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

Rossana Scavelli a,1, Zhaobing Ding a,1, Nenad Blau a, Jan Haavik b, Aurora Martínez b, Beat Thöny a,¤

a Division of Clinical Chemistry, Department of Pediatrics, University of Zürich, Switzerland
b Department of Biomedicine, University of Bergen, Norway

Received 18 August 2005; received in revised form 19 September 2005; accepted 22 September 2005
Available online 11 November 2005

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.

Keywords: PKU; Tetrahydrobiopterin; Phenylalanine hydroxylase; Chemical-chaperone

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 (Km 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

1096-7192/S - see front matter © 2005 Elsevier Inc. All rights reserved.
presence or absence of BH$_4$, the mechanism of cofactor-responsiveness turns out to be multifactorial involving decreased cofactor-binding affinity and stabilization of PAH by BH$_4$, which appears to act as a chemical chaperone [9–12]. An animal model carrying a BH$_4$-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 BH$_4$ 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 $^{13}$CO$_2$ 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 BH$_4$ content at least in rodents appears to be present at sub-saturating concentrations both to form the stabilizing PAH–BH$_4$ 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 BH$_4$ 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 BH$_4$ 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 BH$_4$ loading in vivo. Here we addressed the question of what molecular changes occur in mice with normal PAH upon BH$_4$ administration by performing comparative measurements of BH$_4$ 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 BH$_4$ (Schircks Laboratories, Jona, Switzerland) during a period of 5 weeks with oral doses of 50 mg BH$_4$ 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 BH$_4$ 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 BH$_4$ 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 BH$_4$ 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 BH$_4$ 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 µM/mg enzyme activity; treated: 11.2 ± 1.6 µg/mg protein content and 69.9 ± 8.8 µM/mg enzyme activity; see also Fig. 1 for details on the PAH activity assay). Regarding $Pah$ gene expression, we did not 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 BH$_4$ 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 BH$_4$ on PAH protein stability and/or enzyme activity under in vivo conditions as one of the possible mechanisms to explain BH$_4$-responsiveness in patients with mutant PAH. Enhancement of hepatic PAH activity upon BH$_4$ 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 BH$_4$ 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 BH$_4$ 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 BH$_4$ 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 BH$_4$ 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 BH$_4$ 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.

Acknowledgments

We thank Walter Leimbacher and Randi Svebak for excellent technical assistance. This work was supported by grants from the Swiss National Science Foundation and the Research Council of Norway.

References


