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Genetics

Pah^{hph-5}: A mouse mutant deficient in phenylalanine hydroxylase

(hyperphenylalaninemia/mouse model/phenylketonuria/ethylnitrosourea/interspecific mapping)

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ABSTRACT **Mutant mice exhibiting heritable hyperphenylalaninemia have been isolated after ethylnitrosourea mutagenesis of the germ line. We describe one mutant pedigree in which phenylalanine hydroxylase activity is severely deficient in homozygotes and reduced in heterozygotes while other biochemical components of phenylalanine catabolism are normal. In homozygotes, injection of phenylalanine causes severe hyperphenylalaninemia and urinary excretion of phenylketones but not hypertyrosinemia. Severe chronic hyperphenylalaninemia can be produced when mutant homozygotes are given phenylalanine in their drinking water. Genetic mapping has localized the mutation to murine chromosome 10 at or near the *Pah* locus, the structural gene for phenylalanine hydroxylase. This mutant provides a useful genetic animal model affected in the same enzyme as in human phenylketonuria.**

Disorders of phenylalanine catabolism, resulting in phenylketonuria (PKU) and hyperphenylalaninemia (HPH), were among the first heritable errors of metabolism discovered in the human (1). The rate-limiting step in mammalian phenylalanine catabolism is hydroxylation to produce tyrosine. This reaction, catalyzed by phenylalanine hydroxylase (PAH) (2), requires the reduced pteridine cofactor tetrahydrobiopterin (3), which is synthesized from GTP (4) through a number of intermediates and is maintained in its reduced form by quinonoid dihydropteridine reductase (q-DHPR) (5). Mutations reducing the activity of PAH, q-DHPR, or the enzymes involved in tetrahydrobiopterin synthesis result in HPH because of a block in phenylalanine hydroxylation (6). In humans, PKU is defined as a condition resulting from mutations that abolish or severely reduce PAH activity (7). Other defects in phenylalanine catabolism are termed HPH. Extensive research has been undertaken to characterize these disorders (early work is reviewed in refs. 8 and 9 with recent summations in refs. 10 and 11).

Laboratory mice with defined PKU and HPH mutations would be helpful in evaluating features of these diseases by permitting investigations not acceptable with human subjects. To produce such mutants we have used the alkylating agent *N*-ethyl-*N*-nitrosourea, which induces mutations in the mouse germ line at a frequency near 10^{-3} per locus (12). We refer to all mutants with deficiencies in phenylalanine catabolism by their common phenotype, HPH. We have screened the progeny of 347 gametes and have isolated four mutant pedigrees exhibiting the HPH phenotype. One of these, HPH-1, was detected by its neonatal HPH phenotype and is deficient in GTP cyclohydrolase activity (13–15). The others were detected by their impaired ability to clear a phenylalanine challenge (described in ref. 13). One of these, HPH-5, we now report to be deficient in PAH.

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MATERIALS AND METHODS

Biochemical Determinations. Liver homogenates were prepared for PAH assay as described in ref. 16. A crude liver extract was prepared from approximately 50 mg of fresh liver tissue per ml of 150 mM KCl/0.7 mM 2-mercaptoethanol (pH 7.0) with a Potter–Elvehjem tissue homogenizer on ice and was clarified by centrifugation at 4°C for 15 min at 14,000 × *g*. Total protein levels were determined by the biuret method (17). PAH activities, determined as described in ref. 2 and modified in ref. 18, were measured by the phenylalanine-dependent oxidation of NADH in the presence of excess q-DHPR. This reaction was monitored by the decrease in absorption at 340 nm. Components of the assay system in a total volume of 1 ml were: 100 μmol of potassium phosphate (pH 6.8), 0.2 μmol of NADH, 800 units of catalase, 0.1 unit of DHPR, 1 μmol of L-phenylalanine, 0.04 μmol of 6-methyltetrahydropterin, and the enzyme extract. The background rates of NADH oxidation were determined in the absence of phenylalanine and independently in the absence of the synthetic pteridine cofactor 6-methyltetrahydropterin (purchased from Sigma). The rate of NADH oxidation was linear for at least 15 min and was directly proportional to total protein concentration over a range of at least 0.6–1.4 mg/ml of reaction volume. The activities presented are the total values minus the background rate.

q-DHPR activity in liver homogenates (prepared as above) was determined by measuring the rate of 6-methyltetrahydropterin-dependent reduction of ferricytochrome *c* to ferrocytochrome *c* (19). This reaction was monitored by the increase in absorption at 550 nm. The components of the assay system in a total volume of 2 ml were 80 μmol of Tris·HCl (pH 7.6), 0.1 μmol of ferricytochrome *c*, 0.1 μmol of NADH, 0.02 μmol of 6-methyltetrahydropterin, and the enzyme extract. The rate of ferricytochrome *c* reduction for each sample was corrected for two blank rates, one omitting the homogenate and the other omitting 6-methyltetrahydropterin. The rate of ferricytochrome *c* reduction was linear for at least 5 min and was proportional to the total protein over a range of at least 2–10 μg/ml of reaction volume.

Serum phenylalanine clearance was followed by spectrofluorometric phenylalanine determination (20). Animals were injected intraperitoneally with 1 mg of phenylalanine per g of body weight from a stock solution of 25 mg/ml of aqueous phenylalanine. Serum was obtained by centrifugation of whole blood. The effect of tetrahydrobiopterin (obtained from Shirck's Laboratories, Jona, Switzerland) supplementation was determined as described (13) by using 10 μg of (6*R*)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride per g of body weight, administered by intraperitoneal injection 1 hr after phenylalanine challenge. This solution was prepared immediately before use by dissolving the pteridine compound

Abbreviations: PKU, phenylketonuria; HPH, hyperphenylalaninemia; PAH, phenylalanine hydroxylase; q-DHPR, quinonoid dihydropteridine reductase.

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Table 1. Hepatic PAH activity

Genotype	Addition	NADH oxidized,* μmol/min per mg of total protein
+/+	6-MeH$_4$P	12.6 (1.1)
hph-5/+		4.1 (0.7)
hph-5/*hph-5*		0.4 (0.7)
+/+	L-Phe	12.2 (1.5)
hph-5/+		4.5 (1.1)
hph-5/*hph-5*		0.4 (1.5)

PAH assay done as described in *Materials and Methods* with 0.6 mg of total protein per reaction. Reactions were started by adding 6-methyltetrahydropterin (6-MeH$_4$P) or by adding L-phenylalanine. *Values shown are total values corrected for the blank values shown in parentheses.

in deionized water. *hph-1*/*hph-1* mice, known to respond to tetrahydrobiopterin supplementation with greatly increased phenylalanine clearance, served as a control for PAH cofactor activity of the tetrahydrobiopterin solution.

Southern Blot Analysis. DNA was prepared from frozen mouse tissues as described in ref. 21 and was digested with *Eco*RI (according to the manufacturer's specifications). Southern blot analysis was carried out as in ref. 22. Southern blots were probed with a mouse cDNA clone of the gene for PAH (kindly provided by S. L. C. Woo and F. D. Ledley, Baylor University College of Medicine, Houston).

RESULTS

The ability to clear a phenylalanine challenge administered by intraperitoneal injection at 1 mg/g of body weight is a measure of normal phenylalanine catabolism. In the wild-type mouse, serum phenylalanine increases from 0.12 mM to 1.7 ± 0.1 mM and returns to normal within 2 hr with no detectable urinary phenylketones (as measured by Phenistix, Miles). By contrast *hph-5* homozygotes exhibit almost a 50-fold increase (6.2 ± 0.9 mM) in serum phenylalanine, which returns to normal in about 6 hr. Phenylalanine exposure was accompanied by excretion of phenylketones in the urine of homozygous mutants. Chronic HPH (2.2 ± 0.7 mM) can be maintained in homozygous mutants by adding phenylalanine to drinking water at 25 mg/ml.

In vitro assay of the enzymes involved in phenylalanine hydroxylation revealed that HPH-5 is deficient in hepatic PAH activity (Table 1). Previous claims of murine PAH deficiency in a different mouse strain had been shown to result from inhibition by a phenylalanine metabolite (23). We have tested explicitly for such an artifact: upon mixing homogenates from homozygotes and wild-type animals, the resulting PAH activity was never reduced by more than the dilution factor (data not shown). Reduced activity (about 30% of normal) in heterozygotes correlates well with the PAH levels seen in human PKU carriers (24, 25). These results

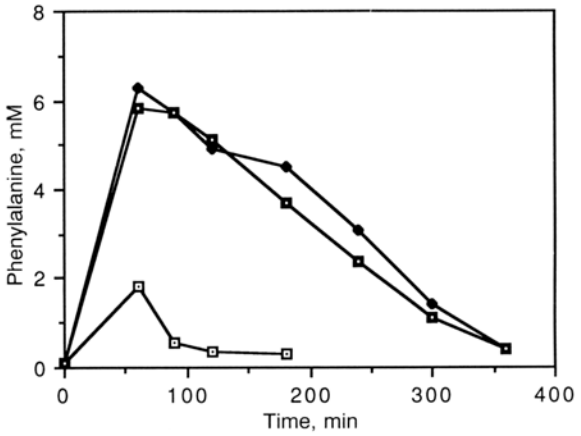

FIG. 1. Phenylalanine challenge with tetrahydrobiopterin (BH$_4$) supplementation. ⊡, Wild-type mouse, with or without BH$_4$ (average of two animals); ◆, homozygous mutant, without BH$_4$ (average of three animals); ■, homozygous mutant, with BH$_4$ (average of two animals).

have been confirmed (data not shown) with PAH determinations based on tyrosine production (26). In contrast, liver q-DHPR activity (see *Materials and Methods*) in homozygotes was comparable to wild type: *hph-5*/*hph-5* scored 5.4 ± 0.3 Δ OD$_{550}$ per min/mg of total protein, whereas wild type scored 6.1 ± 0.4 Δ OD$_{550}$ per min/μg of total protein. Further, the ability of the mutant to clear a phenylalanine challenge is not enhanced by tetrahydrobiopterin supplementation (Fig. 1). Therefore, we conclude that the lack of PAH activity in the mutant is not caused by an inhibitor of PAH, nor is there a deficiency in q-DHPR or tetrahydrobiopterin.

hph-5, although induced in the inbred BTBR/Pas mouse strain, was identified in hybrid animals. The mutant phenotype is fully penetrant and is transmitted as a single autosomal recessive trait (Table 2). *hph-5* has been genetically mapped by using an interspecific cross (as described in ref. 27): complete concordance was observed between *hph-5* and a *Pah*-linked restriction site polymorphism (Table 3 and Fig. 2). We conclude that *hph-5* is located at or near the *Pah* locus on chromosome 10. A mapping cross between inbred mouse lines demonstrated tight linkage between *hph-5* and Steel (*Sl*), a dominant visible mutation (28) on chromosome 10 (Table 3). These results are in accord with previous demonstrations (15, 29) that *Pah* maps to mouse chromosome 10.

DISCUSSION

Based upon the concordance between the biochemical finding of PAH deficiency and the genetic cosegregation of *Pah* and *hph-5*, we have concluded that *hph-5* is an allele of *Pah* and have renamed the mutation *Pah*$^{hph\text{-}5}$.

Table 2. Inheritance of *hph-5*

Cross	Total progeny	Phenotype: HPH Exp.	HPH Obs.	Normal Exp.	Normal Obs.
hph-5/+ × *hph-5*/*hph-5*	115 neonates	57	61	58	54
hph-5/+ × *hph-5*/*hph-5*	208 adults	104	103	104	105
hph-5/+ × *hph-5*/+	20 adults	5	7	15	13

Genetic crosses were carried out in each parental orientation. The HPH phenotype of progeny was determined by measuring blood phenylalanine levels by the Guthrie assay (34). In neonates this determination was made without previous phenylalanine exposure; in adults, the determination was made 3.5 hr after phenylalanine challenge with 1 mg of aqueous phenylalanine per g of body weight injected intraperitoneally. Animals with blood phenylalanine ≤ 0.24 mM were classified as normal, and those with elevated phenylalanine (>0.24 mM) were classed as HPH (typically HPH animals were >0.5 mM). Expected values were calculated by assuming single-locus autosomal recessive inheritance.