

Brief communication

Stimulation of hepatic phenylalanine hydroxylase activity but not *Pah*-mRNA expression upon oral loading of tetrahydrobiopterin in normal mice

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Abstract

Tetrahydrobiopterin (BH₄) supplementation in patients with BH₄-responsive phenylalanine hydroxylase (PAH) deficiency is an alternative to low-phenylalanine diet. To further investigate hepatic BH₄-responsiveness, oral administration of 50 mg BH₄/kg/day for 5 weeks was performed in wild-type mice. We observed a 2-fold increase in PAH protein by quantitative Western blot analysis and a 1.7-fold increase in enzyme activity, but no change in *Pah*-mRNA expression by quantitative real-time PCR analysis in treated mice compared to controls. Our findings support the proposed chemical-chaperone effect of BH₄ to protect PAH.

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Phenylketonuria (PKU; OMIM 261600) or hyperphenylalaninemia is caused primarily (>97% of all cases) by autosomal recessive mutations in the gene coding for phenylalanine hydroxylase (PAH, EC 1.14.16.1). Inactive PAH leads to pathologically elevated blood phenylalanine values (>120 μmol/L) due to insufficient hepatic hydroxylation, which is the major degradation pathway for phenylalanine [1]. PAH-dependent hydroxylation of phenylalanine involves molecular oxygen as co-substrate and the cofactor tetrahydrobiopterin (BH₄). The latter is synthesized and recycled endogenously, and serves as a cofactor also for other enzymes and diverse cellular functions [2]. Elevated serum phenylalanine is screened in newborns by blood spot analysis (Guthrie card). Subsequently, differential diagnosis is performed, including a BH₄ loading test, to discriminate between classical PKU due to mutant PAH and the rare

BH₄ deficiencies due to mutations in one of the cofactor-metabolizing enzymes [1,3]. Treatment of classical PKU requires basically life-long restriction of dietary phenylalanine intake to keep plasma levels to <360 μmol/L [4].

Kure and co-workers reported in 1999, and later other groups, on a new variant of hyperphenylalaninemia, BH₄-responsive PAH deficiency, associated to certain mutations in the *PAH* gene, that can be treated with BH₄ supplementation as an alternative to the low-phenylalanine diet [5–7]. So far reported, these patients have normal values for hepatic BH₄ content and at least one of the two mutant PAH alleles is restricted to a missense mutation with putative residual activity. Several mechanisms were discussed to explain this phenomenon, including a reduced BH₄-affinity for PAH (*K_m* mutants), a chaperone-like effect of BH₄ to protect PAH from misfolding or degradation, BH₄-regulatory changes, and induction of gene expression and/or *PAH*-mRNA stabilization by the cofactor [8].

Based on recent investigations with recombinant mutant and wild-type PAH including in vitro expression in

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presence or absence of BH₄, the mechanism of cofactor-responsiveness turns out to be multifactorial involving decreased cofactor-binding affinity and stabilization of PAH by BH₄, which appears to act as a chemical chaperone [9–12]. An animal model carrying a BH₄-responsive mutant allele for PAH is not available so far. However, several studies with mice have been performed to investigate cofactor-responsiveness in vivo. Kure et al. [13] performed BH₄ loading with a 2-fold intraperitoneal (i.p.) injection of 50 mg/kg in normal mice pre-loaded with phenylalanine and observed a 1.7-fold increase for in vivo PAH activity by measuring ¹³CO₂ production in breath sampling. The molecular basis for this stimulation of the activity was not further investigated, but Kure et al., and previously other authors, have noted that the hepatic BH₄ content at least in rodents appears to be present at sub-saturating concentrations both to form the stabilizing PAH–BH₄ 1:1 complex and to meet the *K_m* concentration needed in the PAH reaction [12–14]. We have recently studied the putative protective effect that BH₄ has on wild-type PAH activity by investigating transgenic mice that had a complete or partial deficiency in the endogenous cofactor biosynthesis and found that PAH enzyme activity and protein content correlated with BH₄ concentration without affecting gene expression or *Pah*-mRNA stability [12]. Nevertheless, no further molecular data were available on the direct effect on hepatic PAH upon BH₄ loading in vivo. Here we addressed the question of what molecular changes occur in mice with normal PAH upon BH₄ administration by performing comparative measurements of BH₄ content, and expression of *Pah*-mRNA, PAH protein, and PAH activity in treated and non-treated animals.

Young adult C57Bl/6 wild-type mice kept under normal diet were loaded with BH₄ (Schircks Laboratories, Jona, Switzerland) during a period of 5 weeks with oral doses of 50 mg BH₄ per kg body weight per day (in a solution containing 10 mg/kg ascorbic acid and 5 mg/kg *N*-acetyl-L-cysteine as antioxidants). As controls, we used age-matched mice that had no treatment. Between 20 and 30 min after the final dose, animals were sacrificed to analyze BH₄ and PAH content in liver by quantitative PCR and Western blot analysis, respectively, as described before [12]. We observed, as depicted in Fig. 1, a BH₄ concentration of 95.6 ± 40.3 pmol/mg liver extract, 5- to 6-fold higher than without treatment (16.6 ± 0.7 pmol/mg untreated). The range of standard deviation was high as orally administered BH₄ seems to be absorbed with high variability, besides that it is rapidly metabolized, thus becoming barely detectable in liver 1–2 h after application (unpublished observation; [15,18]). The presence of PAH was enhanced under BH₄ loading by a factor of 2 for protein content, while the enzyme activity, measured as described [16], was stimulated by a factor of 1.7 (untreated: 5.7 ± 0.4 μg/mg protein content and 41.3 ± 7.7 mU/mg enzyme activity; treated: 11.2 ± 1.6 μg/mg protein content and 69.9 ± 8.8 mU/mg enzyme activity; see also Fig. 1 for details on the PAH activity assay). Regarding *Pah* gene expression, we did not

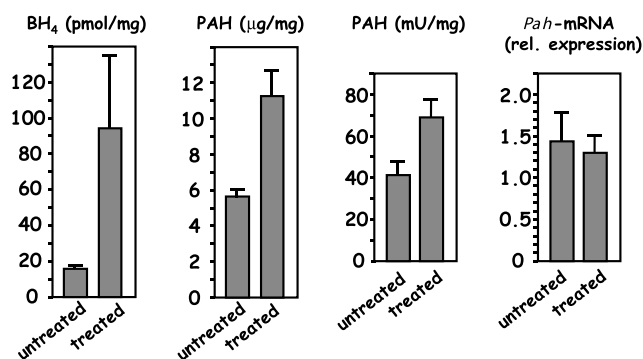


Fig. 1. Effect on hepatic PAH expression in mice supplemented with BH₄ for 5 weeks. Young adult C57Bl/6 wild-type mice ($n = 6$) were treated with daily doses of 50 mg/kg body weight for 5 weeks, sacrificed and analyzed for hepatic BH₄ and PAH protein content, PAH enzyme activity, and *Pah*-mRNA expression by real-time PCR (untreated control mice, $n = 5$). Measurements of BH₄ [17] and quantitative PAH analysis by Western blot [12] were described previously. PAH enzyme activity was assayed as reported in [16]; briefly, the assays (final volume, 50 μl) included 2–5 μl liver homogenate (15–25 mg protein/ml), 0.1 M Na–Hepes, pH 7.0, 10 μM ferrous ammonium sulfate, 1 mM L-phenylalanine, 5 mM dithiothreitol (DTT), 75 μM BH₄ (6(*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin from Schirck's Laboratories, Jona, Switzerland) and 0.04 mg/ml catalase. The enzyme extracts were preincubated 5 min at 25 °C with all components except BH₄ and DTT, which were then added to initiate the reaction. After 2 min at 25 °C the reaction was stopped by adding 1% (v/v) acetic acid in ethanol. L-Tyrosine formation was measured by HPLC and fluorimetric detection.

observe any significant difference in *Pah*-mRNA by quantitative real-time PCR analysis (relative expression of 1.46 ± 0.35 for non-treated versus 1.3 ± 0.21 for treated animals).

The data presented here regarding quantification of *Pah*-mRNA conforms to previous reports on BH₄ not affecting hepatic *Pah* gene expression and/or transcript stability both in animals and in a hepatoma cell line [11,12]. Furthermore, we corroborate the chemical-chaperone effect of BH₄ on PAH protein stability and/or enzyme activity under in vivo conditions as one of the possible mechanisms to explain BH₄-responsiveness in patients with mutant PAH. Enhancement of hepatic PAH activity upon BH₄ loading, now repeatedly shown to be moderate with a factor of ~1.7 for the wild-type enzyme, has obviously a dramatic physiological benefit for some mutant PAH with residual activities, which are conformationally protected by BH₄ and can experience even higher relative stimulation [9]. As mentioned in a previous study [12] oral treatment of normal mice with doses of only 10 mg/kg BH₄ over a period of 10 days did not induce any change in PAH activity or expression. In agreement with this was the observation that a 2-fold i.p. injection of 50 mg/kg BH₄ in wild-type mice did not yield a difference in PAH activity as assessed by an in vivo breath test [13]. On the other hand, PAH stimulation was visible during the first 1 h after BH₄ loading when wild-type mice were pre-loaded with phenylalanine [13]. These previous studies are not directly comparable to what we present here; nevertheless, we hypothesize that the molecular basis for the effect of BH₄ on wild-type PAH is the same in all

cases and does not involve transcriptional up-regulation. Although we have no indication of any adverse effects from *in vivo* studies, potential consequences on other proteins or cellular functions from physiologically elevated BH₄ concentrations cannot yet be ruled out.

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