고신대학교 의과대학 학술지 제21권 제1호
Kosin Medical Journal
Vol. 21. No. 1, pp. 369~374, 2006

페닐케톤뇨증의 효소 치료

김 우 미*

602-702 부산시 서구 암남동 34번지 고신대학교 의과대학 약리학교실

Enzyme therapy for phenylketonuria: future directions

Woomi Kim*

Kosin University College of Medicine, Department of Pharmacology, #34 Amnam-dong, Suh-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).[1] 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/* .[2]

PAH is a non-heme, homo-tetrameric, iron-containing enzyme that needs (*6R*)-L-*erythro*-5, 6, 7, 8-tetrahydrobiopterin (BH_4), molecular oxygen and the active site-bound Fe^{2+}, 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,

교신저자 : 김 우 미
주소: 602-703, 부산광역시 서구 암남동 34번지
고신대학교 의과대학 약리학교실
TEL. 051-990-6437 FAX. 051-51-990-3081
E-mail: kwm@ns.kosinmed.or.kr

tryptophan, threonine, isoleucine, leucine, valine, methionine, and histidine) has proven effective in reducing high Phe levels in the brain with PKU patient.[3] The LNAAs share a common transporter across the blood-brain barrier and will therefore compete with Phe, which is high in patient with PKU.[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[6] and it has been used for the treatment of many cases from classic PKU to mild HPA.[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.[3] 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.[8] NIH Consensus Panel also encouraged research on therapeutics for PKU including the break down Phe and the possibility of gene therapy.[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.[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_4 and O_2 to be present at the site of delivery for PAH enzyme.[10] Gene therapy was also transient and ineffective when it was re-administered due to the presence of neutralizing antibodies against the recombinant vector.[11]

BH_4-responsive PKU

Recently, patients with mild PKU have been shown to respond to BH_4.[12] The patients display a lowering of their blood Phe levels upon an oral load of 10-20 mg/kg of the BH_4 cofactor to the phenylalanine hydroxylase (PAH). Subsequent studies have found that up to 60% of mild PKU patients are BH_4-responsive.[13] The genotype of known PKU/HPA patients that are BH_4-responsive have been gathered in the BH_4 database (http://www.bh4.org/biopku.html). Even though most of the BH_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_4 to a normal diet, similar to vitamin therapies used for other metabolic diseases.[14] The advantage to BH_4-supplementaion is that it can be taken orally, however, some major disadvantages at the moment are that BH_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_4 (3.3-5.1 hours),[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_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.[16] PAL is very stable under a wide temperature ranges, whereas recombinant PAH looses activity rapidly upon production and purification in *E. coli*, and it must be stored at -80℃. Purified PAL from *Rhodotorula glutinis* at a concentration of 20-40 mg/ml showed no loss of activity at -60℃ for at least 6 months.[17]

PAL therapy

A non-mammalian enzyme, PAL is widely distributed in plants[18,19] and some fungi[20] and yeasts[17] and also produced from *Escherichia coli*.

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.[21-23] The purified PAL from the yeast *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 *Rhodotorula glutinis* and *Rhodotorula rubra* were 8.75 and 8.0, respectively.[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 *Rhodosporidium toruloides* was reported to have no activity at pH 2.2 and a half-life in duodenal juice of 3.5 minutes.[22] PAL from *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.[24] 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 proteases. Chang *et al.* immobilized PAL (from *Rhodotorula glutinis)* within artificial cells and the result was an enzymatic PAL system that acted effectively on permanent external substrates, such as Phe.[25] Antibodies (Ab), as well as intestinal proteases are unable to come into directly attack the microencapsulated (ENC) PAL.[25] 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.[26,27] 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.[27,28] Another restriction was that the ENC PAL displayed an activity only 20% of the native enzyme activity. The V_{max} values for ENC PAL and native PAL were 9 mmol/min and 55 mmol/min, respectively.[25] 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 *in vitro*.[29] 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_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.[30] PAL was encased in its original *E. coli* expression cells, and to evaluate the effect of recombinant PAL, PAH ENU2 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 ofthe 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.[31] To overcome these problems, multitubular 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.[32,33] A sustained reduction of Phe was examined in less than 1h, *in vitro*.[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.[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.[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*[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^{ENU2} mice, so it is not know 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_4, either orally, or by addition of the (BH_2 to BH_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,[38-40] the PEGylation method was applied to PAL from *Rhodotorula glutinis* by Wieder *et al*.[41] The half-lives of native PAL and linear PEGylated PAL after the 1st 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 13th injection. Therefore, more advanced PEGylation for the consistent protection from immunological recognition after repetitive injections should be developed.[39]

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 semipermeable

microcapsules can be further encapsulated by enteric-soluble materials to protect it from gastric juice. When the preparation passes into the intestine, the small molecule Phe will rapidly diffuse and equilibrate across the semipermeable membrane and can be converted to non-toxic products by the enveloped enzymes.[42)]

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.

PAL 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.

Acknowledgments

This work was investigated in part by the support of Kosin University College of Medicine, 2005.

References

1. Scriver CR, and Kaufamn S: The Metabolic and Molecular Bases of Inherited Disease, New York, McGraw-Hill press, 2001, 1667-1724
2. Erlandsen H, and Stevens RC: The structural basis of phenylketonuria. Mol Genet Metab 68:103-125, 1999.
3. Matalon R et al: Biopterin responsive phenylalanine hydroxylase deficiency. Genet Med 6(1):27-32, 2004
4. Segawa H, Fukasawa Y, Miyamoto K, Takeda E, Endou H, and Kanai Y: Identification and functional characterization of a Na+-independent neutral amino acid transporter with broad substrate selectivity. J Biol Chem 274(28):19745-19751, 1999
5. Castagna M, Shayakul C, Trotti D, Sacchi VF, Harvey WR, Hediger MA: Molecular characteristics of mammalian and insect amino acid transporters: implications for amino acid homeostasis. J Exp Biol 200(Pt 2):269-286, 1997
6. Woolf LI, Griffiths R, Moncrieff A, Coates S, Dillistone F: The dietary treatment of phenylketonuria. Arch Dis Child 33(167):31-45, 1958
7. Baumeister A: Dietary treatment of destructive behavi,or associated with hyperphenylalaninemia. Clin Neuropharmacol 21(1):18-27, 1998
8. Report of Medical Research Council Working Party on Phenylketonuria. Recommendations on the dietary management of phenylketonuria. Arch Dis Child 68(3):426-427, 1993
9. National Institute of Health Consensus Development Conference Statement. Phenylketonuria: screening and management October 16-18, 2000. Pediatrics 108(4):972-982, 2001
10. Ding Z, Harding CO, and Thony B: "State-of-the-art 2003 on PKU gene therapy". Mol Genet Metabol 81:3-8, 2004
11. Eisensmith RC, and Woo SL: Gene therapy for phenylketonuria. Eur J Pediatr 155(S1): 16-19, 1996
12. Kure S et al: Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. J Pediatr 135(3):375-378, 1999
13. Bernegger C, and Blau N: High frequency of tetrahydrobiopterin-responsiveness among hyperphenylalaninemia : a study of 1,919 patients observed from 1988 to 2002.

Mol Genet Metab 77(4):304-313, 2002
14. Ames BN, Elson-Schwab I, and Silver EA: High-dose vitamin therapy stimulates variant enzymes with decreased coenzyme binding affinity (increased Km): relevance to genetic disease and polymorphisms. Am J Clin Nutr 75:616-658, 2002
15. Fiege B et al: Plasma tetrahydrobiopterin and its pharmacokinetic following oral administration. Mol Genet Metab 81(1):45-51, 2004
16. Hoskins JA, and Gray J: Phenylalanine ammonia-lyase in the management of phenylketonuria: the relationship between ingested cinnamate and urinary hippurate in humans. Res Commun Chem Pathol Pharmacol 35:275-282, 1982
17. Abell CW, and Shen R-S: Phenylalanine ammonia-lyase from the yeast Rhodotorula glutinis. Methods Enzymol 142:242-249, 1987
18. Koukol J, and Conn EE: The metabolism of aromatic compounds in higher plans. IV. Purification and properties of the phenylalanine deaminase of Hordeum vulgare. J Biol Chem 236:2692-2698, 1961
19. Hanson KR, and Havir EA: The Enzymes, New York, Academic Press, 1972, 75
20. Rao PV, Moore K, and Towers GH: Degradation of aromatic amino acids by fungi. II. Purification and properties of phenylalanine ammonia-lyase from Ustilago hordei. Can J Biochem 45(12):1863-1872, 1967
21. Marconi W, Bartoli F, Gianna R, Morisi F, and Spotorno G: Phenylalanine ammonia-lyase entrapped in fibers. Biochimie 62(8-9):575-580, 1980
22. Gilbert HJ, and Tully M: Protection of phenylalanine ammonia-lyase from proteolytic attack. Biochem. Biophys. Res Commun 131(2):557-563, 1985
23. Hoskins JA et al: Enzymatic control of phenylalanine intake in phenylketonuria. Lancet 23: 392-394, 1980
24. Gilbert HJ, and Jack GW: The effect of proteinases on phenylalanine ammonia-lyase from the yeast Rhodotorula glutinis. Biochem J 199(3):715-723, 1981
25. Bourget L, and Chang TM: Artificial cell-microencapsulated phenylalanine ammonia-lyase. Appl Biochem Biotechnol 10: 57-59, 1984
26. Bourget L, and Chang TM: Phenylalanine ammonia-lyase immobilized in microcapsules for the depletion of phenylalanine in plasma in phenylketonuric rat model. Biochim Biophys Acta 883(3):432-438, 1986
27. Bourget L, and Chang TM: Effects of oral administration of artificial cells immobilized phenylalanine ammonia-lyase on intestinal amino acids of phenylketonuric rats. Biomater Artif Cells Artif Organs 17(2):161-181, 1989
28. Chang TM, Bourget L and Lister C: A new theory of enterorecirculation of aminoacids and its use for depleting unwanted amino acids using oral enzyme-artificial cells, as in removing phenylalanine in phenylketonuria. Artif Cells Blood Substit Immobil Biotechnol 23(1):1-21, 1995
29. Inoue S, Matsunaga Y, Iwane H, Sotomura M, Nose T: Entrapment of phenylalanine ammonia-lyase in silk fibroin for protection from proteolytic attack. Biochem Biophys Res Commun. 141(1):165-170, 1986
30. Sarkissian CN et al: A different approach to treatment of phenylketonuria: phenylalanine degradation with recombinant phenylalanine ammonia lyase. Proc Natl Acad Sci USA 96(5):2339-2344, 1999
31. Fritz RR, Hodgins DS, and Abell CW: Phenylalanine ammonia-lyase. Induction and purification from yeast and clearance in mammals. J Biol Chem 251(15):4646-4650, 1976
32. Kalghatgi K, Horvath C, and Ambrus CM: Multitubular reactors with immobilized L-phenylalanine ammonia-lyase for use in extracorporeal shunts. Res Commun Chem Pathol Pharmacol 27(3):551-561, 1980
33. Ambrus CM: Depletion of phenylalanine in the blood of phenylketonuric patients using a PAL-enzyme reactor. An in vitro study. Res Commun Chem Pathol Pharmacol 37(1): 105-111, 1982
34. Larue C et al: An extracorporeal hollow-fiber reactor for phenylketonuria using immobilized phenylalanine ammonia-lyase. Dev. Pharmacol Ther 9(2):73-81, 1986
35. Ambrus CM et al: In vivo safety of hollow fiber enzyme-reactors with immobilized phenylalanine ammonia-lyase in a large animal model for phenylketonuria. J Pharmacol Exp Ther 224(3):598-602, 1983
36. Ambrus CM et al: Extracorporeal enzyme reactors for depletion of phenylalanine in phenylketonuria. Ann Intern Med 106(4):531-537, 1987
37. Gamez A, Wang L, Straub M, Patch MG, and Stevens RC: Toward PKU enzyme replacement therapy: PEGylation with activity retention for three forms of recombinant phenylalanine hydroxylase. Mol Ther 9(1):124-129, 2004
38. Abuchowski A, McCoy JR, Palczuk NC, van Es T, and Davis FF: Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. J Biol Chem 252(11):3582-3586, 1977
39. Veronese FM, Monfardini C, Caliceti P, Schiavon O, Scrawen MD, Beer D: Improvement of pharmacokinetic, immulonogical and stability properties of asparaginase by conjugation to linear and branched monomethoxypoly (ethylene glycol). J Control Release 40: 199-209, 1996
40. Savoca KV, Abuchowski A, van Es T, Davis FF, and Palczuk NC: Preparation of a non-immunogenic arginase by the covalent attachment of polyethylene glycol. Biochim Biophys Acta0 578(1):47-53, 1979
41. Wieder KJ, Palczuk NC, van Es T, and Davis FF: Some properties of polyethylene glycol: phenylalanine ammonia-lyase adducts. J Biol Chem0 254(24):12579- 12587, 1979
42. Wang XL, and Shao JY: New preparation for oral administration of digestive enzyme. Lactase complex microcapsules. Biomater Artif Cells Immobilization Biotechnol 21(5):637- 646, 1993