were stimulated with recombinant human IFN-γ and TNF-α at concentrations of 250 U/mL and 100 U/mL, respectively, in fresh medium. After incubation for 24 hours, cells were harvested by trypsinization, washed with phosphate-buffered saline and immediately lysed for neopterin and biopterin measurement. SR is constitutively expressed in fibroblasts. Therefore its activities were tested in unstimulated cells. Confluent cells in 78 cm^2 plates were harvested, washed with phosphate-buffered saline, and kept at –80°C until analyzed for SR (8).

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Neopterin and biopterin production in fibroblast extracts

Cytokine-stimulated cells were lysed and oxidized with 6 µL of 1 mol/L HCl and 20 µl of 1% (w/v) iodine solution for 15 minutes at room temperature in the dark. Oxidation was stopped by adding 20 µL of 1% ascorbic acid (w/v). Sample was adjust to pH 8.0-9.0 and hydrolysed with alkaline phosphatase (16.8 U) for 60 minutes at 37°C. The reaction was stopped by acidification to pH 2.0 with 7 µL of 2 mol/L HCl. The sample was subsequently deproteinized on Ultrafree-MC and analyzed by HPLC (9). The intracellular concentrations of neopterin and biopterin after 24 hours stimulation with cytokines are expressed as pmol per mg of protein (8).

SR assay

The assay monitors the conversion of sepiapterin to BH_2, which is then measured as the oxidized product, biopterin (8).
Unstimulated cells were lysed by freezing and thawing, and centrifuged for 5 minutes at 15000 x *g*. Twenty microliters of supernatant were incubated in a freshly prepared reaction mixture (final volume 50 µL) containing 0.1 mol/L potassium phosphate buffer, pH 6.4, 125 µmol/L sepiapterin, 250 µmol/L NADPH, at 37°C for 30 minutes in the dark. A blank with cell extract was immediately oxidized and a blank without cell extract (20 µL of lysis buffer in 50 µL of reaction mixture) was incubated with the samples. The reaction was stopped by adding 10 µL of oxidizing solution (0.5% w/v iodine/1% w/v potassium iodide in 1 mol/L HCl) and maintained at room temperature in the dark for 30 minutes. Excess iodine was removed by adding 10 µL of 1% ascorbic acid solution (w/v, freshly prepared). The sample was then deproteinized through an Ultrafree-MC and analyzed by HPLC (9). One unit of enzyme activity produces 1 µmol biopterin per minute at 37°C. Because the same reaction can be catalyzed by CR, this assay measures both SR and CR activities (10).

Mutation analysis

Based on the published cDNA sequence (11) (accession number M76231), a fragment of 756 bp harboring 96% of the coding sequence was amplified by a one-step RT-PCR (OneStep RT-PCR Kit, Qiagen, Germany) using the cDNA-specific primers SR12 (5'-(8)GCGGGCTGGGGCGTGCTGTG-3') and SR13 (5'-(763) GGGCTCCAGACTTGAACTCG-3') (5). The amplification products from cDNA were separated on 1% agarose gels, purified (Concert Gel Extraction Systems, Life Technologies, Gibco BRL), and directly sequenced using

fluorescence-labeled terminator reagents and an automated sequencer (ABI Prism 310, Applied Biosystems).

The mutation was identified simultaneously on genomic DNA by combined denaturing gradient gel electrophoresis (DGGE) and direct sequencing (A. Romstad, to be published).

Western blot analysis of SR in fibroblasts

For Western blot analysis, SDS-polyacrylamide gel electrophoresis was performed according to the method by Laemmli (12) with a pre-stained SDS-PAGE low-range protein standard from Bio-Rad. Proteins were blotted onto nitrocellulose sheets (Bio-Rad) and stained with the rabbit anti-mouse SR antibody (provided by Y.S. Park, Korea) (1:5000 dilution) followed by a second antibody (goat anti-rabbit IgG alkaline phosphatase conjugate from Bio-Rad; 1:3000 dilution)). Immunostaining was carried out by the colorimetric method for alkaline phosphatase using NBT/BCIP solution (Roche).

RESULTS AND DISCUSSION

Biochemical findings are summarized in Table 1.

Table 1 Neopterin and biopterin in cytokine-stimulated fibroblasts and SR activity in non-stimulated fibroblasts from patient with SR deficiency and control persons.

	Patient #373	**Controls**
Neopterin (pmol/mg prot.)	389	18-98
Biopterin (pmol/mg prot.)	12	154-303
SR activity (µU/mg prot.)	<10	99-185

As in two previously described patients (5,6), neopterin levels were elevated and biopterin levels were markedly reduced in the patient's fibroblasts when stimulated with interferon-γ and tumor necrosis factor-α for 24 hours. SR activity in non-stimulated fibroblasts was below detection limit, indicating SR deficiency. Investigation of pterins and enzyme activity is the only reliable method for the diagnosis of SR deficiency. Low neurotransmitter metabolites 5HIAA and HVA and high biopterin and dihydrobiopterin in CSF from our patient (data not shown) were suggestive for the deficient DHPR activity and initial diagnosis was, similar as in two previous patients (13), a "central" form of DHPR deficiency.

The patient described in this study was homozygous for a A to G transition at nucleotide 448, causing a Arg to Gly exchange at codon 150 (R150G) (Figure 2).

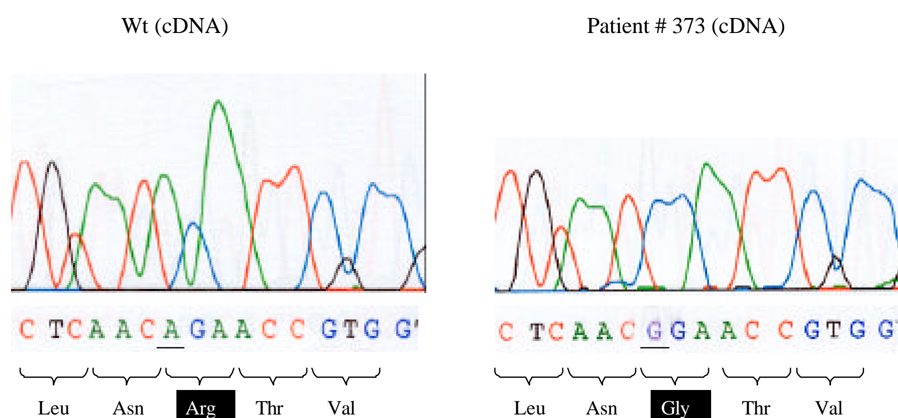

Figure 2. Mutations in the *SPR* gene of the patient with SR deficiency.

The R150G mutation was previously reported in a heterozygous form in a Turkish patient and it has been shown that when expressed recombinantly in *E. coli* the mutant protein was not active (5). As SR protein was hardly visible in the patient's fibroblasts (Figure 3), the inactive mutant SR expressed from these alleles must be degraded.

The exact mechanism of the metabolic impairment in the CNS of this patients is still not clear, but most probably the 1'-oxo-TP intermediate is converted non-enzymatically to sepiapterin which is then reduced to 7,8-dihydrobiopterin by the action of CR. In contrast to peripheral tissues and cells, 7,8-dihydrobiopterin cannot be reduced effectively to BH_4 due to low brain dihydrofolate reductase activity.

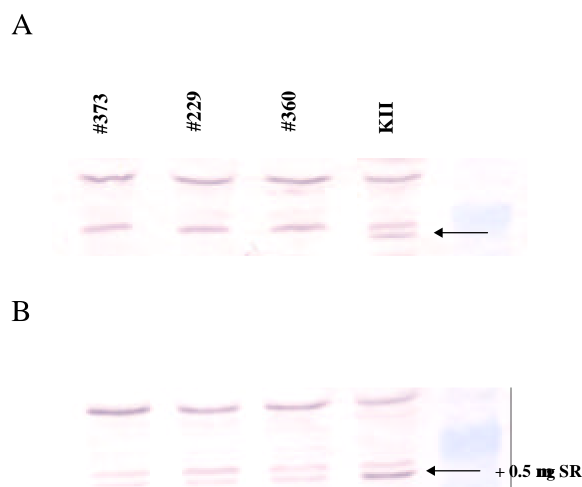

Figure 3. Western blot analysis of SR in primary fibroblasts from three patients with SR deficiency. As control, a fibroblast protein extract from a healthy subject (KII) was used (A). Purified human recombinant SR (0.5 µg) was added to all fibroblast extracts (B).

The expression pattern of SR, CR, AR, and DHFR is different in different tissues and cells. The effect of the R150G mutant SR in the brain is in reduced concentrations of BH_4 and accumulation of dihydrobiopterin. Both of this may impair neuronal cells function. The nitric oxide synthase reaction is known to be uncoupled under conditions of reduced BH_4. It is also known that dihydrobiopterin can replace pre-bound BH_4 and thus potentate superoxide production (14). Superoxide and the NO free radical generate highly cytotoxic peroxynitrite. This may possibly lead to neuronal cell damage (6). In addition, dihydrobiopterin competitively inhibits tyrosine and tryptophan hydroxylases, the two rate-limiting enzymes in the biosynthesis of catecholamines and serotonin (15,16).

In peripheral tissues and cells DHFR is highly active, and we propose that in patients with SR deficiency BH_4 biosynthesis proceeds via the salvage pathway (Figure 1). Neopterin and biopterin levels in urine and plasma from patients with SR deficiency are normal, corroborating this hypothesis. Although these patients present without hyperphenylalaninemia, under loading conditions with phenylalanine (100 mg/kg) hydroxylation reaction is very slow, indicating partially impaired hepatic BH_4 metabolism (6).

In conclusion, we report a new patient with SR deficiency. The diagnosis was made using CSF neurotransmitters metabolites in conjunction with the fibroblasts assay for neopterin and biopterin and SR activity. Finally, SR inactivity was confirmed by mutations analysis. This case further illustrates the importance of systematic investigations (CSF) in patients with suspected impairment of monoamine neurotransmitter biosynthesis. Further investigations are necessary in order to clarify the proposed alternative pathways in patients with SR deficiency and to establish more effective therapy.

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