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Phenylketonuria and Its Variants

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ABSTRACT

The hepatic phenylalanine hydroxylase system is complex, consisting of at least two enzymes and two non-protein cofactors. In classical phenylketonuria (PKU) the affected component has been shown to be the enzyme, phenylalanine hydroxylase. Recently, several variant forms of PKU have been identified which are due to deficiencies of two of the other components of the hydroxylase system, dihydropteridine reductase and tetrahydrobiopterin. In these cases, the defects lead to symptoms which are more severe than in PKU.

Furthermore, since these two components are also required in the biosynthesis of the neurotransmitters, serotonin, dopamine and norepinephrine, treatment with a phenylalanine-restricted diet is not effective. Thus, it is important to distinguish PKU from one of its variant forms at as early an age as possible to institute alternate therapies. The methods for determining the affected component will be discussed.

Introduction

It has been known ever since Fölling's work in 1934 that there is a defect in phenylalanine metabolism in phenylketonuria (PKU). He showed not only that these patients excrete phenylpyruvate, but also that they have elevated levels of phenylalanine in their blood.[6]

In 1947, Jervis reported the results of a series of experiments that led to the correct identification of the affected reaction in PKU. He found that the administration of phenylalanine to normal animals, as well as humans, led to a prompt increase in tyrosine in the blood.[11] When, on the other hand, phenylalanine was given to PKU patients, there was no increase in tyrosine. Jervis concluded that the block lay between phenylalanine and tyrosine, i.e., in the hydroxylation of phenylalanine.

In 1953, Jervis presented direct evidence in favor of this hypothesis when he showed that a liver sample from a PKU patient could not convert phenylalanine to tyrosine *in vitro*.[12]

A more precise delineation of the molecular defect in PKU could not be made until the hepatic phenylalanine hydroxylase system was separated into its individual components. It was shown that the system is a complex one, consisting of several essential components, both protein and nonprotein in nature.[21,22]

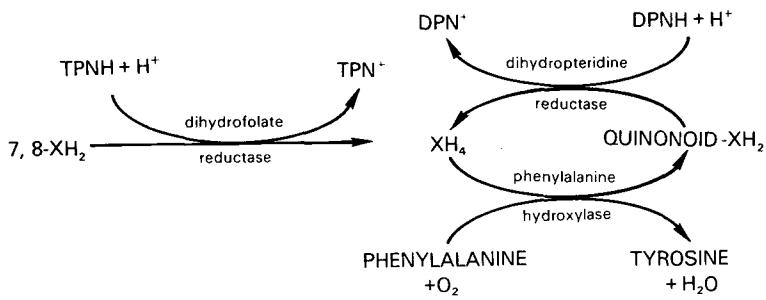

FIGURE 1. The reactions catalyzed by the hepatic phenylalanine hydroxylase system.

Reactions Catalyzed by Enzymes

The reactions catalyzed by the enzymes of the system are shown in figure 1, where XH_4 stands for the pterin cofactor, tetrahydrobiopterin[16] and XH_2 for its oxidation product, dihydrobiopterin. The hydroxylase catalyzes a coupled oxidation reaction in which phenylalanine is oxidized to tyrosine and the tetrahydrobiopterin is oxidized to the quinonoid dihydro derivative; molecular oxygen is the electron acceptor, being reduced to water. In addition to the naturally-occurring cofactor, certain synthetic analogues of tetrahydrobiopterin, such as 6,7-dimethyltetrahydropterin ($DMPH_4$) and 6-methyltetrahydropterin (6-MPH_4), show high cofactor activity in the hydroxylation system.[24]

In the presence of reduced pyridine nucleotide and another enzyme, dihydropteridine reductase, the quinonoid dihydrobiopterin is reduced back to the tetrahydro level. This latter reaction allows the cofactor to function catalytically. The quinonoid dihydropterin can also be reduced to the active, tetrahydro level non-enzymatically by high concentration of reductants such as mercaptans and ascorbate.[15]

The quinonoid dihydrobiopterin is an unstable compound which can rapidly rearrange to the 7,8-dihydro isomer.[21] The reduction of this isomer back to the tetrahydro level requires still another reduced pyridine nucleotide-dependent enzyme, dihydrofolate reductase.[17] An important difference between the behavior of tetrahydrobiopterin and the model cofactors such as $DMPH_4$ and 6-MPH_4 in the phenylalanine hydroxylase system is that the 7,8-derivatives of the latter compounds are not substrates for dihydrofolate reductase.[17] If the quinonoid dihydro derivatives of these synthetic pterins are not rapidly reduced to the tetrahydro level by dihydropteridine reductase, they will rearrange to their respective 7,8-dihydro compounds and will irreversibly lose their activity as hydroxylation cofactors.

Phenylalanine Hydroxylase in PKU

In classical PKU, it has been shown that the only component of the hydroxylase system that is affected is the hydroxylase.[14,28,31] Direct assays have shown that the pterin cofactor levels in liver samples from patients with classical PKU are not decreased.[14]

The conclusion that phenylalanine hydroxylase is the affected component in the classical form of PKU has been confirmed and further strengthened with immunological methods. A specific antiserum has been prepared in sheep, to pure rat liver phenylalanine hydroxylase.[9] The antiserum cross-reacts with normal human phenylalanine hydroxylase.[9] Under conditions where 5 to 10 percent of the normal human liver enzyme could be detected by immunodiffusion, no precipitin line was seen with an extract from a PKU liver sample.[8] These

results provided independent proof that the hydroxylase is the affected component in classical PKU.

Early studies on phenylalanine hydroxylase levels in PKU liver failed to detect any hydroxylase activity. More recently, the observation that certain phospholipids, such as lysolecithin, can markedly stimulate purified rat liver phenylalanine hydroxylase in the presence of tetrahydrobiopterin,[5] have led to the development of a more sensitive assay method for the enzyme. With this technique, very low hydroxylase levels (about 0.27 percent of normal) have been detected in a liver biopsy sample from a classical PKU patient.[7] Analysis of the properties of the hydroxylase in this patient supported the conclusion that this low hydroxylase activity was due to the presence of an unknown level of an altered form of the enzyme rather than to a low level of the normal enzyme.[17]

Using the same assay technique, Bartholomé et al[2] have detected 3 and 5 percent of normal activity in liver samples from two mentally retarded patients with PKU; no activity was found in 13 other patients with classical PKU. (As far as these negative results are concerned, it can be calculated that hydroxylase activities much below 1.0 percent of normal *could not* have been detected under their conditions of assay).

PKU Caused by Mutations

There is evidence that certain cases of mild or atypical PKU are caused by mutations that lead to a structural modification of phenylalanine hydroxylase that is different from the one seen in classical PKU. It was shown that a group of these patients have about 5 percent of the normal amount of phenylalanine hydroxylase.[13,25] There are indications that this low activity is due not to the presence of 5 percent of the normal enzyme but rather to the presence of an altered hydroxylase with kinetic properties that distinguish it from both the normal enzyme and probably from the enzyme in classical PKU. The main difference between it and the normal enzyme is in the K_m value for phenylalanine measured in the presence of the dimethyltetrahydropterin cofactor. In three different hyperphenylalaninemia patients, the K_m values were about 60 percent of those found for the enzyme from three different control patients.[8]

The resolution of the complex phenylalanine hydroxylase hydroxylating system into its separate components provided a logical basis for considering variants of PKU that might be caused by defects in one of the other essential enzymes or coenzymes of the system. In 1967, it was suggested that variants of PKU lacking the pterin cofactor or dihydropterin reductase were the most likely ones to be encountered.[18]

PKU Caused by Enzyme Defect

Although there were hints that atypical PKU could be caused by a defect in an enzyme other than in phenylalanine hydroxylase,[29] the first case of atypical PKU being caused by a defect in a component of the hydroxylase system other than the hydroxylase itself was reported by Kaufman et al.[23]

This child was diagnosed early in life as a classical phenylketonuric (serum levels of phenylalanine 53.3 mg per dl, abnormal phenylalanine tolerance test) and placed on a low phenylalanine diet during the third week of life. Despite excellent control of his serum phenylalanine levels, seizures and retardation became evident at seven months of age.

From the clinical symptoms, it was expected that this patient lacked a component of the hydroxylating system that is shared by the two brain pterin-dependent hydroxylases, tyrosine and tryptophan hydroxylases — either the

pterin cofactor or dihydropteridine reductase. It was anticipated that the symptoms caused by a lack of either of these components might be difficult to distinguish.

Under carefully standardized conditions of assay, (in the presence of $DMPH_4$ as the cofactor,[19] the phenylalanine hydroxylase activity measured on a biopsy liver sample was 15.2 μmoles of tyrosine formed per 60 minute per mg protein. This value is 20 percent of the average normal adult value. Unfortunately, no values for a group of age-matched controls are available.

For this reason, the physiological significance of this low hydroxylase value is difficult to assess. Even without this information, however, it is clear that the patient's hydroxylase activity is higher than that of the classical PKU patient or of hyperphenylalaninemics. As mentioned, under these conditions of assay, the average hydroxylase activity of this latter group is 5 percent of normal.[13,25] The K_m for phenylalanine of the patient's phenylalanine hydroxylase was determined by us and was found to be indistinguishable from that for the normal human liver hydroxylase.

Since the phenylalanine hydroxylase activity in the patient's liver was not low enough to account for his symptoms, the other components of the hydroxylase system were measured. With two independent assay procedures (direct and coupled assays), no dihydropteridine reductase activity was detected. In the coupled assay, an activity of less than 1 percent of normal activity could have been detected so that one can conclude that he has less than this activity. Mixing experiments, i.e., mixing extracts from the patient's liver and control liver extracts, failed to detect any inhibition by the patient's liver extract.

If hepatic dihydropteridine reductase is missing, it can be predicted that the level of tetrahydrobiopterin in this tissue should be lower than normal. This test, therefore, constitutes an independent assessment of the status of the reductase; moreover, it tells us about its status *in vivo*. It was found by us that in normal human liver, as well as in rat liver, the cofactor exists predominantly in the tetrahydro or fully active form. By contrast, in the patient's liver there is little or no detectable active cofactor. In addition, the total cofactor concentration may be lower, although values are not available for age-matched controls. Perhaps relevant to this last point is our previous finding that the newborn rat has only 60 percent of the adult amount of total hepatic cofactor.[3]

Screening Test

In order to devise a practical screening test for possible mutants in dihydropteridine reductase, a search for the enzyme was made in several other tissues that are more readily available than liver. No activity was detectable in normal lymphocytes, leukocytes or erythrocytes. The enzyme was detectable, however, in normal human fibroblasts, where it is present at levels of 20 to 30 percent of the specific activity of liver. Fibroblasts isolated from the patient's skin had no detectable reductase activity under conditions where 0.7 percent of the control activity could have been detected.

Since it would be important to know whether the patient's neurological symptoms are caused by an indirect effect on the brain of a metabolic lesion in his liver or by a direct effect on the brain caused by an enzyme defect in the brain, itself, it was of interest to try to assay for the reductase in brain tissue. Samples of frontal cortex were obtained from controls (both biopsy samples and autopsy samples) and a biopsy sample from the patient. With this tissue, no reductase activity was detectable (1 percent of the normal value could have been detected).

To consolidate further the conclusion that the reductase is the affected component, antibody to highly purified sheep liver dihydropteridine reductase was prepared in rabbits and tested by standard immunodiffusion methods against normal human liver reductase. A single precipitin line was found with extracts from control livers, but no detectable line was found with an equivalent amount of the extract from the patient's liver.[23] These results not only provide independent evidence that the reductase is affected in this patient's liver, but also show that there is no cross-reacting protein present in his liver that can form a precipitin line with the specific antiserum.

It seems plausible to conclude that the neurological deterioration in this patient is related to his lack of dihydropteridine reductase. As mentioned previously, lack of this enzyme in brain would be expected to lead to impairment in neurotransmitter synthesis. These abnormalities, in turn, could account for the neurological disorders of the patient.

Impairment of Dopamine Metabolism

Preliminary studies* support the conclusion that synthesis of certain neurotransmitters is defective in this patient. Thus, lower than normal levels of homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5HIAA) were found in cerebrospinal fluid (CSF) from the right ventricle (obtained at brain biopsy). These findings suggest a decreased turnover of dopamine and serotonin in the patient.

Added support for the conclusion that dopamine turnover is severely impaired was obtained with the probenicid technique.[30] Lumbar CSF was studied before and after an 18 hr period of oral probenicid in high dose (175 mg per kg). Egress of HVA and 5HIAA from CSF is prevented by this drug, and accumulation of these acidic metabolites is believed to reflect turnover of dopamine and serotonin, respectively, during this period of time. A marked impairment in HVA formation was apparent in the patient with an initial value of 26 μg per ml and an increase to 36 μg per ml after 18 hr. This increase is markedly lower than the 5 to 10-fold increase in normals noted by various investigators.[10]

It should be noted that the defect in dopamine metabolism in this patient, as reflected by HVA accumulation after probenicid, is much more severe than that observed with classical PKU patients, in whom, presumably, the hydroxylase is the affected component. Thus, McKean[27] has reported that there is a relatively mild decrease in the accumulation of HVA in CSF of PKU patients during a 24 hr period on probenicid and that this decrease correlates with plasma phenylalanine concentrations. Patients with high plasma phenylalanine (25 to 32 mg per dl) showed a 4.3 to 4.6-fold rise in HVA, whereas patients with low plasma phenylalanine (3 to 5 mg per dl) showed 5.6 to 6.4-fold rise in HVA.

A second case of hyperphenylalaninemia owing to dihydropteridine reductase deficiency has been reported.[4] This child, is also characterized by developmental delay and neurological dysfunction (with onset of seizures at five months of age), has normal hepatic phenylalanine hydroxylase activity. No dihydropteridine reductase was detected (2 percent of the normal value could have been detected). Results of assays of the components of the hydroxylating system in these two cases of dihydropteridine reductase deficiency are summarized in table I.

Approaches to Therapy

Several approaches to the therapy of hyperphenylalaninemia owing to dihyd-

* Carried out in collaboration with Drs. I. J. Butler, A. Krumholz, N. A. Holtzman and S. H. Koslow.

TABLE I

Levels of Components of the Phenylalanine Hydroxylase System in Human Liver

	Hyperphenylalaninemia owing to			
	I PAH deficiency (3-6)	*II DHPR deficiency (2)*	*III Cofactor deficiency (1)*	*IV Other (unknown) (1)*
Phenylalanine hydroxylase activity (PAH)	< 1% of normal	low to normal	normal	normal
Dihydropteridine reductase activity (DHPR)	normal	none detected	high	normal
Concentration of pterin cofactor				
total	normal	<normal	very low	normal
tetrahydro	normal (90-95% of total)	5-50% of normal	<5% of normal	90-95% of total
dihydro	normal (5-10% of total)	50-90% of total	95% of total	5-10% of total

Figure in parenthesis at the head of each column is the number of patients whose livers were analyzed.

ropteridine reductase deficiency have been discussed.[23] One of these, the administration of the products that are beyond the putative metabolic block, i.e., 5-hydroxytryptophan and 3,4-dihydroxyphenylalanine (DOPA), is currently being evaluated on both of these patients.

The first case of hyperphenylalaninemia owing to a lack of the pterin cofactor has recently been encountered (S. Kaufman, S. Milstien, J. Schulman, S. Berlow, unpublished). As with the two cases of hyperphenylalaninemia owing to dihydropteridine reductase deficiency, this child showed persistent developmental delay and neurological dysfunction (severe hypotonia and "spasticity" but no seizures) despite the fact that his hyperphenylalaninemia was controlled by dietary restriction of phenylalanine.

The individual components of the phenylalanine hydroxylase system were assayed on a liver biopsy sample when the patient was five years old. As summarized in table I (column III), the level of phenylalanine hydroxylase was normal, and his level of dihydropteridine reductase was high—normal. On the other hand, the level of pterin cofactor was less than 10 percent of normal. In addition, his urinary excretion of pterin cofactor was less than 10 percent of the amount excreted by adults.

On the basis of these results, it seems likely that this child's neurological dysfunction is due to a defect in his ability to synthesize dopamine, norepinephrine or serotonin, a defect caused by decreased activity of tyrosine and tryptophan hydroxylase, which in this case would be caused by a very low concentration of the pterin cofactor. It is not known whether the low levels of the cofactor are caused by a decreased rate of synthesis or an increased rate of catabolism. It is possible that the child with atypical PKU, reported by Leeming et al,[26] who has low serum levels of biopterin may have the same molecular defect as the patient described previously. A precise identification of the lesion in this child, however, must await the results of assays of the hepatic levels of the components of the hydroxylase system.

Recently, a variant form of PKU has been described in which the primary molecular defect has yet to be established.[1] Hyperphenylalaninemia in this child was detected at the age of three

weeks, at which time he was placed on a phenylalanine-restricted diet. In spite of control of his blood levels of phenylalanine, at 6.5 months he showed signs of neurological deterioration—increased muscle tone, constant myoclonal and oculomotor spasms. Urinary excretion of the metabolite of serotonin, 5HIAA, was near zero, whereas excretion of dopamine, epinephrine and norepinephrine was normal.

Components of the phenylalanine hydroxylase system were assayed on a liver biopsy sample obtained when the child was 13 months old. As can be seen in table I (Column IV), the levels of the hydroxylase, dihydropteridine reductase, and the pterin cofactor are all within the normal range. In addition, the activity of dihydrofolate reductase, and the phenylalanine hydroxylase stimulating protein[20] were normal.

To explain these results, it has been postulated by us that this child suffers from an abnormality which leads to the accumulation of a metabolite that inhibits phenylalanine hydroxylase as well as cerebral tryptophan hydroxylase and, perhaps, tyrosine hydroxylase. Alternatively, this child may suffer from a defect in biopterin metabolism that leads to the accumulation of a pterin derivative that is active *in vitro* under our assay conditions but is inactive *in vivo* as a cofactor for these three pterin-dependent hydroxylases.

Impaired activity of tryptophan hydroxylase is indicated by the decreased excretion of 5HIAA, whereas the Parkinson-like symptoms are suggestive of a defect in the activity of tyrosine hydroxylase. Because of these indications, the child was treated by administration of 5-hydroxytryptophan and DOPA. The results indicate that this treatment led to a marked improvement in the patient's neurological symptoms. Further studies will be required to identify the primary metabolic defect in this form of hyperphenylalaninemia.

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