Enzyme therapy for phenylketonuria: future directions

Woomi Kim*

Kosin University College of Medicine, Department of Pharmacology, #34 Annam-dong, Saha-gu, Busan, Korea 602-702

Abstract

The dietary therapy for phenylketonuria (PKU) is unpalatable and ineffective in controlling systemic phenylalanine (Phe) levels during pregnancy. Alternative therapies are currently being investigated, particularly ones that break down Phe. This review underscores the progress made in enzyme replacement therapy for PKU. Two modalities are discussed, the enzymes phenylalanine hydroxylase (PAH) and phenylalanine ammonia-lyase (PAL). Developing stable and functional forms of both enzymes have proven difficult, but recent success in producing PEG-modified form of active and stable PAH shows promise. In addition, microencapsulation (ENC) could partially protect proteolysis and gastric acidity. If the immunologic problems can be overcome by PEGylation, and the activity of PEGylated enzyme can be protected by additional encapsulation, it may provide a new prospect for both the oral and parenteral enzyme therapies in PKU.

Key words: phenylketonuria, phenylalanine ammonia-lyase, PEGylation, microencapsulation

Introduction

Phenylketonuria (PKU; OMIM 261600) is an inborn error of phenylalanine (Phe) metabolism leading to hyperphenylalaninemia (HPA). Untreated PKU causes mental retardation, microcephaly and seizures, if not treated immediately after birth through a low-Phe diet. PKU is a result of mutations in the gene for phenylalanine hydroxylase (PAH; EC 1.14.16.1), resulting in impaired activity of the mutant PAH enzyme. Inactive PAH enzyme causes accumulation of the essential amino acid Phe. Due to an extensive worldwide screening of newborn Phe blood-levels, more than 400 mutations in the gene for PAH have been found: http://www.pahdb.mcgill.ca/.

PAH is a non-heme, homo-tetrameric, iron-containing enzyme that needs (6R)-L-erythro-5, 6, 7, 8-tetrahydrobipterin (BH4), molecular oxygen and the active site-bound Fe2+, for conversion of Phe to Tyrosine (Tyr). PAH is responsible for the majority of the catabolism of dietary Phe and is located mainly in the liver. Thus Tyr becomes an essential amino acid in PKU patients, and if not added through supplements to the PKU diet, low levels of Tyr may affect the biosynthesis of the neurotransmitters dopamine, noradrenaline and adrenaline. Tyrosine supplementation provided by oral administration of large neutral amino acids (LNAA: Phe, Tyr,
tryptophan, threonine, isoleucine, leucine, valine, methionine, and histidine) has proven effective in reducing high Phe levels in the brain with PKU patient. The LNAAs share a common transporter across the blood-brain barrier and will therefore compete with Phe, which is high in patient with PKU.\textsuperscript{4,5}

The current therapy for PKU/HPA involves decreasing Phe intake by a special synthetic diet. A Phe restriction diet can lower plasma Phe levels and may prevent the mental impairments of PKU patients. The first dietary therapy for PKU was administered in the 1950s\textsuperscript{6} and it has been used for the treatment of many cases from classic PKU to mild HPA.\textsuperscript{7} However, the diet is expensive, unpalatable, and must be maintained for life. The diet has proven difficult to adhere to, in particular in adolescents.\textsuperscript{31}

In particular, pregnant PKU/PHA women have a particular need for keeping the Phe levels low, since high levels of Phe can cause harm to the embryo and fetus (maternal PKU). The UK MRC study group on PKU has concluded that there is a need for an alternative to the low phe diet.\textsuperscript{60} NIH Consensus Panel also encouraged research on therapeutics for PKU including the break down Phe and the possibility of gene therapy.\textsuperscript{9}

Gene therapy

Due to the apparent disadvantages of the synthetic formulas that are used in the treatment of PKU, effort has been directed towards producing alternatives to the unpalatable diet. Probably the most promising is gene therapy. The goal of gene therapy is to permanently restore PAH expression in liver and to eliminate the need for the special diet. Recent studies have utilized gene therapy by using different gene transfer vehicles, and adding the PAH enzyme either in vitro or in vivo and also by use of heterologous non-hepatic gene targeting attempts. A general and up-to date review on PKU gene therapy was recently published.\textsuperscript{10}

Even with all these promising results, all attempts to use gene therapy to treat PKU have failed, potentially due to poor efficiency of gene delivery vehicles, and the need for BH\textsubscript{4} and O\textsubscript{2} to be present at the site of delivery for PAH enzyme.\textsuperscript{10} Gene therapy was also transient and ineffective when it was re-administered due to the presence of neutralizing antibodies against the recombinant vector.\textsuperscript{11}

BH\textsubscript{4}-responsive PKU

Recently, patients with mild PKU have been shown to respond to BH\textsubscript{4}.\textsuperscript{12} The patients display a lowering of their blood Phe levels upon an oral load of 10-20 mg/kg of the BH\textsubscript{4} cofactor to the phenylalanine hydroxylase (PAH). Subsequent studies have found that up to 60% of mild PKU patients are BH\textsubscript{4}-responsive.\textsuperscript{13} The genotype of known PKU/HPA patients that are BH\textsubscript{4}-responsive have been gathered in the BH\textsubscript{4} database (http://www.bh4.org/biopku.html). Even though most of the BH\textsubscript{4}-responsive patients have the milder form of PKU, the diet is still necessary in most cases, and thus an alternative to the PKU-diet would be addition of BH\textsubscript{4} to a normal diet, similar to vitamin therapies used for other metabolic diseases.\textsuperscript{14} The advantage to BH\textsubscript{4}-supplementation is that it can be taken orally, however, some major disadvantages at the moment are that BH\textsubscript{4} is expensive, and can only be used for the mild forms of PKU/HPA. Also, due to the relatively short elimination half-life of BH\textsubscript{4} (3.3-5.1 hours),\textsuperscript{15} it needs to be given in doses at least two or three times a day. Sublingual injection may lower the required dosage of BH\textsubscript{4}, and subsequently the cost. This method will potentially be useful to treat mild HPA patients, however, the severe PKU/HPA patient still needs diet alternatives.

Enzyme replacement therapy

There is an increasing interest in enzyme replacement therapy (ERT) for metabolic diseases. ERT is gaining popularity in the treatment of lysosomal storage disease, thus circumventing the difficulties with gene therapy. Two enzyme systems are being developed for treatment of PKU: the PAH enzyme, and the Phe degrading enzyme
phenylalanine ammonia lyase (PAL; EC 4.3.1.5). In comparison to PAH, PAL therapy for PKU has some advantages. PAL requires no cofactors for degrading Phe, and trans-cinnamate has a very low toxicity and no embryotoxic effects in experimental animals. The PAL-product trans-cinnamic acid is converted to benzoic acid in the liver, which is then excreted via the urine mainly as hippurate.\textsuperscript{16} PAL is very stable under a wide temperature ranges, whereas recombinant PAH looses activity rapidly upon production and purification in \textit{E. coli}, and it must be stored at $-80^\circ$C. Purified PAL from \textit{Rhodotorula glutinis} at a concentration of 20-40 mg/ml showed no loss of activity at $-60^\circ$C for at least 6 months.\textsuperscript{17}

\textbf{PAL therapy}

A non-mammalian enzyme, PAL is widely distributed in plants\textsuperscript{18,19} and some fungi\textsuperscript{20} and yeasts\textsuperscript{21} and also produced from \textit{Escherichia coli}.

\textbf{I. In vivo studies of oral PAL therapy}

PAL (Figure 1B) was investigated to treat PKU as early as 1980 and ERT studies in human PKU patients began with the oral administration of PAL in enteric-coated gelatin capsules.\textsuperscript{22-25} The purified PAL from the yeast \textit{Rhodotorula glutinis} was packed into hard gelatin and enteric-coated capsules (50U each, SA 1.2U/mg). PAL enteric-coated capsules reduced the blood Phe levels in PKU patients by 22%. The pH optima of PAL from \textit{Rhodotorula glutinis} and \textit{Rhodotorula rubra} were 8.75 and 8.0, respectively.\textsuperscript{17,22} These pH ranges, which are close to the average pH of the small intestine, may have potential advantages in oral enzyme therapy of PKU. For investigating oral administration of PAL therapy, both enzymatic activity and its stability should be evaluated in gastrointestinal fluid. PAL from \textit{Rhodotorula toruloides} was reported to have no activity at pH 2.2 and a half-life in duodenal juice of 3.5 minutes.\textsuperscript{22} PAL from \textit{Rhodotorula glutinis} was also inactivated rapidly by duodenal juice. This inactivation of PAL in duodenal juice was due to the enzyme being more susceptible to chymotrypsin than to trypsin.\textsuperscript{26} In order to preserve the activity of PAL in intestinal fluids, PAL has to be protected from intestinal proteolysis and also from pH levels found in the upper gastrointestinal tract. Therefore, pretreatment was necessary to protect the PAL enzyme against gastric acidity and pancreatic protease. Chang \textit{et al.} immobilized PAL (from \textit{Rhodotorula glutinis}) within artificial cells and the result was an enzymatic PAL system that acted effectively on permanent external substrates, such as Phe.\textsuperscript{27} Antibodies (Ab), as well as intestinal proteases are unable to come into directly attack the microencapsulated (ENC) PAL.\textsuperscript{28} Immobilized PAL within artificial cell was more effective than a phenylalanine-free diet in PKU rats and lowered Phe in plasma, intestinal and cerebrospinal fluids more than a low Phe diet.\textsuperscript{29,30} Consequently, the depletion of intestinal phenylalanine by ENC PAL could significantly lower the plasma phenylalanine levels [28]. However, oral administration of ENC PAL would be limited to mild PKU patients and diet control should also be recommended for better results.\textsuperscript{31,32} Another restriction was that the ENC PAL displayed an activity only 20% of the native enzyme activity. The $V_{\text{max}}$ values for ENC PAL and native PAL were 9 mmol/min and 55 mmol/min, respectively.\textsuperscript{35} Accordingly, additional modifications for enhancing enzyme activity are needed in order for immobilization of PAL in artificial cells to work in reducing Phe levels.

An alternative approach has been investigated in order to overcome the reduction of enzyme activity by microencapsulation. PAL was entrapped in silk fibroin to maintain its activity in the intestinal fluids. Entrapped (ENT) PAL was resistant against chymotrypsin and trypsin \textit{in vitro}.\textsuperscript{36} The ENT PAL was injected directly into rat duodenum. The activity of ENT PAL was retained since it circumvents the intestinal proteases. This approach also actively degraded Phe in the intestinal tract. Although the ENT enzyme showed similar $K_{\text{m}}$ for Phe compared to the
native enzyme, there was no discussion on the protective effect that the silk fibroin produced towards gastric acidity.

In a recent study on PAL ERT by Sarkissian et al., recombinant PAL was produced from the yeast gene.\textsuperscript{30} PAL was encased in its original E. coli expression cells, and to evaluate the effect of recombinant PAL, PAH\textsuperscript{30,32} mice were given either enteral or intraperitoneally injected PAL. Orally administered recombinant PAL (25 units) lowered plasma Phe in PKU mice by 31\% in 1 hour (P<0.04) and 44\% in 2 hours (P<0.0004). This formulation also reduced the Phe content significantly in vitro solution, which contains mouse intestinal fluid. Although this treatment has promising effects, it has been stated that low specific activity compared to native PAL and relative inefficiency at pH 7.0 may be offset.

II. In vivo studies of parenteral PAL therapy

Although oral replacement therapy will be more comfortable for the patient, it will also be necessary to investigate a parenteral modality for PAL therapy because of the limitations of oral therapy. The highly immunogenic property of PAL is a serious problem of parenteral PAL therapy, since it may lead to a short half-life of the enzyme in the blood and unwanted immunologic responses.\textsuperscript{33} To overcome these problems, multibladder enzyme-reactors with immobilized PAL (from Rhodotorula glutinis) were investigated, and resulted in a rapid, 77\% removal of Phe in blood samples of PKU patients.\textsuperscript{32,33} A sustained reduction of Phe was examined in less than 1h, in vitro.\textsuperscript{34} A series of experiments were conducted with a large animal model to evaluate its safety for clinical use. Repeated use of PAL (from Rhodotorula glutinis) reactors to artificially induced HPA in animals did not produce unwanted immunological reactions.\textsuperscript{35,36} The PAL reactor was also applied to a PKU patient, and as a result the Phe concentration was decreased from 1.82 to 1.24 mmol/L after 5.5 hours of treatment, without side effects.\textsuperscript{36}

Extracorporeal hollow fibers containing PAL cannot be easily administered to young children, although it may be recommended for PKU management in pregnant women.

**PAH therapy**

A recent report by Gamez et al.\textsuperscript{37} described the first attempts at producing a stable and non-immunogenic form of the PAH enzyme which can be used for ERT. PEGylation increased the in vitro activities of three forms of PAH (full-length, double-truncated and bacterial PAH from Chromobacterium violaceum). The results were promising, but it has not been tested in PAH\textsuperscript{30,32} mice, so it is not known whether it is effective in vivo. Effectiveness may prove to depend upon method of delivery (i.e. oral route versus intraperitoneal). Additionally, for this to work, there will be a need to administer the PAH cofactor BH\textsubscript{4}, either orally, or by addition of the (BH\textsubscript{2} to BH\textsubscript{4}) recycling enzyme dihydropteridine reductase (DHPR).

**Enzyme modification by PEGylation**

So far the use of PAL/PAH as a therapeutic drug of PKU via the oral and parenteral routes has been severely limited due to inactivation by intestinal proteolysis and immunoreaction. To reduce the degree of immunoreaction,\textsuperscript{39-40} the PEGylation method was applied to PAL from Rhodotorula glutinis by Wieden et al.\textsuperscript{41} The half-lives of native PAL and linear PEGylated PAL after the 1\textsuperscript{st} injection were 6 hours and 20 hours, respectively. PEG-PAL had a much longer blood-circulating time in mice than native PAL. However, intravenously injected PEGylated PAL was cleared rapidly from circulating blood after the 13\textsuperscript{th} injection. Therefore, more advanced PEGylation for the consistent protection from immunological recognition after repetitive injections should be developed.\textsuperscript{42}

PEGylated enzyme also needs additional treatment before oral administration. This review infers that complex microcapsules could be used as additional measures to protect the therapeutic enzymes from inactivation in both the stomach and the intestine. The semi-permeable
Concluding remarks

Previously published articles have indicated that ENC-PAL can partially protect against proteolysis and gastric acidity. However, it is also known that a reduced activity of ENC-PAL was not enough to control the Phe level in PKU. Therefore, it needs additional processes to enhance the activity of oral enzyme therapy. To determine whether the PEGylated enzyme can be effectively protected from intestinal proteases by further processing, both PEGylation and encapsulation may be useful.

PEG therapy lowers the level of tyrosine of a PKU patient, since PAL catalyzes the deamination of both Phe to form trans-cinnamic acid and Tyr to trans-coumaric acid. Although the roles of Tyr in the pathogenesis and therapeutics of PKU are a matter of continued debate, Tyr supplement during PAL therapy would be recommended in PKU patients. High concentrations of Tyr may inconsistently affect the activity of PAL for Phe. If Tyr supplement is recommended between PAL therapies, it should not be given at the same time as PAL. Another difficulty with PKU therapy is that the enzymes need to be given on a daily basis. For this reason, the oral administration route offers a greater advantage, since there is no need for surgical intervention.

This review suggests that the PEGylation may be one of the useful pretreatment modalities for enhancing and maintaining its enzyme activity from additional processing. PEGylated enzyme should be evaluated both in vitro and in vivo, in order to assess its potential use in the treatment of human PKU patients. If PEGylation can overcome the immunological problems by further study and the enhanced activity of PEGylated enzyme can be protected by additional encapsulation, it may provide a new prospect for both the oral and parenteral enzyme therapies. There have been considerable advances in discovering an optimistic answer of ERT for PKU.

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References


