Hyperphenylalaninemia in the *hph-1* Mouse Mutant

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ABSTRACT. A mutation, resulting in a deficiency of liver GTP-cyclohydrolase activity, has been induced in the laboratory mouse. Mice homozygous for this mutation exhibit hyperphenylalaninemia under the following conditions: 1) early in life and 2) throughout life when exposed to phenylalanine. A phenylalanine loading regimen was used to discriminate between mutant and wild type mice on the basis of the resultant phenylalanine and tyrosine serum levels. Subjecting mice to this regimen reveals several distinguishing characteristics. Mutant mice exhibit approximately 2-fold higher peak phenylalanine levels than wild-type mice. In wild-type mice the hyperphenylalaninemic state is transient and rapidly abates while in mutant mice it is persistent and remains for a prolonged period. Mutant mice exhibit normal serum tyrosine levels after a loading challenge, while wild-type mice experience an increase in tyrosine levels. The loading regimen was also used to gauge the response of mutant hyperphenylalaninemic mice to exposure to chemical compounds required for normal phenylalanine catabolism (*i.e.* pteridine cofactors of the phenylalanine hydroxylase reaction). Mutant mice exposed to native enzyme cofactor or cofactor precursors exhibit a sharp decline in serum phenylalanine levels relative to their uninjected counterparts coupled with a tyrosine increase. By contrast, mutant mice exposed to nonprecursor compounds that are structurally related to the native cofactor, experience no diminution of serum phenylalanine levels. (*Pediatr Res* 23: 63-67, 1988)

Abbreviations

HPA, hyperphenylalaninemia
ENU, N-ethyl-N'-nitrosourea
PHE, phenylalanine
TYR, tyrosine
THB, tetrahydrobiopterin
QDHPR, quinonoid dihydropteridine reductase
PKU, phenylketonuria
PH, phenylalanine hydroxylase
GTP-CH, GTP-cyclohydrolase

better understanding both a common human affliction and some important aspects of neurotransmitter synthesis.

Normal catabolism of PHE in the mammalian system is initiated by a reaction catalyzed by the enzyme PHE hydroxylase (EC 1.14.16.1) (2). PHE hydroxylase requires the reduced pteridine cofactor THB for this reaction. This cofactor is synthesized, through a number of intermediates, from GTP (3). The resultant pteridine cofactor is kept in the required reduced state by the enzyme, QDHPR (EC 1.6.99.7). Thus, mutations resulting in the phenotypic expression of HPA generally fall into three classes. Mutations that eliminate or reduce PHE hydroxylase activity (HPA types I and II), mutations that eliminate or reduce QDHPR activity (HPA type IV), and mutations that interrupt the synthesis of the required pteridine cofactor (HPA type V) (4).

Because the laboratory mouse has been extremely well characterized both physiologically and genetically mouse modeling systems with blockages of PHE catabolism would be attractive experimental tools in the investigation of the complex system of PHE metabolism in mammals. Although chemically induced animal modeling systems for HPA have been developed (5, 6, 17), there is always a degree of uncertainty inherent in their use due to the potential presence of unknown secondary effects induced by the chemical agents used (7).

Thus, utilizing the potent mouse spermatogonial stem cell mutagen ENU (8) to induce mutations in the mouse genome and the Guthrie assay (9) to screen for mutations that cause HPA, a systematic search was undertaken to isolate mice with heritable errors of PHE metabolism. The first such mutation isolated has been called *hph-1*. The biochemical defect associated with the *hph-1* mutation is a deficiency for GTP-CH activity (10) which catalyzes the initial rate-limiting step in the synthesis of the pteridine cofactor for the PH reaction (3). An especially useful tool for the initial determination of the biochemical nature of *hph-1* was a PHE loading regimen. The resulting PHE clearance profile highlights some of the phenotypic differences between wild type mice and mice that are either homozygous or heterozygous for the *hph-1* mutation. PHE loading also provides a method to determine the effect of administering various pteridine cofactors to mutant mice.

METHODS

ENU mutagenesis of (C57BL/6 × CBA/Ca)F1 male mice was performed by previously published methods (11). The *hph-1* mutation was isolated by screening progeny from mutagenized animals as described by Bode *et al.* (12). Animals referred to herein as mutant or HPH-1 are homozygous for the *hph-1* mutation and those referred to as wild type or normal are nonmutagenized F1 mice from the same pair of inbred lines mentioned above.

For the purposes of clearance experiments, PHE loading of HPH-1 mice was achieved by intraperitoneal injection of a 25 mg/ml aqueous solution of the amino acid, adjusted to neutral pH. Each mouse received a 1 mg PHE per g body weight dose.

The inability to effectively catabolize the amino acid PHE is a common inborn error of metabolism in humans (1). The resultant HPA can give rise to a number of pathological effects. Furthermore, the initial step in the normal catabolism of PHE and the rate-limiting steps in the biosynthetic pathways of several important neurotransmitters share many features. Thus, a more thorough knowledge of PHE metabolism would be helpful in

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Similarly, all cofactor injections were given intraperitoneally with weight-normalized injection volumes to achieve the overall dose rates noted in Table 1 (all pteridine compounds were purchased from Schirks Laboratories, Jona, Switzerland). Mice that were used for the loading and clearance experiments were all less than 1 yr of age.

Serum PHE and TYR levels were determined by quantitative

Table 1. Serum PHE clearance obtained by exposing HPH-1 mice to native PHE hydroxylase cofactor, cofactor precursors, or related pteridine compounds*

Injected compound	Dose (mg/kg)	Clearance ratio (SEM)
None		0.85 (0.06)
THB	10	0.46 (0.33)
THB	2	0.58 (0.14)
7,8-Dihydrobiopterin	10	0.34 (0.27)
7,8-Dihydrobiopterin	2	0.70 (0.07)
L-Sepiapterin	10	0.28 (0.17)
L-Sepiapterin	2	0.53 (0.23)
7,8-Dihydro-D-neopterin	10	0.82 (0.02)
7,8-Dihydro-D-neopterin	2	0.83 (0.07)
6-Biopterin	10	0.89 (0.03)
6-Biopterin	2	0.89 (0.07)
6-Methyl-THB	100	0.80 (†)
6-Methyl-THB	10	0.87 (0.02)
6-Methyl-THB	2	0.89 (0.02)
6,7-Dimethyl-THB	100	0.94 (†)
6,7-Dimethyl-THB	10	0.87 (0.08)
6,7-Dimethyl-THB	2	0.86 (0.02)

* The mice were loaded with PHE, either exposed to a pteridine compound or not, and bled as described in "Methods." Mice were bled immediately prior to pteridine injection and again 1 h after pteridine injection. The ratio of final to initial PHE concentration, the clearance ratio, was taken as a measure of the effectiveness of the particular pteridine compound at reducing plasma PHE levels.

† Indicates that a single reading was taken at this level of cofactor administration. All other clearance ratio values represent the arithmetic means of at least three independent determinations.

spectrophotometric method of Shen and Abell (13). Blood was obtained from adult mice either by retroorbital or tail bleeding into heparinized capillary blood collection tubes (American Scientific Products, McGaw Park, IL). Mice 2 wk old and younger were decapitated and exsanguinated. Mice younger than 4 days old could not be assayed due to insufficient blood volume. The heparinized blood was centrifuged to sediment the red blood cells and the supernatant serum was removed for PHE and TYR determinations. The serum sample was then incubated with the enzyme PHE ammonia-lyase (EC 4.3.1.5) (Sigma Chemical, St. Louis, MO). This enzyme catalyzes the conversion of PHE and TYR to trans-cinnamic acid and trans-coumaric acid, respectively. By measuring the rate of the change in the absorbance at an absorption maximum for each of these compounds, the amount of PHE and TYR was determined in a serum sample. Samples with known amounts of PHE and TYR were carried through each determination as standards. Serum samples not titrated immediately after bleeding were frozen at -20°C for no more than 1 wk before PHE/TYR determination.

RESULTS

The scatter plot in Figure 1 relates serum PHE levels without PHE loading to the age of HPH-1 mice. Serum PHE levels are maximal at about 6 days after birth, at approximately 10-fold normal levels. After this, the HPA gradually abates and serum PHE reaches normal levels at about the age of weaning (21 days after birth). All of the animals used in this experiment were the progeny of HPH-1 parents. Similar values were also obtained when HPH-1 young had suckled wild type foster mothers from birth and when the nursing mother was heterozygous for the mutation (data not shown).

A number of symptoms arise in HPH-1 animals pursuant to PHE exposure. Intense exposures inevitably lead to the death of the animal. Although the precise cause of death is unknown, it follows a chronic wasting syndrome that is more prolonged the greater the age of the animal at the onset of PHE exposure. Low level exposures are usually not lethal but HPH-1 animals remain smaller than heterozygote and wild-type siblings. In addition female HPH-1 mice never become palpably pregnant while under low level PHE exposure. It is unknown whether this results from an inability to conceive or early death of developing embryos.

Fig. 1. Scatter plot relating serum PHE levels to the age of HPH-1 mice. Closed circles of smallest size indicate unique readings and closed circles of intermediate and largest sizes represent the double or triple repeated occurrence of a given reading from different mice, respectively. The arrowhead pointing at the ordinate represents accepted normal adult mouse serum PHE levels (14).

The PHE loading and clearance experimental results comparing HPH-1 and wild-type mice are depicted in Figure 2. The HPH-1 mice exhibited approximately 2-fold higher peak PHE levels compared to wild type mice subjected to the same loading regimen. In addition, HPH-1 mice retained normal TYR levels while their wild-type counterparts exhibited a TYR peak following the PHE peak. Wild type mice had returned to normal serum PHE levels and near normal TYR levels 1.5 h after onset of loading while HPH-1 mice continued to exhibit elevated serum PHE levels for many hours. The half-life for the decay of peak PHE levels in HPH-1 mice was found to be approximately 5½ h.

The histogram in Figure 3 illustrates the different phenotypic responses of the three genotypic classes to the loading regimen described for Figure 2. The serum PHE levels of the three groups are most clearly differentiable at 1.5 h after PHE challenge.

The variation with time of serum PHE and TYR levels of loaded HPH-1 mice in the presence and absence of THB is depicted in Figure 4. On administration of THB to hyperphenylalaninemic HPH-1 mice, the serum PHE levels rapidly diminish relative to mice not receiving THB. In addition a trailing TYR peak is seen. In contrast, mice not receiving the cofactor continue to exhibit HPA with baseline levels of TYR.

Related pteridines were used in the manner described for the

Fig. 2. The variation of serum PHE and TYR levels of HPH-1 and wild-type mice in response to a PHE load with time. The closed (PHE) and open (TYR) circles connected by solid lines represent the clearance curves for HPH-1 mice. The closed and open boxes connected by dashed lines represent the corresponding curves for wild-type mice. Each Data point represents the mean of three independent determinations.

Fig. 3. Variation of the PHE clearance capacity with gene dosage. Animals were pretested to establish that all were exhibiting normal levels of serum PHE. The animals were then given a PHE challenge as described in "Methods." After PHE challenge, 1.5 h, animals were bled and serum PHE levels were determined. Three independent determinations were done for each genotypic class. Error bars indicate $\pm$ SEM for these classes.

experiment depicted in Figure 4 and the results are depicted in Table 1. *L*-Sepiapterin and 7,8-dihydrobiopterin cause reduction of serum PHE in a similar range with THB. Due to the degree of variability seen in the clearance ratios elicited by these three cofactors no order of precedence can be determined. 7,8-Dihydro-D-neopterin, 6-biopterin, 6-methyl-THB, and 6,7-dimethyl-THB did not cause serum PHE reduction.

DISCUSSION

While screening the progeny of ENU mutagenized mice for HPA a mutation, *hph-1*, was isolated. After four generations of outcrossing the mutation continues to exhibit segregation consistent with autosomal recessive inheritance of a single gene defect (12). The biochemical basis of the HPA seen in *hph-1* homozygotes has recently been shown to arise from a deficiency of liver GTP-CH activity (18). The degree of HPA exhibited by unloaded HPH-1 mice early in life, although variable, is pronounced. Serum PHE levels increase after day 4 and peak at about day 6 at nearly 10 times normal; after this stage, the levels gradually abate, reaching normal by the time of weaning. After weaning, mice exhibiting HPA are rare. In addition, some mutant mice show normal PHE levels prior to weaning, but these are also rare and seem to correlate with insufficient nursing.

The diminishing HPA of unloaded HPH-1 mice is apparently consistent with the mutation causing delayed developmental onset of normal PHE metabolism. Not only is this interpretation inconsistent with biochemical findings (18) but it fails to explain many other physiological features of the HPH-1 phenotype. HPH-1 mice exhibit a lifelong inability to metabolize PHE effectively and, whenever they are exposed to extraneous PHE (either in their diet or by injection), HPA results. Prolonged exposure often results in the death of the animal. A possible cause for the decrease of HPA with maturation is the change in the diet of the mouse. Thus early in life, when the animal is totally dependent on the relatively high PHE nursing diet provided by the mother, high levels of serum PHE are noted. Later, when the animal's diet shifts to the predominantly grain laboratory Chow, the serum PHE levels subside to normal.

A PHE loading regimen was developed to compare the clearance response of HPH-1 and wild-type adult mice to a measured PHE exposure. Wild-type mice are able to catabolize the injected PHE in a relatively short period of time. In normal catabolism the initial fate of PHE is conversion to TYR by hydroxylation of the aromatic ring. Thus in wild-type mice the initial PHE peak is always followed by a smaller TYR peak. In contrast, HPH-1 mice exhibit roughly 2-fold higher peak serum PHE levels and no detectable increase in TYR levels. This clearly indicates an interruption of PHE hydroxylation in the HPH-1 mouse. PHE loading also proved useful in correlating gene dosage with PHE clearance capabilities with heterozygous animals exhibiting approximately half PHE clearance capacity of homozygous animals.

On exposure of hyperphenylalaninemic HPH-1 mice to THB elevated serum PHE levels were rapidly reduced relative to untreated control mice and the trailing TYR peak returned. That is, a response very similar to wild-type (as seen in Fig. 2) is induced by injection of THB. This implicates the fully reduced enzyme cofactor as the missing entity. Moreover, the precipitous decline in serum PHE levels pursuant to THB exposure is more characteristic of defective cofactor synthesis than cofactor reductase deficiency (15, 16).

Similarly, related pteridine compounds (some known to be precursors of the native cofactor) were administered to hyperphenylalaninemic HPH-1 mice. All precursor compounds tested induced reduction of HPA and elicited clearance profiles indistinguishable from those seen with the native cofactor. This is in accord with previously published experimental observations involving pteridine administration to mice which have been chemically blocked for THB synthesis (8). Ineffective compounds gave PHE clearance profiles very similar to the untreated control clearance profile. These findings correspond well with GTP-CH deficiency in humans (18).

It is puzzling that HPH-1 animals do not experience the severe pathology usually associated with GTP-CH deficiency. In order to better understand this enigmatic observation it will be important to investigate GTP-CH activity levels in brain and spleen as well as liver as has been described for other mouse mutants (19).

Fig. 4. Serum PHE and TYR levels in PHE loaded HPH-1 mice followed by the administration of the native PHE hydroxylase cofactor. Closed (PHE) and open (TYR) circles represent the PHE and TYR levels of control mice. Closed and open boxes represent the corresponding levels of mice that have received THP. Arrows on the abscissa indicate the time of cofactor administration. Each data point represents the mean of three independent determinations.

The *hph-1* mutation in the mouse may prove useful in a variety of ways. While the somewhat differing developmental profiles of mouse and man must always be taken into consideration it is likely that the *hph-1* mutation can serve as an animal model for pathological syndromes that result from GTP-CH deficiency. In addition, due to the ease in which serum PHE levels can be manipulated either by oral exposures or intraperitoneal injections, it is possible that *hph-1* is suitable to investigate other pathologies that arise as a result of chronic HPA. Importantly, these investigations can be undertaken without the questions concerning the interference from concurrent exposure to enzyme or transport inhibitors which hamper chemically induced animal models for HPA. An added advantage is that when HPH-1 mice are fed regular laboratory Chow they are healthy and fecund and pose no difficulties in the maintenance of homozygous mutant stocks for experimentation.

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