Phenylketonuria: towards mechanism-based treatment

de Groot, Martijn Jonathan

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Chapter 7

Effects of tetrahydrobiopterin on brain neurotransmitter concentrations in C57Bl/6 PKU mice

Karen Anjema¹, Martijn J. de Groot¹,², Vibeke M. Bruinenberg², Danique van Vliet¹,², Els van der Goot², M. Rebecca Heiner-Fokkema³, Ido P. Kema³, Martijn van Faassen³, Tanja Scherer⁴, Beat Thöny⁴, Eddy A. van der Zee², Francjan J. van Spronsen¹

¹ Department of Pediatrics, Section of Metabolic Diseases, Beatrix Children’s Hospital, University Medical Center Groningen, the Netherlands
² Department of Molecular Neurobiology, University of Groningen, Groningen, the Netherlands
³ Department of Laboratory Medicine, University Medical Center Groningen, the Netherlands
⁴ Division of Clinical Chemistry and Biochemistry, University Children’s Hospital, Zürich, Switzerland

Submitted
Abstract

Tetrahydrobiopterin (BH4) is the co-factor for tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH-2), the rate-limiting enzymes for cerebral dopamine and serotonin synthesis. In phenylketonuria (PKU), brain concentrations of both dopamine and serotonin are decreased. BH4 treatment might increase neurotransmitter synthesis and cerebral function in phenylketonuria patients. Wild type (WT) and Pah-enu2 C57Bl/6 PKU mice were treated with 40 mg/kg/day BH4 or placebo by intraoral pipetting. Neurotransmitter and amino acid concentrations were measured in brain homogenates, while amino acid concentrations were also measured in plasma. Brain dopamine and serotonin concentrations were reduced in PKU mice compared to WT mice, both placebo-treated. Brain neurotransmitter concentrations of BH4-treated PKU mice were not significantly different from those of placebo-treated PKU mice. However, in WT mice, brain serotonin concentrations were significantly higher in the BH4-treated group (BH4: median 4.50 (IQR 4.36-4.60) versus placebo 3.88 (3.79-4.42) nmol/g wet weight, p=0.021). Brain dopamine concentrations were not statistically different between both WT groups. We concluded that BH4 treatment under the current conditions did not affect brain neurotransmitter concentrations in PKU mice. However, in WT mice BH4 increased brain concentrations of serotonin, which could indicate increased cerebral TPH-2 activity. Possibly, too high concentrations of brain phenylalanine or too low concentrations of brain tryptophan inhibit proper function of this enzyme and therefore abolish the possible cerebral effect of BH4 in PKU.
Introduction

In phenylketonuria (PKU, OMIM 261600), deficiency of the hepatic phenylalanine hydroxylase (PAH) enzyme results in insufficient conversion of phenylalanine (Phe) to tyrosine (Tyr). Untreated, this deficiency results in markedly elevated plasma Phe concentrations, which are associated with severe mental retardation. Timely diagnosis and initiation of dietary Phe restriction lead to cognitive outcome within normal limits (1,2). Still, early and continuously treated patients show mild neuropsychological deficits (3-5), and have an increased risk of developing anxiety and depressive disorders (6-8).

Reduced cerebral concentrations of dopamine (DA), norepinephrine (NE), and serotonin (5-HT) are believed to contribute to the impairments of brain function in PKU (9,10). One study on neurotransmitters in deceased and untreated PKU patients has shown that concentrations of DA, NE and 5-HT were reduced by 60-70% in the caudate nuclei (11). CSF obtained from both late-treated and early and continuously treated PKU patients shows decreased concentrations of the neurotransmitter metabolites homovanillic acid and 5-hydroxyindoleacetic acid (5-HIAA) compared to controls (12-14), in association with impaired neuropsychological performance (15,16). Phe restriction increases the CSF concentrations of these metabolites (11-13,17). The rate-limiting step of cerebral synthesis of DA, NE and epinephrine is the hydroxylation of Tyr to L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH), whereas in 5-HT synthesis the rate-limiting step is the hydroxylation of tryptophan (Trp) to 5-hydroxytryptophan by tryptophan hydroxylase (TPH-2).

Recently, the PAH co-substrate and co-factor tetrahydrobiopterin (BH4) was found to increase PAH activity in a subset of PKU patients, who are described as BH4-responsive patients (18-21). Some patients being tested for BH4-responsiveness during several weeks reported feeling more relaxed and to have a better ability to concentrate during BH4 treatment, while their blood Phe concentrations did not change (unpublished observations). Possibly, these improvements relate to the fact that in addition to its effect on PAH in the liver, BH4 also serves as a co-factor for TH and TPH-2 in the brain. Although evidence in humans is scarce (22-24), studies in laboratory animals show that BH4 crosses the blood-brain barrier when dosed at 20 mg/kg/day or higher (25-30).

Therefore, we hypothesized that BH4 treatment enhances cerebral TH and TPH-2 activity, thereby increasing brain neurotransmitter concentrations, independent of its effect on blood Phe concentrations. To investigate this hypothesis, we studied neurotransmitter and amino acid concentrations in the C57Bl/6 Pah-enu2 PKU mouse model treated with 40 mg/kg/day BH4.
Chapter 7

Experimental procedures

Animals

The reported experiment was approved by the ethics committee for the use of experimental animals of the University of Groningen, in accordance with national and international laws and standards for animal protection. A breeding colony was initiated using heterozygous founders, generously provided by Prof. B. Thöny (Department of Clinical Chemistry and Biochemistry, University Children’s Hospital, Zurich, Switzerland). Wild type (WT, C57Bl/6) and PKU (C57Bl/6 Pah-enu2) mice of both genders were used for the experiment. Animals were weaned at the age of four weeks and individually housed two weeks before the beginning of the experiment under a 12 hour light/dark cycle at 21 ± 1 ºC. Animals had ad libitum access to water and normal chow (RMH-B food pellets, Arie Block BV, Woerden, The Netherlands).

Genotyping

Genetic characterization was performed on DNA extracted from tail tissue using quantitative PCR analysis. Primers were based on GenBank and designed to replicate an amplicon on exon 7 of the PAH gene according to the guidelines of Eurogentec. We used double-dye probes tagged with a non-fluorescent Black-Hole Quencher1TM (Eurogentec) that transforms the absorbed excitation energy of the reporter into heat (absorbance maximum at 435 nm). WT and Pah-enu2 probes were tagged with a FAM fluorophore and a Yakima Yellow fluorophore (Epoch Biosciences), respectively. Both probes contained locked nucleic acids according to the guidelines of Exiqon to enhance mismatch discrimination. Reactions were carried out on an ABI Prism 7500 sequence detection system, using Applied Biosystems’ standard thermal cycling parameters (10 minutes 95°C followed by 40 cycles of 15 minutes at 95°C and 1 minute at 60°C).

Study design

Mice were divided into four groups: placebo-treated WT, BH4-treated WT, placebo-treated PKU, and BH4-treated PKU, with n=8 animals per group. (6R)-5,6,7,8-Tetrahydro-L-biopterin dihydrochloride was purchased at Schircks Laboratories (Jona, Switzerland). BH4 was administered at a dose of 40 mg/kg/day in two doses by intra-oral pipetting two hours after the beginning of the light phase and two hours after the beginning of the dark phase (under red light conditions). Both the BH4 solution and the placebo solution contained 40 mg/kg ascorbic acid and 20 mg/kg N-acetyl-L-cysteine. Treatment started at age 2.5-4.5 months. At day 31 of the study, approximately three hours after the last morning dose, the mice
were anesthetized with isoflurane. Next, blood was obtained by cardiac puncture, collected in heparinized tubes and centrifuged shortly after collection (12,000 rpm x 10 min). Mice were sacrificed by cervical dislocation, brains were removed and snap-frozen by freeze-clamping in liquid nitrogen. Plasma and brain samples were stored at -80 °C until further processing.

**Biochemical analyses**

Mouse brains were crushed in liquid nitrogen and divided into aliquots. Frozen brain aliquots for amino acid measurements were processed to 20% (weight:volume, mg:µl) homogenates in phosphate-buffered saline (pH 7.4). For neurotransmitter analyses, brain aliquots were processed to 20% (weight:volume) homogenates in acetic acid (0.08 M). Brain homogenates were sonified on ice at 11-12 W for approximately 30 s per sample and centrifuged at 12,800 rpm for 10 min at 4°C. The supernatant was used for further analyses.

For amino acid concentration measurements, brain homogenate supernatants and plasma samples were processed using the same method. Norleucine was used as an internal standard (1:1, volume:volume), with 60 mg/ml sulfosalicylic acid to precipitate the dissolved proteins. Samples were vortexed and centrifuged at 20,800 rcf for 4 min. The supernatant was pipetted into capsules and measured by high-performance liquid chromatography (HPLC) using a cation exchange resin followed by post-column ninhydrin derivatization on a Biochrom 30 apparatus (Pharmacia Biotech, Cambridge, UK).

For neurotransmitters, neurotransmitter metabolites and Trp concentration measurements, the supernatants of the 20% brain homogenates were further diluted to 2% homogenates in acetic acid (0.08 M). An antioxidative solution containing 400 mg/l ascorbic acid and 1,616 g/l ethylenediaminetetraacetic acid was prepared in demineralised water. For catecholamine concentrations measurements, 10 µl of each 2% homogenate was pipetted in a 96 wells plate with 40 µl anti oxidative solution. For indole concentration measurements (including the indole derivative Trp), 25 µl of each 2% homogenate was pipetted in a 96 wells plate with 25 µl antioxidative solution. Analysis was performed using isotope dilution mass spectrometry, essentially as described by Van de Merbel et al. (31).

**Statistical analyses**

Data with a normal distribution are presented as mean ± SD, whereas data with a skewed distribution are presented as medians with interquartile ranges (IQR). The Shapiro-Wilk test was used to test whether the variables in each experimental group were normally distributed. To test whether body weight changed during the
experiment, the Wilcoxon signed rank test was used. To compare normally distributed independent data the two-sample t-test was used. With skewed independent data, the Mann-Whitney U test was used. Statistical analyses were performed using IBM Corp. SPSS Statistics for Windows, Version 20.0. Armonk, NY, USA. A two-tailed p-value <0.05 was considered to be statistically significant.

### Results

**Baseline characteristics and general health**

Gender and age at the start of the experiment were equally distributed among the treatment groups (Table 1). The Kruskal-Wallis test revealed that body weight at the beginning of the experiment differed significantly among treatment groups (p=0.004). Comparison by Mann-Whitney U test showed that PKU mice weighed significantly less than WT mice (p=0.000), while within mice of the same genotype no significant differences were found between the treatment groups (PKU p=0.536 and WT p=0.798). One mouse (PKU, placebo-treated) was excluded from the experiment prematurely, as the animal developed seizures during daily handling. In the remaining mice, treatment as well as the procedures were well tolerated and did not affect general health. In WT mice, median weight did not significantly change during the experiment. In PKU mice, median weight increased slightly (PKU Placebo 0.9 g p=0.018, PKU BH4 0.7 g p=0.017).

<table>
<thead>
<tr>
<th>Table 1 Baseline characteristics of experimental groups.</th>
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<td>Gender, M:F</td>
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<td>Age (months)^a</td>
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<td>Weight (g)^a</td>
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WT: wild type, PKU: phenylketonuric, M: male, F: female. ^a Values show median with interquartile range in parentheses.

**Plasma phenylalanine and tyrosine concentrations**

Median (IQR) plasma Phe concentration in placebo-treated PKU mice was significantly higher than in WT mice (1459 (1392 – 1646) versus 66 (58 – 79) µmol/l, respectively, p=0.000). In both PKU and WT mice, plasma Phe concentrations did not differ between placebo-treated and BH4-treated mice (p=0.241 and p=0.161). Median plasma Tyr concentration was lower in placebo-treated PKU mice compared to placebo-treated WT mice (37 (35 – 38) versus 79 (64 – 106) µmol/l, p=0.001). Both in PKU and WT mice, no differences in plasma Tyr concentration were found
between placebo-treated and BH4-treated groups (p=0.613 and p=0.574).

**Brain phenylalanine, tyrosine and tryptophan concentrations**

Brain homogenate concentrations of Phe, Tyr and Trp are shown in Figure 1. Brain Phe concentrations were significantly higher in placebo-treated PKU mice compared to placebo-treated WT mice (p=0.000). Brain concentrations of both Tyr and Trp were reduced in placebo-treated PKU mice compared to placebo-treated WT mice (p=0.002 and p=0.002). No differences in Phe, Tyr and Trp were found between the treatment groups in both PKU as WT mice.

![Brain homogenate concentrations of phenylalanine (A), tyrosine (B), and tryptophan (C) in wild type (WT) and phenylketonuric (PKU) mice, either placebo- or BH4-treated. Bar graphs show means ± SD. Box plot whiskers represent the 5th and 95th percentiles.](image)

**Brain neurotransmitter and metabolite concentrations**

Regarding DA and NE, brain homogenate concentrations were significantly lower in PKU mice compared to WT mice (Figure 2). This was also true for DA metabolite normetanephrine (PKU 0.10 ± 0.02 and WT 0.15 ± 0.06 nmol/g wet...
weight, p=0.033). The concentration of 3-methoxytyramine (another DA metabolite) did not significantly differ between PKU and WT (0.54 ± 0.08 and 0.62 ± 0.21 nmol/g wet weight, respectively, p=0.352). In both PKU and WT mice, no differences were found between placebo-treated and BH4-treated groups for DA (PKU p=0.683, WT p=0.464), NE (PKU p=0.465, WT p=0.215), normetanephrine (PKU p=0.450, WT p=0.730) and 3-methoxytyramine (PKU p=0.540, WT p=0.927).

Regarding brain homogenate concentrations of 5-HT and its metabolite 5-HIAA, both were found significantly lower in PKU mice compared to WT mice. In PKU mice, no differences were found between placebo and BH4 treated animals for 5-HT (p=0.714) and 5-HIAA (p=0.961). However, in WT mice the median (IQR) 5-HT concentration was significantly higher in the BH4 treated group versus placebo (4.51 (4.36 – 4.60) versus 3.88 (3.79 – 4.42) nmol/g wet weight, p=0.002). For 5-HIAA, this difference was not found (p=0.489).

**Figure 2** Brain homogenate concentrations of serotonin (A), 5-HIAA (B), dopamine (C), and norepinephrine (D) in wild type (WT) and phenylketonuric (PKU) mice, either placebo- or BH4-treated. Bar graphs show means ± SD. Box plot whiskers represent the 5th and 95th percentiles.
Discussion

The main finding of this study is that BH4-treated WT mice showed higher brain concentrations of 5-HT than their placebo-treated counterparts, and that this effect was not seen in PKU mice, contrary to our hypothesis. This finding is important as it shows that BH4 may indeed increase neurotransmitter synthesis (currently only shown for 5-HT), while additional interventions may be required to observe a similar effect in PKU.

Before discussing the results in more detail, we will address the methodological issues of the study. First, brain BH4 concentrations were not measured. In theory, BH4 could have sufficiently reached the brain of the WT mice, but not of the PKU mice. Although the mechanism of transport of BH4 across the blood-brain barrier is not known, BH4 transport does not seem to be influenced by blood Phe concentrations, as BH4 has been shown to reach the brain in BH4 deficient mice with hyperphenylalaninemia (29). Second, it is not known if cerebral BH4 is influenced by high Phe concentrations, for example by oxidation.

The C57Bl/6 Pah-enu2 mouse is homozygous for a null-mutation obtained by chemical mutagenesis. Our results show that blood Phe concentrations in C57Bl/6 Pah-enu2 mice do not respond to BH4 administration as previously shown for BTBR Pah-enu2 mice (32,33). Therefore, any observed effects of BH4 on neurotransmitter concentrations in Pah-enu2 mice are unrelated to changes of blood Phe concentrations. Thus, these animals provide an excellent model to study the brain-specific actions of BH4 treatment in PKU.

Regarding the results of the present study, two issues deserve attention, i.e. 1) the difference in response to BH4 treatment regarding brain 5-HT concentration in WT and PKU mice, and 2) the different responses of 5-HT and dopamine to BH4 in WT mice. With regard to the different response to BH4 in WT mice compared to PKU mice, there may be several explanations. First, BH4 may have reached the brain in insufficient amounts to exert cerebral effects in PKU mice, in contrast to WT mice. However, as reasoned above, differences in transport of BH4 across the blood-brain barrier in PKU mice and WT mice are unlikely. In addition, both the clinical side-effects and the reports of BH4-non-responsive PKU patients using BH4 during longer time periods to test their BH4 responsiveness suggest that BH4 does reach the brain in these patients (34,35). Second, elevated brain Phe concentrations are suggested to decrease the activity of both TH and especially TPH-2 by inhibition (36), although discussion on this topic remains (37). This possible inhibiting effect of high brain Phe concentrations on cerebral neurotransmitter synthesis may have been too strong for the current dose of BH4 to overcome under the conditions of our experiment. Third, elevated blood Phe concentrations decrease the transport of
the neurotransmitter precursors Tyr and Trp across the blood-brain barrier (38,39), supported by the reduced brain concentrations of these precursors in our PKU mice. Therefore, reduced brain Tyr and Trp concentrations may have been limiting factors for the improvement of cerebral neurotransmitter synthesis on BH4 treatment in PKU mice. This is in line with studies of Joseph and Dyer (40) and Pascucci et al (41), which showed that brain neurotransmitter concentrations were not (fully) restored by only decreasing brain Phe concentrations. Therefore, future studies in PKU mice should examine the combined effects of increasing the cerebral availability of BH4 as well as the precursors of DA and 5-HT, possibly in combination with decreasing the Phe concentration in (blood and) brain.

The observation that BH4 increased brain 5-HT concentrations in WT mice, while brain DA concentrations were unaffected, may be explained by different effects of BH4 on the TH and TPH-2 enzymes. Kinetic analyses on these enzymes have shown that Phe acted as an inhibitor more strongly against TPH-2 than against TH (36). Also, in urine of PKU patients, excretion of 5-HT is inhibited at lower Phe concentrations compared to dopamine (42). In BTBR Pah-enu2 mice, reduced precursor cerebral availability seems to be more limiting to TH than TPH-2, whereas inhibition by high Phe concentrations is more distinct for TPH-2 (41,43). In line with our current findings, previous studies in WT mice showed that BH4 did not influence brain DA concentrations. In contrast, BH4 did increase the amounts of TH protein and its activity (30). Possibly, this finding can be attributed to feedback inhibition mechanisms by catecholamines (44,45) and post-translational regulation of TH by phosphorylation by serine kinases (46).

It has been a matter of debate whether disturbed myelination or neurotransmitter abnormalities are the most important in the pathogenesis of brain dysfunction in PKU (9,10). Very recent studies showed that white matter integrity is important in PKU pathogenesis, even in early and continuously treated PKU patients (47-49). These studies support the theory that neuropsychological dysfunction in PKU is not only related to executive function and maybe even ‘just’ a matter of speed of neural transmission (50-52). Those white matter abnormalities have previously been thought to be primarily due to high blood and brain phenylalanine concentrations, but recent studies showed that the white matter abnormalities might be explained by problems in neurotransmitter metabolism rather than high phenylalanine (40,53), suggesting that both processes are involved in cognitive dysfunction in PKU, and interact with one another. Therefore, neurotransmitter metabolism likely has a central role in the pathogenesis of PKU brain dysfunction one way or the other and improving this metabolism in PKU might be the primary treatment goal.

In conclusion, 40 mg/kg/day of orally administered BH4 seems to improve
brain 5-HT concentrations in WT mice, but not in PKU mice. In both WT and PKU mice, BH4 treatment did not affect brain dopamine concentrations. Possibly, changes in the BH4 treatment regime and/or additional interventions are required to observe BH4-mediated treatment effects in PKU mice. Probably, too low cerebral concentrations of Trp and/or too high cerebral concentrations of Phe inhibit proper function of TPH-2 and therefore abolish the possible effect of BH4. Further studies are needed to investigate the cerebral effects of higher doses of BH4 treatment alone or the present dose in combination with other interventions.
Chapter 7

References

Effects of BH4 on brain neurotransmitter concentrations in C57Bl/6 PKU mice


