

RAPID
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Reduced nitric oxide metabolites in CSF of patients with tetrahydrobiopterin deficiency

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Abstract

We investigated CSF concentrations of nitrite and nitrate as indicators of nitric oxide (NO) production in patients with tetrahydrobiopterin (BH₄) deficiencies. Patients with 6-pyruvoyl-tetrahydropterin synthase, sepiapterin reductase and dihydropteridine reductase deficiencies exhibited decreased CSF nitrite + nitrate levels compared with healthy control subjects. Reduced levels of

nitrite + nitrate were not influenced by oral administration of 2.5–5.0 mg/kg tetrahydrobiopterin. Our data indicate impaired NO synthase function in patients with BH₄ deficiency and suggest possible involvement in the neuronal cell dysfunction.

Keywords: dihydropteridine, neurotoxicity, nitric oxide synthase, peroxynitrite, 6-pyruvoyl-tetrahydropterin, sepiapterin. *J. Neurochem.* (2002) **80**, 362–364.

Tetrahydrobiopterin (BH₄) is the essential co-factor for aromatic amino acid hydroxylases and for the NO synthases (NOS; Thöny *et al.* 2000). Classical BH₄ deficiencies are caused by mutations in one of the genes coding for enzymes involved in BH₄ biosynthesis or regeneration (Blau *et al.* 2001b). They are all characterized by severe monoamine neurotransmitters deficiency, mostly accompanied by hyperphenylalaninemia. Typical clinical symptoms usually include neurological disturbances, muscle tone and co-ordination abnormalities, seizures, hypersalivation and delayed motor development. Recently it has been shown that the BH₄ metabolites dihydrobiopterin and sepiapterin competitively inhibit NOS (Jones *et al.* 2001) and that with low BH₄ concentrations the NOS reaction becomes uncoupled (Cosentino *et al.* 1998). Under these conditions highly reactive peroxynitrite (ONOO⁻), generated from superoxide (O₂⁻) and NO[•] radical, may cause oxidative neuronal cell damage and thus contribute to the neurological symptoms. The *hph-1* mouse, a mutant with reduced BH₄ levels and low NOS activity in the brain, suggests that a deficiency of BH₄ may become a limiting factor for NO production (Brand *et al.* 1996). To further study the role of BH₄ in NO production and possible neuronal cell dysfunction, we measured the stable NO metabolites nitrite and nitrate in the CSF of patients with different variants of BH₄ deficiency and found decreased levels compared to controls.

Materials and methods

Patients and control subjects

Cerebrospinal fluid (CSF) samples were obtained from 22 patients (aged 1–25 years) and 20 controls (aged 5 months to 14 years). Twelve patients had 6-pyruvoyl-tetrahydropterin synthase (PTPS) deficiency, nine patients had dihydropteridine reductase (DHPR) deficiency, and one patient had sepiapterin reductase (SR) deficiency. Two patients with PTPS deficiency were untreated and 10 were receiving standard treatment with L-Dopa/Carbidopa (3.0–12.0 mg/kg/day), 5-hydroxytryptophan (2.0–8.0 mg/kg/day), and BH₄ (2.5–5.0 mg/kg/day). All patients with DHPR deficiency were on low-phenylalanine diets and supplemental L-Dopa/Carbidopa (3.5–10.0 mg/kg/day), 5-hydroxytryptophan (2.0–7.0 mg/kg/day), and folic acid (10–20 mg/day). The patient with SR deficiency was receiving L-Dopa/Carbidopa (0.6 mg/kg/day), the MAO inhibitor 'Selegiline' (0.08 mg/kg/day), and folic acid (7.5 mg/day). The bio-

chemical data from all patients are tabulated in the BIODEF database (<http://www.bh4.org/biodef1.html>). No neurological abnormalities were detected in any of the controls. Patients in whom infection was apparent or suspected were excluded from this study.

CSF sampling

Lumbar punctures were performed in the morning, and the first 0.5 mL of CSF was discarded or used for a cell count. The next 1–2 mL of CSF were collected in an EDTA tube and frozen at –80°C until analyzed. The procedures used were in accordance with the current revision of the Helsinki Declaration of 1975.

Nitrite and nitrate measurement

The NOS product NO is extremely reactive and undergoes a series of reactions. The final products *in vivo* are nitrite (NO₂⁻) and nitrate (NO₃⁻). We measured the sum of nitrite and nitrate 'nitrite + nitrate' (the index of total NO production) using a commercial Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). Nitrate was converted to nitrite utilizing nitrate reductase and measured with the Griess reagent. Absorbance was read at 570/630 nm in a MicroELISA Autoreader MR 530 (Dynatech, Chantilly, VA, USA).

Statistical methods

One way ANOVA was used to compare the results between controls and groups with the different BH₄ disorders. Differences were considered significant when *p*-values were < 0.05.

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Abbreviations used: BH₄, tetrahydrobiopterin; DHPR, dihydropteridine reductase; NO, nitric oxide; NOS, NO synthases; PTPS, 6-pyruvoyl-tetrahydropterin synthase; SR, sepiapterin reductase.

Table 1 CSF characteristics of patients with BH₄ deficiency

Mean ± SEM (range)	Nitrite + nitrate μmol/L	Biopterin* nmol/L
PTPS deficiency (n = 12)	0.30 ± 0.21**** (0–2.15)	3.31 ± 0.60 (0–7.2)
SR deficiency (n = 1)	0	70**
DHPR deficiency (n = 9)	0.60 ± 0.24*** (0–1.77)	44.69 ± 3.63** (23.9–60.5)
Controls (n = 20)	2.69 ± 0.26 (1.30–5.30)	23.4 ± 3.4 (10.8–36.3)

*Total biopterin (biopterin + dihydrobiopterin + tetrahydrobiopterin). ** > 70% as dihydrobiopterin and biopterin (< 30% tetrahydrobiopterin). ****p* < 0.005. *****p* < 0.0005.

Results

Patients with BH₄ deficiencies displayed significantly lower levels of nitrite + nitrate in CSF when compared with healthy controls (Table 1). The mean concentrations in patients with DHPR and PTPS deficiency were 0.60 and 0.30 μmol/L, respectively, compared with 2.69 μmol/L in controls. Nitrite + nitrate was not detectable in the CSF from the patient with SR deficiency. In patients with PTPS deficiency displaying low CSF biopterin levels the nitrite + nitrate concentrations were lower than in patients with DHPR deficiency, with high total biopterin and dihydrobiopterin levels, but these differences were not statistically significant (Table 1). The two patients with PTPS deficiency, who were not receiving any treatment, had nitrite + nitrate levels similar to those of the group with BH₄ treatment (data not shown). There were also no differences in the CSF nitrite + nitrate levels when BH₄ was administered, regardless of the daily amount of BH₄ given (data not shown).

Discussion

It has been well documented that BH₄ deficiency states, as seen in patients with inherited disorders of BH₄ metabolism, contributes to cerebral monoamine neurotransmitters depletion (Blau *et al.* 2001b).

Although, some patients with BH₄ deficiency have good outcomes when diagnosed early and treated adequately, there are a number of patients who do not respond to different therapy protocols and who show severe neurodevelopmental retardation despite substitution treatment with BH₄ and the neurotransmitter precursors L-Dopa and 5-hydroxytryptophan (Dudsek *et al.* 2001). One of the reasons for the poor outcome may be the fact that BH₄ is not only the essential co-factor for tyrosine and tryptophan hydroxylases, the rate limiting enzymes in the biosynthesis of catecholamines and serotonin, but is also required for all three isoforms of NOS (Thöny *et al.* 2000). A mouse mutant for the GTP cyclohydrolase I (*hph-1* mouse), the first enzyme in the biosynthesis of BH₄, displayed low BH₄ concentrations and low NOS activity in the brain (Brand *et al.* 1996). Furthermore, Heales *et al.* (1999) demonstrated a link between BH₄ levels and nitrite + nitrate levels in CSF. It has been suggested that under BH₄ deficient conditions, the NOS reaction becomes uncoupled and that reduced NO and increased superoxide production may contribute to neuronal cell pathology through peroxynitrite generation (Fig. 1; Tiefenbacher 2001). Although, peroxynitrite is rather unstable and could also degrade to nitrate, even a small amount of peroxynitrite may be neurotoxic.

Recently, it has been shown that some biopterin metabolites, e.g. dihydrobiopterin and sepiapterin, inhibit NOS *in vitro* (Jones *et al.* 2001). These metabolites accumulate in the CSF of patients with DHPR and SR deficiency (Hyland and Heales 1993; Bonafé *et al.* 2001).

In this study, we documented that patients with BH₄ deficiencies characterized by a deficiency of monoamine neurotransmitters exhibited decreased NO production in the brain as measured by the concentration of nitrite and nitrate in the CSF. The decrease of the CSF nitrite + nitrate levels was similar in different variants of BH₄ deficiency (e.g. PTPS, DHPR and SR deficiency). While in patients with PTPS deficiency reduced NO production may be explained by the low cerebral BH₄ concentrations, in patients with DHPR and SR deficiency dihydrobiopterin probably potentiates the effect of low BH₄ concentrations by inhibiting NOS (Fig. 1). We recently proposed that sepiapterin, a metabolite which accumulates in patients with SR deficiency, exhibits similar effects (Blau *et al.* 2001a). We also showed that the oral administration of low doses of BH₄ (2–5 mg/kg/day) did not alter NO production in the brain. Thus, high dosage BH₄ therapy (10–20 mg/kg/day) and vitamins C and E supplementation may be necessary to improve

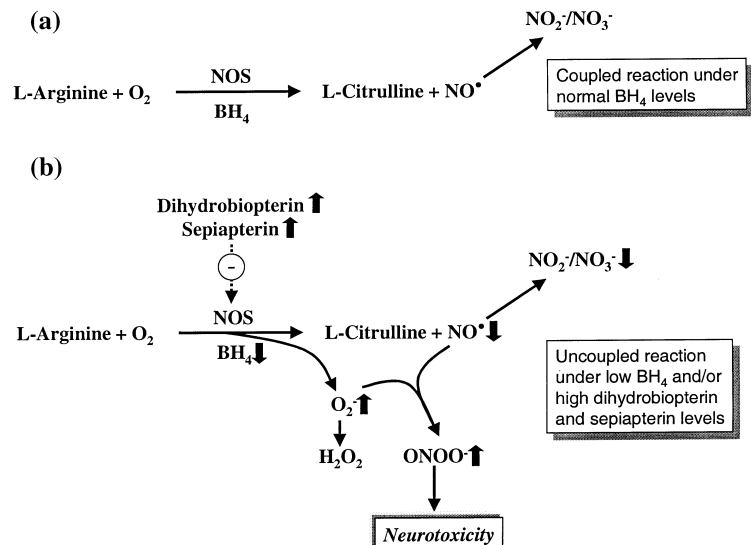


Fig. 1 Proposed mechanism of dihydrobiopterin and sepiapterin-mediated neurotoxicity in patients with BH₄ deficiency. (a) NOS reaction under normal physiological conditions. (b) Under BH₄-deficient conditions NOS reaction becomes uncoupled and generated superoxide (O₂⁻) reacts with nitric oxide (NO) to produce peroxynitrite (ONOO⁻). Peroxynitrite may induce apoptosis of the neuronal cells through oxidation of lipids, proteins and DNA. Furthermore, dihydrobiopterin and sepiapterin displace prebound BH₄ from nitric oxide synthase (NOS) and potentiate the uncoupling (Jones *et al.* 2001).

neuronal NOS function and to prevent superoxide and peroxynitrite generation. In the *hph-1* mouse subcutaneous administration of BH₄ (100 µmol/kg) plus ascorbate (1%, w/v) normalized brain NO metabolism (Canevari *et al.* 1999).

In conclusion, low CSF nitrite + nitrate levels in patients with different forms of BH₄ deficiency have been demonstrated. We would suggest that low BH₄ and high dihydrobiopterin and sepiapterin concentrations in CSF may contribute to the pathophysiology of neurotransmitter deficiency by uncoupling the NOS reaction.

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