Ellagic acid ameliorates learning and memory impairment in APP/PS1 transgenic mice via inhibition of β-amyloid production and tau hyperphosphorylation

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Abstract. β-amyloid (Aβ) aggregation and tau hyperphosphorylation are considered to be the primary pathological hallmarks of Alzheimer’s disease (AD). Targeted inhibition of these pathological processes may provide effective treatments for AD. Accumulating evidence has demonstrated that ellagic acid (EA) exerts neuroprotective effects in several diseases. The present study investigated the effects of EA on AD-associated learning and memory deficits on APP/PS1 double transgenic mice and the underlying mechanisms. APP/PS1 mice or wild-type C57BL/6 mice were intragastrically administered EA (50 mg/kg/day) or vehicle for 60 consecutive days. The learning and memory abilities of mice were investigated using the Morris water maze test. Hippocampal regions were examined for the presence of amyloid plaques, neuronal apoptosis and tau phosphorylation. Expression levels of APP, Aβ, RAC-α serine/threonine-protein kinase and glycogen synthase kinase (GSK)3β in the hippocampus were determined by western blot analysis and ELISA. The results demonstrated that EA treatment ameliorated spatial learning and memory impairment in APP/PS1 mice and significantly reduced neuronal apoptosis and Aβ deposition in the hippocampus (P<0.05 and P<0.01). In addition, EA significantly inhibited the hyperphosphorylation of tau and significantly decreased the activity of glycogen synthase kinase (GSK)3β (P<0.01), which is involved in tau phosphorylation. Overall, these findings indicated that the beneficial effects of EA on AD-associated cognitive impairments may be attributed to the inhibition of Aβ production and tau hyperphosphorylation, and its beneficial action may be mediated in part, by the RAC-α serine/threonine-protein kinase/GSK3β signaling pathway.

Introduction

Alzheimer’s disease (AD) is a chronic neurodegenerative disease that frequently occurs in the elderly population and is characterized by progressive learning dysfunction and memory impairment (1). Pathologically, accumulation of senile plaques, neurofibrillary tangles (NFTs) and neuronal loss have been observed in the brain cortex and hippocampus of patients with AD (2). Although the underlying mechanisms have not been fully elucidated, β-amyloid (Aβ) aggregation and tau hyperphosphorylation have been revealed to exhibit key roles in the pathogenesis of AD (3,4), as they are primary components of senile plaques and NFTs, respectively (5,6) and are associated with neuronal loss and memory deficits (7,8). Therefore, potential pharmacological agents targeting the inhibition of these pathological processes may be promising therapeutic approaches in treating AD.

Ellagic acid (EA) is a natural polyphenol present in several types of fruits and nuts, including strawberries, pomegranates and walnuts (9). EA has been reported to possess multiple pharmacological effects, including antioxidant, anti-inflammatory, antitumor and antifibrotic properties (10,11). A previous study demonstrated the neuroprotective role of EA in several central nervous system diseases (12). Farbood et al (13) reported that
EA alleviates brain injury-induced cognitive dysfunction via inhibition of neuroinflammation. Notably, Sarkaki et al (14) demonstrated that EA mitigates the symptoms of Parkinson's disease induced by 6-hydroxidopamine, and this effect may be attributed to its antioxidant properties (9). Furthermore, Feng et al (15) indicated that EA reduces Aβ42-induced neurotoxicity, which suggested a beneficial role of EA in AD. In accordance with these results, Kiasalari et al (16) further revealed that EA ameliorated learning and memory deficits in a rat model of AD induced by Aβ25-35 and suggested the underlying mechanisms were associated with its inhibition of oxidative stress and the nuclear factor (NF)-κB/NF erythroid 2 like 2/Toll-like receptor 4 signaling pathway. However, whether EA affects Aβ production and tau hyperphosphorylation remains unclear.

In the present study, the APP/PS1 double-transgenic mouse model was employed to evaluate the efficacy of EA in the treatment of AD. Brain hippocampi were examined for alterations in neuronal apoptosis, Aβ plaque formation and tau hyperphosphorylation. In addition, the RAC-α serine/threonine-protein kinase (AKT)/glycogen synthase kinase (GSK) 3β signaling pathway, which is involved in the regulation of tau hyperphosphorylation, was also investigated.

**Materials and methods**

**Animals and treatment.** Male APP/PS1 double-transgenic and wild-type (WT) C57BL/6 mice (6-months-old, weighing 24-28 g, n=12 per group) were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). Prior to the experiment, all the mice were acclimated for 1 week and were kept at a 12-h light/dark cycle at room temperature (21-23°C) with 55% humidity and access to food and water *ad libitum*. Mice were randomly divided into four groups (n=12 mice per group), namely the WT, WT+EA, APP/PS1 and APP/PS1+EA groups. The mice were intragastrically administered EA (50 mg/kg/day, cat. no. E102710; Shanghai Aladdin Bio-Chem Technology Co., Ltd., Shanghai, China) or the same volume of 10% DMSO for 60 consecutively days. The dosage of EA used was based on previous data (14,17), which demonstrated that EA at this dose exerted protective effects in a model of Parkinson's disease. The animal experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Heilongjiang University of Chinese Medicine (Harbin, China).

**Morris water maze (MWM) test.** Following 60 days of EA treatment, the spatial learning and memory abilities of the mice were assessed using the MWM test, as previously described (18). The apparatus included a black circular tank (180 cm in diameter) filled with water (temperature, 22-24°C). A platform (9 cm in diameter) was placed at the targeted quadrant 1.5 cm below the water surface. Mice were randomly placed into one of the four quadrants facing the maze wall and allowed to search for the platform. If the mouse did not find the platform within 120 sec, it was guided to the platform for 30 sec. The training lasted for 5 consecutive days, with four sessions per day. The time required to find the hidden escape platform was recorded. The probe trial was performed following 5 days of hidden platform trials. Mice were allowed to swim freely in the water without the platform for 120 sec. The frequency of crossing the platform location, the time spent in the target quadrant and the swimming tracks were monitored.

**Tissue preparation.** Following the behavioral tests, the mice were euthanized. Part of the resected hippocampal tissues were fixed in 10% formalin at 4°C for 24 h, embedded in paraffin and sliced into 5-μm sections. The remaining tissues were homogenized for western blot analysis and ELISA.

**Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay.** TUNEL staining (In Situ Cell Death Detection kit; Roche Diagnostics, Indianapolis, IN, USA) was used to assess neuronal apoptosis in the hippocampus. The TUNEL assay was performed according to standard procedures. Hippocampal slices (5-μm thick) were deparaffinized, rehydrated, permeabilized and blocked in 3% H2O2 for 10 min at room temperature. Following washing, the slices were incubated in TUNEL reaction mixture for 60 min at 37°C and processed with 50 μl converter-peroxidase for 30 min at 37°C. Sections were stained using the diaminobenzidine substrate kit (cat no. DA1010; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 3 min at room temperature for color development and counterstained with hematoxylin for 3 min at room temperature. Apoptotic cells were assessed under a light microscope (magnification, x400). The number of apoptotic cells was calculated in six different visual fields of each section.

**Thioflavin-S staining.** Amyloid plaque formation was measured by thioflavin-S staining (cat no. TI892; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. Hippocampal slices (5-μm thick) were deparaffinized, rehydrated and stained in thioflavin-S solution at room temperature for 8 min. Subsequently, the slices were successively rinsed in 50% alcohol and distilled water. Amyloid deposition was evaluated under a fluorescent microscope (magnification, x100 and x400).

**Immunohistochemical analysis.** The expression of tau protein in the hippocampus was determined using immunohistochemical analysis. The procedures were performed as previously described (19) with slight modifications. Briefly, hippocampal sections were subjected to heat-induced epitope retrieval by immersing slides in a boiling solution for 10 min, followed by 3% H2O2 treatment to block endogenous peroxidases for 15 min at room temperature. The sections were then blocked with 10% goat serum (cat no. SL038; Beijing Solarbio Science & Technology Co., Ltd.) for 15 min at room temperature, followed by incubation with anti-tau antibody (cat no. D155045; 1:50; Sangon Biotech, Co., Ltd., Shanghai, China) at 4°C overnight. Following washing three times in PBS, the sections were incubated with a biotinylated anti-rabbit IgG antibody (cat no. A0277, 1:200; Beyotime Institute of Biotechnology, Shanghai, China) for 30 min at 37°C, followed by incubation with the avidin

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biotinylated horseradish peroxidase (cat no. A0303; 1:200; Beyotime Institute of Biotechnology) for 30 min at 37°C. Color was developed using the diaminobenzidine substrate kit (cat no. DA1010; Beijing Solarbio Science & Technology Co., Ltd.) for 3 min at room temperature and counterstained with hematoxylin for 3 min at room temperature. Images of the stained sections were analyzed under a light microscopy (magnification, x400).

ELISA. The concentration of Aβ40 and Aβ42 in the hippocampus was quantified using commercial ELISA kits (cat no. CEA864Mu and cat no. CEA946Mu; USCN Business Co., Ltd., Wuhan, China). ELISA was performed according to the manufacturer's protocol.

Western blot analysis. Western blot analysis was performed according to standard protocols with some modifications (20). Briefly, total protein from each tissue sample was lysed with radiolabeled precipitation assay lysis buffer (Beyotime Institute of Biotechnology) following the manufacturer's instructions. Protein concentrations were measured using the BCA method. A total of 20 µg proteins were separated by SDS-PAGE (8, 11 or 15% gels) and transferred to polyvinylidene difluoride membranes. Following blocking with 5% skimmed milk at room temperature for 1 h, the membrane was incubated with a specific primary antibody at 4°C overnight. The primary antibodies used in the study included anti-cleaved caspase-3 antibody (cat no. ab2302; 1:1,000), anti-pSer199-tau antibody (cat no. ab81268; 1:10,000) (both from Abcam, Cambridge, UK), anti-amyloid precursor protein (APP) antibody (cat no. D26097; 1:500; Sangon Biotech, Co., Ltd.), anti-pThr668-APP antibody (cat no. D155082; 1:500; Sangon Biotech, Co., Ltd.), anti-β secretase (BACE) 1 (cat no. D220305; 1:500; Sangon Biotech, Co., Ltd.), anti-pSer396-tau antibody (cat no. D155045; 1:500; Sangon Biotech, Co., Ltd.), anti-pSer473-AKT antibody (cat no. KG11054; 1:500; Nanjing KenGen Biotech, Co., Ltd., Nanjing, China), anti-AKT antibody (cat no. KG21004; 1:500; Nanjing KenGen Biotech, Co., Ltd.), anti-pThr216-GSK3β (cat no. KG11301-2; 1:500; Nanjing KenGen Biotech, Co., Ltd.), anti-pSer21-GSK3β antibody (cat no. KG21002-2; 1:500; Nanjing KenGen Biotech, Co., Ltd.), anti-tau antibody (cat no. KG21099-2; 1:500; Nanjing KenGen Biotech, Co., Ltd.) and anti-β-actin antibody (cat no. ab33139M; 1:500; Bioss Antibodies, Beijing, China). The membrane was subsequently incubated with goat anti-rabbit IgG horseradish peroxidase-conjugated antibody (cat no. A0208; 1:5,000; Beyotime Institute of Biotechnology) at 37°C for 45 min. An enhanced chemiluminescence detection reagent (Beyotime Institute of Biotechnology) was used for enhanced blot detection. The band intensity was assessed and analyzed with Gel-Pro-Analyzer software 4.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Data are presented as the mean ± standard deviation. Analyses were performed using one-way analysis of variance and the Bonferroni post-hoc test with GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

EA improves learning and memory deficits in APP/PS1 transgenic mice. APP/PS1 transgenic mice begin to exhibit memory impairment and amyloid plaque deposition at ~6 months of age (21). In the present study, the MWM test was performed to evaluate the learning and memory abilities of the mice. In the hidden platform trials, the APP/PS1 model group exhibited significantly longer escape latencies at days 1, 2, 4 and 5 of the trial compared with the WT group (P<0.01; Fig. 1A). EA treatment significantly decreased the escape latencies at day 4 and 5 of the trial compared with the APP/PS1 group (P<0.05), reflecting the improved learning ability with EA treatment. In the probe trials, following removal of the platform, the mice randomly swam in the pool for 120 sec. In addition, the number of platform crossings and the time spent in the target quadrant for the APP/PS1-vehicle treated mice was significantly decreased compared with the WT group (P<0.01; Fig. 1B and C). By contrast, the EA-treated APP/PS1 mice crossed the position of the platform more frequently and spent more time searching for the platform in the target quadrant compared with the APP/PS1-vehicle treated mice (P<0.05). Furthermore, the swimming tracks of EA-treated APP/PS1 mice were similar to those of the WT group (Fig. 1D). Taken together, the MWM test results suggested that EA treatment improved the spatial learning and memory abilities in APP/PS1 transgenic mice.

EA reduces neuronal cell apoptosis in the hippocampus of APP/PS1 transgenic mice. Neuronal loss is responsible for the learning and memory deficits in patients with AD (22). The present study further evaluated the effects of EA on neuronal apoptosis in the hippocampus. TUNEL staining indicated that the APP/PS1 group exhibited an increased proportion of apoptotic cells in the hippocampus compared with the WT group, whereas EA treatment reduced the numbers of apoptotic cells compared with the APP/PS1-vehicle group (Fig. 2A). Furthermore, the differences between the percentage of apoptotic cells exhibited in the hippocampus of mice in these groups were statistically significant (P<0.01; Fig. 2B). Western blot analysis also revealed that the expression of cleaved caspase-3 was upregulated in APP/PS1 mice, and EA treatment reduced the expression level of caspase-3 in the hippocampus compared with that in the APP/PS1-vehicle group (Fig. 2C). Statistical analysis indicated these differences were significant (P<0.01; Fig. 2D).

EA reduces Aβ deposition and Aβ protein expression levels in the hippocampus of APP/PS1 transgenic mice. Accumulation of Aβ in the brain is considered to be a primary hallmark of AD (23). Deposited amyloid plaques in the hippocampus may further induce neuronal loss and cognitive impairment deficits (20,24). The present study further evaluated the effect of EA on Aβ deposition. Aβ plaques were stained with thioflavin-S (Fig. 3A). Compared with the WT group, the APP/PS1 group exhibited increased amyloid staining in the hippocampus, whereas EA treatment reduced the amyloid aggregates in the hippocampus. ELISA also revealed significantly increased levels of Aβ40 and Aβ42 in the APP/PS1 group, whereas EA treatment effectively reversed this increase (P<0.01; Fig. 3B and C).
Figure 1. EA treatment improves learning and memory impairment in APP/PS1 mice. Mice were intragastrically administered EA (50 mg/kg/day) or vehicle for 60 consecutive days. The learning and memory abilities of the mice were evaluated using the Morris Water Maze test. (A) Escape latencies of mice for finding the platform in the hidden platform trials. (B) Number of mice crossing the platform location in the probe test. (C) Time spent in the target quadrant in the probe test. (D) Swimming tracks of mice in the probe test. The circle in the left lower quadrant represents the location of the hidden platform. Data are presented as the mean ± standard deviation. *P<0.05 or **P<0.01 vs. WT mice; †P<0.05 or ‡P<0.01 vs. APP/PS1 transgenic mice. EA, ellagic acid; WT, wild-type.

Figure 2. Effects of EA on neuronal apoptosis in the hippocampus of APP/PS1 transgenic mice. (A) TUNEL staining of the hippocampus from WT, WT+EA, APP/PS1 and APP/PS1+EA groups. Magnification, x400; scale bar, 50 µm. (B) Percentage of apoptotic cells in the hippocampus. (C) Western blotting and (D) quantitative analysis of cleaved caspase-3 expression in the hippocampus; β-actin served as an internal control for grayscale analysis. Data are presented as the mean ± standard deviation. **P<0.01 vs. WT mice; ‡P<0.01 vs. APP/PS1 transgenic mice. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; EA, ellagic acid; WT, wild-type.
Aβ peptides are produced from the APP; phosphorylation of APP and BACE1 kinase are responsible for Aβ generation (25,26). To evaluate whether EA affects Aβ production, the protein expression levels of pThr668-APP and BACE1 were determined. The western blot analysis results revealed that pThr668-APP and BACE1 protein expression levels in the APP/PS1 group were increased compared with the WT group, whereas EA treatment reduced these expression levels (Fig. 3D). Notably, these differences were indicated to be statistically significant (P<0.01; Fig. 3E). These results suggested that EA reduced Aβ deposition by suppressing Aβ production.

EA inhibits tau hyperphosphorylation in the hippocampus of APP/PS1 transgenic mice. In addition to Aβ deposition, tau hyperphosphorylation is another characteristic feature of the brain in APP/PS1 mice (27). In the present study, the extent of Tau hyperphosphorylation in the hippocampus was determined by immunohistochemical staining and western blot analysis. Immunohistochemical results revealed high expression of pSer396-tau in the APP/PS1 group compared with the WT group. Notably, EA treatment reduced the expression of pSer396-tau in the hippocampus (Fig. 4A). Furthermore, western blot analysis also determined that EA downregulated the protein expression levels of pSer199-tau and pSer396-tau in the APP/PS1 group (Fig. 4B). Furthermore, the difference between the protein expression levels of pSer199-tau and pSer396-tau in the APP/PS1 group compared with the APP/PS1+EA group was indicated to be statistically significant (P<0.01; Fig. 4C). Taken together, these results indicated that the beneficial role of EA in AD may be associated with the downregulation of tau phosphorylation.

The AKT/GSK3β signaling pathway has been demonstrated to have an important role in tau hyperphosphorylation. Activated GSK3β has been demonstrated to facilitate tau phosphorylation and NFT formation (28). To further investigate
The underlying mechanism of action of EA, the effect of EA on the regulation of the AKT/GSK3β signaling pathway was examined by western blot analysis. As presented in Fig. 4D, mice in the APP/PS1 group exhibited low protein expression levels of pSer473-AKT and high expression levels of pTyr216-GSK3β compared with those in the WT group. Notably, these differences were indicated to be statistically significant (P<0.01; Fig. 4E). These results suggested GSK3β activation. Conversely, EA treatment significantly upregulated the protein expression levels of pSer473-AKT and decreased the protein expression levels of pTyr216-GSK3β in APP/PS1 mice (P<0.05 and P<0.01, respectively; Fig. 4E), which indicated activation of AKT and deactivation of GSK3β following EA treatment. These results suggested that EA inhibited tau phosphorylation in the hippocampus of APP/PS1 mice and that this inhibitory effect may be partially mediated by the AKT/GSK3β signaling pathway.

Discussion

AD, which is the most common form of dementia, severely affects the life quality of elderly patients, and there is currently no effective treatment (29). To further understand the underlying mechanisms and develop novel pharmacological agents for AD, animal models of AD have been employed. A growing body of evidence has demonstrated that APP/PS1 transgenic mice exhibit similar pathological alterations to those of patients with AD (30). In APP/PS1 transgenic mice, the memory impairment and amyloid deposition appear at 4-6 months of age and progress in an age-dependent manner (31,32). In the present study, APP/PS1 mice were selected to investigate the therapeutic efficacy of EA in AD. The results demonstrated that administration of EA (50 mg/kg/day for 60 days) ameliorated the learning and memory deficits in APP/PS1 mice, significantly reduced neuronal apoptosis and amyloid deposition, and also significantly inhibited tau hyperphosphorylation in the hippocampus, which suggested a beneficial role of EA in AD. A previous study also reported the beneficial role of EA in an Aβ-induced AD mouse model (16). Notably, the present findings further demonstrated the therapeutic efficacy of EA in APP/PS1 mice and the beneficial effects were indicated to be associated with the inhibition of Aβ production and tau hyperphosphorylation. Aβ aggregates exhibit crucial roles in AD-associated cognitive impairment and neuronal loss (24,33). Targeted
inhibition of Aβ production has been indicated to exert beneficial effects on APP/PS1 mice (34). In the present study, APP/PS1 mice exhibited significant deficits in their learning and memory abilities alongside increased neuronal apoptosis and Aβ deposition. Learning and memory impairments of APP/PS1 mice in the present study were in line with those reported by other studies (35,36). Notably, EA treatment improved the learning and memory abilities, and significantly decreased Aβ deposition and Aβ expression levels in the hippocampus. Furthermore, EA treatment significantly reduced the expression levels of pThr668APP and BACE1. As previously reported, the phosphorylation of APP at the Thr668 site promotes Aβ generation (25) and BACE1 is the key enzyme for Aβ generation through cleavage of APP (26,37). The present results revealed that EA treatment alleviated cognitive impairment and decreased Aβ production. These results were consistent with a previous study reporting the anti-amyloidogenic effect of EA on potassium sorbate and glucose-induced human serum albumin fibril formation (38).

Hyperphosphorylated tau is the primary component of intracellular NFTs, which have an important role in AD pathology (2,39). It has been demonstrated that hyperphosphorylated tau may also result in neuronal death and memory impairment (5,40,41). A number of serine phosphorylation sites of tau are elevated in AD mice, including Ser199 and Ser396 (42). Targeting inhibition of tau phosphorylation may therefore provide an effective therapeutic approach to AD (43).

In the present study, phosphorylation of tau at Ser199 and Ser396 was significantly increased in the hippocampus of APP/PS1 mice, whereas EA treatment efficiently inhibited tau phosphorylation. Since the phosphorylation of tau is regulated by multiple signals, including AKT/GSK3β signaling (35,44), the present study further investigated the effect of EA on the AKT/GSK3β signaling pathway. The results demonstrated that EA significantly increased the phosphorylation of AKT at the Ser473 site and significantly decreased the phosphorylation of GSK3β at the Tyr216 site, indicating de-activation of GSK3β (45). These results suggested that EA inhibited tau phosphorylation in APP/PS1 mice and that the inhibitory effect of EA may be partially mediated by the AKT/GSK3β signaling pathway. However, several other kinases are involved in the regulation of tau phosphorylation and further investigation is required to identify the precise mechanisms of action of EA in AD.

In conclusion, the present study demonstrated the beneficial effects of EA on spatial learning and memory in APP/PS1 mice, which included inhibition of neuronal death, Aβ production and tau hyperphosphorylation. Furthermore, the beneficial effects of EA were indicated to be partially mediated by the AKT/GSK3β signaling pathway. These findings suggest that EA may be a promising therapeutic agent for the treatment of AD.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LZ, HL and ZS contributed to the conception and design of the study, wrote and revised the manuscript. LZ, HL and WZ conducted the studies and analyzed and interpreted the data. XL, BJ and HF analyzed the data and interpreted the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Heilongjiang University of Chinese Medicine (Harbin, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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