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A new therapy prevents intellectual disability in mouse with phenylketonuria

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ABSTRACT

Untreated phenylketonuria (PKU) results in severe neurodevelopmental disorders, which can be partially prevented by an early and rigorous limitation of phenylalanine (Phe) intake. Enzyme substitution therapy with recombinant *Anabaena variabilis* Phe Ammonia Lyase (r*Av*PAL) proved to be effective in reducing blood Phe levels in preclinical and clinical studies of adults with PKU. Aims of present study were: a) to gather proofs of clinical efficacy of r*Av*PAL treatment in preventing neurological impairment in an early treated murine model of PKU; b) to test the advantages of an alternative delivering system for r*Av*PAL such as autologous erythrocytes. BTBR-Pah$^{enu2-/-}$ mice were treated from 15 to 64 post-natal days with weekly infusions of erythrocytes loaded with r*Av*PAL. Behavioral, neurochemical, and brain histological markers denoting untreated PKU were examined in early treated adult mice in comparison with untreated and wild type animals. r*Av*PAL therapy normalized blood and brain Phe; prevented cognitive developmental failure, brain depletion of serotonin, dendritic spine abnormalities, and myelin basic protein reduction. No adverse events or inactivating immune reaction were observed. In conclusion present study testifies the clinical efficacy of r*Av*PAL treatment in a preclinical model of PKU and the advantages of erythrocytes as carrier of the enzyme in term of frequency of the administrations and prevention of immunological reactions.

1. Introduction

Phenylketonuria (PKU; OMIM#261600) is a hereditary metabolic disorder due to the deficiency of the enzyme phenylalanine hydroxylase (PAH), which converts L-phenylalanine (Phe) into L-tyrosine (Tyr), resulting in the accumulation of neurotoxic levels of Phe and severe neurodevelopment impairment. The natural history of PKU has been dramatically improved by the discovery that dietary restriction of Phe intake can prevent mental disability in early treated patients [1]. However, although normal, in general term, early and continuously treated PKU patients reach lower intelligent quotients than expected [2], may show specific deficits in the higher-order cognitive processes [3,4], and report more frequently psychopathological symptoms [5]. In order to improve the outcome of the disease, a more restricted and lifelong diet has become mandatory [6], which implies a demanding, and sometimes exceeding, burden for many adult patients. With the

Abbreviations: PKU, phenylketonuria; PAH, phenylalanine hydroxylase; Phe, L-phenylalanine; Tyr, L-tyrosine; BH4, tetrahydrobiopterin; r*Av*PAL, recombinant Phenylalanine Ammonia Lyase from *Anabaena variabilis*; RBC, erythrocytes or red blood cells; pFC, prefrontal cortex; NAc, nucleus accumbens; CP, caudate putamen; Amy, amygdale; Hipp, hippocampus; 5-HT, serotonin; NE, norepinephrine; DA, dopamine; DOPAC, 3-4-Dihydroxyphenylacetic acid; HVA, homovanillic acid; MOPEG, 3-methoxy-4 hydroxyphenylethyleneglycol; 5-HIAA, 5-hydroxyindoleacetic acid; MBP, myelin basic protein; NFL, neurofilament light chain protein; EPM, elevated plus maze; ORT, object recognition test; TPH, tryptophan hydroxylase; TH, tyrosine hydroxylase

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exception of tetrahydrobiopterin (BH4) in BH4-responsive patients, no alternative treatment has been proved to be as effective as Phe-restricted diet in lowering blood Phe and in preventing mental disability and/or neurocognitive dysfunctions in early treated patients [6,7].

Proofs of pharmacological efficacy of the enzyme substitution therapy (EST) with recombinant enzyme Phenylalanine Ammonia Lyase from *Anabaena variabilis* (r*Av*PAL) [8] have been obtained and this treatment is currently under intensive preclinical [9,10] and clinical investigation [11]. However, proofs of the clinical efficacy of this treatment on immature brain are so far lacking.

As for other parenteral administrations of recombinant enzymes, the occurrence of local adverse effects [11,12], the inactivating immunological reaction and the requirement of frequent treatments to maintain a stable metabolic control, are some of the constraints of this promising new approach. These criticalities could be overcome by using an alternative delivery system able to protect the enzyme from proteolytic degradation and immunogenic response. Erythrocytes (red blood cells, RBC) proved to be an excellent vehicle for a number of pharmaceutical compounds [13,14]. RBC are able to rapidly accumulate Phe by means of saturable transport according to a Michaelis-Menten kinetics [15]. Once inside RBC, Phe may be transformed to trans-cinnamic acid and trace amounts of ammonia by r*Av*PAL previously internalized into these cells. Preclinical proof of pharmacological efficacy of r*Av*PAL-loaded RBC have been obtained in vitro and in vivo in adult BTBR-Pah$^{enu2-/-}$ (ENU2) mice, the best mouse model of PKU [16].

With present study we aimed at a) gathering proofs of clinical efficacy of enzyme substitution therapy with r*Av*PAL in diverting the natural course of the disease in early treated adult ENU2 mice (ENU2-r*Av*PAL-RBC); b) testing clinical advantages and effectiveness of adopting erythrocytes as vehicle of the enzyme.

2. Methods

2.1. Animals

Developing homozygous Pah$^{enu2-/-}$ (ENU2) and Pah$^{enu2+/+}$ (wild type; WT) male mice of BTBR background strain were issued from heterozygous mating Pah$^{enu2+/-}$. Genetic characterization was performed as described [17]. Two groups of ENU2 mice (ENU2-r*Av*PAL-RBC, n = 9; ENU2-veh, n = 7) and one group of healthy genetic background (WT-veh, n = 13) mice were used for behavioral, biochemical, morphological and molecular analyses as described below. Animals were housed in standard cages, 3 to 6 mice per cage, on a 12 h light: dark cycle and in controlled conditions (temperature 22 ± 1 °C, humidity 60%, air change every 12 h); all mice were fed on Teklad global 18% protein rodent diet (Harlan Laboratories Inc., Madison, WI) and water *ad libitum*.

2.2. Study approval

All experiments were conducted in accordance with European legislation (2010/63/UE), with Italian national legislation (DL26/2014) governing the use of animals for research and with the guidelines of the National Institute of Health on the use and the care of laboratory animals (Authorization n° 486/2017-PR).

2.3. rAvPAL

r*Av*PAL was prepared at 110 mg/ml in Tris-buffered saline containing 2 mM Phe and provided by the BioMarin Protein Sciences Research group. Specific Activity (SA) was 1.85 IU/mg.

2.4. Development of murine rAvPAL-RBC and treatment

Blood was collected by beheading anesthetized adult BTBR-WT and Pahenu2+/− mice in heparinized tubes and r*Av*PAL was loaded into murine RBC by means of hypotonic dialysis, isotonic resealing and "reannealing", essentially according to Rossi et al. [16]. The amount of entrapped r*Av*PAL was quantified essentially by a kinetic assay as previously described [8,16]. Percent RBC recovery was calculated from the number of RBC submitted to the dialysis step and those recovered at the end of the loading procedure. Final packed r*Av*PAL-loaded RBC were re-suspended in Hepes solution at approx. 25% Ht and *iv* infusions of these suspensions, ranging 50–250 µl, were performed based on the weight gain of developing animals in order to administer 0.03 IU r*Av*PAL/g body weight. For the experiment, untreated ENU2 mice (ENU2-veh) (n = 7) and untreated healthy mice (WT-veh) (n = 13) were subjected to the same manipulations and received repeated *iv* injections of saline solution (NaCl 0.9% W/V) following the same schedule as the ENU2-r*Av*PAL-RBC mice (n = 9).

Mice were aged 15 days at the beginning of the treatment. Briefly, ENU2-r*Av*PAL-RBC mice were treated with *iv* injections of r*Av*PAL-loaded RBC (0.03 IU/g BW) from PND 15 to PND 64. On the bases of a previous study [16], to obtain a stable value of blood Phe the time interval between subsequent infusions was 7 days.

2.5. Phe and Tyr evaluation in dried blood spot (DBS)

Mouse whole blood was collected on Whatman TM 903, dried under ambient conditions and stored at 4–8 °C in plastic bags. A 3-mm diameter dot was punched from the DBS into a single well of 96-well micro plate. The analysis of Phe and Tyr in the DBS was performed using a previously published method [18] with some modifications [16].

2.6. Behavior

The apparatus of Elevated Plus Maze (EPM) and the Object Recognition Test (ORT) were previously described [19] and behaviors were analyzed by Video-based EthoVision System (Noldus, The Netherlands). Three groups of male mice (WT-veh, n = 12; ENU2-veh, n = 7; ENU2-r*Av*PAL-RBC, n = 9) were submitted to EPM and ORT, in this order.

2.6.1. Behavioral assay in EPM apparatus

The moved distance in the apparatus (cm), the velocity (cm/s), the number of total entries in the arms (*sec*), the percentage of time spent in open arms (time in open/open closed × 100), the percentage of entries in the open arms (open entries/open closed × 100) were evaluated. One-way ANOVA, followed by post-hoc Duncan's test for multiple comparisons, was used for statistical analysis of the effects of groups (WT-veh, ENU2-veh, ENU2-r*Av*PAL-RBC) on all parameters.

2.6.2. Behavioral assay in ORT apparatus

Each mouse was individually submitted to three successive 6-min sessions (Open Field, Pretest and Test sessions).

During the first session (Open Field) the distance moved and velocity were analyzed by one-way ANOVA, followed by post-hoc Duncan's test for multiple comparisons (group: three levels = WT-veh, ENU2-veh and ENU2-r*Av*PAL-RBC as factor).

In the second session (Pretest) the total time spent exploring objects on pretest session was analyzed by one-way ANOVA (group: three levels = WT-veh, ENU2-veh and ENU2-r*Av*PAL-RBC as factor), followed by post-hoc Duncan's test.

In the Test session the time exploring each object on the test session was evaluated by two-way ANOVA for repeated measure ("object" as within factor: two levels = familiar and novel, and group: three levels = WT-veh, ENU2-veh and ENU2-r*Av*PAL-RBC as between factor).