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A Murine Model for Type III Tyrosinemia: Lack of Immunologically Detectable 4-Hydroxyphenylpyruvic Acid Dioxygenase Enzyme Protein in a Novel Mouse Strain with Hypertyrosinemia

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Summary

We have characterized a new mutant strain of mouse that has hypertyrosinemia. The blood tyrosine level was persistently high, and increased amounts of 4-hydroxyphenylpyruvic acid and its derivatives were excreted into the urine. Succinylacetone was not detected in urine samples from these mice. All the animals were apparently healthy, and there was no evidence of hepatorenal dysfunction. The hypertyrosinemia was transmitted through an autosomal recessive inheritance. Analyses of hepatic enzymes related to tyrosine metabolism revealed that 4-hydroxyphenylpyruvic acid dioxygenase activity was virtually absent, while fumarylacetoacetase and tyrosine aminotransferases (cytosolic and mitochondrial forms) were normal in these mutant mice. Immunoblot analysis of 4-hydroxyphenylpyruvic acid dioxygenase protein in the liver indicated that the subunit protein of the enzyme was absent. It would appear that hypertyrosinemia in this mutant strain was caused by a genetic defect in 4-hydroxyphenylpyruvic acid dioxygenase. These features are similar to type III tyrosinemia in humans. Analysis of this mutant strain of mouse is expected to provide valuable information on the pathogenesis of human type III tyrosinemia and can also serve as a useful system for studies on tyrosine metabolism.

Introduction

4-Hydroxyphenylpyruvate dioxygenase (E.C.1.13.11.24), an enzyme that participates in the catabolism of tyrosine in most organisms, is present in the liver and kidney of mammals. This enzyme catalyzes the formation of homogentisic acid from 4-hydroxyphenylpyruvic acid, and it has been purified from pig liver (Roche et al. 1982), human liver (Lindblad et al. 1977), and avian liver (Wada et al. 1975). The properties of the enzyme have been extensively characterized (Buckthal et al. 1987; Fellman 1987; Lindstedt and Odelhog 1987).

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The enzyme activity is low or absent in type I tyrosinemia (McKusick 27670) and in type III tyrosinemia (McKusick 27671). Recent studies have indicated that the type I disease is caused by a genetic deficiency of fumarylacetoacetase (Berger et al. 1981), an enzyme which catalyzes the hydrolysis of fumarylacetoacetic acid into fumaric and acetoacetic acids. Type III disease, described by Endo et al. (1983), is characterized by a deficiency of 4-hydroxyphenylpyruvic acid digoxygenase with normal fumarylacetoacetase. Mild mental retardation and mild hypertyrosinemia are the apparent biochemical and clinical features of the disease. A few cases in the literature are considered to be linked to a similar genetic defect (Goldsmith and Laberge 1989).

Animal models for human disease facilitate examinations of disease processes and provide the way for evaluating therapeutic strategies. We describe here our initial biochemical analyses of a novel mouse strain

with hypertyrosinemia. This mutant mouse strain has a CRM (immunologically cross-reacting material)-negative mutation of 4-hydroxyphenylpyruvic acid dioxygenase.

Material and Methods

Animals

All animals used in the present study were from The Central Institute for Experimental Animals (Kawasaki, Japan). The mutant strain, strain III, and a control strain, strain IST, were derived from strain ICR mice and were maintained through brother-sister mating by H.K. From the time of weaning, the mice were fed a standard chow diet. The protein intake of these mice was 50–100 g/kg/d.

Amino Acid and Organic Acid Analyses

Serum amino acids levels were determined by automated amino acid analyzer. Fifteen mice (eight males and seven females) of different ages (1 mo, 3 mo, and 12 mo) were used for amino acid analysis. Six strain III mice (4 mo old) and six strain IST mice (4 mo old) were used for enzymic analysis. Organic acids in the urine were analyzed by gas chromatography–mass spectrometry (Tanaka et al. 1980) using a silica capillary column (DB-17, 0.25 mm × 30 m; J&B Scientific, CA).

Enzyme Assay

Livers were homogenized in 50 mM ice-cold potassium phosphate buffer, pH 7.4, and were centrifuged at 10,000 *g* for 20 min. The supernatants were used for enzyme assay. The activity of fumarylacetoacetase was measured according to a method described, elsewhere (Edwards and Knox 1955), with slight modifications (Endo et al. 1983). Fumarylacetoacetase was prepared and stored according to a method described elsewhere (Endo et al. 1983). Tyrosine aminotransferase was measured by the method of Diamondstone (1966). Cytosolic and mitochondrial forms of tyrosine aminotransferases were separated by DEAE column chromatography (Endo et al. 1983). The radiochemical assay of 4-hydroxyphenylpyruvic acid was carried out by the method of Lindblad (1971), with slight modifications (Endo et al. 1983). Proteins were measured by the dye-binding method using a kit from BioRad. [1-^{14}C]-tyrosine was purchased from New England Nuclear.

Purification of 4-Hydroxyphenylpyruvic Acid Dioxygenase and Preparation of Antibody

Purification of 4-hydroxyphenylpyruvic acid dioxygenase from pig liver was carried out according to the method of Buckthal et al. (1987), through step 4. DEAE-cellulose chromatography (step 5) and Sephacryl S-200 chromatography (step 6) were replaced by Mono Q column (Pharmacia) and Superose column (Pharmacia), respectively. 4-Hydroxyphenylpyruvic acid dioxygenase activity was eluted from the Mono Q column as three peaks of isozymes, as described for human liver enzyme (Lindstedt and Odelhog 1987). The enzyme in the first peak was further purified on a Superose column. The final preparation was analyzed by SDS-PAGE by the method of Laemmli (1970). A protein of M_r 43,000 was detected.

The purified protein was used to immunize rabbits, to obtain antiserum. Specific IgG was isolated on an immunoaffinity column in which 10 mg of purified protein was immobilized on 5 ml of Sepharose 4B (Pharmacia). Coupling of the protein to cyanogen bromide–activated Sepharose 4B was carried out according to the manufacturer's instructions.

Serum (100 ml) was incubated with the gel suspension for 2 h at 4°C, and then the gel was packed onto a column, after which it was washed with 50 mM Tris-HCl buffer, pH 7.4, containing 1 M NaCl. The IgG was eluted with 0.1 M Na_2CO_3, and the elute was immediately neutralized with 1 M Tris-HCl buffer, pH 7.0. The purified IgG was stored at 4°C.

Immunoblot Analysis of pHPP Dioxygenase

The subunit protein of 4-hydroxyphenylpyruvic acid dioxygenase was analyzed by immunoblot, after SDS-PAGE. An aliquot of the crude extracts of the liver (100 μg protein) was subjected to SDS-PAGE using a 10% acrylamide gel. The proteins were then transferred to a nitrocellulose filter (Towbin et al. 1979). Immunological staining of protein was carried out according to a method described elsewhere (Endo et al. 1990). The purified IgG was used as the first antibody, at a concentration of 5 μg/ml.

Alternatively, 4-hydroxyphenylpyruvic acid dioxygenase in the crude extracts was partially purified, prior to SDS-PAGE. In this experiment, an aliquot of the crude extracts (~1 mg protein) was incubated with 3 μg of purified IgG at room temperature for 1 h, and then the immune complex was precipitated with protein A agarose. The agarose gel was treated according to a method described elsewhere (Endo et al.

Table 1

Blood Tyrosine Levels (μmol/liter) in Mouse Mutant Strain III

	Blood Tyrosine Level at Age of		
	1 mo	3 mo	12 mo
Male	868	814	1,098
	995	760	1,096
	1,052	728	
Female.....	717	732	891
	758	677	1,116
	978		

NOTE.—Tyrosine levels in strain IST mice were 51–150 (mean 82) μmol/liter, at all ages.

1990) and then was subjected to SDS-PAGE and immunoblotting.

Results

Screening of blood samples for amino acids revealed elevated levels of tryrosine in mouse strain III (Katoh 1989), one of the colonies maintained in The Central Institute for Experimental Animals (Kawasaki, Japan). The hypertyrosinemia in this strain became evident shortly after birth and persisted throughout the life span (table 1). The phenotype of hypertyrosinemia in the mutant strain was considered to be transmitted through autosomal recessive inheritance. The mean ± SD serum phenylalanine levels in the tyrosinemic mice (age 3–12 mo) were not high (89.9 ± 26.6 μmole/liter).

The chromatographic profile of the urine obtained from these mutant mice is shown in figure 1. Large peaks of 4-hydroxyphenylpyruvic acid, 4-hydroxyphenylacetic acid, and 4-hydroxyphenyllactic acid appeared. Excretion of these substances was markedly increased, compared with findings in the control urine (table 2). Succinylacetone and delta-aminolevulinic acid were not detected.

Hepatic enzymes related to tyrosine metabolism were analyzed to determine the cause of hypertyrosinemia in strain III mice; results are summarized in table 3. The activity of 4-hydroxyphenylpyruvic acid dioxygenase in the livers from strain III mice was virtually absent, and the other enzymes were apparently normal. We also investigated the 4-hydroxyphenylpyruvic acid dioxygenase protein by using immune blots.

A homogeneous preparation of purified pig liver

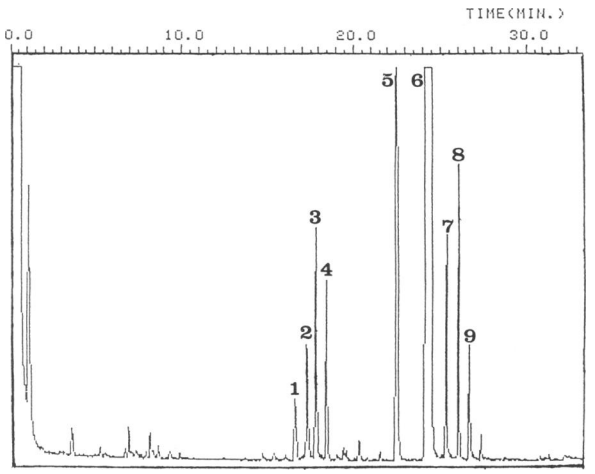


Figure 1 Gas chromatographic analysis of urine samples from tyrosinemic mice. The urine samples from five male mice with an inherited tyrosinemia were analyzed for organic acids. 1 = 2-ketoglutaric acid; 2 = unknown peak derived from 4-hydroxyphenylpyruvic acid; 3 = 4-hydroxyphenylacetic acid; 4 = phenylpyruvic acid; 5 = 4-hydroxyphenyllactic acid; 6 = 4-hydroxyphenylpyruvic acid; 7 = hippuric acid; 8 = internal standard; 9 = phenylacetylglycine. Succinylacetone and delta-aminolevulinic acid were not detected.

4-hydroxyphenylpyruvic acid dioxygenase contained a subunit protein with a M_r 43,000, which was similar to findings in the literature (Buckthal et al. 1987). Antiserum developed against the purified protein gave a single line on Ouchterony double immunodiffusion (not shown). Purified IgG from the antiserum was used for immunoblot analysis (fig. 2*A*). When the purified enzyme was analyzed by SDS-PAGE and immune blot analysis, a single polypeptide of M_r 43,000 was specifically stained (fig. 2*A*, lane 1). Similarly, crude extracts of the liver from mouse stain III (tyrosinemic) and control mouse strain IST were analyzed (fig. 2*A*,

Table 2

Urinary Excretion of Tyrosl Compounds (in μmol/liter) Tyrosinemic Mice (Strain III) and Control Mice (Strain IST)

	Strain III	Strain IST[a]
4-Hydroxyphenylpyruvic acid.....	330.65	1.78
4-Hydroxyphenyllactic acid.....	10.07	.04
4-Hydroxyphenylacetic acid	1.93	.06

NOTE.—For the organic acid analysis, urine samples were collected from five male mice.

[a] Those with normal blood tyrosine levels were fed the same diet.

Table 3

Hepatic Enzymes Related to Tyrosine Metabolism in Tyrosinemic Mice (Strain III) and Control Mice (Strain IST)

	Strain III	Strain IST
Fumarylacetoracetase (nmol/min/mg protein):		
Female	7.23 ($N=2$)	7.33 ($N=2$)
Male	7.15 ($N=2$)	6.91 ($N=2$)
Cytosolic tyrosine aminotransferase (μmol/min/g tissue):		
Female	1.31 ($N=2$)	1.51 ($N=2$)
Male	1.38 ($N=2$)	1.49 ($N=2$)
Mitochondrial tyrosine aminotransferase (μmol/min/g tissue):		
Female	.52 ($N=2$)	.30 ($N=2$)
Male	.34 ($N=2$)	.44 ($N=2$)
4-Hydroxyphenylpyruvic acid dioxygenase (μmol/h/g tissue):		
Female	<.2 ($N=3$)	26.4 ($N=3$)
Male	<.2 ($N=3$)	41.2 ($N=3$)

lanes 2–5). Although nonspecifically stained proteins were visualized, a protein band with M_r 43,000 appeared when the extracts from the control mice were analyzed (fig. 2*A*, lanes 4 and 5). On the other hand, a corresponding protein was not visualized when the extracts from the mutant mice were analyzed (figs 2*A*, lanes 2 and 3).

To confirm these results, we partially purified the enzyme protein by immunoprecipitation, and the fractions were analyzed by SDS-PAGE and immune blots (fig. 2*B*). This system of immunoprecipitation was able to concentrate ≤2 μg of purified protein, under the conditions described. Similarly, the crude extracts from the IST mice (fig. 2*B*, lanes 2 and 4) and the strain III mice (fig. 2*B*, lane 7 and 9) were analyzed. Again, a protein band corresponding to the subunit of 4-hydroxyphenylpyruvic acid dioxygenase was absent in the crude extracts of the liver from the strain III mice.

Discussion

Elevated levels of blood tyrosine and increased amounts of urinary 4-hydroxyphenylpyruvic acid and its derivatives are common features among hereditary tyrosinemias (Goldsmith and Laberge 1989). Type I disease is characterized by liver and renal tubular dysfunctions and is caused by a genetic deficiency of fumarylacetoacetase (Berger et al. 1981; Kvittingen et al. 1981), the enzyme which catalyzes the hydrolysis of fumarylacetoacetic acid into fumaric and acetoacetic acids. Type II disease is associated with eye and skin lesions and is caused by a genetic deficiency of cytosolic tyrosine aminotransferase (Fellman et al. 1969). 4-Hydroxyphenylpyruvic acid dioxygenase activity would seem to be functionally defective in type I disease (La Du 1967). The precise mechanism which led to the secondary deficiency of the enzyme activity has not been elucidated. A classical patient of Medes (1932) may represent another distinctive entity of tyrosinemia, since the clinical features of the patient differ from those of type I and type II disease.

We reported familial cases of tyrosinemia that had atypical clinical courses (Endo et al. 1983). The proband was an infant who was a product of brother-sister mating. The infant and the mother showed elevated levels of blood tyrosine, and increased amounts of tyrosyl compounds were excreted into the urine. Detailed analysis of hepatic enzymes from the infant revealed that the infant had isolated 4-hydroxyphenylpyruvic acid dioxygenase deficiency with normal fumarylacetoacetase. The activity of fumarylacetoacetase in the peripheral leukocytes from the parents was normal. It was proposed that a primary 4-hydroxyphenylpyruvic acid dioxygenase deficiency in humans is characterized by moderate hypertyrosinemia without hepatorenal dysfunctions. Mild mental retardation was found in the mother, suggesting it might be a symptom of the disease. The isolated 4-hydroxyphenylpyruvic dioxygenase deficiency was later classified as type III tyrosinemia (McKusick 1986).

An enzymic diagnosis of type III tyrosinemia requires analyses of hepatic tyrosine aminotransferases, 4-hydroxyphenylpyruvic acid dixoygenase, and fumarylacetoacetase. There are case reports describing tyrosinemic patients who have similar biochemical

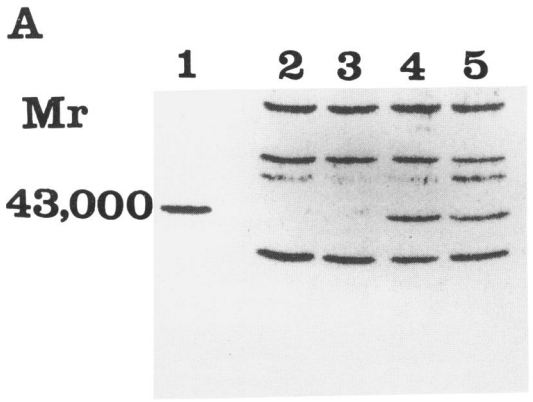
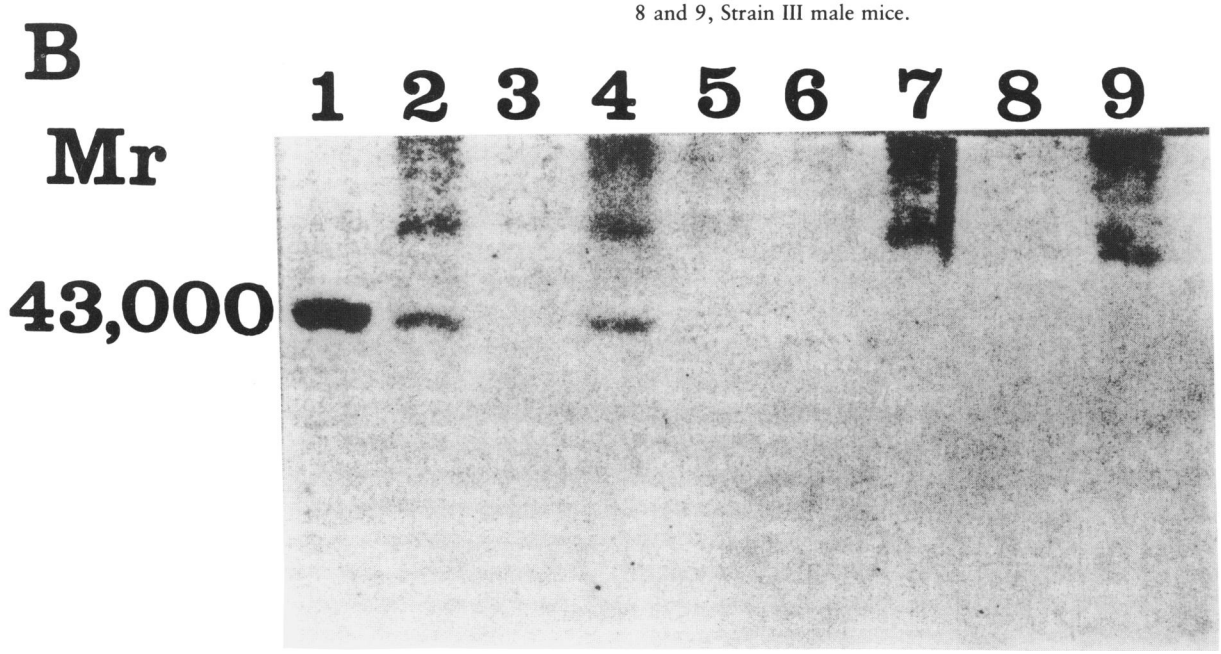


Figure 2 Immune blot analysis of 4-hydroxyphenylpyruvic acid dioxygenase. *A*, Immune blot analysis of crude extracts of liver. Purified enzyme from pig liver (300 ng; lane 1) and crude extracts of liver from strain III female mouse (lane 2), strain III male mouse (lane 3), strain IST control female mouse (lane 4), and strain IST male mouse (lane 5) were analyzed by SDS-PAGE and immune blot analysis, as described in Material and Methods. *B*, Immune blot analysis of partially purified 4-hydroxyphenylpyruvic acid dioxygenase. Crude extracts of liver (500 µl) were incubated with 3 µg of purified IgG (anti-4-hydroxyphenylpyruvic acid dioxygenase) for 2 h at room temperature; then the protein A agarose (1:1 suspension, 100 µl) was added. Incubation was continued for 1 h, after which the agarose gel was collected. Proteins absorbed on the protein A agarose were analyzed on SDS-PAGE and immunoblot. Lane 1, Purified enzyme. Lanes 2, 4, 7, and 9, Treatment with IgG and protein A agarose. Lanes 3, 5, 6, and 8, Treatment with protein A agarose. Lanes 2 and 3, Strain IST female mice. Lanes 4 and 5, Strain IST male mice. Lanes 6 and 7, Strain III female mice. Lanes 8 and 9, Strain III male mice.



features and some neurological symptoms (Gardini et al. 1983; Goldsmith and Laberge 1989). However, the enzymic studies on some of these cases were incomplete, and the case reports were too few to establish definitively the clinical and biochemical features of type III tyrosinemia.

We analyzed a novel strain of hypertyrosinemic mice and noted that the clinical features were compatible with findings in the case of the human type III disease (Endo et al. 1983). Enzymic analysis of the liver from these mice led to the diagnosis of type III tyrosinemia. Chemical analysis of the urine from these mice supported the diagnosis, in that there were increased amounts of tyrosyl compounds but no succinylacetone. In addition, the enzyme protein of 4-hydroxyphenylpyruvic acid dioxygenase was absent in the liver. On the basis of the data presented here, it appears that mouse strain III can serve as an appropriate model for human type III tyrosinemia. Enzyme deficiencies can have very different phenotypes in mouse and man (DeMars et al. 1976; Bulfied et al. 1984). Detailed pathologic and metabolic studies will be needed.

This novel mutant strain of mice with hereditary tyrosinemia can be used to gain insight into the role of 4-hydroxyphenylpyruvic acid dioxygenase in hereditary tyrosinemias. It seems likely that a deficiency in the enzyme activity alone will not cause hepatorenal

dysfunctions. Eye and skin lesions observed in the type II disease seem to be caused by high levels of blood tyrosine, not by 4-hydroxyphenylpyruvic acid and its derivatives. Impairment of the catabolism of 4-hydroxyphenylpyruvic acid is not a life-threatening condition.

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