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Arctigenin Attenuates Learning and Memory Deficits through PI3k/Akt/GSK-3β Pathway Reducing Tau Hyperphosphorylation in Aβ-Induced AD Mice

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ABSTRACT

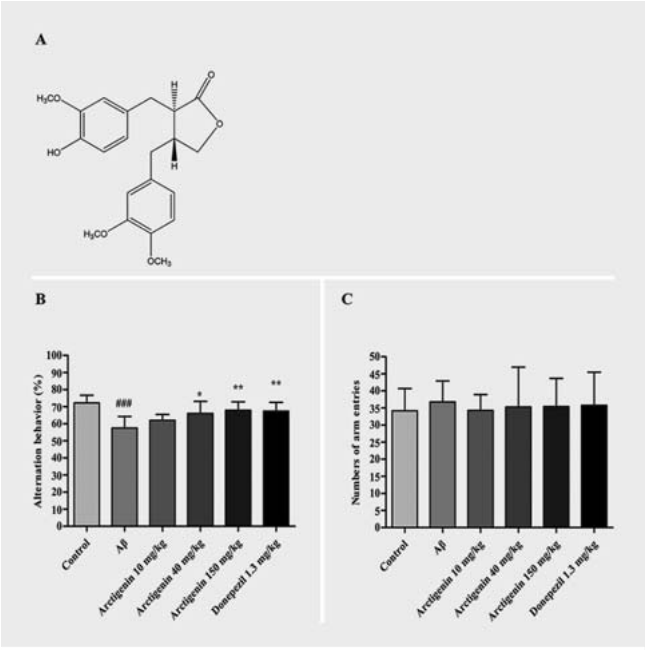
Arctigenin is a phenylpropanoid dibenzylbutyrolactone lignan compound possessing antitumor, anti-inflammatory, anti-influenza, antioxidant, antibacterial, and hypoglycaemic activities. Our previous study demonstrated that arctigenin exerts neuroprotective effects both *in vitro* and *in vivo* in a Parkinson's disease model. However, the exact mechanism through which arctigenin improves amyloid beta-induced memory impairment by inhibiting the production of the hyperphosphorylated tau protein is unknown. Amyloid β_{1-42} was slowly administered via the intracerebroventricular route in a volume of 3 µL (≈ 410 pmmol/mouse) to mice. The mice were administered arctigenin (10, 40, or 150 mg/kg) or vehicle starting from the second day after amyloid β_{1-42} injection to the end of the experiment. Behavioural tests were performed from days 9 to 15. On day 16 after the intracerebroventricular administration of amyloid β_{1-42}, the mice were sacrificed for biochemical analysis. Arctigenin (10–150 mg/kg) significantly attenuated the impairment of spontaneous alternation behaviours in the Y-maze task, decreased the escape latency in the Morris water maze test, and increased the swimming times and swimming distances to the platform located in the probe test. Arctigenin attenuated the level of phosphorylated tau at the Thr-181, Thr-231, and Ser-404 sites in the hippocampus, and increased the phosphorylation levels of phosphatidylinositol-3-kinase, threonine/serine protein kinase B, and glycogen synthase kinase-3β. Arctigenin effectively provides protection against learning and memory deficits and in inhibits hyperphosphorylated tau protein expression in the hippocampus. The possible mechanism may occur via the phosphatidylinositol-3-kinase/protein kinase B-dependent glycogen synthase kinase-3β signalling pathway.

Introduction

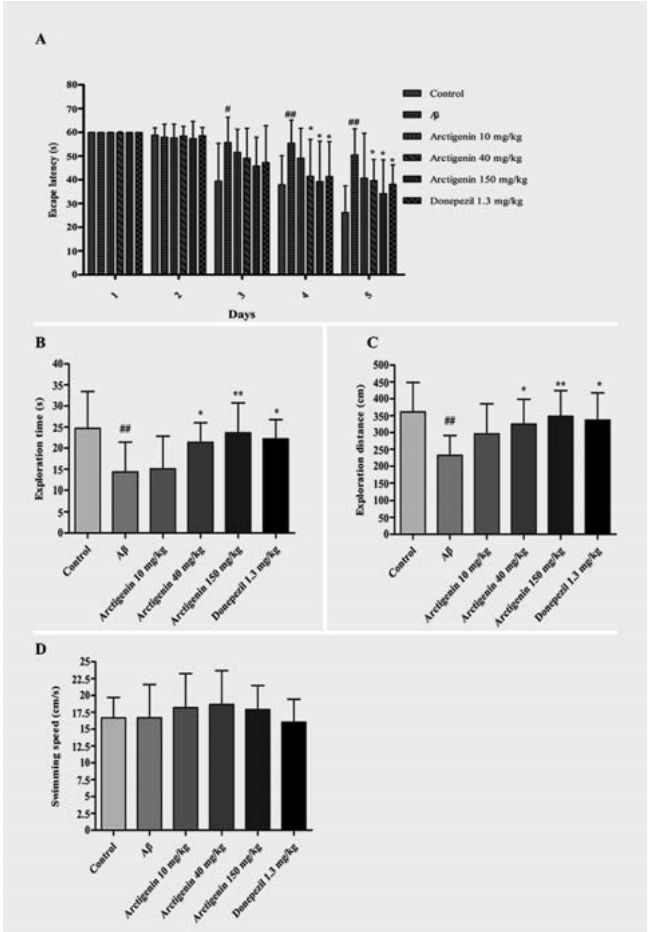
Alzheimer's disease (AD) is a neurological condition characterised by progressive cognitive deterioration and loss of memory. Extracellular β-amyloid (Aβ) deposits are proposed to serve as initial pathological hallmarks of AD [1, 2]. The deposition of Aβ in AD may lead to the formation of senile plaques (SPs) followed by intraneuronal neurofibrillary tangles (NFTs) composed of aggregates of hyperphosphorylated tau [3]. Tau proteins are microtubule-associated proteins mainly expressed in neurons. Physiologically, their primary function is to stabilise the neuronal cytoskeleton [4]. However, tau phosphorylation may affect the ability of the tau protein to bind microtubules and promote their assembly, resulting in cognitive impairments [5, 6]. Based on these observations, inhibiting Aβ-induced tau phosphorylation is of great importance for the development of therapeutic strategies in AD.

Various kinases and phosphatases are involved in the regulation of tau phosphorylation [7]. Glycogen synthase kinase-3β (GSK-3β), a major tau kinase, is a downstream target of the phosphatidylinositol-3-kinase (PI3K)/threonine/serine protein kinase B (Akt) signalling pathway. Substantial evidence shows that PI3K/Akt activation prevents Aβ-induced neurotoxicity in cells [8] and in mouse models of AD [9]. GSK-3β regulates both tau phosphorylation and Aβ production [10, 11] through inactivation of the PI3K/Akt pathway. Therefore, the PI3K/Akt/GSK-3β signalling pathway is an important target for the diagnosis and treatment of AD.

Arctigenin is a phenylpropanoid dibenzylbutyrolactone lignan compound that occurs naturally in *Arctium lappa* L. (Asteraceae), Bardanae Fructus, *Ipomoea cairica* (L.) Sweet (Convolvulaceae), *Saussurea medusa* Maxim. (Asteraceae), and *Torreya nucifera* (L.) Siebold & Zucc. (Taxaceae) [12, 13]. Previous studies have shown that arctigenin possesses antitumour, anti-inflammatory, anti-influenza, antioxidant, antibacterial, and hypoglycaemic activities [14, 15]. Our previous study demonstrated that arctigenin exerts



▶ **Fig. 1** Effects of arctigenin on learning and memory deficits induced by ICV Aβ in the Y-maze test. **A** Chemical structure of arctigrenin. **B** Alternation (%) from Y-maze test. **C** The total number of arm entries from the Y-maze test. All of the results are expressed as the means ± SD; n = 8 animals; $^{###}p < 0.001$ vs. control; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Aβ.



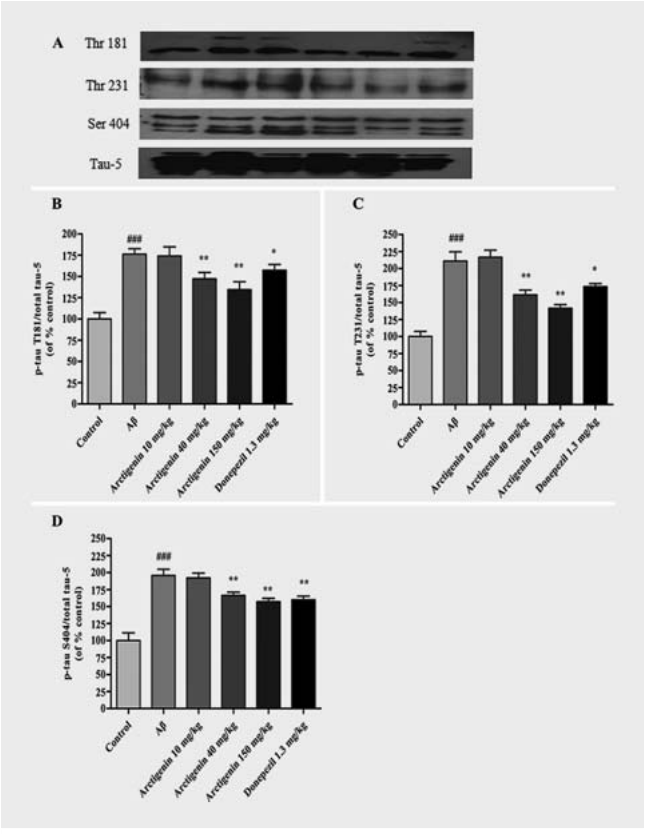
▶ **Fig. 2** Effects of arctigenin on learning and memory deficits induced by ICV Aβ in the Morris water maze test. **A** Changes in the latency to reach the platform during the training period. Changes in the swimming time (**B**) and swimming distance (**C**) in the target quadrant in the probe trial. Aβ-treated mice exhibited a decrease in the swimming time and swimming distance in the target quadrant compared to the control mice. These effects were reversed by treatment with arctigenin. **D** No significant differences in swim speed were observed among the groups. All of the results are expressed as the means ± SD; n = 8 animals; $^{#}p < 0.05$, $^{##}p < 0.01$ vs. control; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Aβ.

neuroprotective effects both *in vitro* and *in vivo* in a Parkinson's disease model [16]. This present study examines the effect of arctigenin on learning and memory impairments induced by an injection of amyloid beta ($A\beta_{1-42}$) in a mouse model. Furthermore, its effects on hyperphosphorylated tau proteins and the possible mechanisms were investigated.

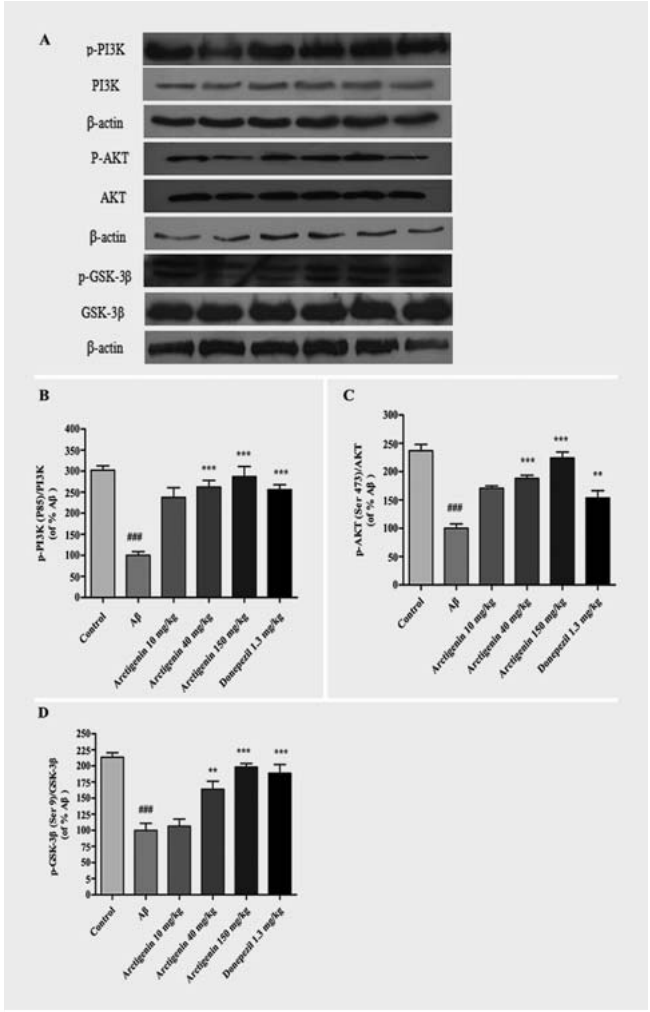
Results

The effect of arctigenin on spontaneous alternation behaviour was evaluated using a Y-maze test. As shown in ▶ **Fig. 1 B**, the model group exhibited significantly reduced spontaneous alternation behaviour compared to the control group [$F(5,42) = 6.986$, $p < 0.001$]. Arctigenin (40 and 150 mg/kg) attenuated the impairment of spontaneous alternation behaviour. LSD post hoc analysis showed that the 40 and 150 mg/kg groups treated with arctigenin presented significantly alleviated effects ($p < 0.05$; ▶ **Fig. 1 B**). The group administered donepezil (1.3 mg/kg) was used as a positive control. The total number of arm entries was not significantly different among the groups [$F(5,42) = 0.114$, $p > 0.05$; ▶ **Fig. 1 C**].

To test whether $A\beta_{1-42}$ induces spatial memory deficits in mice, we assessed the behavioural performance of the mice using the Morris water maze test. During the training period, a significant difference in the performance of six different mouse groups was observed [$F_{group} = 5.596$, $p < 0.001$; $F_{day} = 37.349$, $p < 0.001$; $F_{group \times day} = 1.178$, $p = 0.276$; ▶ **Fig. 2 A**]. The mice in the Aβ group consistently took longer to find the platform than those in the control group (▶ **Fig. 2 A**). Post hoc analyses indicated a significant decrease in the escape latency of mice treated with 150 mg/kg and 40 mg/kg arctigenin ($p < 0.05$). In the probe test, the exploration time spent and the distance travelled by the Aβ mice in searching for the target quadrant were significantly lower compared with the control mice, and the mice treated with arctigenin (10, 40, and 150 mg/kg) spent more time [$F(5,42) = 3.361$, $p < 0.05$; ▶ **Fig. 2 B**] and travelled a longer distance [$F(5,42) = 2.846$, $p < 0.05$; ▶ **Fig. 2 C**] in their attempt to search for the target quadrant compared with the Aβ-treated mice. LSD post hoc analyses showed that the arctigenin-treated mice in all three dose groups demonstrated a significant improvement ($p < 0.05$). No significant differences in swim speed were observed among



▶ **Fig. 3** Effects of arctigenin on the expression of Thr-181, Thr-231 Ser-404, and Tau-5 in the hippocampus (**A**). Arctigenin (40 mg/kg and 150 mg/kg) significantly decrease Thr-181 (**B**), Thr-231 (**C**), and Ser-404 (**D**), as determined by Western blotting. All of the results are expressed as the means ± SD; n = 3 animals; ###p < 0.001 vs. control; *p < 0.05, **p < 0.01 vs. Aβ.



▶ **Fig. 4** Effects of arctigenin on the expression of p-PI3K, p-AKT, and p-GSK-3β in the hippocampus (**A**). Arctigenin (40 mg/kg and 150 mg/kg) significantly increase p-PI3K (**B**), p-AKT (**C**), and p-GSK-3β (**D**) protein expression levels, as determined by Western blotting. All of the results are expressed as the means ± SD; n = 3 animals; ###p < 0.001 vs. control; **p < 0.01, ***p < 0.01 vs. Aβ.

the animal groups [F(5,42) = 0.471, p = 0.796; ▶ **Fig. 2 D**]. Donepezil also significantly increased the time and distance spent searching for the target quadrant. Taken together, these data suggest that arctigenin ameliorates learning and memory deficits induced by intracerebroventricular (ICV) Aβ_{1-42} in mice.

We found that arctigenin inhibits the production of phosphorylated tau and measured the expression of phosphorylated tau through Western blot analysis. The results indicate that the level of phosphorylated tau at the Thr-181, Thr-231, and Ser-404 sites in the hippocampus was significantly higher in the Aβ group than in the control group [Thr-231: F(5,12) = 74.542, p < 0.01; Ser-231: F(5,12) = 35.584, p < 0.01; Ser-404: F(5,12) = 62.515, p < 0.01; ▶ **Fig. 3**], but was significantly lower in the group treated with arctigenin than in the Aβ group (p < 0.01, ▶ **Fig. 3**).

We examined the phosphorylation levels of PI3K, Akt, and GSK-3β to investigate the potential involvement of the PI3K/Akt pathway in the arctigenin-induced protection against phosphorylated tau. Aβ injection significantly decreased the phosphorylation levels of PI3K [F(5,12) = 60.413, p < 0.001; ▶ **Fig. 4**], Akt [F(5,12) = 92.591, p < 0.001; ▶ **Fig. 4**], and GSK-3β [F(5,12) = 4.328, p < 0.05; ▶ **Fig. 4**]. Treatment with arctigenin at doses of 150 mg/kg and 40 mg/kg significantly increased the phosphorylation levels of PI3K (p < 0.01; ▶ **Fig. 4**), Akt (p < 0.01; ▶ **Fig. 4**), and GSK-3β (p < 0.05; ▶ **Fig. 4**).

Discussion

Extracellular Aβ deposits and intracellular neurofibrillary tangles in the brain parenchyma involved in cognitive functions are assumed to be pathological hallmarks of AD. Abnormal fibres composed of hyperphosphorylated tau protein can form NFTs in neuronal cell bodies or other cell types of the brain [17]. Arctigenin treatment was reported to be effective in reversing the deficits in memory and learning by using APP/PS1 transgenic AD mice models. These improvements are hypothesised to be regulated by both the inhibition of Aβ production and promoting Aβ clearance through the AKT/mTOR and AMPK/Raptor signalling pathways [18], but the

mechanism through which arctigenin improves Aβ-induced memory impairment by inhibiting tau hyperphosphorylation *in vivo* has not been elucidated. In this study, we demonstrated that arctigenin effectively provides protection against learning and memory deficits and in inhibiting tau hyperphosphorylation in the hippocampus. The possible mechanism involves inhibition of the PI3K/Akt-dependent GSK-3β signalling pathway.

Because AD patients display learning and memory disabilities, in this study, we investigated the behavioural changes in $A\beta_{1-42}$-treated mice administered arctigenin using the Y-maze test and the Morris water maze test. The results showed that the ICV injection of $A\beta_{1-42}$ caused impairments in learning and memory performance of the mice and that treatment with arctigenin significantly attenuated the $A\beta_{1-42}$-induced spatial learning and memory impairments in a dose-dependent manner.

Tau is a major microtubule-associated protein that plays a key role in the outgrowth of neuronal processes and the development of neuronal polarity. Abnormal levels and hyperphosphorylation of the tau protein are the underlying cause of a group of neurodegenerative diseases, including AD. Among all of the phosphorylation sites on the tau protein, the majority are serines (53% of phosphorylation sites of tau) and threonines (41%) [19]. On the basis that Thr-231, Thr-181, and Ser-404 were reported to be identified in AD patients [20], the role of tau phosphorylation sites in $A\beta_{1-42}$-injected mice were investigated by using Western blot analysis. As shown in ▶ **Fig. 3**, compared with the control group, $A\beta_{1-42}$ increased the levels of tau protein phosphorylated at the three sites. However, arctigenin attenuated the above-described level of the tau protein. The result indicated that arctigenin effectively protects against tau phosphorylation.

Considering that GSK-3β plays a central role in the pathogenesis of AD and regulates tau phosphorylation, we studied the effect of GSK-3β on AD [21–25]. Phosphorylation of Ser-9 (inhibitory) and Tyr-216 (activating) for GSK-3β is regulated by multiple mechanisms, including the PI3K/Akt pathway [26, 27]. Hyperactivation of the PI3K/AKT/GSK-3β pathway is linked to the disrupted clearance of Aβ and tau, synaptic loss, and cognitive decline in AD. Nevertheless, the PI3K/AKT pathway is downregulated in AD transgenic mice and AD patients [28, 29]. Our data showed that arctigenin increases the GSK-3β (Ser-9) phosphorylation level, suppresses PI3K/Akt activation, and increases the PI3K p85 and Akt Ser 473 phosphorylation levels. These results suggest that the Aβ-induced tau hyperphosphorylation is attenuated by arctigenin via the PI3K/Akt/GSK-3β cascade. Furthermore, the point that Aβ promotes tau phosphorylation, and arctigenin reduces the accumulation of Aβ [18], may be in correlation with the reduction in the levels of tau phosphorylation.

In this study, we provide the first demonstration that arctigenin exerts protective effects against ICV $A\beta_{1-42}$-induced learning and memory deficits by the reduction of tau hyperphosphorylation through the PI3K/Akt-dependent GSK-3β signalling pathway. These data demonstrate that arctigenin possesses anti-tau hyperphosphorylation activity, which may be a potential therapeutic target in AD treatment.

Materials and Methods

Reagents and instruments

$Amyloid_{1-42}$ and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich. The total protein extraction kit was purchased from Nanjing KeyGen Biotech. Co., Ltd., and a PageRuler prestained protein ladder (10–170 kDa) was purchased from Thermo Fisher Scientific Chemical Reagent Co., Ltd. The BCA protein assay kit, SDS-PAGE gel preparation kit, SDS-PAGE sample loading buffer (5 ×), HRP-labelled goat anti-rabbit IgG (H+L), HRP-labelled goat anti-mouse IgG (H+L), and BeyoECL Plus (ECL-like Western reagent) were obtained from the Beyotime Institute of Biotechnology. Antibodies against Ser-404, Thr-231, Thr-181, PI3K, p-PI3K (P85), AKT, p-AKT (Ser-473), GSK-3β, p- GSK-3β (Ser-9), and β-actin were purchased from Santa Cruz Biotechnology, Ltd. Antibodies against Tau-5 were obtained from Invitrogen Biotechnology, Ltd. Arctigenin, with a purity higher than 98%, was prepared by our laboratory, and its chemical structure is shown in ▶ **Fig. 1 A** [30]. Donepezil, with a purity higher than 98%, was obtained from Eisai, Ltd. All other chemicals were of analytical grade.

Experimental animals

Equal numbers of adult male and female ICR mice (n = 48; 8 weeks of age) weighing 20–22 g were obtained from the Beijing HFK Bioscience Co., Ltd., and used in the study. The animals were housed in a polyacrylic cage with four mice per cage and maintained under standard housing conditions (22 °C ± 2 °C and humidity 50% ± 5%) with a 12-h light/12-h dark cycle. Food and water were available *ad libitum*. All efforts were made to minimise the number of animals and their suffering throughout the experiments. The food was withdrawn 16–18 h prior to the surgical procedure. All animal studies were performed in strict accordance with the P. R. China legislation on the use and care of laboratory animals and the guidelines established by the Institute for Experimental Animals at Liaoning University of Traditional Chinese Medicine (131/2010; approved on November 1, 2011).

Animal model and amyloid beta administration

$A\beta_{1-42}$ was dissolved in sterile physiological saline and aggregated by incubation at 37 °C for 5 days prior to injection, according to a previous report [31].

The mice were anaesthetised with chloral hydrate (300 mg/kg i. p.) and placed on a stereotaxic apparatus (David Kopf Instruments) using a mouse adaptor (Kopf 921), and the skull was then exposed. $A\beta_{1-42}$ was injected via the ICV route slowly in a volume of 3 µL (≈ 410 pmol/mouse) on day 1, as described by Chi et al. [32]. The coordinates from the bregma were the following: AP − 0.5 mm, ML − 1.0 mm, and DV − 3 mm [33]. The control animals were injected with equivalent volumes of sterile physiological saline. The mice were orally administered arctigenin (10, 40, or 150 mg/kg), donepezil (1.3 mg/kg), or vehicle by gavage immediately after the second $A\beta_{1-42}$ injection. The mice were then treated once daily or 1 h before the behavioural tests.

Behavioural analysis

The Y-maze test was performed 9 days after Aβ injection. The maze had three arms, and the dimensions of each arm were 40 × 12 × 10 cm (length × height × width). Each mouse was placed at the terminus of one arm and allowed to move freely through the maze for 5 min. The total number of arm entries (N) and the sequence of entries were recorded. Alternation behaviour was defined as entries into all three arms on consecutive occasions. The alternation rate (%) was calculated as follows: alternation behaviour (%) = number of alternations/(N − 2) × 100.

The Morris water maze test was performed 10 days after Aβ injection. The Morris water maze consisted of a circular pool (100 cm in diameter and 50 cm in height) that was filled with water (25 °C ± 1 °C and 30 cm in depth). A platform (10 cm in diameter) was placed 1 cm below the water surface in one of four identical quadrants. The mice were trained twice a day for 5 consecutive days with an inter-trial interval of 3 h. The mice were allowed to escape by swimming to the platform, and the escape latency was recorded for 60 s. After 60 s, if a mouse failed to locate the platform, it was then placed on the platform for 20 s. On the sixth day, the mice were administered a probe test. In this test, the platform was removed, and each mouse was allowed to explore the pool for 60 s. The escape latency (time spent in the target quadrant), swimming distance, and swimming speed were measured using a computer system with a video camera. All trials were completed between 08 : 00 and 18 : 00 h (on days 1, 2, 3, 4, and 5 of the test).

Western blotting analysis

Sixteen days after the ICV Aβ_{1-42} injection, the rats were sacrificed. Brain tissues were rinsed twice with cold PBS, disrupted with 400 µL of cold hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCL, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 M PMSF) for 15 min on ice, and incubated for an additional 5 min after the addition of 25 µL of 10 % NP-40. The samples were then sonicated for 15 s and centrifuged at 12 000 × *g* and 4 °C for 10 min. The protein levels were determined using the Bradford assay (Beyotime). Protein extracts (50 µg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then electrophoretically transferred to polyvinylidene difluoride membranes (Millipore). After incubation at room temperature with 5 % nonfat milk in PBS for 2 h, the membranes were incubated overnight at 4 °C with the indicated primary antibodies against Tau-5 (Invitrogen), Ser-404, Thr-231, Thr-181, PI3K, p-PI3K (P85), AKT, p-AKT (Ser-473), GSK-3β, p- GSK-3β (Ser-9), and β-actin (Santa Cruz). The membranes were then incubated with secondary anti-mouse IgG or anti-rabbit IgG antibodies (Santa Cruz) for 2 h at room temperature. Protein bands were developed using electrochemiluminescent Western blot reagents (Cwbiotech). The intensity was quantified by densitometry using Quantity One 4.6.2 software (Bio-Rad) and corrected with the corresponding β-actin level. The results are expressed as percentages of the control or Aβ levels.

Statistical analysis

The data are expressed as the means ± SD. The statistical significance was determined through one-way or two-way ANOVA followed by Fisher's LSD multiple comparisons test. A p value less than 0.05 was considered to indicate statistical significance. The data were analysed using SPSS 17.0.

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Conflict of Interest

The authors declare no conflicts of interest.

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