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The fate of intravenously administered tetrahydrobiopterin and its implications for heterologous gene therapy of phenylketonuria

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Abstract

Tetrahydrobiopterin (BH₄) is a required cofactor for the enzymatic activity of phenylalanine hydroxylase (PAH) and is synthesized de novo from GTP in several tissues. Heterologous expression of PAH in tissues other than liver is a potential novel therapy for human phenylketonuria that is completely dependent upon BH₄ supply in the PAH-expressing tissue. Previous experiments with liver PAH-deficient transgenic mice that expressed PAH in skeletal muscle demonstrated transient correction of hyperphenylalaninemia only with hourly parenteral BH₄ administration. In this report, the fate of intravenously administered BH₄ is examined. The conclusions are that (1) BH₄ administered intravenously is rapidly taken up by liver and kidney, and (2) uptake of BH₄ into muscle is relatively low. The levels of BH₄ achieved in skeletal muscle following IV injection are only 10% of the amount expected were BH₄ freely and equally distributed across all tissues. The half-life of BH₄ in muscle is approximately 30 min, necessitating repeated injections to maintain muscle BH₄ content sufficient to support phenylalanine hydroxylation. The efficacy of heterologous muscle-directed gene therapy for the treatment of PKU will likely be limited by the BH₄ supply in PAH-expressing muscle.

Introduction

Phenylalanine hydroxylase (PAH, EC 1.14.16.1) deficiency is the most common cause of hyperphenylalaninemia in humans and is one of the most common inborn errors of metabolism (IEM) with an incidence of approximately 1:16,000 live births in the US [1]. PAH is an iron-containing, molecular oxygen-requiring homo-tetramer that is expressed primarily in liver but also in kidney and pancreas. PAH catalyzes the hydroxylation of phenylalanine to tyrosine, and this reaction requires the participation of the unconjugated pterin cofactor (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH₄). BH₄ is synthesized de novo from GTP and is abundant in liver [2]. BH₄ is also required for the activities of the other aromatic amino acid hydroxylases (tryptophan and tyrosine hydroxylases) and types I, II, and III nitric oxide synthases.

Chronic untreated hyperphenylalaninemia causes mental retardation, microcephaly, and seizures. Successful therapy of classical phenylketonuria (PKU) due to inherited PAH deficiency requires the reduction of body phenylalanine levels. Contemporary therapy for PKU

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¹In memory of Krzysztof Wild, now deceased.

is based upon decreasing dietary phenylalanine intake; this special synthetic diet however, is expensive, unpalatable and must be maintained for life. Gene therapy is a promising novel approach to the treatment of PKU (and other IEM) with the goal of permanently restoring PAH expression and eliminating the need for a special diet. Liver is the obvious target organ for gene therapy of PKU, but we propose that circulating phenylalanine may be effectively cleared and the PKU phenotype influenced by PAH expressed in tissues other than liver (so-called heterologous gene therapy). We have previously demonstrated that PAH expression in skeletal muscle can correct hyperphenylalaninemia in *Pah^{enu2}* mice, a murine model of human PAH deficiency, if the enzyme is supplied with sufficient BH₄ to support physiologically meaningful phenylalanine hydroxylation [3]. Our conclusion however was that the clinical efficacy of muscle-directed gene therapy for PKU would be limited by an inability to maintain sufficient intramuscular BH₄ supply through exogenous injection. In this paper, we describe experiments, using both in vitro and in vivo approaches, performed to investigate the fate of BH₄ administered to mice. Our preliminary data comparing liver and muscle BH₄ uptake following intravenous BH₄ injection have been presented previously [4]; these investigations were subsequently repeated and extended to other tissues as reported here. We discovered that the capacity of muscle to take up BH₄ from blood is relatively limited in comparison to liver and that a large portion of injected BH₄ is rapidly excreted through the kidney.

Methods

Animal care

All experiments were reviewed and approved by the OHSU Committee on Animal Care and Use. Mouse colonies were maintained in accordance with current IACUC guidelines. Most experiments were performed with LCRPAH mice, a line of transgenic mice on the 129/Sv background that were developed to investigate PAH expression in erythrogenic bone marrow. Investigations of BH₄ uptake were carried out in these mice during ongoing experiments evaluating the effects of bone marrow PAH expression upon hyperphenylalaninemia [5]. Bone marrow PAH expression should not affect the uptake of BH₄ in other tissues such as liver and muscle. Some control data for bipterin levels in tissues were collected on C57Bl/6J mice. Phenylalanine loading studies were carried out on C57Bl/6J and on *Pah^{enu2}* mice, a model of human PKU. Mice were 3–6 months age at the time of the experiments. Standard mouse chow and water were provided ad libitum.

Reagents

BH₄ was purchased from Schircks Laboratories, Jona, Switzerland, stored sealed under liquid nitrogen at –20 °C, and dissolved in 1% ascorbic acid just prior to injection. Tissue culture supplies were purchased from Gibco Life Technology, Rockville, MD. Protein concentration was measured using a commercially available bicinchonic acid-based kit (Pierce Chemical, Rockford, IL). All other reagents were of the highest available commercial grade and purity.

BH₄ uptake in cultured primary mouse myotubes

Primary mouse myocytes were isolated from 1- to 5-day-old newborn C57Bl/6J or ICR wild-type mice according to established methods [6]. Myocytes were maintained in an undifferentiated state in 50% F10/50% Ham's media with 20% fetal bovine serum. To induce fusion and differentiation of the cells, fetal bovine serum was replaced with 10% calf serum in standard media. Numerous multinucleate linear myotubes were typically observed in the plates following 5–7 days on calf serum.

Differentiated myotube cultures were exposed to BH₄-containing media for varying time periods. Cultured myotubes in 60 mm dishes were first washed with sterile PBS then exposed to 1 ml MEM media containing freshly dissolved BH₄. The final media BH₄ concentration

ranged from 1 to 10 μM . The cultures were then incubated at 37 °C until harvest. Prior to cell harvest, an aliquot of BH_4 -containing media was removed from the plate and frozen for later BH_4 analysis. The cells were washed twice with PBS, then lysed in 1 ml of 0.1 M potassium phosphate buffer, pH 7.4, 0.1 M DTT, and 0.1% Triton X-100. Following a 10 min incubation at room temperature, supernatants were collected and frozen at -20 °C for later BH_4 analysis. BH_4 content of the cells was expressed relative to total cellular protein in the lysates.

BH_4 or phenylalanine administration to mice

Anesthesia (ketamine/xylazine/promethazine) was administered by intraperitoneal injection prior to BH_4 administration. BH_4 (0.1 μmol BH_4 /gm body weight, total volume = 200–300 μl in 1% ascorbic acid) was injected intravenously via the tail vein. At various time points following BH_4 injection, the mice were euthanized under anesthesia by exsanguination via cardiac puncture, perfused via the left ventricle with phosphate-buffered saline (PBS) and tissue samples were collected. Tissues were also collected from mice injected with only 1% ascorbic acid. All solid tissue samples were weighed and homogenized in five volumes of 50 mM Tris/1 mM EDTA/1 mM DTT, pH 7.4. Supernatants were removed and frozen at -20 °C until BH_4 analysis. Whole blood samples were drawn into EDTA-containing Eppendorf tubes and centrifuged to separate the plasma from erythrocytes. Plasma was frozen at -20 °C and used as is for BH_4 analysis.

Phenylalanine (1 mg/gm body weight = 6 μmol /gm) was administered by intraperitoneal injection without anesthesia. At various time points following injection, the animals were anesthetized, euthanized by exsanguination via cardiac puncture, and perfused with PBS. Solid tissue samples were homogenized in 10% trichloroacetic acid and centrifuged at 3000g for 10 min at room temperature. Phenylalanine concentration was measured in serum samples and in supernatants of tissue homogenates using a fluorometric method [7].

Measurement of BH_4 concentration

Total biopterin concentration was analyzed by HPLC according to established methods [2]. The reduction state of biopterin (that is, BH_4 vs. dihydrobiopterin (BH_2)) in tissues was not evaluated in all samples. For solid tissues, the intracellular BH_4 concentration was estimated by dividing the BH_4 content measured in the supernatant by the wet weight of the tissue sample.

Results

BH_4 uptake in vivo

BH_4 uptake into cultured Differentiated primary mouse myotubes was measured over 2 h with culture media BH_4 concentration at 1 or 10 μM . A plot of intracellular BH_4 content vs. time for cultures with 10 μM BH_4 in the culture media is presented in Fig. 1. The amount of intracellular BH_4 increased linearly over the first 10 min then stabilized. No further significant increase in BH_4 uptake was seen over the remainder of the experiment. This result suggests that intracellular and extracellular BH_4 concentration had reached equilibrium and that no further uptake occurred after 10 mins. These data likely do not support an active transport model given that BH_4 uptake does not continue to increase against the intracellular/extracellular gradient. BH_4 uptake was not saturated at least up to an extra-cellular concentration = 10 μM , five times the normal plasma BH_4 concentration.

Previous investigators have suggested that BH_4 up-take occurs only by passive diffusion [8]. Although our in vitro data do not suggest a transporter-mediated model in cultured myotubes and are consistent with passive diffusion, we can estimate the capacity of the sarcolemma to allow BH_4 uptake by assuming a first order kinetic relationship between extracellular BH_4 concentration and the rate of BH_4 intake into the cells. Creation of a Lineweaver–Burke type

plot (not shown) of $1/\text{velocity}$ of BH_4 uptake vs. $1/\text{BH}_4$ concentration yields a crude $K_{\text{transport}} = 15 \mu\text{M}$ and $V_{\text{max}} = 4.7 \text{ pmol/mg protein/min}$. In comparison, measurement of neutral amino acid uptake into cultured cells, which is known to occur via sodium-independent facilitated transport (reviewed in Shotwell et al. [9]), yields $K_m = 1.3\text{--}1.8 \text{ mM}$ and $V_{\text{max}} = 7000\text{--}20,000 \text{ pmol/mg protein/min}$ [10].

Native BH_4 supply

Previous measurements by other laboratories of native tissue biopterin content in rats [2] and mice [11] demonstrated the highest biopterin levels in pineal gland, pituitary gland, liver, adrenal gland, spleen, bone marrow, and whole blood with negligible amounts in other tissues including kidney and muscle. Prior to performing IV BH_4 injections, we sought to confirm these results in select tissues from mice in our colony. Tissues were harvested from wild-type mice for measurement of tissue biopterin concentration (Fig. 2). In agreement with the previously published data, biopterin was abundant in liver of our mice; much less biopterin was present natively in kidney, plasma or skeletal muscle.

BH_4 uptake in vivo

To study tissue uptake of BH_4 from the circulation, BH_4 ($0.1 \mu\text{mol/gm}$ body weight in 1% ascorbic acid) was injected into mice via the tail vein. Animals were euthanized at various time points following injection for analysis of tissue biopterin concentration (Fig. 3). Plasma biopterin levels increased from approximately $1.5 \mu\text{M}$ to almost $600 \mu\text{M}$ immediately postinjection. Biopterin was rapidly cleared from plasma with kinetics that approximate a single compartment pharmacologic model with exponential decay ($y = 211e^{-0.0481x}$, $R^2 = 0.938$) (Fig. 3A). The half-life of biopterin in plasma was only 14.4 min. Were BH_4 freely distributed throughout all tissues and not immediately excreted, then based upon the dose of BH_4 given and the estimated weight of total body water, the maximal BH_4 concentration in any tissue should have been approximately $150 \mu\text{M}$. Yet, BH_4 concentration approached $450 \mu\text{M}$ in kidney, $200 \mu\text{M}$ in liver, but only $40 \mu\text{M}$ in skeletal muscle. We also measured erythrocyte biopterin content in a subset of ten animals following IV BH_4 injection; erythrocyte biopterin increased from a mean preinjection concentration of $7.0 \mu\text{M}$ (range = $2.2\text{--}12.0 \mu\text{M}$, $n = 4$) up to $1200 \mu\text{M}$ immediately after injection as measured in a single animal. At subsequent time points and again measured in single animals, erythrocyte biopterin content decreased, down to $150 \mu\text{M}$ at 120 min postinjection, but always remained greater than the plasma biopterin. Correlation between plasma and erythrocyte BH_4 content has been previously reported [12].

The half-life of BH_4 in muscle was approximately 30 min. Because the HPLC method used measures total biopterin including the oxidized form of BH_4 , namely 7,8-dihydrobiopterin, the relatively rapid disappearance of biopterin suggests actual movement of BH_4 back out of muscle rather than loss through oxidation. These data indicated significantly lower uptake of BH_4 into muscle in comparison to liver or kidney.

In comparison to BH_4 uptake, phenylalanine transport into muscle is much more robust (Fig. 4A). Following intraperitoneal injection into wild-type mice, phenylalanine is rapidly taken up by muscle and liver; tissue phenylalanine levels then gradually decrease over 120 min. The serum phenylalanine level remains elevated (normal = $100\text{--}150 \mu\text{M}$) during this time period. We propose that after the first 30 min following injection, phenylalanine, which had rapidly distributed throughout the body, is transported back into blood and flows to the liver for metabolism. As the total body load is gradually cleared by the liver, the serum phenylalanine level begins to fall. In PAH-deficient $\text{Pah}^{\text{enu2}}/\text{Pah}^{\text{enu2}}$ mice (Fig. 4B), phenylalanine injection yields much higher tissue phenylalanine content because liver lacks the capability to metabolize

the phenylalanine load. The liver and muscle phenylalanine content parallel each other suggesting that the distribution of phenylalanine is equal between the two tissues.

Discussion

BH₄ is a required cofactor for phenylalanine hydroxylase (PAH) activity. One mole of BH₄ is oxidized for each mole of phenylalanine that is hydroxylated to tyrosine. In liver, the supply of BH₄ is maintained both through recycling of qBH₂ back to BH₄ via pterin-4a-carbinolamine dehydratase (EC 4.2.1.96) and dihydropteridine reductase (DHPR) (EC 1.6.99.7) activity and through de novo synthesis of BH₄ from GTP. In any liver-directed gene therapy protocol for PKU, the BH₄ content of liver should be sufficient to support meaningful phenylalanine hydroxylation once PAH activity is expressed within hepatocytes. PAH expressed in other tissues such as muscle will not enjoy such a robust BH₄ supply. The success of heterologous gene therapy for PKU using PAH is dependent upon BH₄ supply to the target tissue. We have previously demonstrated reduced serum phenylalanine levels in muscle PAH-expressing, liver PAH-deficient mice when BH₄ is administered repetitively and in large amounts [3]. The goal of the experiments presented here was to further our understanding of the fate of exogenously administered BH₄. To our knowledge, this study also represents the first attempt to directly measure BH₄ uptake into skeletal muscle following IV injection.

BH₄ is administered clinically to individuals with BH₄ deficiency secondary to inborn errors of pterin synthesis or recycling. This medical use has stimulated the pharmacologic investigation of parenterally administered BH₄. For instance, penetration of BH₄ across the blood–brain barrier following IV injection has been shown to be low [13]. In another study, the fate of intravenous 2-¹⁴C-BH₄ was examined in rats by whole body autoradiography [14]. At 4 h following BH₄ injection, the majority of radioactivity was detected in liver, renal cortex, and renal medulla. By 16 h, most radiolabel was retained in liver. Very little radiolabel was detected in skeletal muscle. A similar pattern of radiolabel distribution has been reported following IV injection of 2-¹⁴C-BH₄ into mice [15]. To our knowledge, the tissue distribution of intravenously administered BH₄ in humans has not been systematically studied, however, oral administration of BH₄ yields substantial accumulation of biopterin in urine within 4 h [16].

The results of our experiments demonstrate that the majority of injected BH₄ is taken up by liver and kidney. Urine biopterin content in a single mouse following IV BH₄ injection was very elevated. Although we did not systematically evaluate urinary biopterin excretion in these animals, we propose that a large fraction of injected BH₄ is rapidly filtered and excreted by the kidney. Much less BH₄ is taken up by skeletal muscle. The muscle BH₄ level is generally only about 5% of the plasma BH₄ concentration following IV BH₄ injection. In contrast, the tissue phenylalanine concentration achieved following phenylalanine injection is much closer to the plasma phenylalanine level at all time points. Following injection, the half-life of muscle biopterin content is short, approximately 30 min. In our previous work, hourly intraperitoneal administration of 0.1 μmol BH₄/gm body weight was required to support physiologically meaningful phenylalanine hydroxylation in muscle PAH-expressing mice. Under this regimen, plasma BH₄ levels peak at approximately 800 μM but decline to 50 μM by 60 min following injection. Muscle BH₄ never exceeded 5 μM following IP BH₄ administration. After IV injection, muscle BH₄ peaked at approximately 30 μM 5 min after injection but then rapidly declined. The *K_m* for BH₄ in native rat liver phenylalanine hydroxylase is 2 μM [17]. Therefore, muscle BH₄ levels have either met, in the case of IP BH₄ injection, or exceeded (IV injection) the enzyme *K_m* for BH₄ and therefore would be expected to support physiologically significant phenylalanine hydroxylation. However, the required muscle BH₄ level was not sustained; muscle BH₄ content dropped rapidly with a half-life of approximately 30 min. Even following IV injection, the muscle BH₄ content fell below 2 μM by 2 h postinjection.

BH₄ uptake across mammalian cell membranes is thought to occur via passive diffusion [8]. Uptake into pheochromocytoma PC12 cells and rat brain synaptosomes is non-saturable, concentration-dependent, and does not require either NaCl or glucose. There is no published evidence for a specific BH₄ transporter in these neuronal cell types or any other mammalian tissue. However, a putative high affinity bipterin transporter, distinct from the folate transporter, has been described in the trypanosome *Leishmania tarentolae* [18]. The fact that BH₄ in blood is rapidly taken up by mouse liver while muscle uptake is limited suggests the possibility that the mechanism of BH₄ uptake might differ among different tissues. Further investigation into the mechanism of BH₄ uptake into liver will be necessary to fully explain the tissue specific differences we found.

The results of these experiments suggest that multiple repeated BH₄ injections would be required to sustain physiologically significant phenylalanine hydroxylation in animals with muscle PAH expression and explains why hourly intraperitoneal BH₄ injection was necessary to lower serum phenylalanine levels in our muscle PAH-expressing transgenic mice [3]. The efficacy of muscle-directed, PAH-mediated gene therapy for the treatment of PKU will be limited unless a method to insure continuous, sufficient intramuscular BH₄ supply is developed. Expression of BH₄ synthetic enzymes, including GTP cyclohydrolase (GTPCH) and 6-pyruvoyltetrahydropterin synthase (PTPS), in cultured fibroblasts yields BH₄ production [19] and phenylalanine hydroxylation in cultured fibroblasts has been demonstrated following retrovirus-mediated expression of PAH along with GTPCH and PTPS [20]. Perhaps, muscle coexpression of PAH, GTPCH, and PTPS via a multicistronic vector would yield sufficient PAH activity and BH₄ supply to support physiologically significant phenylalanine hydroxylation. Alternatively, muscle expression of a cofactor independent, phenylalanine-metabolizing enzyme such as yeast phenylalanine ammonia lyase (PAL, EC 4.3.1.5) [21] could potentially clear circulating phenylalanine and avoid cofactor delivery problems altogether.

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References

1. Scriver, CR.; Kaufman, S. Hyperphenylalaninemia: phenylalanine hydroxylase deficiency. In: Scriver, CR.; Beaudet, AL.; Sly, WS.; Valle, D., editors. *The Metabolic and Molecular Bases of Inherited Disease*. McGraw-Hill; New York: 2001. p. 1667-1724.
2. Fukushima T, Nixon JC. Analysis of reduced forms of bipterin in biological tissues and fluids. *Anal Biochem* 1980;102:176–188. [PubMed: 7356152]
3. Harding CO, Wild K, Chang D, Messing A, Wolff JA. Metabolic engineering as therapy for inborn errors of metabolism—development of mice with phenylalanine hydroxylase expression in muscle. *Gene Ther* 1998;5:677–683. [PubMed: 9797873]
4. Harding, C.; Neff, M.; Wild, K.; Milstien, S. The fate of intravenously administered tetrahydrobiopterin and its implications for heterologous gene therapy of phenylketonuria. In: Milstien, S.; Kapatoss, G.; Levine, R.; Shane, B., editors. *Chemistry and Biology of Pteridines and Foliates, Proceedings of the 12th International Symposium on Pteridines and Foliates*. Kluwer Academic Publishers; Boston: 2002. p. 305-308.
5. Harding C, Neff M, Jones K, Wild K, Wolff J. Expression of phenylalanine hydroxylase (PAH) in erythrogenic bone marrow does not correct hyperphenylalaninemia in *Pah^{enu2}* mice. *J Gene Med*. 2003in press
6. Rando TA, Blau HM. Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. *J Cell Biol* 1994;125:1275–1287. [PubMed: 8207057]
7. McCaman MW, Robins E. Fluorimetric method for the determination of phenylalanine in serum. *J Lab Clin Med* 1962;59:885–890.

8. Anastasiadis PZ, Kuhn DM, Levine RA. Tetrahydrobiopterin uptake into rat brain synaptosomes, cultured PC12 cells, and rat striatum. *Brain Res* 1994;665:77–84. [PubMed: 7882022]
9. Shotwell MA, Kilberg MS, Oxender DL. The regulation of neutral amino acid transport in mammalian cells. *Biochim Biophys Acta* 1983;737:267–284. [PubMed: 6303424]
10. Foster DO, Pardee AB. Transport of amino acids by confluent and nonconfluent 3T3 and polyoma virus-transformed 3T3 cells growing on glass cover slips. *J Biol Chem* 1969;244:2675–2681. [PubMed: 4306034]
11. Duch DS, Bowers SW, Woolf JH, Nichol CA. Biopterin cofactor biosynthesis: GTP cyclohydrolase, neopterin and biopterin in tissues and body fluids of mammalian species. *Life Sci* 1984;35:1895–1901. [PubMed: 6492998]
12. Ponzzone A, Guardamagna O, Spada M, Ponzzone R, Sartore M, Kierat L, Heizmann CW, Blau N. Hyperphenylalaninemia and pterin metabolism in serum and erythrocytes. *Clin Chim Acta* 1993;216:63–71. [PubMed: 8222274]
13. Kapatos G, Kaufman S. Peripherally administered reduced pterins do enter the brain. *Science* 1981;212:955–956. [PubMed: 7233193]
14. Hennings G, Rembold H. Regional and subcellular distribution of biopterin in the rat. *Int J Vitam Nutr Res* 1982;52:36–43. [PubMed: 7085198]
15. Hoshiga M, Hatakeyama K, Watanabe M, Shimada M, Kagamiyama H. Autoradiographic distribution of [¹⁴C]tetrahydrobiopterin and its developmental change in mice. *J Pharmacol Exp Ther* 1993;267:971–978. [PubMed: 8246172]
16. Blau N, de Klerk JB, Thony B, Heizmann CW, Kierat L, Smeitink JA, Duran M. Tetrahydrobiopterin loading test in xanthine dehydrogenase and molybdenum cofactor deficiencies. *Biochem Mol Med* 1996;58:199–203. [PubMed: 8812740]
17. Kaufman, S. Basic biochemistry and role in human disease. Johns Hopkins University Press; Baltimore: 1997. Phenylalanine hydroxylase, in: Tetrahydrobiopterin; p. 86
18. Kundig C, Haimeur A, Legare D, Papadopoulou B, Ouellette M. Increased transport of pteridines compensates for mutations in the high affinity folate transporter and contributes to methotrexate resistance in the protozoan parasite *Leishmania tarentolae*. *EMBO J* 1999;18:2342–2351. [PubMed: 10228149]
19. Laufs S, Blau N, Thony B. Retrovirus-mediated double transduction of the GTPCH and PTPS genes allows 6-pyruvoyltetrahydropterin synthase-deficient human fibroblasts to synthesize and release tetrahydrobiopterin. *J Neurochem* 1998;71:33–40. [PubMed: 9648848]
20. Christensen R, Kolvraa S, Blaese RM, Jensen TG. Development of a skin-based metabolic sink for phenylalanine by overexpression of phenylalanine hydroxylase and GTP cyclohydrolase in primary human keratinocytes. *Gene Ther* 2000;7:1971–1978. [PubMed: 11175307]
21. Sarkissian CN, Shao Z, Blain F, Peevers R, Su H, Heft R, Chang TM, Scriver CR. A different approach to treatment of phenylketonuria: phenylalanine degradation with recombinant phenylalanine ammonia lyase [see comments]. *Proc Natl Acad Sci USA* 1999;96:2339–2344. [PubMed: 10051643]

Fig. 1. Plot of intracellular biopterin concentration versus time following addition of BH_4 to the media in cultured primary mouse myotubes. Media BH_4 concentration = $10 \mu\text{M}$. Two separate myotube cultures were assayed at each time point and the intracellular biopterin concentration was measured in duplicate for each culture. The data are displayed as the mean biopterin concentrations $\pm$ one standard error of the mean.

Fig. 2. Total biopterin concentration in select tissues of wild-type mice. Data represent the mean tissue biopterin concentration with the number of mice assayed for each tissue given in the figure. The error bars represent one standard error of the mean.

Fig. 3. Biopterin concentration vs. time following BH₄ injection. (A) Plasma and kidney biopterin. (B) Liver and muscle biopterin. For plasma, liver, and muscle, the data represent the mean tissue biopterin of 3–5 mice euthanized at each time point. Error bars display the standard error for each time point. For kidney, the data represent the mean of two to three mice at each time point and the error bars display the full range of the data.

Fig. 4. Phenylalanine concentration vs. time following phenylalanine injection. (A) Tissue phenylalanine concentration following IP phenylalanine injection in wild-type C57Bl/6J mice. Data represent the mean of 3 determinations at each time point. Error bars display the standard error for a single time point. (B) Tissue phenylalanine concentration following IP phenylalanine injection in hyperphenylalaninemic *Pah^{enu2}/Pah^{enu2}* mice. Each data point represents a single animal sacrificed at each time point.