Phenylketonuria: A new look at an old topic, advances in laboratory diagnosis, and therapeutic strategies

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ABSTRACT

Disorders of protein metabolism are the most common diseases among discovered inherited metabolic disorders. Phenylketonuria (PKU), a relatively common disorder that is responsive to treatment, is an inherited autosomal recessive disorder caused by a deficiency in phenylalanine hydroxylase (PAH) or one of several enzymes mediating biosynthesis or regeneration of the PAH cofactor tetrahydrobiopterin. The objective of this review is to discuss therapeutic strategies that have recently emerged for curing patients with PKU, which have demonstrated promising improvements in managing these patients. Data sourcing included a systematic literature review of PubMed with a focus on emerging knowledge pertaining to this well-studied disease. Recent advances in laboratory diagnosis and therapeutic strategies were described. Collectively, promising and rapid enhancements in neonatal diagnostic technologies and recently emerged therapeutic strategies are paving the way for early diagnosis and treating many inborn errors of metabolism, such as PKU.

Keywords: BH4, gene therapy, molecular diagnosis, phenylalanine hydroxylase, phenylketonuria

Introduction

Inborn errors of metabolism (IEM) involve a heterogeneous group of rare genetic disorders, which are commonly caused by mutant genes, resulting in abnormal proteins that interrupt a metabolic pathway, with clinical manifestations. Almost all known IEMs are inherited in an autosomal-recessive or X-linked-recessive manner. The protein expressed from a defective gene may cause partial deficiency in enzyme activity or a complete loss of function. Moreover, problems in most IEMs result from the toxic effects of accumulated substances, their interference with normal functions, or a reduced capacity for synthesizing essential compounds.

Conventionally, inherited metabolic diseases have been divided into 4 categories: Disorders of amino acid metabolism, carbohydrate metabolism, lysosomal storage, and organic acid metabolism. The classification of these diseases has been expanded to include more than 10 categories, based on the recent discovery of several inherited metabolic disorders. The most common diseases among these new categories are disorders of protein metabolism. Remarkably, phenylketonuria (PKU), a treatment-responsive and comparatively common disorder, is considered the most important disease among the known disorders of amino acid metabolism.

In this review, we discuss emerging knowledge on this well-studied disease and describe recent advances in therapeutic strategies.

Phenylalanine (Phe) Metabolism Requires Several Components

Characteristics of Phe and Tyrosine (Tyr)

Phe (C9H11NO2) is an essential α-amino acid with a nonpolar side chain. Like all other nonpolar amino acids, Phe is an electrically neutral amino acid that facilitates hydrophobic interactions. As shown in Figure 1, Phe is a precursor of Tyr, highlighting its importance in metabolic processes. In contrast to Phe, Tyr is a nonessential α-amino acid with a molecular formula of C9H11NO3. This proteinogenic amino acid is uncharged but has a polar side chain. Tyr is a critical amino acid owing to its various functions. For example, Tyr residues are often phosphorylated on the side chain hydroxyl group, which alters the activity of the target protein. Thus, Tyr phosphorylation is a key step in regulating enzymatic activity and signal transduction. Furthermore, the normal metabolism of Tyr results in its conversion into other compounds, as discussed.

Phenylalanine Hydroxylase (PAH)

Phe hydroxylation is catalyzed by PAH, which generates Tyr. PAH belongs to a class of monooxygenases named pterin-dependent amino acid hydroxylases; this class contains 3 members (PAH, tryptophan hydroxylase, and tyrosine hydroxylase) that utilize tetrahydrobiopterin (BH4, a pteridine cofactor) and a non-heme iron for catalysis.
The PAH gene

The human PAH gene (hPAH) is located on chromosome 12q22-12q24, as confirmed by mapping studies. Kwok et al. isolated full-length hPAH complementary DNA (cDNA) from a human liver cDNA library. In addition, digestion of the cDNA clone as a probe for full-length hPAH by several restriction enzymes showed that this gene is comprised of 13 exons and spans 90 kb. As shown in Figure 2, the entire PAH chromosomal sequence spans 171 kb, including its flanking regions. Within this sequence, approximately 92 kb comprises the un-translated regions (UTRs), with 27 kb in the 5′-UTR, and 65 kb in the 3′-UTR.

Structure and function of the PAH enzyme

Homotetrameric and homodimeric forms of eukaryotic PAH have been shown to exist in a pH-dependent equilibrium. Both forms are catalytically active, have different kinetics, and undergo different regulation. However, the 51.9-kDa PAH monomer protein (452 amino acid residues; National Center of Biotechnology Information Reference Sequence: NP_000268.1) contains 3 domains: An NH₂-terminal regulatory domain that represents approximately 26% of the total amino acid residues (117 residues); a catalytic domain that represents 69% of the entire molecule (310 residues); and a COOH-terminal domain containing 25 residues (<6%) responsible for oligomerization (Figure 2).

The NH₂-terminal domain of PAH has a βαββαβ topology comprised of an interlocking double-βαβ arrangement, which confers structural flexibility and facilitates the regulatory nature of the domain. Structure-function studies of the human PAH have shown that the activity of the entire enzyme can be affected by alterations in hydrophobic interactions in the NH₂-terminal domain. Interestingly, the auto regulatory sequence of the NH₂-domain (residues 19-33) extends across the catalytic domain at the active site although the regulatory domain is located far from the active site pocket of the catalytic domain. Data from several studies have shown that the conformation of PAH is altered globally by Phe allosteric binding and that a 2-fold increase in Phe affinity, along with elimination of the lag time, is induced by removal of the PAH NH₂-terminus. The serine (Ser) residue at position 16 (Ser16) within the PAH NH₂-terminal residues also regulates the enzyme. The Phe concentration required for allosteric activation was reduced by post-transcriptional Ser16 phosphorylation by cyclic adenosine monophosphate-dependent protein kinase. Furthermore, data from an in vitro study of human PAH have shown that the basal activity of
PAH and its affinity for the substrate Phe were increased by substitution of Ser16 with a polar amino acid having an acidic side chain, such as glutamic acid (Glu) or, to some extent, aspartic acid.18 The NH₂-terminus is attached to the catalytic domain through a hinge region (residues 111-117).19 Analysis of the crystal structure of the PAH catalytic domain showed that this region consists of a basket-like structure formed by 13 α-helices and 8 β-strands. The active site within this structure contains a pocket lined by 34 amino acid residues and is situated at the center. Six of these 34 residues are polar, including 3 Glu residues, 2 histidine residues, and 1 Tyr residue; however, most residues are nonpolar (hydrophobic). Pterin- and iron-binding residues are also contained in this region.11,20 The COOH-terminal domain is responsible for oligomerization of PAH monomers.13

With respect to its expression and activity, PAH is distinct among the aromatic amino acid hydroxylases. The central nervous system is the principal site of tryptophan (Trp) and Tyr hydroxylase expression, whereas the liver and kidney are major sites of PAH expression.21 PAH catalyzes catabolic processes, while Trp and Tyr hydroxylases catalyze rate-limiting steps in neurotransmitter and hormone biosynthesis.19

Several biological functions are performed by PAH. For example, PAH plays a critical role in Phe metabolism. Moreover, PAH converts most ingested Phe to Tyr, some of which is utilized for protein synthesis.2 Phe turnover is an alternative pathway through which ingested Phe is converted into phenylpyruvate and finally excreted in the urine. The rate-limiting step, which generates CO₂ and water, is also catalyzed by PAH.22

BH₄ is an important cofactor
BH₄ is an essential, naturally occurring cofactor that plays various roles in human biochemistry. BH₄ is biosynthesized de novo from its precursor molecule guanosine triphosphate through Mg²⁺-, Zn²⁺-, and nicotinamide adenine dinucleotide phosphate-dependent chemical reactions. As shown in Figure 3, dihydropteridine reductase uses the quinoid form of 7,8-dihydrobiopterin to catalyze the regeneration of BH₄.23 Functionally, BH₄ acts as a cofactor in the conversion of Phe to Tyr through the catalytic activity of PAH24 and in the production of neurotransmitters. BH₄ also serves as a catalyst for nitric oxide synthase during synthesis of the messenger molecule nitric oxide from the amino acid arginine.25 Moreover, BH₄ catalyzes the hydroxylation of alkylglycerol by alkylglycerol monoxygenase.26 Thus, insufficient biosynthesis and/or regeneration of this critical cofactor may lead to the development of PKU,27 hereditary progressive dystonia with marked diurnal fluctuation,28 or neurovascular dysfunction.29

Normal and abnormal Phe/Tyr metabolism
Figure 3 illustrates the metabolic pathways of Phe. The production of Tyr is the terminal step in the normal metabolism of Phe, catalyzed by PAH. In turn, Tyr participates in a network of biological processes. Many metabolites, including catecholamines, melanin, and thyroid hormones (triiodothyronine and thyroxine), are involved in this network. Transamination eliminates the excess of dietary Phe by converting it into phenylpyruvate. In the context of protein metabolic diseases, however, abnormalities in Phe metabolism and its products may occur due to either a deficiency in the substrate or a defect in PAH or its cofactors.

PKU as an Inherited Disorder
PKU is a disorder of Phe metabolism that is mainly caused by deficiencies in PAH or one of the enzymes involved in the synthesis or regeneration of its cofactor BH₄, as shown in Figure 3.30 PKU is an inherited autosomal recessive disorder, meaning that the biochemical or clinical features of PKU do

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**Figure 3:** Phenylalanine metabolic pathways. A schematic representation of the normal Phe metabolic pathway is shown in (a). (b) The alternative pathway of Phe metabolism, due to abnormalities in the normal Phe metabolic pathway or an excess of dietary Phe.
not appear in individuals in whom only 1 allele carries the mutation (disease carriers); instead, the mutation must be present in both alleles for PKU to manifest.\(^{31}\)

**PKU classification**

Tolerance to dietary Phe or blood Phe concentrations provides a basis for PKU classification, as summarized in Table 1.\(^1\),\(^3\),\(^31\) High blood Phe concentrations (hyperphenylalaninemia [HPA]) have also been demonstrated in approximately 1-2\% of patients with PKU and occurs as a result of mutations in 1 of the genes coding for enzymes involved in BH\(_4\) biosynthesis or regeneration.\(^3\),\(^32\) However, HPA has been reported to be absent in some patients with PKU.\(^33\)

**Incidence**

The incidence of PKU varies depending on ethnicity, and 1 in 13,500-19,000 newborns in the United States of America (USA) has PKU,\(^34\) whereas the African-American population exhibits PKU at a rate of 1 in 50,000 individuals.\(^35\) Finland and Japan have been reported to show the lowest incidence of PKU (<1 in 100,000 and 1 in 108,823 individuals, respectively).\(^36\),\(^37\) In contrast, high rates of PKU have been reported in populations such as Yemenite Jews in Israel (1 in 5,300 individuals), Scottish populations (1 in 5,300 individuals), Arabic populations (up to 1 in 6,000 individuals), and Czechoslovakian populations (1 in 7,000 individuals). The highest rate of PKU was reported for the Turkish population (1 in 2,600 newborns).\(^38\)

**Diagnosis**

**Clinical presentation**

Infants with PKU are often asymptomatic before consuming food containing Phe and may not be identified by newborn screening,\(^39\) explaining the insidious onset of PKU, where symptoms may not appear until early infancy. In general, untreated infants and children with PKU develop a range of impairments, including behavioral, mental, neurological, and physical symptoms. Almost all untreated PKU patients demonstrate behavioral impairments, including aggressiveness, anxiety, hyperactivity, purposeless movements, social withdrawal, and stereotypy. Furthermore, severe mental retardation is the most common outcome and is usually associated with eczema, growth reduction and microcephaly, reduced blond hair, pigmentation of the iris and skin, and a mousy urine odor. The mental retardation observed in patients with PKU is also associated with neurological impairments, such as electroencephalogram abnormalities, epilepsy, gait and tic abnormalities, hyperreflexia, Parkinsonian signs, pyramidal signs, limb spasticity, and tremors. In addition, the brains of untreated PKU patients exhibit disrupted myelination, impaired synaptogenesis, and reduced dendrite arborization.\(^40\)

**Biochemical and molecular genetic diagnosis**

PKU diagnosis is performed by detecting an elevated serum Phe concentration; this is the standard method for confirming the positive neonatal screening results.\(^32\) Tandem mass spectrometry is one of the most useful laboratory tools for measuring various analytes, including Phe and Tyr, in a single sample.\(^41\) PKU diagnosis is also supported by normal or low Tyr levels, in addition to high serum Phe concentrations. Table 1 summarizes the different severities of HPA. All neonates with high serum Phe concentrations should also be evaluated for Pterin disorders by analysis of the urine or blood.\(^32\),\(^34\) Enzyme activity analysis can be performed to determine the activities of the enzymes involved in BH\(_4\) synthesis and regeneration. However, PAH activity cannot be analyzed effectively in the urine or blood because PAH activity is typically only observed in hepatic and renal cells.\(^21\),\(^43\)

PKU diagnosis should be confirmed by molecular analysis. In families with confirmed affected individuals, carriers can be identified by genetic testing. The mutation profile of the gene(s) involved in PKU is limited to certain regions, which are distributed among the structural domains. However, the position and nature of any of these mutations determine the effects of the mutations on enzyme activity, yielding patients with different clinical and biochemical phenotypes.\(^30\),\(^44\),\(^45\)

Several approaches have been established for molecular genetics testing. However, variations in the number of mutations within a population have been observed in different countries, and diagnosis based on molecular genetics requires knowledge of the specific mutations present in a certain population. Thus, Sanger sequencing is the method of choice for detecting causative mutations. Because of the large number of known mutations in suspected genes, confirmation of such a diagnosis can be achieved by high-throughput analysis using advanced molecular genetic testing technologies, such as next generation sequencing (NGS). NGS can also be applied as a discovery tool at the exome and genome levels.\(^46\)-\(^50\)

**Current Strategies for PKU Treatment**

PKU is the first metabolic disorder found to cause mental retardation.\(^51\) Numerous therapeutic strategies for treating PKU have emerged in recent years.\(^52\) In this section, we present current knowledge regarding these therapeutic strategies.

**Treatment with a Phe-restricted diet**

The management of PKU with dietary habits was established in the mid-1900s.\(^53\) Patients with PKU were restricted to diets containing

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**Table 1: PKU classification**

| Class               | Blood Phe concentration* | Phe dietary tolerance |
|---------------------|--------------------------|-----------------------|
| Mild PKU            | 600-900 µmol/L           | 250-400 mg/day        |
| Moderate PKU        | 900-1200 µmol/L          | <250 mg/day           |
| Severe (classic) PKU| Above 1200 µmol/L        |                       |

*Normal range: 50-110 µmol/L.
low Phe concentrations for life. Severe mental retardation can be effectively prevented by following this diet plan. However, this approach is also associated with a risk for nutritional deficiencies. For example, growth retardation and early-onset osteoporosis can be caused by deficiencies in specific substances, such as minerals or vitamins, in a Phe-restricted diet. Moreover, huge social and economic burdens are imposed by the Phe-restricted diet. In addition, compared with age-matched individuals without PKU, patients with PKU who consume a Phe-restricted diet may not achieve their full neurodevelopment potential. Variations in PKU supplementation with LNAAs have been proposed for PKU treatment.

**Treatment with a BH₄ chaperone**

Although the vast majority of patients with PKU are successfully treated through dietary changes, treatment with a Phe-restricted diet is ineffective in patients with PKU exhibiting BH₄ deficiencies. The low activity of a defective enzyme in a metabolic pathway can be increased and the flux can be improved after stabilizing the enzyme conformation with chaperones, and enzyme cofactors can typically function as chaperones. Furthermore, BH₄ can help stabilize misfolded mutant enzymes and prevent their proteolysis, and increase enzyme activity was demonstrated by changing the dose and formulation of BH₄. In patients designated as BH₄-responsive patients, high BH₄ doses correlate with increased PAH activity. These patients exhibit improved responses to BH₄ therapy and milder disease severity than patients lacking mutations. Thus, the patient’s phenotype can form the basis of his/her responses to BH₄ therapy.

A BH₄-loading test was performed to distinguish patients with BH₄-related metabolic defects from those with deficiencies in PAH activity. Measurement of the enzymatic activity of some recycling enzymes responsible for BH₄ help to identify the former group of patients. BH₄ responses have been found in 10–60% of patients with a deficiency in PAH activity. Moreover, BH₄ is commercially available and is prepared synthetically. In addition, in 2007, sapropterin dihydrochloride was approved in the USA as an adjuvant therapy for PKU. Furthermore, when administered at doses of 5-25 mg/(kg·day) for up to 22 weeks, BH₄ caused a decrease in the plasma Phe concentration in approximately 32-50% of treated participants, as illustrated by analyzing the effects of cofactor treatment in clinical trials.

**Large neutral amino acid (LNAA) therapy**

Another encouraging approach for treating PKU is dietary supplementation with LNAAs. LNAAs can be administered to adult patients with PKU in whom a Phe-restricted diet is not tolerable. Four L-type amino acid transporters are present in the transport system through which certain molecules, such as LNAAs, are transported to the brain. LNAAs, including the branched-chain amino acids valine, leucine, and isoleucine; the aromatic amino acids Tyr, Trp, and Phe; and some other amino acids, such as threonine, methionine, and His, are transported by these L transporters. Thus, to cross the blood–brain barrier, these molecules compete with each other to bind the transporters based on their plasma concentrations. High LNAAs supplementation thus inhibits plasma Phe transport and reduces brain Phe concentrations. The impact of LNAAs supplementation has been shown in multiple studies.

**Enzyme therapy**

Another approach for treating PKU is enzyme therapy. Irrespective of the disease genotype, according to this approach, alterations in the PAH metabolic phenotype can reduce harmfully high Phe levels in the plasma. Enzyme substitution with Phe ammonia lyase (PAL) or PAH are the 2 available types of enzyme therapy. Replacement with PAH-fusion proteins has been shown effective in mouse studies. However, PAL-replacement therapy appears to be a more promising approach. In addition to substitution to complement a PAH deficiency, this therapy also converts excess Phe into readily excreted and less toxic products. Moreover, mouse models of PKU have shown that administering injectable or oral version of the PAL enzyme has some limitations.

For example, injectable PAL activates immune responses, whereas oral administration of PAL is associated with enzyme degradation, thereby reducing the effectiveness of the therapy. Notably, conjugation of PAL with polyethylene glycol (PEG) decreased PAL-induced immune responses. Moreover, clinical trials on PKU patients are promising. Phase I and II trials using injectable recombinant PAL conjugated with PEG have shown a reduction in the Phe levels.

**Gene therapy**

PKU gene therapy has been a major focus of various research groups for the past 2 decades. Profound progress in hepatic gene therapy with adenoarial vectors was demonstrated in PKU mouse models. However, in contrast to studies of hepatocytes in PKU-model mice, adenoivirus-associated vectors could not integrate into the DNA of human hepatocytes, in some studies. While previous studies have not shown success in incorporating the PAH gene into human hepatocytes using adenoivirus-associated vectors, other vectors such as lentiviral vectors have demonstrated a stable transduction of the gene of interest in various cell types, which can be used to transduce and stably express PAH in human hepatocytes. Muscle cells may be attractive targets for gene therapy. Successful gene delivery into murine skeletal muscle cells has been demonstrated in several studies. Indeed, normal hepatic Phe metabolism has been mimicked in a system involving gene delivery to muscle cells. Surprisingly, success regarding other IEMs has been shown in gene therapy trials by exploiting advancements in viral vector design. For example, gene therapy has been shown effective in treating aminoaclase-2 deficiency (known as Canavan disease), alpha
I-antitrypsin deficiency, late infantile variant of neuronal ceroid lipofuscinosis, and familial chylomicromenina syndrome (also known as lipoprotein lipase deficiency). Human PKU gene therapy trials may emerge as a result of promising and rapid enhancements in gene therapy studies.

Conclusions

Newborn screening helps in the early establishment of a Phe-restrictive diet and the possibility of avoiding brain damage resulting from HPA in patients with PKU. However, the difficulties of maintaining a strict life-long diet and the occurrence of other complications (despite treatment) make the investigation of new therapeutic strategies of great importance. Moreover, information regarding PKU pathogenesis has increased over the past decade, enabling the development of various novel therapeutic strategies. New treatments, therefore, will eventually become available for addressing the observed HPA in patients with PKU, enabling personalized therapy based on individual genotypes and other specific conditions. Molecular diagnosis by high-throughput sequencing techniques also helps confirm neonatal laboratory-screening results and facilitates the discovery of novel mutations associated with PKU. However, many concerns regarding existing therapeutic strategies need to be addressed and much effort has to be exerted before many of these new therapies can become available for patients. The increasingly competitive pricing and accuracy of NGS for molecular diagnostics has begun to show promise in replacing confirmatory biochemical screening for PKU in newborns in the near future.

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