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New insights into tetrahydrobiopterin pharmacodynamics from *Pah*^{enu1/2}, a mouse model for compound heterozygote tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency

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Abbreviations:

PKU, phenylketonuria; PAH, phenylalanine hydroxylase; BH₄, 6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin; MIM, Mendelian Inheritance in Man number; EC, Enzyme Commission number; MHP, mild hyperphenylalaninemia; enu, N-ethyl-N-nitrosourea; BTBR, black and tan brachyuric mouse strain; DTT, dithiothreitol; K_e, elimination constant; c₀, initial concentration; c_{max}, peak concentration; t_{max}, time to peak concentration; AUC, area under the concentration vs. time curve at 0 to 180 minutes; t_{1/2}, elimination half-life; ¹³C-phenylalanine, L-[1-¹³C]-phenylalanine; DOB, delta over baseline; DOB_{BH₄}, delta over baseline after BH₄ treatment; DOB_{Placebo}, delta over baseline after Placebo; Phe₀, initial phenylalanine concentration; Phe₁₈₀, phenylalanine concentration at 180 minutes.

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 (BH₄) that acts as a pharmacological chaperone and rescues enzyme function in vivo. Here we identified *Pah*^{enu1/2} bearing a mild and a severe mutation (V106A/F363S) as a new mouse model for compound heterozygous mild PKU. Although BH₄ treatment has become established in clinical routine, there is substantial lack of knowledge with regard to BH₄ 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 ¹³C-phenylalanine breath tests. We compared pharmacodynamics in wild-type, *Pah*^{enu1/1}, and *Pah*^{enu1/2} 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*^{enu1/1} and *Pah*^{enu1/2} indicating that the differences in pharmacodynamics were not induced by divergent pharmacokinetic behavior of BH₄. In conclusion, our findings show a significant impact of the genotype on the response to BH₄ 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.

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 6*R*-L-erythro-5,6,7,8-tetrahydrobiopterin (BH₄) 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 concentrations > 1,200 μM) to mild PKU (600 to 1,200 μM) and mild hyperphenylalaninemia (MHP, 120 to 600 μM). 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 sixty 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 ten 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 (EMA) 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 *Pah*^{enu1/1} leads to a MHP phenotype [17], whereas *Pah*^{enu2/2}, 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 *Pah*^{enu1/1} 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 *Pah*^{enu1/1} [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 *Pah*^{enu1/2} is a model for BH₄-responsiveness in mild PKU and (ii) to characterize pharmacodynamics and pharmacokinetics of BH₄ treatment in the

mouse models $Pah^{enu1/1}$ and $Pah^{enu1/2}$. Our results show that $Pah^{enu1/2}$ 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, $Pah^{enu1/1}$ and $Pah^{enu2/2}$ mice were purchased from Jackson Laboratory (Bar Harbor, USA). The compound heterozygous hybrids $Pah^{enu1/2}$ were crossbred in our animals' facility. The animals were housed under controlled temperature conditions and maintained on a cycle of 12 hours 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 hour 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 to 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

Unlabelled 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 intraperitoneally (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 minutes 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 minutes using a single exponential function:

$$Y = (Y_0 - Plateau) \times (e^{-K \times X}) + Plateau$$

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 minute intervals over a 104 minute period. For each sample the airflow was discontinued for 90 seconds 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 x 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 hours before or simultaneously to ^{13}C -phenylalanine. Subsequently, the cumulative recovery of $^{13}\text{CO}_2$ was determined at 104 minutes.

2.3 *BH₄ pharmacokinetics*

In order to determine pharmacokinetics, BH_4 at a dose of 20 $\mu\text{g/g}$ 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 minutes after injection. Total biopterin (BH_4 , 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, $\text{Pah}^{\text{enu1/1}}$, $\text{Pah}^{\text{enu2/2}}$) or co-transfection ($\text{Pah}^{\text{enu1/2}}$). A total amount of 3 μg DNA per 1 million cells was applied using the Amaxa electroporation system (Lonza, Basel, Switzerland). Cells were cultured for 24 and 72 hours under two different conditions: i) basic medium (as described above), ii) basic medium with 43 μM BH_4 , 5 $\mu\text{g/ml}$ ascorbic acid, and 1 mM L-phenylalanine. Culture medium was changed every 24 hours. 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 minutes 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 obtained from cell culture were preincubated with 1 mM L-phenylalanine and catalase 1 mg/ml (Sigma Aldrich, St. Louis, USA) for 5 minutes (25 °C) in 15 mM Na HEPES pH 7.3, followed by 1 minute incubation with 10 µM ferrous ammonium sulphate (Sigma Aldrich). The reaction was initiated by the addition of 75 µM BH₄ stabilized in 2 mM dithiothreitol (DTT; Fluka Chemie AG, Buchs, Switzerland), carried out for 60 minutes at 25 °C and stopped by acetic acid followed by 10 minutes incubation at 95 °C. All concentrations mentioned refer to the final concentration in a 100 µl reaction mixture. The amount of L-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^{enu1/2}* is a model for compound heterozygous BH₄-responsive PAH deficiency

The heteroallelic *Pah^{enu1/2}* was previously reported as an orthologue for human hyperphenylalaninemia [20] with plasma phenylalanine levels of 147 to 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*^{enu1/1}, 284 +/- 24.9 μM for *Pah*^{enu1/2}, and 1115 +/- 54.2 μM for *Pah*^{enu2/2}, in ascending order (Fig. 1A). Phenylalanine oxidation, which is a measure of in vivo PAH enzyme activity, was assessed by a ¹³C-phenylalanine oxidation test [7, 8, 16]. The cumulative recovery of ¹³CO₂ (the product of ¹³C-phenylalanine oxidation) after 104 minutes reached 38 +/- 2.8 % for the wild-type, whereas *Pah*^{enu1/1} (12 +/- 1.0 %), *Pah*^{enu1/2} (9 +/- 0.8 %), and *Pah*^{enu2/2} (5 +/- 0.6 %) showed lower values in descending order (Fig. 1B).

Results obtained from ¹³C-phenylalanine breath tests were substantiated in vitro 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*^{enu1/1}, or V106A/F263S, the in vitro model for *Pah*^{enu1/2}, 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*^{enu1/2} to pharmacological doses of BH₄. A single dose of BH₄ (20 μg/g bw i.p.) significantly reduced blood phenylalanine concentrations of *Pah*^{enu1/2} from 284 +/- 24.9 μM to 135 +/- 17.2 μM and of *Pah*^{enu1/1} from 177 +/- 25.7 μM to 70 +/- 17.2 μM, whereas phenylalanine values remained unchanged in wild-type mice and in *Pah*^{enu2/2}. *Pah*^{enu1/1} reached blood phenylalanine concentrations close to that of wild-type mice, while *Pah*^{enu1/2} still displayed mild hyperphenylalaninemia after treatment (Fig. 1D). In addition, BH₄

injection led to an increase in phenylalanine oxidation in *Pah*^{enu1/1} and *Pah*^{enu1/2}. The cumulative recovery of ¹³CO₂ after 104 minutes attained wild-type level in both *Pah*^{enu1/2} and *Pah*^{enu1/1}. In agreement with the missing effect on blood phenylalanine concentrations, BH₄ treatment did not affect phenylalanine oxidation rates in *Pah*^{enu2/2} (Fig. 1E). In wild-type mice, however, BH₄ led to a reduction in the cumulative ¹³CO₂ 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 hours 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*^{enu1/2} displays more severe PAH deficiency than *Pah*^{enu1/1} and identified *Pah*^{enu1/2} 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 ^{13}C -phenylalanine applied was almost completely oxidized to $^{13}\text{CO}_2$ within the first hour (Fig. 2C).

In $Pah^{enu1/1}$ and $Pah^{enu1/2}$ 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 hours (Table 1). In agreement with the respective biochemical (blood phenylalanine concentrations) and functional (phenylalanine oxidation) phenotypes, $Pah^{enu1/2}$ showed more severe alterations of blood phenylalanine elimination and phenylalanine/tyrosine ratio elevations than $Pah^{enu1/1}$.

The effect of BH_4 on phenylalanine elimination and on the phenylalanine/tyrosine ratio was different from that on phenylalanine oxidation, where both $Pah^{enu1/1}$ and $Pah^{enu1/2}$ reached wild-type levels after treatment (Fig. 1E). Only $Pah^{enu1/1}$ showed normalization of phenylalanine elimination and the phenylalanine/tyrosine ratio within 120 and 60 minutes, respectively. Both parameters did not significantly decrease in $Pah^{enu1/2}$ and the blood phenylalanine concentration and the phenylalanine/tyrosine ratio remained elevated 3 hours after BH_4 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 minutes post BH_4 injection. However, $Pah^{enu1/1}$ reached maximum oxidation rates at 13 minutes, whereas $Pah^{enu1/2}$ displayed a delayed maximum at 26 minutes (Fig. 2C). The resulting elimination constants (K_e) for blood phenylalanine again showed lower response to the drug for $Pah^{enu1/2}$ (0.014) than for $Pah^{enu1/1}$ (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 *Pah*^{enu1/1} and from 17.3 +/- 2.5 to 558.8 +/- 68.3 nmol/g Hb in *Pah*^{enu1/2}. The maximum was reached after 17.5 minutes and concentrations decreased rapidly thereafter. About 70 % of exogenous BH₄ was eliminated 60 minutes post application followed by a second slow elimination phase that reached initial values (*Pah*^{enu1/1} 51.2 +/- 6.2, *Pah*^{enu1/2} 55.6 +/- 6.7) at 180 minutes. None of the pharmacokinetic parameters (*C*₀, *C*_{max}, *t*_{max}, AUC, *t*_{1/2}) significantly differed between *Pah*^{enu1/1} and *Pah*^{enu1/2} (Table 2).

3.4 Effect duration of BH₄

We subsequently aimed to analyze, whether BH₄ effect duration diverges between *Pah*^{enu1/1} and *Pah*^{enu1/2}. 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 hours.

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 hours in *Pah*^{enu1/1}, while in *Pah*^{enu1/2} phenylalanine oxidation decayed to placebo levels within 18 hours (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 hours and 72 hours of cell incubation with BH₄. At overexpression of V106A the major part of the response occurred within 24 hours (1.54-fold increase) and cultivation for additional 48 hours 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 hours and not after 24 hours (1.15-fold) (Fig. 3C).

In conclusion, *Pah*^{enu1/1} and *Pah*^{enu1/2} showed pronounced differences in effect duration of BH₄ treatment. The in vivo effect of BH₄ was shorter in *Pah*^{enu1/2} than in *Pah*^{enu1/1}. 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 *Pah*^{enu1/2}.

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 minutes upon single dose BH₄ treatment revealed a non-dose-dependent inhibitory effect in the range of 5 to 40 µg/g bw i.p in wild-type mice (Fig. 4A).

Pah^{enu1/1} showed a positive dose response starting at 5 µg/g and reached a plateau at 20 µg/g with no further increase in phenylalanine oxidation at higher doses (Fig. 4B). In the compound heterozygous mouse *Pah*^{enu1/2} we also identified a positive

dose effect. However, BH₄ doses of 30 and 40 µg/g resulted in a lower activity as compared to the peak effect at 10 to 20 µg/g (Fig. 4C). With respect to the effect size *Pah*^{enu1/2} revealed a stronger response as compared to *Pah*^{enu1/1} at 5, 10, and 20 µg/g, respectively (Fig. 4B, C).

In conclusion, *Pah*^{enu1/1} and *Pah*^{enu1/2} showed a dose dependent and increasing response to BH₄ at low doses up to 10 µg/g with no further benefit at higher dosages. Moreover, the results observed for *Pah*^{enu1/2} are in line with a negative dose effect for concentrations above 20 µg/g BH₄.

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 *Pah*^{enu1/1} (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 K_m 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 *Pah*^{enu1/2} (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 1). 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, *Pah*^{enu1/2} exhibits an intermediate phenotype between *Pah*^{enu1/1} and *Pah*^{enu2/2}. Hyperphenylalaninemia in vivo is more severe than in *Pah*^{enu1/1} and less severe than in *Pah*^{enu2/2}, whereas PAH activity in vivo and in vitro is lower than in *Pah*^{enu1/1} but higher than in *Pah*^{enu2/2}.

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 *Pah*^{enu1/1} and of only 35.5 % in *Pah*^{enu1/2}. 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 *Pah*^{enu1/1}.

Analysis of the effect kinetics further added to dissect the distinct patterns of BH₄-response in the two strains. At the biochemical level, *Pah*^{enu1/2} showed slower elimination of pathologically elevated phenylalanine concentrations than *Pah*^{enu1/1}.

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 *Pah*^{enu1/2} in comparison to *Pah*^{enu1/1}. Moreover, the duration of the BH₄ effect after a single dose was considerably shorter in *Pah*^{enu1/2} than in *Pah*^{enu1/1}.

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, *Pah*^{enu1/2} exhibited a response inhibition in the presence of higher dosages, while *Pah*^{enu1/1} 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 the 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 *Pah*^{enu1/2} 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 behaviour 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 positive 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 heterogenous condition.

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Table 1. Phenylalanine kinetics and phenylalanine/tyrosine ratios

	BH ₄	Phe ₀ [μmol/l]	Phe ₁₈₀ [μmol/l]	<i>P</i> value	K _e	Phe/Tyr ₀	Phe/Tyr ₁₈₀	<i>P</i> value
<i>Pah</i> ^{enu1/1}	-	109.10 ± 5.4	153.33 ± 19.3	0.0097	-	2.62 ± 0.1	4.79 ± 0.4	0.0035
	+	120.74 ± 16.5	61.64 ± 3.9		0.023	3.65 ± 0.3	1.73 ± 0.1	
<i>Pah</i> ^{enu1/2}	-	187.29 ± 19.8	209.50 ± 35.1	n.s.	-	4.71 ± 0.6	5.65 ± 1.0	n.s.
	+	174.94 ± 20.6	136.9 ± 17.3		0.014	5.38 ± 0.8	3.65 ± 0.9	
BTBR	-	53.70 ± 1.8	56.61 ± 5.0	-	0.015	2.15 ± 0.2	2.49 ± 0.2	-

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.) to wild-type (BTBR), *Pah*^{enu1/1} and *Pah*^{enu1/2}. Phenylalanine concentrations and phenylalanine/tyrosine ratios are given as means ± s.e.m. The elimination constant (K_e) 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 2. Determination of pharmacokinetic parameters.

	<i>Pah</i> ^{enu1/1}	<i>Pah</i> ^{enu1/2}	<i>P</i> value
<i>c</i> ₀ (nmol/g Hb)	19.4 ± 3.1	17.3 ± 2.5	0.5
<i>c</i> _{max} (nmol/g Hb)	467.6 ± 47.5	558.8 ± 68.3	0.3
<i>t</i> _{max} (min)	17.5 ± 2.5	17.5 ± 2.5	1
AUC _{180min}	23769 ± 2341	24522 ± 2638	0.8
<i>t</i> _{1/2} (min)	26.3 ± 2.8	26.3 ± 2.7	0.98

The following pharmacokinetic parameters were determined in *Pah*^{enu1/1} and *Pah*^{enu1/2}: *c*₀ initial concentration, *c*_{max} peak concentration, *t*_{max} time to peak concentration, AUC area under the concentration vs. time curve at 0 to 180 minutes, *t*_{1/2} elimination half-life.

Figure captions

Fig. 1. Characterization of the biochemical and molecular phenotype of wild-type and PAH deficient mice and evaluation of BH₄-responsiveness.

(A) Basal levels of blood phenylalanine in the wild-type (WT), *Pah*^{enu1/1} (enu^{1/1}), *Pah*^{enu1/2} (enu^{1/2}), and *Pah*^{enu2/2} (enu^{2/2}). (B) ¹³C-phenylalanine oxidation *in vivo* assessed in wild-type, *Pah*^{enu1/1}, *Pah*^{enu1/2}, and *Pah*^{enu2/2} expressed as cumulative ¹³CO₂ recovery at 104 minutes after application of ¹³C-phenylalanine (15 µg/g bw i.p.). (C) *In vitro* PAH activity in COS-7 cells transiently expressing murine wild-type and variant PAH. Values were normalized to wild-type activity without BH₄ treatment. (D) Blood phenylalanine before (open circles) and 120 minutes after a single dose of 20 µg/g bw i.p. BH₄ (closed circles). (E) ¹³C-phenylalanine oxidation *in vivo* assessed in wild-type (black), *Pah*^{enu1/1} (dark grey), *Pah*^{enu1/2} (light grey), and *Pah*^{enu2/2} (white). Data points represent cumulative ¹³CO₂ recovery after application of ¹³C-phenylalanine (15 µg/g bw i.p.) and a simultaneous injection of placebo (sodium chloride 0.9%, ascorbic acid 1%) or BH₄ (20 µg/g bw i.p.). Samples were collected in 6.5 minute intervals over a 104 minute period. (F) *In vitro* PAH activity in COS-7 cells transiently expressing murine wild-type and variant PAH upon 24 hours incubation with BH₄ (43 µM, grey bars) and without BH₄ supplementation (black bars). Values were normalized to wild-type activity without BH₄ supplementation. All data are given as means ± s.e.m. and the significance is indicated (* *P*<0.05, ** *P*<0.01, *** *P*<0.001).

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*^{enu1/1} and *Pah*^{enu1/2} mice in comparison to

untreated wild-type mice. The effect of simultaneous challenge of unlabelled phenylalanine (15 $\mu\text{g/g}$ bw) and BH_4 (20 $\mu\text{g/g}$ bw) versus placebo (sodium chloride 0.9 %, ascorbic acid 1 %) was measured over a 180 minute 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 $^{13}\text{CO}_2$ recovery in ^{13}C -phenylalanine oxidation tests (delta over baseline, DOB) of wild-type mice (WT), $\text{Pah}^{\text{enu}1/1}$ and $\text{Pah}^{\text{enu}1/2}$. The measurement was performed in 6.5 minute intervals over a 104 minute period after simultaneous injection of BH_4 (20 $\mu\text{g/g}$ bw) and phenylalanine (15 $\mu\text{g/g}$ bw) (closed circles) or placebo (open circles). To depict the immediate onset of the BH_4 effects, the differences of fractional recovery ($\text{DOB}_{\text{BH}_4} - \text{DOB}_{\text{placebo}}$) within the first four time points are given in the insets. All data are given as means \pm s.e.m.

Fig. 3. *In vivo* and *in vitro* characterization of BH_4 effect duration.

(A) Effect duration of BH_4 in $\text{Pah}^{\text{enu}1/1}$ and $\text{Pah}^{\text{enu}1/2}$ mice expressed as relative recovery of $^{13}\text{CO}_2$ at 104 minutes. BH_4 was administered 90, 24, 18, 9, 6, 4.5 or 3 hours before or simultaneously to ^{13}C -phenylalanine and recovery of $^{13}\text{CO}_2$ 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 hours incubation with BH_4 (43 μM , grey bars) and without BH_4 supplementation (black bars). Values were normalized to wild-type activity without BH_4 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 hours (black bars) and 72 hours (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 minutes measured in wild-type (WT) (A), *Pah*^{enu1/1} (B), and *Pah*^{enu1/2} (C). Data are given as means ± s.e.m. and the significance is indicated (* *P*<0.05, ** *P*<0.01, *** *P*<0.001).

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Figures

Figure 1

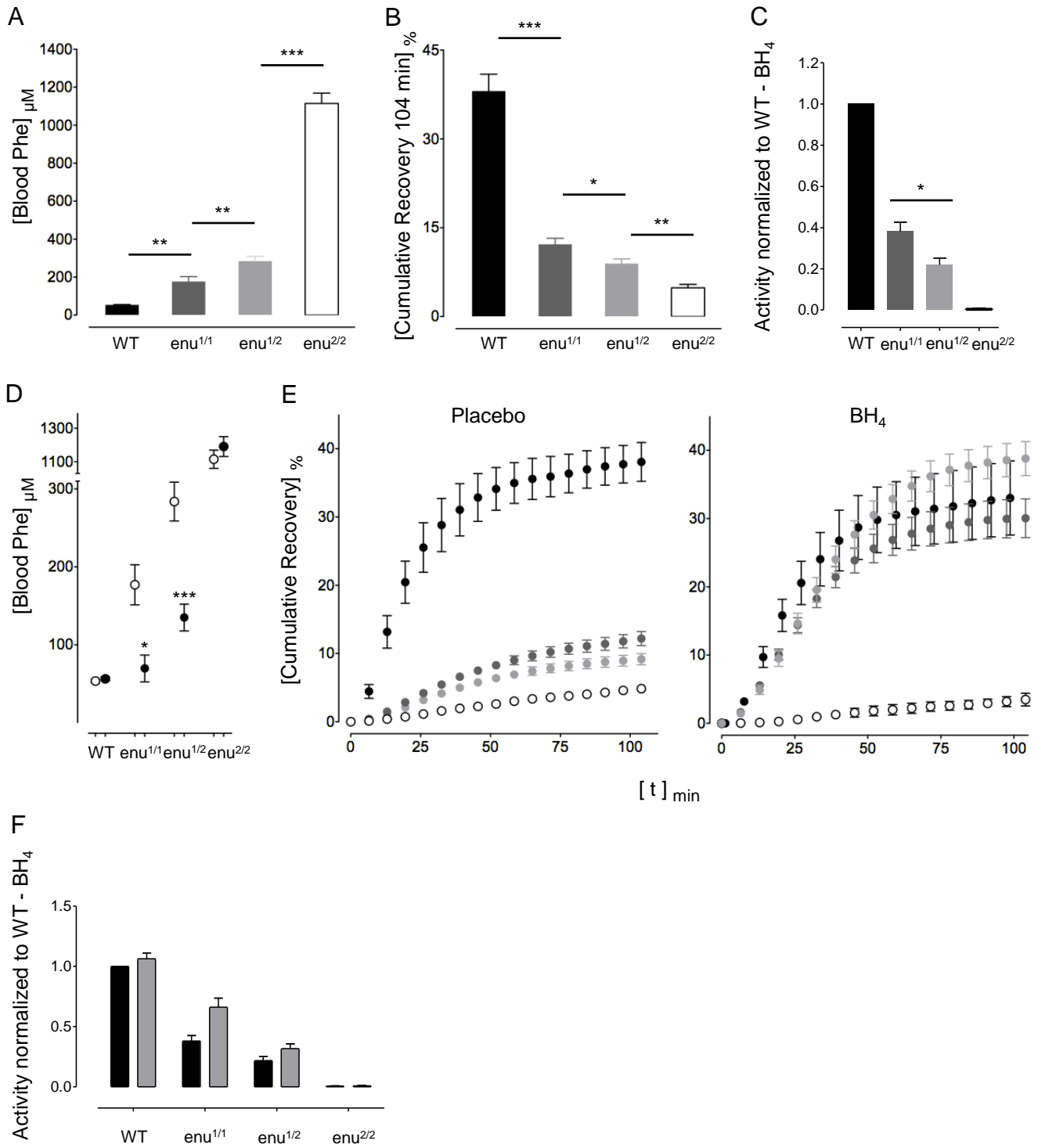


Figure 2

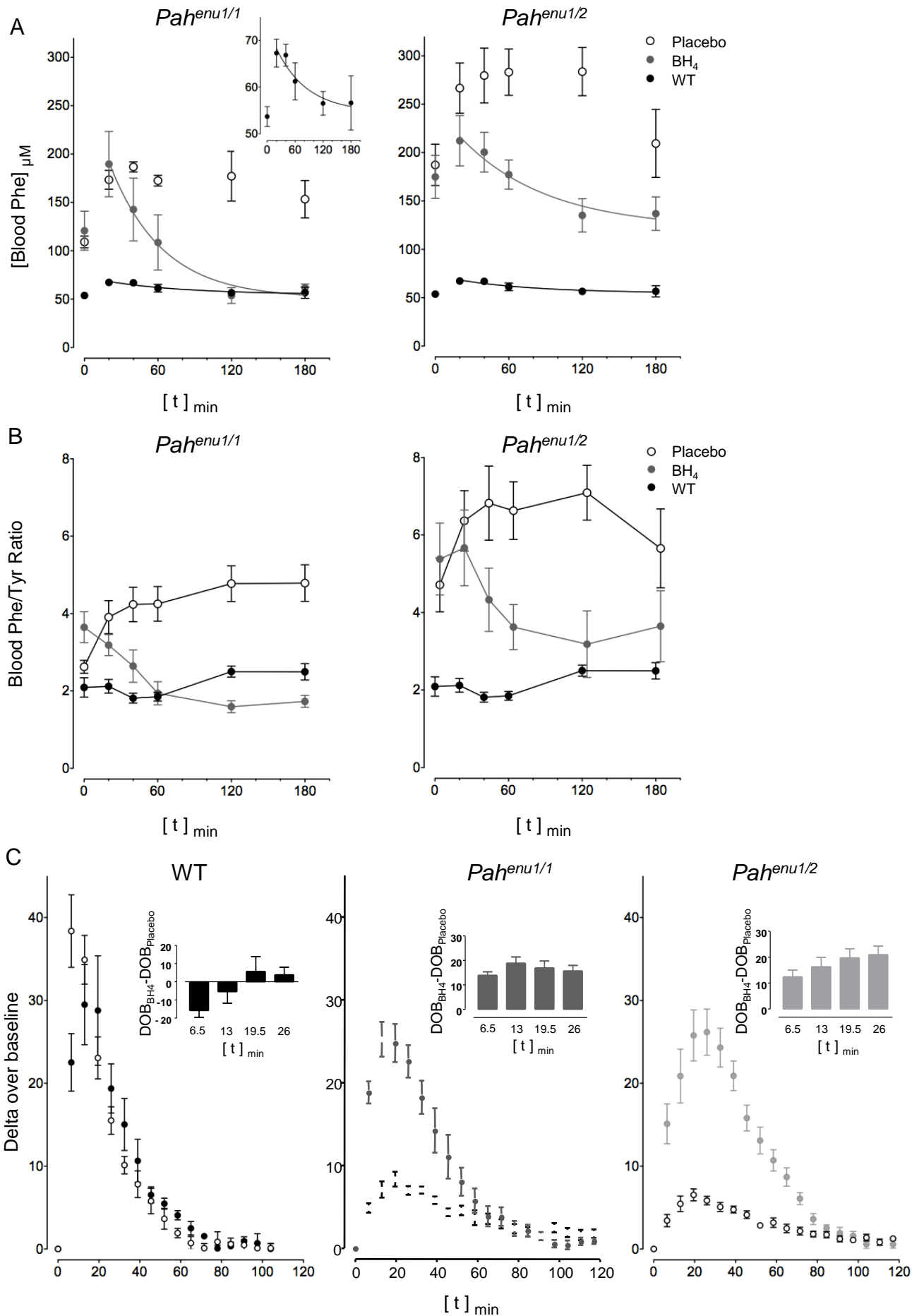


Figure 3

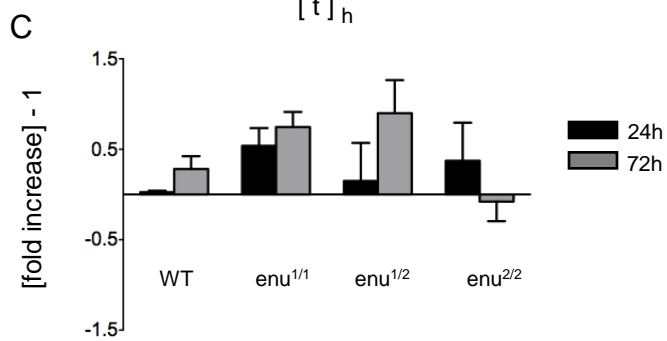
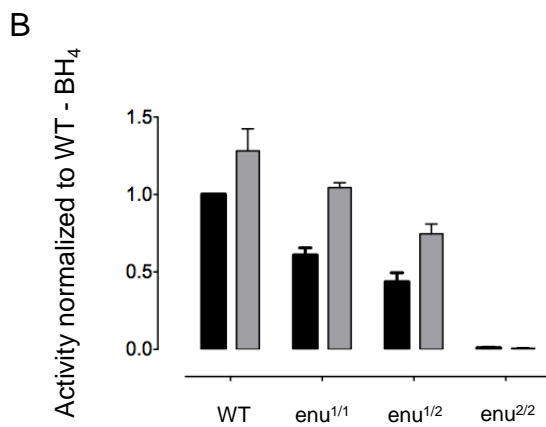
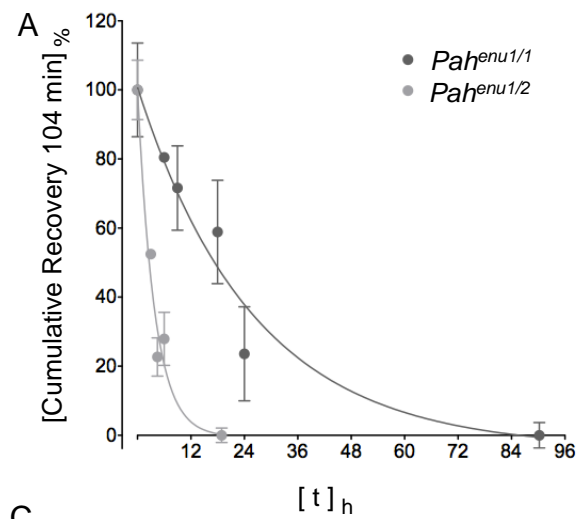


Figure 4

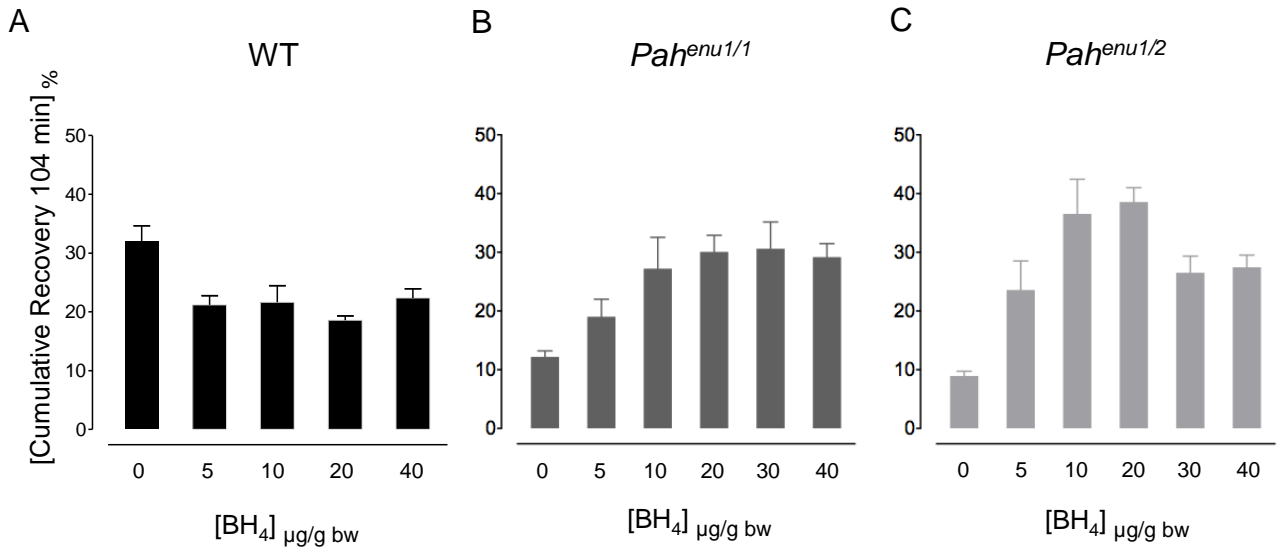


Table S1. Steady state enzyme kinetic parameters of wild-type and variant murine PAH proteins

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

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.

Pah^{enu1/2} is a mouse model for compound heterozygous phenylalanine hydroxylase deficiency and the pharmacological chaperone 6*R*-L-erythro-5,6,7,8-tetrahydrobiopterin rescues in vivo enzyme activity in *Pah^{enu1/1}* and in *Pah^{enu1/2}* but shows genotype-specific pharmacodynamics.

