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REVIEW ARTICLE

Tetrahydrobipterin-responsive phenylalanine hydroxylase deficiency

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Introduction

Nineteen years have passed since discovery of tetrahydrobiopterine (BH_4) responsiveness in a group of patients with phenylalanine hydroxylase (PAH) deficiency in 1999 [1]. More than 400 literatures describing about this topic have been published since 1999 and more than five thousands of patients with hyperphenylalaninemia (HPA)/phenylketonuria (PKU) are now under BH_4 therapy. In this review, we will summarize background of the discovery and recent topics about BH_4-responsive PAH deficiency.

Metabolism of phenylalanine and tetrahydrobiopterine (BH_4)

In mammals L-phenylalanine (Phe) is mainly catabolized by phenylalanine 4-monooxygenase (PAH, EC 1. 14. 16. 1). Approximately 75% of the L-Phe, which is contained in the diet and protein, is catabolized by PAH under normal diet [2]. *Para*-hydroxylation of L-Phe produces L-Tyr in the presence of (6R)-L-erythro-5, 6, 7, 8-BH_4 as a cofactor and O_2 as additional substrate (Fig. 1) [3]. After the catalytic reaction of L-Phe by PAH, pterin-4a-carbinolamine (4-OH-BH_4) is regenerated to its functional tetrahydro-form by two enzymes, pterin carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR). PCD catalyzes dehydration of 4-OH-BH_4, which produces the quinonoid 7,8-dihydrobiopterin (q-BH_2). The NAD(P)H-dependent DHPR reduces q-BH_2 back to BH_4. Therefore, the PAH system is considered to include these two BH_4-regenerating enzymes, PCD and DHPR, in addition to PAH. The rate limiting in the PAH system is the enzymatic activity of PAH.

Proper supply of BH_4 to the PAH system is crucial for Phe catabolism. BH_4 is synthesized *de novo* from guanosine triphosphate (GTP) by three enzymes, GTP cyclohydrolase I (GTPCH), 6-pyruvoyl-5,6,7,8-tetrahydropterin synthethase (PTPS) and sepiapterin reductase (SR) (Fig. 1). The BH_4 biosynthetic pathway in hepatocytes is regulated through GTPCH activity, which is up-regulated by intracellular L-Phe level and down-regulated by intracellular BH_4 level. In BH_4 regenerating and biosynthetic pathways, deficiency of PCD, DHPR, GTPCH, and PTPS, but not SR, leads to impairment of the PAH activity and accumulation of L-Phe in body fluids. These conditions are called as BH_4 deficiency.

HPA and PKU

PAH deficiency results in intolerance to the dietary intake of L-Phe, an essential amino acid, and causes HPA, more severely PKU (OMIM 261600). About 98% of the patients with elevated blood L-Phe level have a deficiency in PAH activity caused by *PAH* mutations [3]. Many PAH mutations have been so far reported (the Phenylalanine hydroxylase locus knowledge base (Pahdb)) [4]. Chronic elevation of blood L-Phe level causes impairment of functions of the central nervous system, which results in development of profound and irreversible intellectual disability. Patients' behavior and psychomotor function may worsen progressively. The coloration of skin and hair may also be influenced since the PAH deficiency causes deficiency of tyrosine that is a precursor of melanin biosynthesis. For prevention of these symptoms the L-phe-restricted diet should be started as soon as the diagnosis is established. The risk of adverse outcome varies based on the degree of PAH deficiency, which is usually evaluated blood L-Phe level on unrestricted diet. Most patients with severe PAH deficiency (blood L-Phe > 1200 μmol/L), known as classic PKU, develop profound and irreversible intellectual

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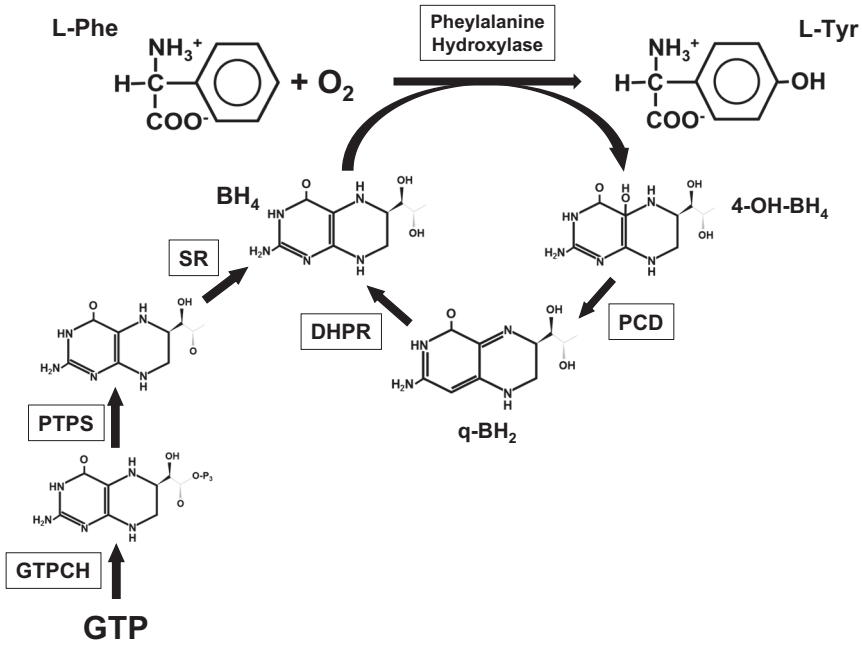

Fig. 1 Metabolism of L-Phe and BH_4. L-Phe is converted to L-Tyr in the presence of BH_4. Two enzymes (PCD and DHPR) are involved in BH_4 regeneration pathway while three enzymes (SR, PTPS, and GTPCH) work as *de novo* synthesis pathway of BH_4. Enzyme names are enclosed by rectangles. PCD, pterin carbinolamine dehydratase; DHPR, dihydropteridine reductase; SR, sepiapterin reductase; PTPS, 6-pyruvoyl-5,6,7,8-tetrahydropterin synthethase; GTPCH, GTP cyclohydrolase I:

disability without effective therapy. Most patients with blood L-Phe < 1200 µmol/L on unrestricted diet (non-PKU HPA) are at much lower risk for impaired cognitive development. PKU may also occur in individuls without the PKU genotype. If the mother with PKU has been treated so as to be asymptomatic, high levels of L-Phe in the maternal blood circulation may affect the non-PKU fetus during pregnancy [5]. Woman with PKU who plans to be pregnant are advised to be under strict restriction of the L-phe intake during pregnancy.

To prevent irreversible brain damage we should make the diagnosis as having HPA/PKU as soon as possible. For early diagnosis, blood L-Phe concentrations of neonates are routinely screened in many countries by the Guthrie test or tandem mass spectrometry, in which a few drops of blood are taken from the heel of the neonate. A Phe-restricted diet can ameliorate the effects of high serum Phe on cognitive function but the life-long Phe restriction presents a heavy burden to patients and their families.

Discovery of BH_4 responsive HPA/PKU

Chronic elevation of blood L-Phe level is caused by a deficiency of either PAH or its cofactor, BH_4 [2]. The two disorders require accurate differentiation since patients with BH_4 deficiency should be treated with the cofactor and/or neurotransmitter as early as possible to prevent brain damages. For that purpose BH_4 loading test is used, since the blood L-Phe level decreases after BH_4 administration in BH_4 deficiency, but not in PAH deficiency. Sapropterin hydrochloride could be used for the BH_4 loading test in Japan because sapropterin was approved for therapy of BH_4 deficiency in 1992. In 1999, we encountered several HPA patients whose elevated blood L-Phe level gradually decreased after sapropterin administration in the BH_4 loading test [1]. Urinary pteridines and DHPR activities were normal, suggesting that the patients were deficient in PAH rather than BH_4. Mutational analysis revealed that all the patients had biallelic PAH mutations, indicating that they were PAH deficiency with response to BH_4. Mutational analysis of the PAH gene was essential for establishment of this novel this entity. Although measurement of urinary pteridines and DHPR activity could exclude the inborn errors of biopterin metabolism, there was no evidence of PAH deficiency without the mutational analysis of *PAH*.

It was surprising that the disease entity had remained unrecognized for decades. A BH_4-loading test has been widely performed worldwide since 1980s [6]. One of the difficulties was probably the small and gradual reduction of L-Phe level after BH_4 administration: L-Phe level was not fully normalized by single BH_4 administration. Also the majority of the patients with BH_4-responsive PAH deficiency had mild elevation of L-Phe and, furthermore, their Phe levels were fluctuating from time to time. These conditions had probably delayed the identification of the disease entity, which turned out to be not so rare among HPA patients.

After we encountered the index case, we started to collect additional patients with mild PAH and performed clinical tests. First, we designed a modified BH_4 loading test with BH_4 loading for four times over 52 h. The patients on this protocol were able to maintain low Phe level for more than

24 h. Second, we performed the BH_4 loading test on two patients with identical PAH mutations. The two patients responded to BH_4 similarly, suggesting that responsiveness to BH_4 was mainly determined by the nature of PAH mutations. These observations prompted us to believe that "BH_4-responsive PAH deficiency" is indeed a novel clinical entity. We considered other names such as "BH_4-responsive HPA" or "BH_4-responsive PKU". The former may be, however, confused with BH_4 deficiency such as PTPS deficiency, while the latter appears inappropriate since the majority of patients with BH_4-responsive PAH deficiency were not classified as classical PKU with high L-Phe in blood.

Characteristic features of BH_4-responsive/PKU

BH_4-responsive/PKU had mutations in the PAH gene, and most of them had mild phenotype mutations in at least one allele. These mild phenotype mutations produced the mutant PAH molecules with a high Michaelis–Menten constant (K_m) for BH_4, and resulted in requiring a higher BH_4 concentration [7]. The responsiveness to BH_4 in PAH deficiency depends on the substantial residual PAH activity, and is characterized by the PKU phenotype. In PKU patients the major cause of phenotypic variability is genotype, of which more than 1000 have been identified in the PAH gene, such as missense, nonsense, splice site, small, or large insertions and deletions [4]. Depending on the effect of a mutation, the activity of mutant protein ranges from 0% to 100% compared to the normal PAH enzyme. Most missense mutations result in misfolding of the PAH protein, increased protein turnover, and loss of enzymatic function. Most PAH proteins with missense mutations are decomposed early after synthesis and has a short life span, which have a decrease in enzyme activity through the abnormality of various PAH proteins to abnormality in the polymerization of subunits, increase in the K_m value of the substrate, increase in the K_m value of the coenzyme and decrease in the activity due to disturbance of the allosteric effect. Therefore, one of the mechanisms for BH_4 responsiveness is derived from the mutant PAH molecules with a high K_m for BH_4 requiring a higher BH_4 concentration. Because PAH activity increased in vivo in response to supplementation of exogenous BH_4 and Phe PAH may not be fully active in vivo under physiological concentration of BH_4 [8]. The other is derived from the stabilization of the mutant PAH molecules, which would be unstable and lead to a shorter half-life. The blood Phe level is considered to decrease in BH_4-responsive HPA/PKU through improving the PAH activity by acting on these abnormalities.

Correlation of PAH genotype and BH_4 responsiveness

As BH_4-responsive HPA/PKU had at least one mild PKU mutation or missense mutation [7]. The BH_4-responsive genotype is characterized by substantial residual PAH activity. However, in compound heterozygous patients a particular combination of two PAH alleles may produce a phenotype that is different from the one expected. Shen et al. described the co-expression of two distinct PAH variants, which revealed positive or negative dominant effects by one of the variants on residual PAH activity. The mechanism is indicated as a result of inter-allelic complementation through the co-expression of 17 variant alleles [9]. Therefore, an accurate prediction of the PKU phenotype based on genotype becomes more complex in some mutations. Blau et al. presented "A tool for allelic and genotype based prediction of metabolic phenotypes in PKU" at the 13th International Congress of Inborn Errors of Metabolism (ICIEM) 2017 in Rio de Janeiro [10]. He described correlation of the PAH genotype and BH_4 responsiveness based on the PKU phenotype by the new allelic phenotype values (APV) and genotypic phenotype values (GPV) system through the analysis of the mutation spectrum of the HPA candidate gene in 9484 PKU patients with full genotype and metabolic phenotype from the BIOPKU database (www.biopku.org) [10]. Expression patterns of PAH in 34 PAH variants transfected into COS-7 cells were compared with in vitro PAH activity and APVs that evaluated the phenotype from severe to mild at 10 levels (0, 5, and 10 as classic PKU, mild PKU, mild HPA, respectively) (Fig. 2). The APV is classified as 0 point for PKU, 5 points for mild PKU, 10 points for mild HPA, so the mutation found only

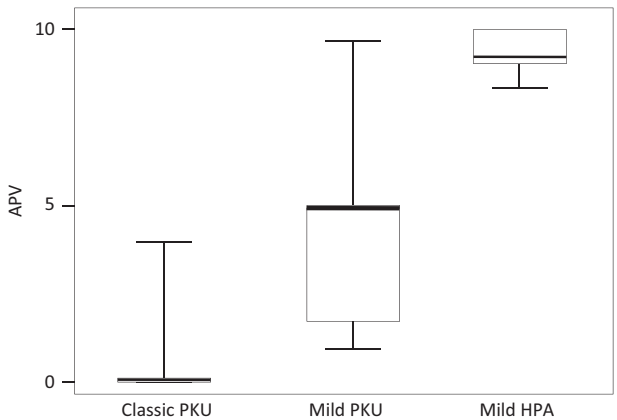

Fig. 2 Comparison of APV between three PKU phenotype classes [10]. APV evaluated the phenotype from severe to mild at 10 levels (0, 5, and 10 as classic PKU, mild PKU, mild HPA, respectively) and express the distribution of data in each group by a box plot. The interquartile range (IQR) shown as a box plot was almost 0 in classic PKU, and between 9 and 10 in mild HPA. However, IQR in mild PKU was widely distributed from 2 to 5

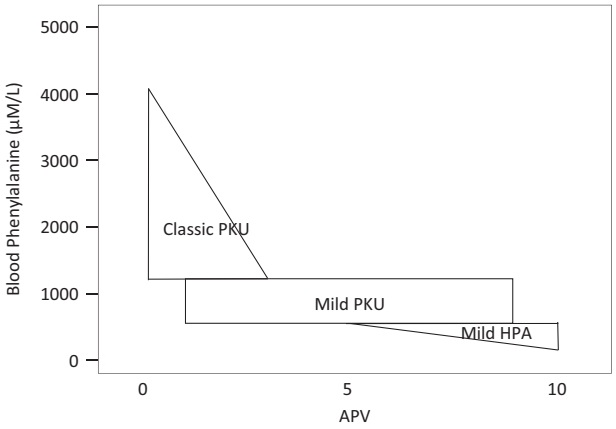

Fig. 3 Comparison of APV (max) with blood Phe levels in patients with PKU ($n = 1325$) [10]. APV values were from 0 to 3 in classic PKU (phenylalanine, more than 1200 μmol/L), from 1 to 9 in mild PKU (phenylalanine, 600–1200 μmol/L), and from 5 to 10 in mild HPA (phenylalanine, less than 600 μmol/L). Although the APV value in classic PKU was mainly 0, APV values from 1 to 3 were shown both in classic PKU and mild PKU. The same in mild phenotype too, APV values between 5 and 9 were shown in both mild PKU and mild HPA. Therefore, APV values showed only weak prediction

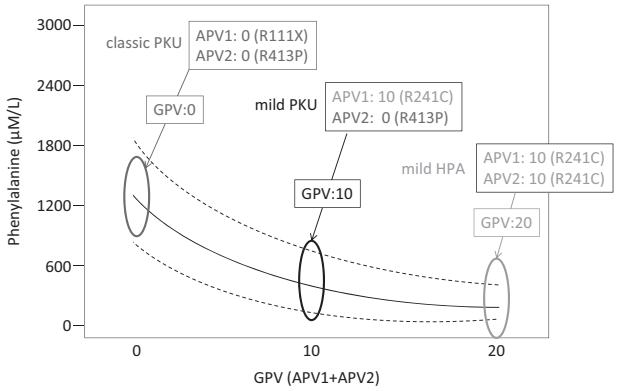

Fig. 4 Correlation between GPV with blood Phe levels in patients with PKU. Typical examples of PHA genotypes and GPVs in classic PKU, mild PKU, and mild HPA GPV were shown. GPV is calculated as, GPV = APV1 + APV2. Note that blood Phe levels correlate with GPV

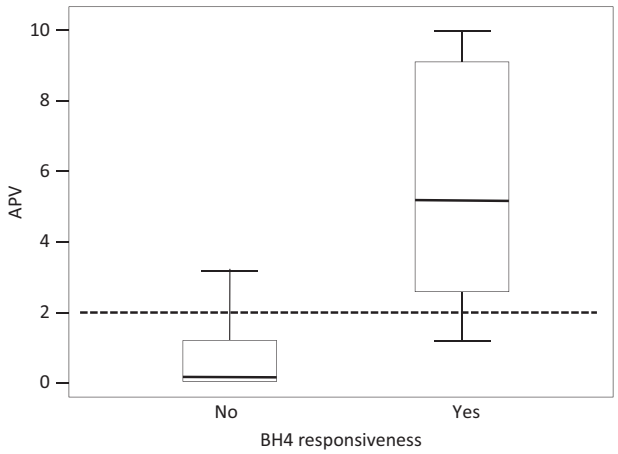

Fig. 5 Comparison of APV (max) between PKU patients tested for BH_4 responsiveness. [10]. This box plot showed that the IQR of BH_4-responsive PKU was higher than APV value 2 and that of BH_4-non-responsive PKU was lower than APV value 2. Therefore, BH_4 reactivity in PKU might be divided by APV value 2 (shown in dotted line)

in classical PKU is 0 point or the mutation only found in mild PKU is 5 points. However, if a mutation is found both classical PKU and mild PKU, it will be a score between 0 points of classical PKU and 5 points of mild PKU depending on its frequency. Similarly, a certain mutation found only in mild HPA is 10 points, but when it can be seen as both mild PKU and mild HPA, it will be a score between 5 points of mild PKU and 10 points of mild HPA depending on its frequency.

In vitro PAH activity and APVs were 52.1 ± 8.5% and 6.7–10.0 in mild HPA, 40.2 ± 7.6% and 2.8–6.6 in mild PKU, and 21.1 ± 7.0% and 0–2.7 in classic PKU, respectively [11]. Those were significantly higher ($P < .01$) in mild HPA and ($P < .048$) mild PKU than in classic PKU. However, there was no significant difference between mild HPA and mild PKU. Therefore, blood phenylalanine levels and APVs showed weak prediction (Fig. 3). GPV was expressed as a numerical value from 0 to 20 in the sum of the two APVs (GPV = APV1 + APV2) (Fig. 4). A comparison of APV (max) between PKU patients tested for BH_4 responsiveness indicated that BH_4 reactivity might be divided by APV 2 (Fig. 5). The new APV and GPV system in PKU is probably highly beneficial for predicting not only clinical phenotypes but also BH_4 responsiveness. Garbade et al. described APV as a model for genotype-based phenotype prediction in 9336 PKU patients from the BIOPKU database [12]. They investigated 588 variants in 2589 different genotypes using an APV algorism. The GPVs were set equal to the higher-APV allele, which determine the metabolic phenotype. GPVs and genotype-based phenotype prediction in 8872 patients were 0.0–2.7 and 99.2% for classic PKU, 2.8–6.6 and 46.2% for mild PKU and 6.7–10.0 and 89.5% for mild HPA, respectively. GPVs were significantly correlated with not only blood Phe levels before treatment ($n = 4217$, $P < 0.001$), but also BH_4 responsiveness ($n = 3488$, $P < 0.001$). Wang et al. described correlation of the PAH genotype and BH_4 responsiveness based on the PKU phenotype by the APV and GPV systems through the analysis of the mutation spectrum of PAH in 1020 Chinese patients with HPA/PKU [13]. The APV and GPV for predicting clinical phenotypes for PKU were calculated based on a recently developed formula. Although level 0, 5, and 10 was expected to correspond to classic PKU, mild PKU, and mild HPA, respectively (Fig. 2), blood Phe levels were poorly predicted by APV alone (Fig. 3). The GPV was expressed as a numerical value from 0 to 20 in the sum of the two APVs (GPV = APV1 + APV2), and predicted blood Phe levels more correctly than

APV (Fig. 4). The APV (max) in HPA/PKU patients with or without BH_4 responsiveness indicated that BH_4 responsiveness may be clearly predicted by APV (Fig. 5). The new APV and GPV system in HPA/PKU is probably highly beneficial for predicting not only clinical phenotypes but also BH_4 responsiveness.

BH_4 for maternal PKU

Maternal PKU is a well-known syndrome in the offspring of women with poorly controlled PKU. HPA >360 µmol/L during pregnancy is teratogenic and may result in maternal PKU syndrome, such as congenital heart disease, microcephaly, growth retardation, and significant developmental delay. To prevent maternal PKU a strict control of maternal phenylalanine concentration is necessary before conception and continued throughout pregnancy. Although there are not so many reports, women of childbearing age with BH_4-responsive PKU received BH_4 therapy instead of diet therapy for the purpose of maintaining their serum Phe level low during pregnancy. There are two cohort studies in Europe and the US, 7 and 5 patients were treated with BH_4 prior to pregnancy, and 1 and 16 patients received BH_4 post-conception, respectively [14, 15, 16]. The Kuvan® Adult Maternal Paediatric European Registry reported four pregnancies. Overall, the dosage varied between 3 and 20 mg/kg of body weight. Neither adverse events nor fetal developmental problems related to the pregnancies were observed [17]. In the Maternal Phenylketonuria Observational Program (PKU MOMS) sub-registry intermediate analysis, sapropterin was generally well tolerated during pregnancy although the severe adverse events possibly related to sapropterin use were found in two cases, premature labor and spontaneous abortion, respectively, among 21 of maternal PKUs treated with sapropterin [18]. Sapropterin has been used for more than 35 years in BH_4 deficiency and 10 years in BH_4-responsive PKU and no major side effects have been reported. Therefore, sapropterin should be considered for a treatment in pregnant women with BH_4-responsive PKU who cannot maintain blood Phe levels within the recommended ranges with dietary therapy alone.

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