Original Article
Phenylketonuria-related cognitive dysfunction and its mechanism in a BTBR-*Pah*[enu2] mouse model

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Abstract: Objective: Phenylketonuria is the most common inherited aminoacidopathy, characterized by elevated level of phenylalanine in the plasma and cerebrospinal fluid of patients, which eventually leads to cognitive dysfunction. However, the underlying mechanisms are not clear yet. In the present study, we aimed to examine the spatial cognitive function in the phenylketonuria mouse model and explore the underlying molecular mechanisms. Methods: We chose BTBR-Pah[enu2] mouse, the most classical model of phenylketonuria, for our study of cognitive function. Genotyping was performed by blood phenylalanine measurement and genetic methods. We examined the spatial cognitive function in eight-arm maze test by comparing the performance of wild type and homozygous BTBR-Pah[enu2] mice. Subsequently we investigated the expression and phosphorylation of Akt/GSK-3β/β-Catenin pathway in the prefrontal cortex and CA1 region of the hippocampus by western blot. Results: Homozygous BTBR-Pah[enu2] mice showed impaired spatial cognitive function in the eight-arm maze test. Subsequent experiments confirmed that the phosphorylation of Akt and GSK-3β was significantly lower in the prefrontal cortex and CA1 of the homozygous BTBR-Pah[enu2] mice, together with a decrease of β-catenin expression. There was no change in the expression of Akt and GSK-3β. Conclusions: The activity of the Akt/GSK-3β/β-Catenin pathway was inhibited in the prefrontal cortex and CA1 of the homozygous BTBR-Pah[enu2] mice. Considering the important role of Akt/GSK-3β/β-Catenin pathway in cognition, these changes may be critical pathological mechanisms underlying cognitive dysfunction in phenylketonuria. These findings provide direct evidence that Akt/GSK-3β/β-Catenin pathway is involved in the brain injury of phenylketonuria.

Keywords: Akt/GSK-3β/β-catenin pathway, BTBR-*Pah*[enu2], cognition, phenylketonuria

Introduction

Phenylketonuria (PKU) is the most frequent inborn aminoacidopathy caused by deficiency of phenylalanine hydroxylase, resulting in an accumulation of phenylalanine in the brain, which disturbs brain development and leads to progressive mental retardation and cognitive defects [1]. Low phenylalanine dietary treatment is the main effective therapy. Extensive research has been done regarding the neuropsychological performance of patients. Although strict dietary adherence prevents profound neurologic impairment, cognitive outcome remains suboptimal. Subtle cognitive deficits remain persistent and are detectable in patients even treated early and continuously [2]. Individuals with early-treated phenylketon-

uria most often present with average intelligence or impairment of cognitive function, compared to the general population [3, 4]. The deficits in cognition include executive functioning, academic abilities, speed of response, interhemispheric transfer of information, attention, and visual-spatial and visual-motor abilities [5, 6]. Their non-executive function is also compromised, such as information processing speed, fine motor skills, and perception and visual-spatial abilities [4]. Although the incidence of cognitive deficits is reported extensively, the underlying molecular mechanisms of brain damage are still poorly understood. It is reported that the obstinate cognitive deficits may relate to the deficiency of dopamine in the prefrontal cortex (PFC), abnormalities of myelination [4], or impaired white matter integrity [7].

These cannot fully explain the general disturbance of neuronal function, and other convergent mechanisms of neuronal modulation may underlie the cognitive deficits of PKU patients.

The Akt/GSK-3β/β-catenin pathway is well known for its role in cell cycle and apoptosis. Accumulating evidence implicates its role in tumor genesis, neurogenesis and neuronal differentiation [8]. Besides, it is also involved in neuropsychiatric disorders, modulating long term memory and cognition [9, 10].

Akt, also named PKB or Rac, plays a critical role in controlling cell growth and apoptosis. Akt promotes cell survival by inhibiting apoptosis through phosphorylation and inactivation of target proteins, such as GSK-3β. The activity of Akt is enhanced after phosphorylation at Ser473 [11]. Glycogen synthase kinase-3β (GSK-3β) is a serine/threonine protein kinase, densely expressed in the brain. It is a critical downstream protein of the Akt cell survival pathway. Its activity can be inhibited by Akt-mediated phosphorylation at Ser9 [10]. GSK3β plays an important role in brain development and memory formation by influencing neuronal growth and differentiation [12]. It is also involved in several pathophysiological processes, including autophagy [13], neural inflammation and oxidative stress [14]. Additionally, GSK3ß is a centre-staged kinase in neuropsychiatric disorders. Abundant evidence implicates deregulation of GSK3β is a key pathological event in neuropsychiatric disorders such as Alzheimer's disease [10]. GSK-3β is also involved in the Wnt signaling pathway. It is the key enzyme regulating β-catenin stability, and its translocation to the nucleus, as well as the transcriptional activity. GSK-3β destabilizes β-catenin by phosphorylation. Besides the well-known role in early embryonic development and tumorigenesis, β-catenin has also been implicated in synapse regulation and remodeling, playing a general role in memory consolidation. It associates with other proteins and links to the actin cytoskeleton. The complex of β-catenin and actin cytoskeletion is localized in synaptic junctions. Alteration of the complex influences synaptic size and strength. The complex is involved not only in the development of synapse but also in the modulation of synaptic connectivity and activity [9].

The hippocampus is a key structure of memory formation and storage in cognition. The pre-frontal cortex (PFC) is also a crucial locus for the control of cognitive function [15], and PFC is extremely sensitive to phenylalanine-induced disturbances [16]. Animal model is a useful tool for identifying the metabolic mechanisms underlying cognitive deficits induced by phenylalanine. The BTBR-Pah[enu2] mouse is characterized by the classic biochemical phenotypes closely resembling untreated human PKU, providing an excellent animal model to assess the cognitive consequence of high phenylalanine [17].

In the present study, we tested the cognitive ability of the wild type mice (WT, +/+) and homozygous BTBR-Pah[enu2] mice (PKU, -/-) by eight arm maze test. Further, we examined whether the Akt/GSK-3β/β-Catenin pathway, which is closely related to cognitive function and synapse development, shows any abnormalities in the PFC and CA1 of the BTBR-Pah[enu2] mouse model.

Materials and methods

Animals

All procedures were carried out in accordance with the National Regulations on the Administration of Laboratory Animals. Ethical approval was obtained from the Ethics Committee of Xin Hua Hospital. BTBR-Pah[enu2] breeding pairs were purchased from the Jackson Laboratory (USA), with a T835C point mutation in exon 7 of the gene *PAH* [17]. The mutation leads to classic PKU with a reduced activity of phenylalanine hydroxylase, exactly mimicking human PKU. WT, heterozygous type (HET), and PKU pups were produced by the breeding pairs. 2-Month-old (2 M) and 8-month-old (8 M) PKU and WT mice were selected. Only age-matched male mice were used in our experiments. A minimum of six mice were used per group.

Genotyping

Two methods were used to identify their genotypes: blood phenylalanine assay and analysis of the restriction fragment length polymorphism.

Blood from the tail of each mouse was dropped onto a standard filter paper used in the newborn screening program. Blood phenylalanine concentration was determined by fluorescence detection method performed by the Shanghai

Newborn Screening Program at the Shanghai Xin Hua Hospital. The blood phenylalanine concentrations of the PKU, HET, and WT mice were 18.57±0.99 mg/dl, 0.80±0.07 mg/dl and 0.66±0.07 mg/dl, respectively. The PKU mice had an approximately 20 times higher blood phenylalanine level than the HET and WT mice. The HET mice, like humans who are heterozygous for PKU, had a blood phenylalanine level as low as the WT mice. The data was in consistence with previous reports.

To distinguish HET mice from WT ones, we used restriction fragment length polymorphism analysis. Genomic DNA was extracted from 1cm of tail (Gentra Puregene Mouse tail Kit, Qiagen, Germany). Exon 7 of the gene Pah was amplified with specific primers (F: 5'-ACTTGTACTG-GTTTCCGCCT-3'; R: 5'-CTTGGCTACTCTTGTTCC-TGTC-3'). A 421 bp PCR product was then subjected to digestion with restriction endonuclease BsmAI (New England Biolabs, USA) and the fragments were separated by electrophoresis. The digested fragment combinations were: WT: 372 bp and 49 bp; HET: 372 bp, 338 bp, 49 bp, and 34 bp; PKU: 338 bp, 49 bp, and 34 bp.

Eight-arm radial cognitive maze test

The apparatus of the eight-arm radial cognitive maze used was in accordance with that previously described by Gazit V [18]. Seven days before the start of pre-training, the mice were housed individually and given restricted access to food until their body weight was reduced to 80-85% of their free-feeding weight. Throughout the experiment, the reduced weight would be maintained by restricted feeding. Pretraining started on the eighth day of the whole test. Once all the mice had learned to run freely through the maze and to consume the pellet rewards readily, the actual maze acquisition trials began. All eight arms were baited with pellets. The mice were placed individually on the central platform. They were given 15 min to find and consume all eight pellets. A trial was terminated immediately after all eight pellets had been consumed, or the 15 min had elapsed. An "arm visit" was defined as traveling more than 5cm into the arm. Each mouse performed one trial per day, for eight consecutive days. For each trial the numbers of different entries during the first eight choices and the latency to obtain all pellets were recorded manually. A minimum of ten mice were used per group.

Western blot

After the mice had been euthanized, the PFC and CA1 strata radiate were collected and homogenized. The supernatant containing the peptides was collected by centrifugation. The protein concentration was determined by BCA assay. After denaturation, 40 μg protein for each sample was subjected to SDS-PAGE for electrophoresis at 90 V for 2 hours, and then electrotransferred onto a polyvinylidene difluoride membrane at 250 mA for 2 hours. After having been blocked for 1 hour, the membrane was immunolabeled with the following specific primary antibodies overnight at 4°C: anti-Akt (1:1000, Cell Signaling Technology, USA), anti-pSer473-Akt (1:1000, Cell Signaling Technology, USA), anti-GSK-3β (1:1000, Cell Signaling Technology, USA), anti-pSer9-GSK-3β (1:1000, Cell Signaling Technology, USA), anti-β-catenin (1:1000, Cell Signaling Technology, USA) and GAPDH (1:10,000, Kangchen, China). Then, the membrane was incubated for 1 hour at room temperature with corresponding secondary antibodies: Peroxidase-Conjugated Goat Anti-Rabbit IgG (1:10000, Jackson ImmunoResearch, USA) and Peroxidase-Conjugated Goat Anti-Mouse IgG (1:5000, Jackson Immuno-Research, USA). Subsequently, the membranes were added with the enhanced chemiluminescence (Pierce Biotechnology, USA). Finally, the bands were recorded and quantified using the Image Lab software package (Bio-Rad, USA). The GAPDH blots served as internal standards. The optical density of phosphorylated proteins was normalized for each sample by their respective GAPDH. A minimum of six mice were used per group.

Statistical analysis

The statistical analysis was performed using the SPSS 17.0 software package (SPSS, USA). All the data were analyzed using Student's t-test for comparison of two groups.

Differences were considered significant at $P < 0.05$.

Results

Behavioral observations of the two groups of mice

In the eight-arm maze, the PKU mice exhibited a lower number of correct entries among the
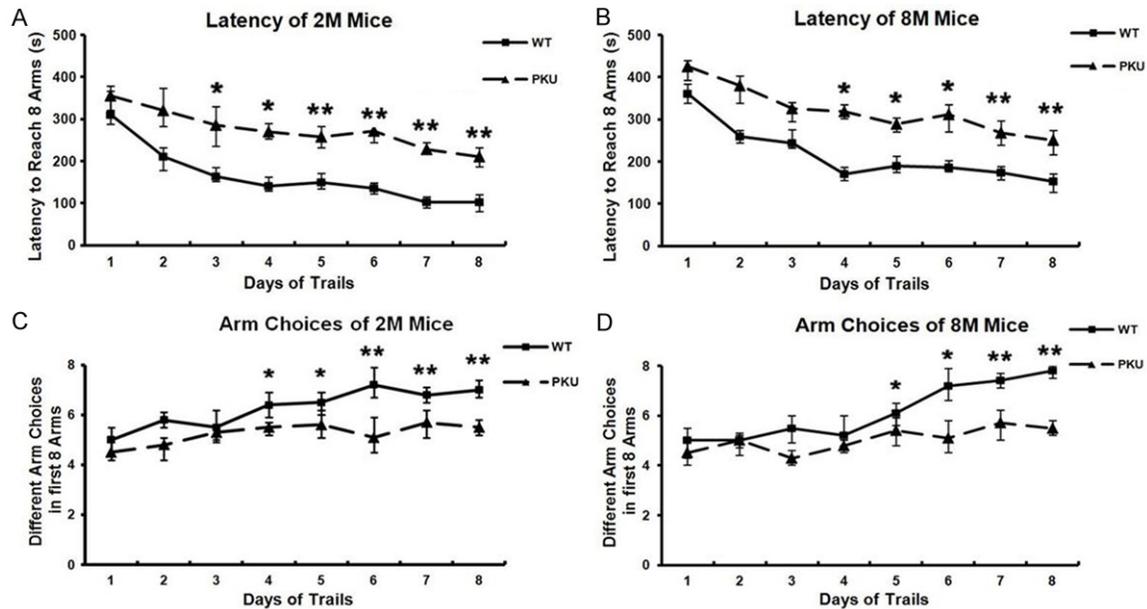
Figure 1. Behavioral responses in the eight arms maze by WT and PKU mice of two ages. (A, B) The latency to obtain all the pellets for the two groups of mice (A: 2 M; B: 8 M). (C, D) The number of correct entries into eight-arm maze among the first eight attempts for the two groups of mice (C: 2 M; D: 8 M). *$P < 0.05$, **$P < 0.01$, compared with the WT group. —▲— PKU mice; —■— WT mice.
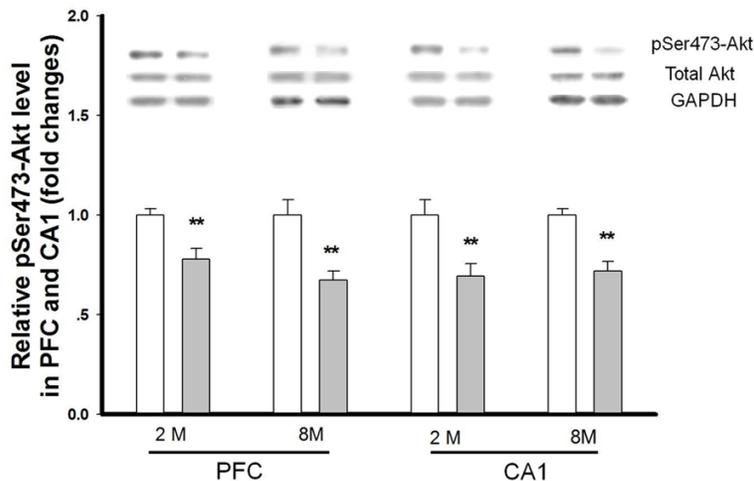
Figure 2. Level of pSer473-Akt in the CA1 and PFC of the WT and PKU mice of two ages. The optical density of pSer473-Akt in the PFC and CA1 is decreased in the 2 M and 8 M PKU mice. Upper bands: pSer473-Akt; middle bands: total Akt; lower bands: GAPDH (□ WT; ▨ PKU). Data are expressed as mean ± S.E.M. *$P < 0.05$, **$P < 0.01$, compared with the WT group.

The PKU mice displayed longer latencies than the WT mice from the third day for the 2 M mice and from the fourth day for the 8 M mice. The detailed data of every day of the trial has been summarized in **Figure 1**. This observation manifested the defective spatial cognition in the PKU mice.

Investigation of phosphorylation of the Akt/GSK-3β/β-catenin signaling pathway in the PFC and CA1 of the two groups of mice

Normalized by GAPDH in westernblot, the signal of pSer473-Akt strongly decreased in both the PFC and CA1 of PKU mice, compared to the WT mice (PFC: 78% 2 M, 67% 8 M; CA1: 69% 2 M, 71% 8 M; **Figure 2**). The normalized optical density of pSer9-GSK-3β was also strongly decreased in the two encephalic regions of PKU mice, compared to the WT mice (PFC: 70% 2 M, 65% 8 M; CA1:

first eight attempts and an obviously longer latency to obtain all the pellets compared with the WT controls. The PKU mice showed significantly fewer correct choices than the WT mice from the fourth day of the experiment for the 2 M mice and from the fifth day for the 8 M mice.
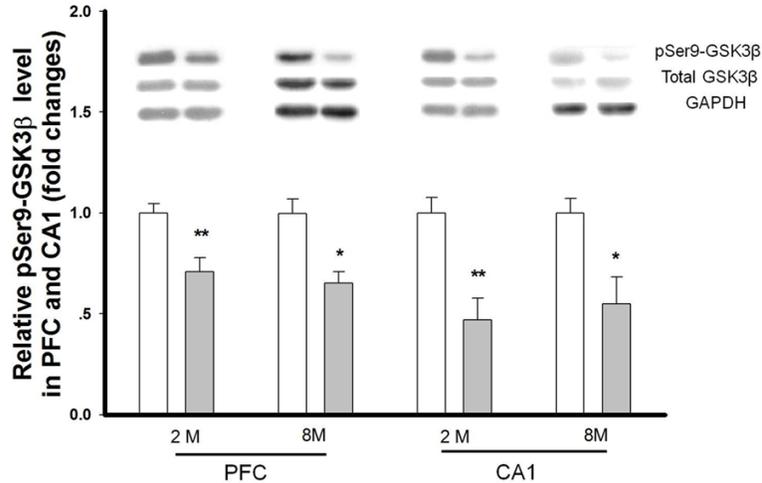
Figure 3. Level of pSer9-GSK3β in the CA1 and PFC of the WT and PKU mice of two ages. The optical density of pSer9-GSK-3β in the PFC and CA1 is decreased in the 2 M and 8 M PKU mice. Upper bands: pSer9-GSK-3β; middle bands: total GSK-3β; lower bands: GAPDH (☐ WT; ▨ PKU). Data are expressed as mean ± S.E.M. *P < 0.05, **P < 0.01, compared with the WT group.
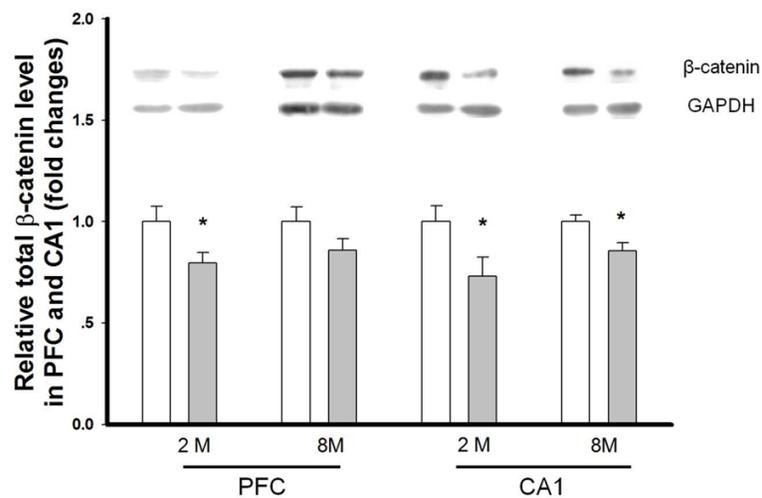
Figure 4. Expression of β-catenin in the CA1 and PFC of the WT and PKU mice of two ages. The optical density of the total β-catenin is decreased in the two encephalic regions of 2 M PKU mice and the CA1 of the 8 M PKU mice. Upper bands: β-catenin; lower bands: GAPDH (☐ WT; ▨ PKU). Data are expressed as mean ± S.E.M. *P < 0.05, **P < 0.01, compared with the WT group.

46% 2 M, 55% 8 M; **Figure 3**). The normalized optical density of the total Akt and total GSK-3β was nearly the same between the two groups, while the total β-catenin decreased in the two encephalic regions of 2 M PKU mice and the CA1 of the 8 M PKU mice, compared to the WT mice (PFC: 80% 2 M; CA1: 73% 2 M, 85% 8 M; **Figure 4**). The expression of total

β-catenin in the PFC of the 8 M PKU mice was also slightly decreased compared with the WT mice, but without significant statistical differences.

Discussion

Our study revealed impaired spatial cognition and abnormal phosphorylation of Akt/GSK-3β/β-catenin signaling pathway in BTBR-Pah[enu2] mice by behavioral test and biochemical analysis.

In the behavioral test, compared with the WT controls, PKU mice showed poor performance in the eight-arm maze, with a lower number of correct entries among the first eight attempts and an obviously longer latency to obtain all the pellets. Just as reported in PKU patients, motor deficits also exist in PKU mice [19]. However, the motor deficits are not accountable for the poor performance in the cognitive tests, as the impaired basic locomotion dose not influence the choice of the arms.

In our study, the expression of total proteins of Akt and GSK-3β were not different between the two groups of any age. Besides expression, phosphorylation also plays an essential role in protein function. As shown in the result, the phosphorylation of Akt and GSK was dramatically decreased and the expression of β-catenin was also decreased in the brain of PKU mice. It was consistenet with our prior study, in which the phenylalanine induced rat cortical neurons showed defects of neurite outgrowth and lower expression of BDNF, accompanied by a decrease in Akt phosphorylation activity [20]. In the Akt/GSK-3β/β-catenin signaling pathway, β-catenin is under control of GSK-3β. Under baseline con-

ditions, GSK-3β is constitutively active and destabilizes β-catenin by phosphorylation, which leads to its proteasomal degradation [21]. In addition, GSK-3β can be inactivated by phosphorylation of the Ser 9 residue through phosphorylated Akt. As a result, phosphorylation of GSK-3β stabilizes β-catenin, which then migrates into the nucleus and mediates gene transcription, and thereby influences the development of synapse and cognitive function. The activity status of GSK-3β and its influence on β-catenin levels are consistent with the findings in our study, with decreased pSer9-GSK-3β increased GSK-3β activity and decreased β-catenin levels in the CA1 and PFC of PKU mice, when compared with WT mice. As shown in our results, pSer473-Akt and pSer9-GSK-3β were decreased in the PFC and CA1 of the 2 M and 8 M PKU mice. Therefore, more active GSK-3β would destabilize β-catenin by phosphorylation and then lead to decreased β-catenin expression level. For the β-catenin can then migrate into the nucleus and mediate neural development related genes transcription, the decreased β-catenin would influence development of neuron and cognitive function afterwards. On the other hand, besides β-catenin, Akt/GSK3β affect other downstream proteins with different functions. The decreased phosphorylated Akt GSK3β might lead to the cognitive consequence via other downstream molecules and pathways as well.

Early development of the hippocampus, which is essential for spatial memory and learning, is controlled by secreted Wnt gene family and by Wnt/β-catenin signaling [22]. The Wnt gene family is critically involved in neurogenesis and synaptic plasticity. They play important roles in postsynaptic dendritic spine morphogenesis and neurotransmitter release at presynaptic terminals [23], through a set of direct target genes including β-catenin [22]. In our prior research, abnormalities of the dendritic spine and synapse ultrastructure were found in neurons of BTBR-Pah^{enu2} mice, such as reduced density of dendritic spines, shortened length of the presynaptic active zone, a widened synaptic cleft, and decreased thickness of postsynaptic density [24]. We suspect that Wnt signaling dysfunction may exist in BTBR-Pah^{enu2} mice, and the impaired β-catenin function may be partially due to that. This hypothesis needs data support in further investigation.

The current study is the first report to discover the dysfunction of the Akt/GSK-3β/β-catenin signaling pathway in PKU. The results may suggest a possible mechanism for the development of PKU-induced cognitive deficits. We presumed that this pathomechanism may be responsible at least in part for the severe cognitive deficit. This may help identify the pathway as a potential therapeutic target in preserving cognition during PKU.

Conclusion

The present study described the impaired spatial cognition of the PKU mouse by eight arm maze test for the first time. The phosphorylation and expression of the Akt/GSK-3β/β-catenin signaling pathway were decreased in the PFC and CA1 of the PKU mouse model. Our results suggest that the disturbed Akt/GSK-3β/β-catenin signaling pathway may be one of the mechanisms contributing to the cognitive dysfunction observed in PKU.

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Disclosure of conflict of interest

None.

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