Animal Models Reveal Pathophysiologies of Tyrosinemas1,2

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ABSTRACT The activity of the enzyme 4-hydroxyphenylpyruvic acid dioxygenase (HPD) is regulated by transcription factors. Mutations in the HPD locus are related to two known distinct diseases: hereditary tyrosinemia type 3 and hawkinsinuria. HPD-deficient mice are a good model with which to examine the biological effects of 4-hydroxyphenylpyruvic acid, which is a keto acid that causes no apparent visceral damage. In contrast, hereditary tyrosinemia type 1, a genetic disease caused by a deficiency of fumarylacetoacetate hydrolase (FAH), induces severe visceral injuries. Mice with FAH deficiency are lethal after birth; thus, efforts to elucidate the mechanisms of the disease process have been impeded. The use of Fah−/−, Hpd−/− double-mutant mice has enabled studies on tyrosinemas, and essential features of visceral injury have been revealed. J. Nutr. 133: 2063S–2067S, 2003.

KEY WORDS: • tyrosinemia • apoptosis • animal model mice • hereditary tyrosinemia

Several disorders are related to the tyrosine catabolic pathway (1), and three such diseases manifest hyper tyrosinemia [hereditary tyrosinemia (HT) types 1, 2 and 3; see Fig. 1]. However, the clinical features of these disorders differ apparently due to the influence of metabolites in cells and body fluids. The use of animal models to investigate these diseases is important. We discuss herein genetic models for deficiencies of 4-hydroxyphenylpyruvic acid dioxygenase [HPD2, to study HT type 3 (HT3)] and fumarylacetoacetate hydrolase [FAH, to investigate HT type 1 (HT1)].

The HPD enzyme

The enzyme HPD participates in the oxidation of keto acids of tyrosine. Homogentisate (HGA) is produced from 4-hydroxyphenylpyruvate by this enzyme, and the reaction involves decarboxylation, oxidation and rearrangement. In mammals, HPD activity has been detected mostly in liver with small amounts in kidney (2). Purification studies suggest that the human enzyme is a homodimer of identical subunits with a Mr of 43,000. The enzyme contains iron, and part of the enzymic activity is restored by the addition of Fe2+. We cloned cDNA for human, porcine and mouse HPD and determined the complete amino acid sequences of the enzymes. The subunit of the human enzyme is composed of 392 amino acid residues and the mature human enzyme is a homodimer of identical subunits with a Mr of 43,000 (3).

The gene for human HPD is separated into 14 exons with between 27 (exon 2) and 313 (exon 14) bases and 13 introns that range in size from 88 bases (intron 13) to 3.2 kb or longer (intron 7) (4). Using a full-length cDNA as a probe, Southern blot analysis of high-molecular-weight DNA from peripheral blood samples of healthy individuals suggested that the human HPD gene spanned ~30–40 kb. The nucleotide sequence of the 5′ flanking region of the human HPD gene contains a TATA element (TTAAATA), and there are sequences that resemble binding sites for several liver-specific or liver-enriched transcription factors including hepatocyte nuclear factors (HNF)-1 and -4, CCAAT/enhancer binding protein (C/EBP) and PPAR (4).

Transient tyrosinemia is a condition that is commonly seen in the newborn. Activity of the enzyme HPD is undetectable during early fetal life and increases at the late stage of gestation (2). Transient tyrosinemia in the newborn is likely to be due to a delay in a rapid increase in the enzyme activity in the liver during the perinatal period. Transcription factors such as HNF-1 and -4 and C/EBP are possibly related to the developmental delay of HPD gene expression (4). Excretion of tyrosyl compounds into the urine can be increased in patients with various pathological conditions of the liver. Reduced expression of the
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HPD gene is a central feature of the derangement of tyrosine metabolism under these conditions.

**Genetic deficiency of HPD**

Mutations in the HPD locus can cause two distinct genetic diseases, HT3 and hawkinsinuria, and HPD activity is markedly reduced in the liver of patients with HT1.

HT3 is characterized by elevated blood concentrations of tyrosine and massive urinary excretion of 4-hydroxyphenylpyruvic acid, 4-hydroxyphenyllactic acid and 4-hydroxyphenylacetic acid. In our study, we used mice with deficiency of HPD and mice with deficiency of fumarylacetate oxidase (FAO) hydroxase (FAH). The double-mutant (Fa hpd) mice carry metabolic blocks at the steps of HPD and FAH.

HPD gene is a central feature of the derangement of tyrosine metabolism under these conditions.

**Genetic deficiency of FAH**

HT1 is caused by a genetic deficiency of FAH, which is the last enzyme in the catabolic pathway of tyrosine. The complete amino acid sequence of FAH and the structure and organization of the gene for human FAH, located at region q23-q25 of chromosome 15, have been reported. HT1 patients have severe phenotypes that are characteristic of progressive liver failure during infancy, renal tubular dysfunction and early development of hepatocellular carcinomas.

The clinical course of this severe disease is modified by HPD activity in the liver. In patients with a slowly progressive disease, HPD activities are more reduced than in patients with severe and acute types of the disease. This suggests that reduced activities of HPD may result in a better prognosis. Based on these investigations, administration of N-trifluoromethylacetone (NTBC, a potent inhibitor of HPD) has been prescribed for patients with FAH deficiency and has shown clinical benefits. Recent experience in many countries indicates that NTBC is very effective in protecting against progression of liver disease in HT1 patients. These clinical investigations suggest that metabolites derived from fumarylacetate oxidase (FAO) or FAA itself are toxic to hepatocytes and renal tubular epithelial cells. Mechanisms for development of cellular injury have been investigated in animal models.

Mice homozygous for albino deletions (the c14cos mice) die within several hours of birth. The deletion in c14cos mice disrupts the Fa hpd gene and results in the absence of exon 1 and 2 sequences (19,20). The homozygous mice are characterized by impairment of expression of hepatocyte-specific genes in the liver during perinatal periods. The promoter regions of these genes contain binding sites for liver-specific and liver-enriched transcription factors that include HNF-1 and -4 and C/EBP. These transcription factors are reduced in the liver of homozygous albino-lathyric mice, and reduction in these factors is attributable to the low expression of hepatocyte-specific genes.

Grompe et al. (23,24) generated FAH mutant mice by gene targeting. These mice died with hypoglycemia within 12 h of birth. In addition, the same pattern of altered liver mRNA expression that is found in albino-deletion mutant mice was observed in the affected animals (23,24).

**Experiments with double-mutant mice**

Because FAH-deficient mice are lethal after birth, there are limits to the use of these animals for studies of the mechanisms...
of the liver disease in HT1. To overcome these problems, we developed a mouse model that carries defects in *Fah* and *Hpd* genes (25). The double-mutant (*Fah<sup>+/−</sup> Hpd<sup>−/−</sup>) mice are viable and the clinical and biochemical phenotypes are indistinguishable from those of strain III mice (*Fah<sup>+/−</sup> Hpd<sup>+/−</sup>). The livers of *Fah<sup>−/−</sup> Hpd<sup>−/−</sup> mice were normal and similar to those from *Fah<sup>+/−</sup> Hpd<sup>−/−</sup> mice and strain III mice. Long-term investigations of the *Fah<sup>−/−</sup> Hpd<sup>−/−</sup> mice (up to 18 mo) revealed no evidence of hepatocellular carcinomas or preneoplastic lesions (25).

Sensitivity of hepatocytes from the double-mutant mice to HGA, an intermediate metabolite of the tyrosine catabolic pathway (Fig. 1), was investigated using cultured cells. Primary cultures of hepatocytes were prepared from strain III and *Fah<sup>−/−</sup> Hpd<sup>−/−</sup>* mice, and after plating, HGA was added to the culture medium. Alternatively, the cultured cells were transfected with recombinant adenovirus that expressed human HPD to reopen the tyrosine catabolic pathway at the HPD step. These manipulations demonstrate the time-dependent progress of apoptotic death of cultured cells from the *Fah<sup>−/−</sup> Hpd<sup>−/−</sup>* mice (24). Fragmentation of genomic DNA from the cultured hepatocytes of *Fah<sup>−/−</sup> Hpd<sup>−/−</sup>* mice was evident 6 h after the addition of HGA to the medium. Most of the cells from liver of double-mutant mice were positive for the apoptosis signal as detected by a terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick-end labeling (TUNEL) assay after treatment with HGA (Fig. 2A).

Similarly, in vivo administration of recombinant adenovirus that expressed human HPD or HGA into the *Fah<sup>−/−</sup> Hpd<sup>−/−</sup>* double-mutant mice caused acute liver failure. When the liver was examined, most of hepatocytes were abnormal with fragmentation of nuclei, and ~30–80% yielded positive signals by in situ detection of DNA fragmentation (25–27). DNA samples obtained from the HGA-treated double-mutant mice showed fragmentation on agarose gel electrophoresis (Fig. 2B). Thus, in the absence of FAH activity, hepatocytes are vulnerable to HGA. Loss of HPD is a pivotal factor in preventing apoptosis of the cells. These results revealed the presence of toxic metabolites derived from HGA that cause a rapid apoptosis of hepatocytes. Similar apoptosis was observed in proximal renal tubular epithelial cells (28,29).

To investigate the in vitro protective effects of apoptosis inhibitors, newly isolated hepatocytes were incubated for 1 h with YVAD or DEVD (inhibitors for caspase; TaKaRa, Tokyo, Japan) at various concentrations and were then treated with 1 mM HGA. The caspase inhibitors effectively prevented apoptosis of hepatocytes from the *Fah<sup>−/−</sup> Hpd<sup>−/−</sup>* double mutants in vitro and in vivo (Table 1).

Cytochrome c is a key molecule that plays a pivotal role in the process of apoptosis. In experiments using cultured hepatocytes from *Fah<sup>−/−</sup> Hpd<sup>−/−</sup>* double-mutant mice, cytochrome c was released from mitochondria after the addition of HGA and before the appearance of fragmented DNA (26). In another experiment, mitochondria (1 mg) from the control mouse were incubated in the presence of purified FAA. In this cell-free system, purified FAA reacted with the mitochondria, and cytochrome c was released. In addition, accumulation of FAA in the soluble fraction of liver was confirmed by HPLC.

### Table 1

<table>
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<tr>
<th>Caspase inhibitor μg</th>
<th>0</th>
<th>0</th>
<th>10</th>
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<tr>
<td>Homogenisate mg</td>
<td>0</td>
<td>20</td>
<td>20</td>
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<tr>
<th>Aspartate aminotransferase (IU/L)</th>
<th>89.2 ± 11.5</th>
<th>2,509.9 ± 3,116.1</th>
<th>793.4 ± 467.4</th>
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<tr>
<td>Alanine aminotransferase (IU/L)</td>
<td>28.8 ± 9.5</td>
<td>1,487.3 ± 2,691.0</td>
<td>505.6 ± 397.1</td>
</tr>
<tr>
<td>Succinylacetone&lt;sup&gt;2&lt;/sup&gt; (nmol/mmol Cr)</td>
<td>150</td>
<td>205,000</td>
<td>14,300</td>
</tr>
<tr>
<td>Apoptosis of hepatocytes (%)</td>
<td>&lt;1</td>
<td>20–80</td>
<td>2–4</td>
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<sup>1</sup> HGA-treated *Fah<sup>−/−</sup> Hpd<sup>−/−</sup>* mice showed extensive abnormalities in serum transaminases, whereas untreated mice had normal values. However, preadministration of the inhibitor significantly suppressed elevation of the aminotransferase.

<sup>2</sup> Succinylacetone in pooled urine.
analyses of liver extracts from HGA-treated *Fah<sup>−/−</sup> Hpd<sup>−/−</sup> double-mutant mice. These results suggest that the process of apoptosis was thus triggered. As described above, YVAD or DEVD prevented fragmentation of DNA; however, cytochrome c was released from the mitochondria in these animals (26).

Thus, investigations with *Fah<sup>−/−</sup> Hpd<sup>−/−</sup> mice provided important information concerning the disease process involving apoptosis of hepatocytes and renal tubular epithelial cells related to visceral injury in patients with HT1.

**Alterations of gene expression in liver of HGA-treated double-mutant mice**

We analyzed the altered expression of genes after the administration of HGA to the *Fah<sup>−/−</sup> Hpd<sup>−/−</sup> double-mutant mice by using gene chips. In this experiment, gene-expression patterns in liver of double-mutant and strain III mice (Hpd<sup>−/−</sup>) were compared. Total RNA was isolated from liver 24 h after the administration of HGA, and the RNA was pooled from three animals for each investigation. RNA sample integrity was assessed by running denaturing formaldehyde gels. The arrays were scanned using a Hewlett-Packard confocal laser scanner and were visualized using GeneChip 3.1 software (Affymetrix, Santa Clara, CA). The RNA of interest were analyzed using real-time quantitative reverse transcriptase–polymerase chain reaction. Genes, the expressions of which are markedly reduced, are listed in Table 2. Reduction in the expression of metabolic enzymes related to carbohydrate and fatty acids is prominent, and gene expression of coagulation factors is also notable. These results imply that in patients with HT1, abnormality of metabolism and blood coagulation factors are specific events related to the accumulation of FAA in hepatocytes, yet there is no evidence of liver damage. Ongoing investigations are expected to reveal changes in gene expression in hepatocytes that undergo the apoptosis triggered by FAA.

In summary, we used double-mutant (*Fah<sup>−/−</sup> Hpd<sup>−/−</sup>) mice to elucidate the mechanism of visceral injury in HT1. These investigations provided important information that is summarized as follows: 1) the complete block of the tyrosine catabolic pathway at the step of HPD activity prevents development of the visceral injury that is seen in HT1; 2) the hepatocytes and renal tubular epithelial cells undergo apoptosis when a toxic substance (FAA) from the tyrosine catabolic pathway is accumulated; and 3) caspase inhibitors prevent apoptosis of hepatocytes and renal tubular cells; however, they do not prevent release of cytochrome c.

We employed microarray techniques to investigate changes in gene expression during the development of visceral injury in double-mutant (*Fah<sup>−/−</sup> Hpd<sup>−/−</sup>) mice. Application of this technique is valuable for investigating the consequences of metabolic disturbance at the level of gene expression. Disease processes seen in various inborn errors of amino acid metabolism are often considered to be relatively simple events. However, the disease process may be more complex and microarray techniques may be useful in identifying and studying this complexity. Our approaches with animal models and microarray techniques should pave the way for the study of new aspects of inborn errors of amino acid metabolism.

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**LITERATURE CITED**


