New insights into tetrahydrobiopterin pharmacodynamics from Pah\textsuperscript{enu1/2}, a mouse model for compound heterozygous tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency

Florian B. Lagler\textsuperscript{a,1}, Søren W. Gersting\textsuperscript{b,1}, Clemens Zsifkovits\textsuperscript{a}, Alice Steinbacher\textsuperscript{b}, Anna Eichinger\textsuperscript{b}, Marta K. Danecka\textsuperscript{b}, Michael Staudigl\textsuperscript{b}, Ralph Fingerhut\textsuperscript{c,d}, Hartmut Glossmann\textsuperscript{a}, Ania C. Muntau\textsuperscript{b,a}

\textsuperscript{a}Department of Medical Genetics, Molecular and Clinical Pharmacology, Innsbruck Medical University, 6020 Innsbruck, Austria
\textsuperscript{b}Department of Molecular Pediatrics, Dr. von Hauner Children’s Hospital, Ludwig-Maximilians-University, 80337 Munich, Germany
\textsuperscript{c}Laboratory Becker, Olgemöller, and Colleagues, 81671 Munich, Germany
\textsuperscript{d}Newborn Screening Laboratory, University Children’s Hospital, 8032 Zurich, Switzerland

ABSTRACT
Phenylketonuria (PKU), an autosomal recessive disease with phenylalanine hydroxylase (PAH) deficiency, was recently shown to be a protein misfolding disease with loss-of-function. It can be treated by oral application of the natural PAH cofactor tetrahydrobiopterin (BH\textsubscript{4}) that acts as a pharmacological chaperone and rescues enzyme function in vivo. Here we identified Pah\textsuperscript{enu1/2} bearing a mild and a severe mutation (V106A/F263S) as a new mouse model for compound heterozygous mild PKU. Although BH\textsubscript{4} treatment has become established in clinical routine, there is substantial lack of knowledge with regard to BH\textsubscript{4} pharmacodynamics and the effect of the genotype on the response to treatment with the natural cofactor. To address these questions we applied an elaborate methodological setup analyzing: (i) blood phenylalanine elimination, (ii) blood phenylalanine/tyrosine ratios, and (iii) kinetics of in vivo phenylalanine oxidation using \textsuperscript{13}C-phenylalanine breath tests. We compared pharmacodynamics in wild-type, Pah\textsuperscript{enu1}, and Pah\textsuperscript{enu2} mice and observed crucial differences in terms of effect size as well as effect kinetics and dose response. Results from in vivo experiments were substantiated in vitro after overexpression of wild-type, V106A, and F263S in COS-7 cells. Pharmacokinetics did not differ between Pah\textsuperscript{enu1} and Pah\textsuperscript{enu2} indicating that the differences in pharmacodynamics were not induced by divergent pharmacokinetic behavior of BH\textsubscript{4}. In conclusion, our findings show a significant impact of the genotype on the response to BH\textsubscript{4} in PAH deficient mice. This may lead to important consequences concerning the diagnostic and therapeutic management of patients with PAH deficiency underscoring the need for individualized procedures addressing pharmacodynamic aspects.

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1. Introduction
Phenylketonuria (PKU; [MIM 261600]) is an autosomal recessive inborn error of metabolism caused by deficiency of hepatic phenylalanine-4-hydroxylase (PAH; EC 1.14.16.1). PAH catalyzes the rate-limiting step in phenylalanine catabolism and is regulated by binding of its l-phenylalanine substrate and the cofactor 6R-\textsuperscript{L}-erythro-5,6,7,8-tetrahydrobiopterin (BH\textsubscript{4}) and by phosphorylation [1–3]. Mutations in the PAH gene lead to loss-of-function of the PAH protein, which is often induced by protein misfolding [4]. The clinical phenotypes of hyperphenylalaninemia due to PAH deficiency are classified from classic PKU (blood phenylalanine (Phe) concentrations > 1200 \textmuM) to mild PKU (600–1200 \textmuM) and mild hyperphenylalaninemia (MHP, 120–600 \textmuM). Patients with classic and mild PKU need lifelong treatment to prevent mental retardation, whereas the necessity of MHP treatment is under...
debate [5,6]. In the last 60 years the only treatment available for patients with PAH deficiency was dietary phenylalanine restriction, a burdensome treatment associated with significant risks of malnutrition. A paradigm change occurred 10 years ago when researchers observed that pharmacological doses of the natural PAH cofactor, BH4, can reduce blood phenylalanine concentrations in a significant number of patients that do not display one of the rare forms of BH4 deficiency [7–9]. This led to definition of a new clinical phenotype, BH4-responsive PAH deficiency. Efficacy and safety of sapropterin dihydrochloride, the synthetic form of BH4, was subsequently demonstrated in clinical trials [10–13] and the Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) approved Kuvan® (Merck Serono) as an orphan drug to treat BH4-responsive PAH deficiency. However, at the time of market approval the molecular mode of action of the new drug was not well understood. By now, analyses of the pharmacological BH4 effect in vitro performed by different groups have provided evidence for structural stabilization of misfolded PAH indicating a pharmacological chaperone mode of action of BH4 [14–16].

In addition, an animal model with the specific clinical and biochemical phenotype of BH4-responsive PAH deficiency was not available for drug approval. PAH deficiency in mice has previously been generated by germline mutagenesis [17,18]. The V106A mutation in Pahenu1 leads to a MHP phenotype [17], whereas Pahemu2/2, harboring the null-mutation F263S, shows classical PKU (www.pahdb.mcgill.ca) [18–20]. We recently showed that mice homozygous for the V106A mutation display a molecular phenotype of protein misfolding with loss-of-function and that Pahemu1/1 is an animal model for the clinical phenotype of BH4-responsive MHP [16]. However, patients that most benefit from treatment with BH4 are those with mild PKU [8,21,22]. Moreover, the majority of patients (87%) with BH4-responsive PAH deficiency are compound heterozygous carrying two different mutations on the maternal and the paternal allele [23] with about 26% of the alleles being putative null-mutations. Recent studies showed a marked influence of the patient’s genotype on the intensity and time frame of response to BH4 [24–26].

Previous clinical studies mostly analyzed the effect of BH4 on the patients’ blood phenylalanine level, an endpoint with limited functional value. We showed that the effect of BH4 on blood phenylalanine concentrations in humans is accompanied by an increase in PAH function in vivo [8] and recently replicated this in the mouse model Pahemu1/1 [16]. However, comprehensive pharmacodynamic and pharmacokinetic studies in a specific mouse model displaying the clinical phenotype of BH4-responsive PAH deficiency are still not available.

Thus, the aims of this study were: (i) to investigate whether the compound heterozygous strain Pahemu1/2 is a model for BH4 responsiveness in mild PKU and (ii) to characterize pharmacodynamics and pharmacokinetics of BH4 treatment in the mouse models Pahemu1/1 and Pahemu1/2. Our results show that Pahemu1/2 is a model for compound heterozygous mild PKU with BH4-responsiveness. Pharmacodynamics of BH4 in particular with respect to effect size and effect kinetics differed significantly in both strains, while pharmacokinetics was congruent.

2. Materials and methods

2.1. Animals

BTBR, Pahemu1/1 and Pahemu2/2 mice were purchased from Jackson Laboratory (Bar Harbor, USA). The compound heterozygous hybrids Pahemu2/2 were crossbred in our animals’ facility. The animals were housed under controlled temperature conditions and maintained on a cycle of 12 h light/dark period. Between experiments water and food were available ad libitum. Basic blood phenylalanine concentrations and response to a single dose of BH4 20 μg/g body weight (bw) without phenylalanine load were assessed after 1 h fasting from food to minimize postprandial phenylalanine fluctuations. All blood samples were taken from the tail and collected on filter cards. All animal experiments were approved by the Austrian Ministry of Science. Tests were carried out in adult animals at 3–6 months of age.

2.2. Combined phenylalanine-BH4-loading tests and 13C-phenylalanine breath tests

In order to minimize the influence of naturally occurring 13C from food and to standardize phenylalanine supply, the experiments were performed after an overnight deprivation of food, whereas free access to water was allowed.

2.2.1. Combined phenylalanine-BH4-loading test

Unlabeled L-phenylalanine (Sigma–Aldrich, St. Louis, USA) 15 μg/g bw and BH4 (Cayman Chemicals, Tallin, Estonia) 20 μg/g bw or placebo (sodium chloride 0.9%, ascorbic acid 1%) were simultaneously injected intraperitoneal (i.p.). The compounds were dissolved in a sodium chloride 0.9% (B. Braun, Melsungen, Germany), ascorbic acid (Merck, Darmstadt, Germany) 1% solution at concentrations of 3 μg/ml (L-phenylalanine) and 4 μg/ml (BH4) resulting in an injection volume of 5 μl/g. Venous blood samples of approximately 40 μl were collected on filter cards before and 20, 40, 60, 120, and 180 min after injection. Blood phenylalanine and tyrosine were quantified by electron spray ionization–tandem mass spectrometry. Blood phenylalanine elimination was analyzed by non-linear curve fitting of the concentrations at 20–180 min using a single exponential function:

\[
Y = (Y_0 - \text{Plateau}) \times (e^{-k_Xt}) + \text{Plateau}
\]

2.2.2. 13C-phenylalanine breath tests

L-[1-13C]-phenylalanine (13C-phenylalanine, Eurisotop, Saint-Aubin Cedex, France) was injected in a dose of 15 μg/g bw i.p. simultaneously with BH4 or placebo. Preparation and application of the compounds were performed as described above. Mice were placed in individual 100 ml breath chambers with a continuous flow of CO2-free air. Breath samples were collected in 6.5-min intervals over a 104-min period. For each sample the airflow was discontinued for 90 s to allow CO2 levels to accumulate above 0.8%. At the end of accumulation the sample was insufflated into an infrared spectrometer (IRIS Wagner Analysen Technik, Bremen, Germany) for online isotope analysis. The cumulative recovery of 13C was calculated based on the ratio of 13CO2 to 12CO2 as previously described [27], assuming a total CO2 production rate of 94 ml per min per g bw × m2 body surface area [28]. For dose response studies, BH4 was given in doses of 5, 10, 20, 30, and 40 μg/g bw, respectively. To analyze the effect duration, 40 μg/g bw of BH4 were given 90, 24, 18, 9, 6, 4.5 or 3 h before or simultaneously to 13C-phenylalanine. Subsequently, the cumulative recovery of 13CO2 was determined at 104 min.

2.3. BH4 pharmacokinetics

In order to determine pharmacokinetics, BH4 at a dose of 20 μg/g bw was prepared and injected as described above. Venous blood samples (approximately 40 μl) were collected on filter cards before and 20, 40, 60, 120, and 180 min after injection. Total biopterin (BH4, dihydrobiopterin, and biopterin) was quantified by High Pressure Liquid Chromatography (HPLC) as previously described [29,30]. Biopterin clearance was determined by non-
linear regression of a double exponential Bateman function as described by Koch et al. [31].

2.4. Transient expression of PAH in COS-7

COS-7 cells were maintained in basic RPMI 1640 medium (PAA Laboratories, Pasching, Austria) with stable glutamine supplemented with 10% fetal bovine serum (PAA) and 1% antibiotics (Antibiotic–Antimycotic; PAA). For transient expression of the murine wild-type and variant PAH pEF-DEST51 (Invitrogen, San Diego, USA) cDNA constructs coding for wild-type, V106A, and F263S PAH were used in single transfection (wild-type, PAH<sup>em1/2</sup>, PAH<sup>em2/2</sup>), or co-transfection (PAH<sup>em1/2</sup>). A total amount of 3 μg DNA per 1 million cells was applied using the Amaxa electroporation system (Lonz, Basel, Switzerland). Cells were cultured for 24 and 72 h under two different conditions: (i) basic medium (as described above), (ii) basic medium with 43 μM BH4, 5 μg/ml ascorbic acid, and 1 mM l-phenylalanine. Culture medium was changed every 24 h. The cells were harvested and lysed by three freeze-thaw cycles in a lysis buffer containing 1% Triton X-100 and proteinase inhibitors, followed by 20 min centrifugation at 14,000 rpm, 4 °C. Recovered supernatants were subsequently used for activity assays [4].

2.5. PAH activity assay

PAH enzyme activity was determined as previously described [4,32,33] with modifications. 20 μL of total lysates were diluted in cell culture supernatants were preincubated with 1 mM L-phenylalanine and catalase 1 mg/ml (Sigma–Aldrich, St. Louis, USA) for 5 min (25 °C). Incubations were initiated by the addition of 75 μM BH4 stabilized in 2 mM dithiothreitol (DTT; Fluka Chemie AG, Buchs, Switzerland), carried out for 60 min at 25 °C and stopped by acetic acid followed by 10 min incubation at 95 °C. All concentrations mentioned refer to the final concentration in a 100 μL reaction mixture. The amount of tyrosine production was measured and quantified by HPLC, assayed as triplicates. Three independent experiments were performed.

2.6. Statistics

Group mean values were compared by Student’s unpaired two-tailed t-test. Statistical analyses were performed using GraphPad Prism 4.0c (GraphPad Software, San Diego, USA).

3. Results

3.1. PAH<sup>em1/2</sup> is a model for compound heterozygous BH4-responsive PAH deficiency

The heteroallelic PAH<sup>em1/2</sup> was previously reported as an orthologue for human hyperphenylalaninemia [20] with plasma phenylalanine levels of 147–200 μM and a residual enzyme activity of about 5% as compared to wild-type mice. We revisited this mouse model in order to reevaluate the biochemical phenotype and PAH enzyme function in vivo and in vitro and to test for responsiveness to BH4. In our experimental setup mean blood phenylalanine concentrations were 54 ± 2.1 μM for the wild-type, 177 ± 25.7 μM for PAH<sup>em1/2</sup>, 284 ± 24.9 μM for PAH<sup>em1/2</sup>, and 1115 ± 54.2 μM for PAH<sup>em2/2</sup>, in ascending order (Fig. 1A). Phenylalanine oxidation, which is a measure of in vivo PAH enzyme activity, was assessed by <sup>13</sup>C-phenylalanine oxidation test [7,8,16]. The cumulative recovery of <sup>13</sup>CO2 (the product of <sup>13</sup>C-phenylalanine oxidation) after 104 min reached 38 ± 2.8% for the wild-type, whereas PAH<sup>em1/2</sup> (12 ± 1.0%), PAH<sup>em1/2</sup> (9 ± 0.8%), and PAH<sup>em2/2</sup> (5 ± 0.6%) showed lower values in descending order (Fig. 1B).

Results obtained from <sup>13</sup>C-phenylalanine breath tests were substantiated in vivo by overexpression of wild-type, V106A, F263S, and combined overexpression of V106A and F263S PAH, respectively. COS-7 cells transiently transfected with V106A, the in vitro model for PAH<sup>em1/2</sup>, or V106A/F263S, the in vitro model for PAH<sup>em1/2</sup>, showed decreased PAH activity in comparison to the wild-type (Fig. 1C). In line with the in vivo experiments, co-transfection of V106A/F263S resulted in a significantly lower enzyme activity than transfection of V106A only. As expected, transfection of F263S resulted in almost no residual enzyme activity.

As a next step, we aimed to determine the response of PAH<sup>em1/2</sup> to pharmacological doses of BH4. A single dose of BH4 (20 μg/g bw i.p.) significantly reduced blood phenylalanine concentrations of PAH<sup>em1/2</sup> from 284 ± 24.9 to 135 ± 17.2 μM and of PAH<sup>em1/2</sup> from 177 ± 25.7 to 70 ± 17.2 μM, whereas phenylalanine values remained unchanged in wild-type mice and in PAH<sup>em2/2</sup>. PAH<sup>em1/2</sup> reached blood phenylalanine concentrations close to that of wild-type mice, while PAH<sup>em1/2</sup> still displayed mild hyperphenylalaninemia after treatment (Fig. 1D). In addition, BH4 injection led to an increase in phenylalanine elimination in PAH<sup>em1/2</sup> and PAH<sup>em2/2</sup>. The cumulative recovery of <sup>13</sup>CO2 after PAH enzyme activity attained the wild-type level in both PAH<sup>em1/2</sup> and PAH<sup>em1/2</sup>. In agreement with the missing effect on blood phenylalanine concentrations, BH4 treatment did not affect phenylalanine oxidation rates in PAH<sup>em2/2</sup> (Fig. 1E). In wild-type mice, however, BH4 led to a reduction in the cumulative <sup>13</sup>CO2 recovery, which is in line with the known inhibitory effect of the cofactor on enzyme activity [34].

In COS-7 cells, treatment with BH4 over 24 h led to an increase in enzyme activity in cells overexpressing wild-type PAH as well as V106A and V106A/F263S (Fig. 1F) with the effect being most pronounced in cells expressing V106A-PAH.

Taken together, determination of phenylalanine oxidation mirrored the biochemical phenotype and allowed for sensitive discrimination between all genotypes tested. Moreover, combined analysis of the biochemical and molecular phenotype showed that PAH<sup>em2/2</sup> displays more severe PAH deficiency than PAH<sup>em1/2</sup> and identified PAH<sup>em2/2</sup> as a compound heterozygous model for human BH4-responsive PAH deficiency.

3.2. Pharmacodynamic characterization of BH4 treatment in wild-type and PAH deficient mice

To characterize the pharmacodynamic effects of BH4 on wild-type and variant PAH in vivo we selected a combined set of three different endpoints: (i) blood phenylalanine elimination, (ii) blood phenylalanine/tyrosine ratios, and (iii) kinetics of in vivo phenylalanine oxidation.

In wild-type mice a phenylalanine challenge (15 μg/g bw i.p.) led to no significant changes in blood phenylalanine concentrations (Fig. 2A) or the phenylalanine/tyrosine ratio (Fig. 2B). Accordingly, the <sup>13</sup>C-phenylalanine applied was almost completely oxidized to <sup>13</sup>CO2 within the first hour (Fig. 2C).

In PAH<sup>em1/1</sup> and PAH<sup>em1/2</sup> the phenylalanine challenge induced a marked increase in both blood phenylalanine concentrations (Fig. 2A) and blood phenylalanine/tyrosine ratios (Fig. 2B) that did not recover to initial values within 3 h (Table 1). In agreement with the respective biochemical (blood phenylalanine concentrations) and functional (phenylalanine oxidation) phenotypes, PAH<sup>em1/1</sup> showed more severe alterations of blood phenylalanine elimination and phenylalanine/tyrosine ratio elevations than PAH<sup>em1/2</sup>.

The effect of BH4 on phenylalanine elimination and on the phenylalanine/tyrosine ratio was different from that on phenylalanine oxidation, where both PAH<sup>em1/1</sup> and PAH<sup>em1/2</sup> reached wild-
type levels after treatment (Fig. 1E). Only Pahenu1/1 showed normalization of phenylalanine elimination and the phenylalanine/tyrosine ratio within 120 and 60 min, respectively. Both parameters did not significantly decrease in Pahenu1/2 and the blood phenylalanine concentration and the phenylalanine/tyrosine ratio remained elevated 3 h after BH4 administration (Table 1).

In both animal models the rates of phenylalanine oxidation, assessed by determination of delta over baseline, exceeded half maximum values as early as 6.5 min post-BH4 injection. However, Pahenu1/1 reached maximum oxidation rates at 13 min, whereas Pahenu1/2 displayed a delayed maximum at 26 min (Fig. 2C). The resulting elimination constants (Ke) for blood phenylalanine again showed lower response to the drug for Pahenu1/2 (0.014) than for Pahenu1/1 (0.023) (Table 1).

In summary, the application of the three endpoints presented here allowed for a clear discrimination in pharmacodynamics between the two mouse strains. Differences in the response to the drug were not only confined to the effect size but also observed in terms of effect kinetics.

### 3.3. Pharmacokinetics of BH4

In order to determine, whether differences in pharmacodynamics were induced by discrepant pharmacokinetic behavior, we quantified total biopterin blood concentrations over time after intraperitoneal injection of 20 μg/g bw of BH4. We observed an increase from 19.4 ± 3.1 to 467.4 ± 47.5 nmol/g Hb in Pahenu1/1 and from 17.3 ± 2.5 to 558.8 ± 68.3 nmol/g Hb in Pahenu1/2. The maximum was reached after 17.5 min and concentrations decreased rapidly thereafter. About 70% of exogenous BH4 was eliminated 60 min post-application followed by a second slow elimination phase that reached initial values (Pahenu1/1 51.2 ± 6.2, Pahenu1/2 55.6 ± 6.7) at 180 min. None of the pharmacokinetic parameters (C0, cmax, tmax, AUC, t1/2) significantly differed between Pahenu1/1 and Pahenu1/2 (Table 2).
Fig. 2. Characterization of BH4 effects on PAH function in vivo. (A) Blood phenylalanine elimination and (B) blood phenylalanine/tyrosine ratio (Phe/Tyr ratio) were assessed in Pah<sup>enu1/1</sup> and Pah<sup>enu1/2</sup> mice in comparison to untreated wild-type mice. The effect of simultaneous challenge of unlabelled phenylalanine (15 µg/g bw) and BH4 (20 µg/g bw) vs. placebo (sodium chloride 0.9%, ascorbic acid 1%) was measured over a 180-min period and data points representing the decrease in blood phenylalanine concentrations were fitted using a one-phase exponential function. The inset represents blood phenylalanine elimination in wild-type mice at a different scale. (C) Fractional 13CO2 recovery in 13C-phenylalanine oxidation tests (delta over baseline, DOB) of wild-type mice (WT), Pah<sup>enu1/1</sup> and Pah<sup>enu1/2</sup>. The measurement was performed in 6.5-min intervals over a 104-min period after simultaneous injection of BH4 (20 µg/g bw) and phenylalanine (15 µg/g bw) (closed circles) or placebo (open circles). To depict the immediate onset of the BH4 effects, the differences of fractional recovery (DOB<sub>BH4</sub>–DOB<sub>placebo</sub>) within the first four time points are given in the insets. All data are given as means ± s.e.m.
3.4. Effect duration of BH4

We subsequently aimed to analyze, whether BH4 effect duration diverges between Pahenu1/1 and Pahenu1/2. For this purpose we monitored the time course of 13C-phenylalanine oxidation after a single dose of BH4 and analyzed the effect of BH4 on PAH activity in cultured cells over 72 h.

In both PAH deficient strains phenylalanine oxidation reached its maximum immediately after BH4 application (Fig. 2C). In order to compare effect durations we normalized the data defining the highest values as 100% and the placebo level as 0%. A single dose of 40 μg/g bw BH4 induced enhanced phenylalanine oxidation for more than 48 h in Pahenu1/1, while in Pahenu1/2 phenylalanine oxidation decayed to placebo levels within 18 h (Fig. 3A).

These results were confirmed by assessing the long-term effect of BH4 on PAH enzyme activity in COS-7 cells (Fig. 3B). In cells expressing V106A, BH4 treatment normalized enzyme activity, while cells expressing V106A/F263S reached 75% of wild-type activity. BH4 did not affect enzyme activity in cells transfected with F263S. In addition, we compared the fold increase of enzyme activity over the control (no BH4 treatment) after 24 and 72 h of cell incubation with BH4. At overexpression of V106A the major part of the response occurred within 24 h (1.54-fold increase) and cultivation for additional 48 h did not substantially further increase enzyme activity (1.75-fold). By contrast, cells expressing V106A/F263S showed their main increase in enzyme activity (1.9-fold) after prolonged cultivation of 72 h and not after 24 h (1.15-fold) (Fig. 3C).

In conclusion, Pahenu1/1 and Pahenu1/2 showed pronounced differences in effect duration of BH4 treatment. The in vivo effect of BH4 was shorter in Pahenu1/2 than in Pahenu1/1. This was demonstrated by showing that the functional effect of the drug on phenylalanine oxidation leveled off more rapidly. In vitro, a prolonged treatment was needed to reach the maximum treatment effect in Pahenu1/2.

3.5. Dose effects of BH4

To further characterize genotype-specific pharmacodynamics we studied the dose response to BH4. Analysis of the cumulative recovery of 13C-phenylalanine oxidation at 104 min upon single dose BH4 treatment revealed a non-dose-dependent inhibitory effect in the range of 3–40 μg/g bw i.p. in wild-type (BTBR), Pahenu1/1 and Pahenu1/2. Phenylalanine concentrations and phenylalanine/tyrosine ratios are given as means ± s.e.m. The elimination constant (K1/2) was determined using a one-phase exponential function. Unpaired two-tailed students t-tests were applied to test the difference between untreated and treated animals (n.s., not significant).

Table 1

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<th>Phe180 [μmol/l]</th>
<th>P value</th>
<th>K1/2</th>
<th>Phe/180</th>
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<td>Pahenu1/1</td>
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<td>61.64 ± 3.9</td>
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Phenylalanine kinetics and phenylalanine/tyrosine ratios were determined after a phenylalanine load of 15 μg/g bw i.p. with and without simultaneous BH4 load (20 μg/g bw i.p.) in wild-type (BTBR), Pahenu1/1 and Pahenu1/2. Phenylalanine concentrations and phenylalanine/tyrosine ratios are given as means ± s.e.m. The elimination constant (K1/2) was determined using a one-phase exponential function. Unpaired two-tailed students t-tests were applied to test the difference between untreated and treated animals (n.s., not significant).

4. Discussion

The approval of BH4 marked a paradigm change in the management of phenylketonuria treatment. Clinical studies performed to analyze BH4-responsiveness in patients often focused on the surrogate marker blood phenylalanine concentration as an endpoint. However, this method produced high intraindividual variability and discrepant findings among similar genotypes [23] and therefore proved to be of limited value. Moreover, an animal model with the specific clinical and biochemical phenotype of BH4-responsive PAH deficiency was not available during the development of the drug. Thus, important insights into pharmacodynamics of BH4 were still lacking, in particular with regard to pharmacogenetics as well as effect size and effect kinetics.

We recently characterized Pahenu1/1 (V106A/V106A) as the first animal model for BH4-responsive MHP [16]. This opened up the opportunity for first in vivo investigations in order to elucidate the BH4 mode of action and to substantiate the view of the cofactor being a pharmacological chaperone. The genetic alteration in this mouse does not lead to changes in PAH affinity to the cofactor [16] reflecting the human situation where only few K0 variants were found [4,14,15,35–37]. Thus, the mode of action of BH4 in PAH deficiency was not limited to its cofactor action. We showed that pharmacological doses of BH4 attenuate the pathophysiological triad of misfolding, aggregation, and accelerated degradation of the PAH enzyme by conformational stabilization augmenting the effective PAH concentration. This led to the rescue of the biochemical phenotype and enzyme function in vivo. Notably, the pharmaceutical action of the cofactor was confined to the pathological metabolic state of hyperphenylalaninemia [16].

Here we present Pahenu1/2 (V106A/F263S) as a second mouse model for BH4-responsive PKU. This strain displays compound heterozygosity representing the genotype of the large majority of PKU patients (87%) [23] that are dealt with in daily clinical routine.
Amino acid residues V106 in the regulatory domain and F263 in the catalytic core are conserved between mouse and human but neither V106A nor F263S have so far been identified in human PKU patients. However, the F263L amino acid substitution was described to be also associated with a severe phenotype. A comparison of specific activities of murine and human PAH variants V106A, F263S, and F263L confirmed that amino acid substitutions at the respective loci result in similar effects in both species (Supplementary Table S1). Thus, murine V106A and F263S variants are good models for human BH4-responsive PAH deficiency.

As previously shown [20] and confirmed in our current study, Pahenu1/2 exhibits an intermediate phenotype between Pahenu1/1 and Pahenu1/2. Hyperphenylalaninemia in vivo is more severe than in Pahenu1/1 and less severe than in Pahenu1/2, whereas PAH activity in vivo and in vitro is lower than in Pahenu1/1 but higher than in Pahenu1/2. Analyses of phenylalanine oxidation by 13C-phenylalanine breath tests mirrored these differences in severity and were in good agreement with biochemical (blood phenylalanine) and molecular (PAH activity) data allowing for high-sensitivity discrimination of genotypes.

We compared pharmacodynamics of BH4 treatment in the two mouse models and observed crucial differences. Heterogeneous results were obtained with respect to the effect size. In the combined phenylalanine-BH4-loading test a single load of 20 μg/g bw BH4 induced a decrease of blood phenylalanine of 67.5% in Pahenu1/1 and of only 35.5% in Pahenu1/2. This value is in close proximity of the threshold of 30%, an arbitrary but accepted measure for BH4-responsiveness in humans considered to be associated with clinical benefit. By contrast, the effect on in vivo phenylalanine oxidation, the direct measure of PAH activity, was striking in this strain. Treatment with BH4 completely normalized in vivo enzyme activity with the effect being even stronger than in the milder phenotype Pahenu1/1.

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**Fig. 3.** In vivo and in vitro characterization of BH4 effect duration. (A) Effect duration of BH4 in Pahenu1/1 and Pahenu1/2 mice expressed as relative recovery of 13CO2 at 104 min. BH4 was administered 90, 24, 18, 9, 6, 4.5 or 3 h before or simultaneously to 13C-phenylalanine and recovery of 13CO2 was measured subsequently. Data were normalized defining the maximum recovery as 100% and the placebo level as 0%. Lines depict the difference in phenylalanine oxidation and are merely to guide the eye. (B) In vitro PAH activity in COS-7 cells transiently expressing murine wild-type and variant PAH upon 72 h incubation with BH4 (43 μM, grey bars) and without BH4 supplementation (black bars). Values were normalized to wild-type activity without BH4 supplementation. (C) Comparison of in vitro PAH enzyme activities. Fold increase describes the ratio between enzyme activities determined with and without addition of BH4 (43 μM) after 24 h (black bars) and 72 h (grey bars) incubation. All data are given as means ± s.e.m.

**Fig. 4.** Dose effect of BH4 in vivo. Effect of BH4 on phenylalanine oxidation expressed as cumulative recovery at 104 min measured in wild-type (WT) (A), Pahenu1/1 (B), and Pahenu1/2 (C). Data are given as means ± s.e.m.
Analysis of the effect kinetics further added to dissect the distinct patterns of BH4-response in the two strains. At the biochemical level, Pahmut1/2 showed slower elimination of pathologically elevated phenylalanine concentrations than Pahmut1/1. This was mirrored by a less pronounced and delayed reduction of the phenylalanine/tyrosine ratio, an important parameter to judge the extent of metabolic derangement. At the functional level, both strains showed an immediate response to BH4-loading in the breath test, but the peak phenylalanine oxidation upon treatment again was delayed in Pahmut1/2 in comparison to Pahmut1/1. Moreover, the duration of the BH4 effect after a single dose was considerably shorter in Pahmut1/2 than in Pahmut1/1.

In addition, the two animal models displayed important divergences concerning dose response. Both strains attained maximum in vivo phenylalanine oxidation at a dose of 20 μg/g bw BH4. Yet, Pahmut1/2 exhibited a response inhibition in the presence of higher dosages, while Pahmut1/1 reached a plateau. This data is in line with well known features of pharmacological chaperones which show inhibitory effects at higher concentrations [38].

Our findings may lead to important conclusions concerning the diagnostic and therapeutic management of patients with PAH deficiency. First, extending the test procedures to assess BH4-responsiveness may allow to draw a more complete picture of a drug response in the single individual. The methods presented in this work can easily and safely be transferred into clinical routine and provide useful endpoints beyond determination of blood phenylalanine concentrations. Second, some patients show a delayed reduction in blood phenylalanine after BH4-loading, these are often referred to as slow responders [8,22,25,39]. Our results indicate that this may be rather due to a limited pharmacodynamic effect than to a delayed onset of drug action. Cell culture experiments pointed to a beneficial effect of prolonged treatment for slow responder genotypes and hence suggest appreciation of this fact when testing for and treating BH4-responsive PAH deficiency. Third, both animal models bear the same mutation responsible for BH4-responsiveness (V106A), however, the null-mutation on the second allele in the compound heterozygous genotype Pahmut1/2 had substantial impact on pharmacodynamics. Thus, response to treatment is not exclusively related to the putative milder mutation [23,24,39] and effects induced by interallelic complementation may necessitate careful dose finding procedures in compound heterozygous patients. Fourth, the individual behavior with regard to effect size, onset of maximum drug action, and effect duration underscores the demand of individual therapeutic regimes for different genotypes. Some patients may for instance benefit from a treatment scheme with several BH4 administrations to translate the effect of the drug on enzyme activity into a sustained effect on biochemical markers. Moreover, in certain patients, higher dosages may diminish the possible treatment effect at a higher risk of possible adverse effects.

In conclusion, considerable clinical and research effort has been devoted to identifying the conditions for optimal testing for BH4-responsiveness in PAH deficiency. Our in depth pharmacological analyses of two mouse models with different genotypes are in line with the notion that genotype-driven complexity will require comprehensive evaluation instruments addressing different pharmacodynamic and pharmacokinetic aspects. For this purpose, the diagnostic package including time-dependent blood phenylalanine elimination and phenylalanine/tyrosine ratios as well as kinetics of in vivo phenylalanine oxidation can easily be transferred to and implemented in patients and by this may contribute to individualized diagnostics and treatment of patients suffering from a genetically heterogeneous condition.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.07.042.

References


<table>
<thead>
<tr>
<th>Genotype</th>
<th>Specific activity (nmol Tyr/min x mg protein)</th>
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<tbody>
<tr>
<td>WT hsPAH</td>
<td>3115 ± 149</td>
</tr>
<tr>
<td>WT MmPAH</td>
<td>2628 ± 293</td>
</tr>
<tr>
<td>V106A HsPAH</td>
<td>2947 ± 260</td>
</tr>
<tr>
<td>V106A MmPAH</td>
<td>3125 ± 101</td>
</tr>
<tr>
<td>F263S HsPAH</td>
<td>235 ± 51</td>
</tr>
<tr>
<td>F263L HsPAH</td>
<td>159 ± 51</td>
</tr>
<tr>
<td>F263S MmPAH</td>
<td>166 ± 16</td>
</tr>
</tbody>
</table>

Recombinant tetrameric human (HsPAH) and murine (MmPAH) PAH were expressed as MBP-PAH fusion proteins in *E. coli*. Specific activity was determined at standard L-phenylalanine (1 mM) and BH₄ (75 µM) concentrations with L-phenylalanine preincubation. Values are given as means ± SEM of *n* = 3 experiments.