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Enhanced brain distribution of modified aspartoacylase

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ABSTRACT

Canavan disease is a fatal neurological disorder caused by defects in the gene that produces the enzyme aspartoacylase. Enzyme replacement therapy can potentially be used to overcome these defects if a stable enzyme form that can gain access to the appropriate neural cells can be produced. Achieving the proper cellular targeting requires a modified form of aspartoacylase that can traverse the blood–brain barrier. A PEGylated form of aspartoacylase that shows dramatic enhancement in brain tissue access and distribution has been produced. While the mechanism of transport has not yet been established, this modified enzyme is significantly less immunogenic than unmodified aspartoacylase. These improved properties set the stage for more extensive enzyme replacement trials as a possible treatment strategy.

1. Introduction

A defect in the human *acy2* gene has been identified as the cause of Canavan disease (CD)[1] [1], a neurodegenerative disorder for which there is currently no effective treatment or cure. This defective enzyme, aspartoacylase (ASPA), plays a critical role in brain metabolism, the deacetylation of N-acetylaspartate (NAA), to produce acetate and aspartate. ASPA is the only brain enzyme that has been shown to be capable of metabolizing NAA [1]. Over 50 different mutations including numerous deletions, missense mutations, and terminations, have been reported in the enzyme from Canavan patients. In most cases these mutations result in an altered enzyme that is either not expressed or is expressed but retains little catalytic activity [2]. Recent studies have shown correlations between aspartoacylase activity, protein stability and disease severity [3]. Our functional studies have shown that ASPA is a glycoprotein in which the glycan stabilizes the protein structure [4]. A bound zinc atom was identified and showed to play an essential catalytic role [5], and the function of many of the substrate binding and catalytic groups in the enzyme active site has also been determined [6]. Our detailed understanding of the mechanism and function of this enzyme is the basis for our studies to prepare stable and non-immunogenic forms of this enzyme for replacement therapy.

In addition to its role in acetate production, it has been proposed that NAA can be converted to glutamate in the brain and that this conversion is energetically favorable [7]. This possibility has important consequences given the well documented findings that high levels of glutamate are excitotoxic to cells [8]. In fact, we have shown that high levels of glutamate contribute to the neurotoxic effects of the drug of abuse, methamphetamine, through oxidative and metabolic stress [9–11]. NAA and glutamate are inherently linked through a series of metabolic reactions, mainly the tricarboxylic acid (TCA) and the glutamate–glutamine cycle [12]. NAA is proposed to be a reservoir for regulating the concentration of glutamate, maintaining low levels of glutamate yet having the capability to rapidly produce it when needed [7]. Several additional studies also support this proposed connection between NAA and glutamatergic neurotransmission, showing a strong correlation in different brain regions between NAA and Glx syntheses [13,14].

The introduction of ASPA into the brain not only may serve to supply much needed acetate to the brain for myelination, but also could buffer the brain against extremely high levels of NAA and consequently, excitotoxic levels of glutamate. Therefore, enzyme replacement therapy (ERT) may be effective for the treatment of diseases associated with deficiencies in fatty acid biosynthesis such as CD, and may also provide a therapeutic approach for the mitigation of brain injury produced by stimulant drugs of abuse.

ERT has shown success for a number of different metabolic disorders, including phenylketonuria [15] and a glycogen storage disease called Pompe disease [16] where lost catalytic function was restored in animal models of these disorders. ERT trials have been approved for the treatment of a number of metabolic disorders, including some that lead to neurological defects. ERT has shown varying levels of success in the

Abbreviations: ASPA, aspartoacylase; BBB, blood–brain barrier; CD, Canavan disease; CNS, central nervous system; ERT, enzyme replacement therapy; GFAP, glial fibrillary acidic protein; IBA, ionized calcium binding adaptor protein-1; NAA, *N*-acetyl-L-aspartate; NECA, 1-(6-amino-9H-purin-9-yl)-1-deoxy-*N*-ethyl-β-D-ribofuranuronamide; PEG, polyethylene glycol.

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treatment of these disorders, including patients with lysosomal storage diseases [17] such as Gaucher and Fabry diseases as well as Hunter and Hurler syndromes. Treatment with recombinant human enzymes has been used to minimize adverse immune responses in these patients thereby leading to enhanced *in vivo* stability. While these disorders each cause significant neurological symptoms, the underlying causes are genetic defects that are manifested both in neural and in non-neural cells. For disorders such as CD in which the defects are found exclusively in neural cells there is an additional hurdle that must be overcome in order for ERT to succeed, the blood–brain barrier.

The presence of a blood–brain barrier (BBB) was first identified because injected dyes failed to penetrate into cells in the central nervous system. The BBB serves as the gatekeeper controlling access to the neurological system in higher organisms. This structure provides a physical barrier, by means of tight junctions composed of membrane proteins and lipids that seal the gaps between endothelial cells, a chemical barrier that regulates the transport of material through these cells and pumps foreign substances away from the brain, and a metabolic barrier that hydrolyzes and inactivates toxic compounds. Thus the BBB effectively protects the brain against foreign substances, but it also limits access to many therapeutic agents designed to treat neurological disorders. This barrier can potentially be overcome either by delivering drugs behind the barrier through intracerebral injections or implants, or by increasing the flux across the barrier. While these approaches have led to the successful delivery of certain drugs and therapeutic agents, the existence of a BBB makes ERT significantly more difficult to achieve for the treatment of neurological disorders. The potential importance of effective approaches for the delivery of materials to the CNS has led to several patents for different proposed therapies [18–20]. However the relative merits and potential effectiveness of these approaches have not yet been fully examined.

Since replacement enzymes do not typically cross the BBB, severely affected patients with central nervous system symptoms would not be expected to show significant improvements through this approach unless this issue of limited brain access can be overcome. So, while ERT is proving to be a viable approach for the treatment of certain genetic disorders, this promising new therapeutic approach will have only limited applications and modest successes for most neurological disorders until the issues of enzyme stability, protein immunogenicity, and bioavailability are addressed. We have begun to explore approaches that address each of these issues for the application of ERT in the treatment of CD. Preliminary studies have shown that administration of PEGylated forms of ASPA causes increased enzyme activity and decreased substrate accumulation in brain homogenates from an animal model of CD [21]. However, it has not been established if this treatment leads to uptake and transport of the modified enzyme into brain tissue. These issues are the focus of this study.

2. Materials and methods

2.1. Materials

The plasmid containing the *acy2* gene was transformed into *Pichia pastoris* KM71H cells following the directions in the Easy Select Pichia Transformation kit (Invitrogen). Aspartoacylase (ASPA) was purified by a previously published protocol [21]. Reactive polyethylene glycol (PEG) reagents were purchased from NOF (Japan), amine-reactive AlexaFluor® 594 carboxylic acid, succinimidyl ester was from Life Technologies, polyclonal rabbit anti GFAP antibodies were from Millipore (catalog no. AB5804) and anti-Rb-IBA antibody was purchased from Wako (catalog no. 019-19741). Fluoromount-G mounting media were purchased from Southern Biotech (catalog no. 0100-01). NECA was purchased from Tocris (catalog no. 1691) and hyaluronidase was purchased from Sigma (catalog no. H3884).

2.2. Production of modified aspartoacylase

ASPA samples were treated with a methoxy-PEG reagent containing terminal activating aldehyde or ester groups attached with a carboxymethyl linker. Linear 5 kDa PEG molecules were added to the reaction mixture in varying enzyme to polymer ratios and incubated at 25 °C. Aliquots were removed from the reactions at different time points and quenched by addition of excess lysine. The samples were then treated by using a spin concentrator with a 30 kDa molecular weight cut-off to remove the excess PEG, and concentrated to 0.8 to 3.0 mg/ml in a buffer containing 50 mM Hepes, pH 8.3, 1 mM DTT, and 0.1 M NaCl. PEGylated enzyme samples for the animal studies were analyzed by SDS-PAGE by using Coomassie dye or barium-iodide staining to determine the extent of PEGylation and to detect the presence of any unmodified enzyme. For protein staining gels were soaked in Coomassie brilliant blue R-250 solution (0.1% v/v in 50% v/v methanol and 10% v/v acetic acid) for 10 min at ambient temperature with gentle shaking, followed by de-stained using a solution containing 50% v/v methanol and 10% v/v acetic acid. For PEG staining gels were soaked in 5% glutaraldehyde solution for 15 min, followed by a 0.1 M perchloric acid treatment for an additional 15 min. Treatment with a 5% barium chloride solution and 0.1 M iodine solution was used to detect the protein–PEG conjugates [22]. If necessary, the enzyme samples were purified by elution from an anion-exchange column (Source 15Q) with a linear NaCl gradient to remove any residual unmodified enzyme.

The ASPA samples were pre-treated with AlexaFluor® 594 to provide a covalently attached fluorescent probe. This labeling dye was dissolved in anhydrous DMSO and reacted with the enzyme (1:10 w/w ratio) at ambient temperature for 2 to 3 min. The reaction was quenched by adding excess lysine, and the excess dye and lysine were subsequently removed using a spin concentrator of the appropriate molecular weight cut-off. The degree of labeling was determined following the equation in the amine-reactive probe manual by Life Technologies. The labeled samples were concentrated as described above and stored at −80 °C until used for the animal studies.

2.3. Brain distribution of aspartoacylase

To examine the brain distribution of the modified enzyme, 0.8, 1.3, 2.0 or 3.0 mg/kg of fluorescently-labeled modified (PEGylated) enzyme was injected i.p. into adult male Sprague–Dawley rats (200–250 g), with labeled unmodified enzyme and saline serving as controls. After 4 h, the rats were sacrificed, and brains were extracted, frozen in dry ice and sliced at a thickness of 25 μm through the striatum and hippocampus. The sections were then mounted onto subbed slides, coverslipped using Fluromount-G mounting media and air dried overnight in the dark and imaged the next day. To determine if the enzyme was localized to the capillaries, and whether PEGylation increased extravasation of the enzyme from the capillaries, fresh brain sections rather than perfused and fixed sections were used for imaging, since perfusing the brain would remove the enzyme in the capillaries. The dentate region of the hippocampus and the region adjacent to the lateral ventricle in the striatum were imaged using the Olympus Fluoview FV1000 confocal scanning laser microscope system. The fluorophores were excited using argon laser at 561 nm and images were obtained using 20× objective. The gain, offset, voltage, aperture size and laser power were each kept constant between treatment groups in order to allow for unbiased comparisons.

Vehicle saline or 0.08 mg/kg NECA was injected i.p. into a separate group of rats 5 h prior to PEGylated enzyme treatment, or 20 mg/kg hyaluronidase was injected 20 min prior to PEGylated enzyme injections. Four hours after enzyme treatment, brains were extracted and sectioned as described above.