Icarin isolated from Epimedium brevicornum Maxim attenuates learning and memory deficits induced by D-galactose in rats

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The effects of icarin (ICA), a major constituent of flavonoids from the Chinese medical herb Epimedium brevicornum Maxim, on spatial memory performances and expressions of hippocampus brain-derived neurotrophic factor (BDNF) and tyrosine kinase TrkB (tropomyosin receptor kinase B) were investigated in D-galactose (D-gal)-treated rats. Subcutaneous injection of D-gal (500 mg/kg/d) for four months caused memory loss as detected by the Morris water maze, morphologic abnormalities of neurons in hippocampus region and the reduced expression of BDNF and TrkB were observed. ICA (60 mg/kg/d) given orally 1 h after subcutaneous injection of D-gal daily for 4 months markedly attenuated D-gal-induced rats behavioral dysfunction and neurodegeneration, as evidenced by shortened escape latency and searching distance and rescued morphologic abnormalities, and also elevated the mRNA levels and the protein expressions of BDNF and TrkB in hippocampus, as evidenced by quantitative real-time RT-PCR and Western blotting analysis. But ICA had no significant influence on normal rats which were not injected D-gal. These results clearly demonstrated that D-gal produced learning and memory deficits after chronic administration, and ICA can protect neuron from D-gal insults and improve the memory loss.

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1. Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by the presence of senile plaques and neurofibrillary tangles, accompanied by the decrease of number of neurons. Although the etiology and pathogenesis of AD have not been understood clearly, the close relationship between aging and the development of AD has been identified. Amounts of brain derived neurotrophic factor (BDNF) are reduced in the hippocampus in AD (Hock et al., 2000; Nagahara et al., 2009). A decrease in BDNF and/or its receptors in normal aging animals was also evident (Tapia-Arancibia et al., 2008). BDNF and its receptor, tyrosine kinase TrkB (tropomyosin receptor kinase B) reduction may contribute to synaptic and cellular loss and memory deficits of AD (Peng et al., 2009). It is widely believed that the long-term injection of D-galactose (D-gal) contributes to the aging progress and slight neuronal damage and memory deficits which are the prominent changes of AD in the early stage (Chen et al., 2006; Hua et al., 2007). Because D-gal-induced senescence is accompanied by neurodegeneration, it could be an ideal model for studying the molecular mechanisms involved with aging and age-associated neurodegeneration, and for testing new therapeutics (Cui et al., 2006; Wei et al., 2005).

Now the therapeutics for AD is still a challenge, many approved AD drugs have limitations, thus the search for new drugs is the hot topic of research. Fortunately, basic pharmacological researches have provided unprecedented opportunities for the development of new therapeutics aimed at prevention and treatment of AD.

Our previous studies have shown that icarin (ICA), a quinlizidine flavone, extracted from the Chinese traditional herb (Berberidaceae, epimedium L), can protect neuron from oxygen-glucose deprivation, oxidative stress and enhance neuronal viability (Wang et al., 2009). However, whether ICA can attenuate D-gal-induced rat learning and memory deficits is still unknown, and the underlying mechanisms of the protection of ICA against D-gal remain unclear. In the present study, we employed the chronic rat model of AD induced by D-gal to evaluate effects of ICA on spatial learning and memory function by the Morris water maze. Hippocampus morphological changes were determined by the HE stain. Furthermore, the levels of BDNF and TrkB mRNA and protein were detected as potential mechanisms of neuroprotective effect of ICA.

2. Materials and methods

2.1. Reagents

ICA (TCM020-071102, purity ≥ 90%, via HPLC) was purchased from Nanjing TCM Institute of Chinese Materia Medica (Nanjing, China), D-gal from Sigma (St. Louis, MO, USA), β-actin, BDNF and TrkB
primers were the products of TaKaRa Biotechnology (Dalian, China). BCA protein assay kit and BeyoECL Plus were from Beyotime Institute of Biotechnology (Jiangsu, China). The antibodies of BDNF, TrkB and β-actin (rabbit-anti-rat) were purchased from BOSTER Biological Engineering Co. (Wuhan, China) and Santa Cruz Biotechnology (California, USA), respectively. HRP-labeled goat-anti-rabbit IgG from Zhongshan Goldenbridge Biotechnology (Beijing, China). All other reagents were of reagent grade.

The Morris water maze apparatus and “MT-200” image analysis system were purchased from Chengdu Taimeng Co. (Chengdu, China). quantitative real-time RT-PCR instrument and semi-quantitative analysis of protein expression instrument were from BIO-RAD (Alfred, CA, US).

2.2. Animals

Healthy, male Sprague-Dawley rats weighing 200–250 g were purchased from the Animal Center of the Third Military Medical University (Chongqing, China) (Certificate: No SCXK (Jun) 2007017). All animals were caged individually with ad libitum access to water and food, on a 12-h-light/dark cycle and at 22 ± 1 °C. All procedures have complied with the WHO guidance for Animal Use and Care and our Institutional Animal Use and Care Committee approved the animal procedures.

2.3. Drug administration

After one week acclimatization, all rats were screened by the Morris water maze. Through 5 days (2 trials per day) of training, the criteria for selecting qualified rats is the time of searching the hidden platform within 2 min limit (Vorhees and Williams, 2006). Forty qualified rats were randomly divided into 4 groups of 10 each: control group (Control); ICA alone (ICA); d-gal alone (d-gal); and d-gal plus ICA (d-gal + ICA). Rats were injected subcutaneously d-gal (500 mg/kg/d, for d-gal and d-gal + ICA groups) or normal saline (0.1 ml/100 g, for Control and ICA groups) consecutively for 4 months. The next day following d-gal administration, the rats of ICA administration were given ICA by gavage, the others were received equi-volume of distilled water. Based on our previous study (Xu et al. 2009) and our pilot experiment, we have used ICA at the dose of 30, 60, 120 mg/kg/d, and found the dose of 60 mg/kg/d is effective and appropriate.

2.4. The water maze performance (Morris, 1984; Gong et al., 2005)

The Morris water maze apparatus consists of a large circular pool (120 cm in diameter, 38 cm in height) and a clear perspex platform (120 cm in diameter, 38 cm in height) and a clear perspex platform (120 cm in diameter, 38 cm in height). The water temperature was adjusted to 22 ± 1 °C. After 4 min acclimatization, all rats were screened by the Morris water maze. Through 5 days (2 trials per day) of training, the criteria for selecting qualified rats is the time of searching the hidden platform within 2 min limit (Vorhees and Williams, 2006). Forty qualified rats were randomly divided into 4 groups of 10 each: control group (Control); ICA alone (ICA); d-gal alone (d-gal); and d-gal plus ICA (d-gal + ICA). Rats were injected subcutaneously d-gal (500 mg/kg/d, for d-gal and d-gal + ICA groups) or normal saline (0.1 ml/100 g, for Control and ICA groups) consecutively for 4 months. The next day following d-gal administration, the rats of ICA administration were given ICA by gavage, the others were received equi-volume of distilled water. Based on our previous study (Xu et al. 2009) and our pilot experiment, we have used ICA at the dose of 30, 60, 120 mg/kg/d, and found the dose of 60 mg/kg/d is effective and appropriate.

2.5. Brain sample preparation

After 4 months, the animals were anesthetized with chloralhydrat (350 mg/kg) and decapitated. The brain was immediately taken out, three of each group were post-fixed in 4% formaldehyde for 24 h, then embedded in paraffin and coronally sectioned into 4-μm slices for HE stain, and the region of hippocampus CA1 adjacent CA2 was selected to investigate. The other rats’ hippocampi were removed on an ice-plate, and quickly frozen in liquid nitrogen.

2.6. Real-time RT-PCR analysis

Total RNA of hippocampus was isolated using Trizol reagent and purified with the RNeasy mini kit. RNA was spectrophotometrically quantified by measuring the optical density of sample at 260/280 nm, dissolved in 30–50 μl DEPC water and adjusted RNA concentration of each sample to 50 ng/μl. Total RNA was then reverse transcribed with MvLV reverse transcriptase and Oligo-(dT) primers. The SYBR green DNA PCR Master Mix (Applied ABI Company, Foster City, CA USA) was used for the real-time PCR analysis. Primers were designed according to the sequence searched on GeneBank. The nucleotide sequences of the primers used in this experiment were as follows: (1) BDNF (GeneBank NM_012513): sense 5′-GAA GGG CCA GGT CGA TTA GGT G-3′; antisense 5′-GAC GGA AAC AGA ACG AGA AAC AGA AC-3′; (2) TrkB (GeneBank NM_012731): sense 5′-GAG ACG AAA TCC TTC AGC CCC GAC AC-3′; antisense 5′-CAC AGA CCT TCC TTC CTC CAC CG-3′; (3) β-actin (GeneBank NM_011444): sense 5′-GGA GAT TAC TGC CCT GCC TCT TA-3′; antisense 5′-GAC TCA TCG TAC TCC TTG CG-3′.

The relative differences in expression among the groups were expressed using cycle time (CT) values as follows: the CT values of the interested genes were first normalized with β-actin of the same sample, and then the relative difference between control and other groups was calculated and expressed as a relative change, setting the control at 100%.

2.7. Western blotting analysis

Preparation of protein tissue lysate: hippocampi of each group were cut into pieces and homogenized in RIPA lysis buffer containing protease inhibitors using a manual tissue homogenizer, centrifugation at 20,000 g at 4 °C for 15 min, supernatant was used for protein quantification by BCA protein assay, and then Western analysis. Total protein from each sample (approximately 80 μg) was heat denatured at 100 °C for 5 min, electrophoretically separated on 5–10% gradient SDS-PAGE gels, and then transferred to a PVDF membrane. The membrane was blocked with 5% nonfat dry milk in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH2PO4, and 1.4 mM K2HPO4, pH 7.4) for 2 h at room temperature and incubated with a primary rabbit anti-rat BDNF or TrkB (1:400) or β-actin (1:2 000) antibody in 5% nonfat dry milk in PBS buffer at 4 °C overnight. Membranes were then washed three times for 10 min each with PBSTM buffer (1×PBS/ 5% nonfat milk/0.1% Tween 20) and incubated with HRP-labeled goat anti-rabbit IgG (1:5 000) at room temperature for 2 h with gentle rotation. After further washing, the membranes were taken to a Kodak X-ray film developing station in the presence of PBSTM to prevent the membrane from drying. Bound secondary antibodies were incubated with chemiluminescence reagent BeyoECL Plus. The image was scanned and band densities were quantified using Quantity One 1-D analysis software v4.52 (BioRad).

2.8. Statistical analysis

All quantitative data were expressed as mean ± SEM and evaluated using SPSS 13.0 software. Because there were two factors: d-gal and ICA, their effects were evaluated by two-way ANOVA. If there was interaction, the differences between groups were analyzed by
multiple analysis through fixing one factor to analyze the effect of another factor, and $P<0.05$ was considered as statistically significant.

3. Results

3.1. Behavior examination

Following 5 days of training, qualified 40 rats were divided into 4 groups, with similar performance in escape latency and searching distance. The model rats were injected 500 mg/kg d-gal and fed with distilled water for 4 months, rats in ICA group were only given 60 mg/kg ICA by gavage, treatment group rats were received d-gal and ICA. After 4 months of administration, spatial memory was assayed with the Morris water maze. The administration with d-gal and ICA was continued during the water maze performance. The water maze testing lasted for 3 days. The same pattern of behavior was observed on all three days, but the variability of performance occurred by day 3. The main effects of d-gal and ICA on escape latency were statistically significant (d-gal: $F(1,22) = 21.842, P = 0.000$; ICA: $F(1,22) = 21.109, P = 0.000$), the significant interaction of d-gal and ICA was observed ($F(1,22) = 45.477, P = 0.000$); the main effects of the two factors (d-gal and ICA) on searching distance were statistically significant (d-gal: $F(1,22) = 27.498, P = 0.000$; ICA: $F(1,22) = 16.663, P = 0.000$), the significant interaction of d-gal and ICA was observed ($F(1,22) = 36.901, P = 0.000$). On the third day of water maze trials, the d-gal rats had significantly longer escape latency and searching distance than control group (latency: $F(1,22) = 65.176, P = 0.000$; distance: $F(1,22) = 64.054, P = 0.000$). The administration with ICA resulted in a remarkable decrease in escape latency and searching distance in d-gal + ICA group compared to d-gal group (latency: $F(1,22) = 69.634, P = 0.000$; distance: $F(1,22) = 55.877, P = 0.000$) (Fig. 1 A, B).

3.2. Morphologic observation

HE stain showed that hippocampal neurons of control group rats were in order, the nucleus were big, round, or oval, and the nucleoli were observed clearly (Fig. 2 A). ICA alone group was similar with control group (Fig. 2 B). In d-gal group, the evidence remarkably indicated that neuronal abnormalities, including neuronal arrangement disorder, acidophily degeneration or even loss (Fig. 2 C). These pathological changes caused by d-gal were greatly attenuated by treatment with ICA (Fig. 2 D).

3.3. Real-time RT-PCR analysis

The main effect of d-gal on BDNF mRNA was statistically significant ($F(1,12) = 18.940, P = 0.010$), the main effect of ICA on BDNF mRNA was no significant ($F(1,12) = 0.308, P = 0.589$), the significant interaction of d-gal and ICA on BDNF mRNA was observed ($F(1,12) = 9.775, P = 0.009$). The main effects of d-gal and ICA on TrkB mRNA were no significant ($F(1,12) = 1.903, P = 0.193$; $F(1,12) = 0.669, P = 0.429$, respectively), but the significant interaction of d-gal and ICA on TrkB mRNA was observed ($F(1,12) = 15.371, P = 0.002$). Compared with control group, the expression of BDNF and TrkB mRNA in d-gal group decreased significantly ($F(1,12) = 27.694, P = 0.000$; $F(1,12) = 14.046, P = 0.003$, respectively). ICA treatment prevented such decreases, and the expression of BDNF and TrkB was markedly higher than d-gal group ($F(1,12) = 6.778, P = 0.023$; $F(1,12) = 11.227, P = 0.006$, respectively) (Fig. 3).

3.4. Western blotting analysis

Results of Western blotting suggested that the main effect of d-gal on BDNF protein was statistically significant ($F(1,8) = 5.581, P = 0.046$), the main effect of ICA on BDNF protein was no significant ($F(1,8) = 0.266, P = 0.620$), the significant interaction of d-gal and ICA on BDNF protein was observed ($F(1,8) = 8.037, P = 0.022$); the main effect of d-gal on TrkB protein was marginally significant ($F(1,8) = 3.522, P = 0.097$), the main effect of ICA on TrkB protein was no significant ($F(1,8) = 0.984, P = 0.350$), but the significant interaction of d-gal and ICA on TrkB protein was observed ($F(1,8) = 11.949, P = 0.009$). The protein expressions of BDNF and TrkB in hippocampus in d-gal group were obviously lower than that in control group ($F(1,8) = 13.506, P = 0.006$; $F(1,8) = 14.222, P = 0.005$, respectively), showing that high dose injection of d-gal induced a distinct decrease of the expression of BDNF and TrkB protein. ICA could reverse the decrease of the expression of BDNF and TrkB protein caused by d-gal ($F(1,8) = 5.613, P = 0.045$; $F(1,8) = 9.895, P = 0.014$, respectively). ICA alone had no apparent influence on the two protein expressions as compared to normal control (BDNF: $F(1,8) = 2.690, P = 0.140$; TrkB: $F(1,8) = 3.038, P = 0.120$) (Fig. 4 A, B).

4. Discussion

This study clearly demonstrated that: (1) consecutive injection subcutaneous d-gal (500 mg/kg/d) for 4 months produced AD-like disorders, including neurodegenerative evidence, learning and memory deficits, hippocampus neuron abnormalities and decreased expressions of BDNF and TrkB mRNA and protein levels in hippocampus. (2) ICA (60 mg/kg/d) significantly ameliorated complex behavioral deficits induced by d-gal, shortened the escape latency and searching distance, improved pathology in hippocampus and enhanced the expressions of BDNF and TrkB mRNA and protein.

D-gal, a normal sugar in the mammalian body, is converted into glucose in the presence of galactose-1-phosphate uridylytransferase and galactokinase. At a higher level, however, it may lead to the formation of reactive oxygen species by galactose oxidase (Ho et al., 2003). An increasing evidence indicates that long-term systemic exposure of d-gal to rodents causes progressive decline in cognitive function and mimics aging progress, such as hippocampal-dependent cognitive dysfunction (Cui et al., 2006), neurodegeneration (Zhang et al., 2005), and impairments in antioxidant capacity (Lu et al., 2006).
However, little is known about Δ-gal's effect on the levels of BDNF and TrkB. Mizisin et al. (1997) suggested that galactose metabolism by aldose reductase influenced axonal function and structure by altering production of nerve and muscle BDNF. This study revealed that long-time exposure of Δ-gal to rats caused the decreased expression of hippocampus BDNF and TrkB, but the mechanism is still unknown.

Fig. 2. Effect of ICA on neuron morphology in rat hippocampus. The region of hippocampus CA1 adjacent CA2 were selected to investigate by HE stain. Arrows indicate the degenerative neurons. Hippocampal neurons of control group were in order, the nucleus were big, round, or oval, and the nucleoli were observed clearly (A). ICA alone group was similar with control group (B). Long-term exposure to Δ-gal showed remarkably neuronal arrangement disorder, acidophily degeneration or even loss (C). Treatment with ICA significantly attenuated the damages subjected to Δ-gal (D). (magnification ×400).

Fig. 3. Real-time RT-PCR analysis of BDNF and TrkB mRNA levels. Exposure to Δ-gal resulted in significantly the decreased expression of BDNF and TrkB mRNA compared with control group. Administration with ICA to Δ-gal-induced rats prevented such decreases, and the expression of BDNF and TrkB was markedly higher than Δ-gal group, but the level of BDNF was still lower than control group. Data are presented as % change relative to the control. And Y-axis represents Relative Transcript Levels. (n = 4, mean ± SEM), *P<0.05 vs control, #P< 0.05 vs Δ-gal.

Fig. 4. Western blot assay of BDNF and TrkB protein. The protein expressions of BDNF and TrkB of hippocampus in Δ-gal group were obviously lower than that in control group. Administration with ICA notably increased the expressions of BDNF and TrkB protein in Δ-gal-induced rats, but had no effects when given alone. (A) BDNF and TrkB immunobLOTS; (B) densitometric analysis of BDNF and TrkB levels. (n = 3, mean ± SEM), *P<0.05 vs control, #P< 0.05 vs Δ-gal.
ICA has a wide range of pharmacological and biological activities, including the amelioration of learning and memory deficits in aluminum-intoxicated rats, possibly through its anti-oxidant effects and through decreased lipid peroxidation and Aβ1–40 deposits in rat hippocampus (Luo et al., 2007). ICA also has other beneficial effects, such as regulating cardiovascular, circulatory, genital, and bone marrow systems, increasing cerebral blood flow, stimulating neurite growth, retarding senility, and possessing estrogenic activity (Chen et al., 2005; Wu et al., 2003). Recent studies have demonstrated that ICA can protect neuron from cerebral ischemia/reperfusion injury via scavenging oxygen free radical and the inhibition of lipid peroxidation (Li et al., 2005; Takizawa et al., 2003). Furthermore, ICA can improve the abilities of spatial learning and memory of vascular dementia model rats by permanent bilateral ligation of the common carotid arteries method, possibly through its anti-oxidant effects, as well as effects on the circulatory and cholinergic systems (Xu et al., 2009). Overall, ICA is an important remedy for brain dysfunction to prevent neuron degeneration. This study demonstrated for the first time that ICA is also effective against D-gal-induced learning and memory deficits, probably by modulating the expressions of hippocampus BDNF and TrkB. But the potential mechanisms of effect of ICA on D-gal-induced model is still obscure, which could be secondary to its antioxidant effect.

BDNF, a small dimeric protein, is an important neurotrophic factor, and plays a key role in regulating not only neuronal development, maintenance and survival, but also in cognition, formation and storage of memory. In 1991, a decreased BDNF expression in hippocampus samples from AD donors was first demonstrated, suggesting that this decrease may contribute to the progressive cell death characteristic of AD (Phillips et al., 1991). This finding has been replicated (Hock et al., 2000). While some researchers showed that BDNF and TrkB play a critical role in long-term synaptic plasticity in the adult brain (Dwivedi, 2009; Schinder and Poo, 2000). Furthermore, BDNF has been found to promote the survival of all major types of neurons related to functional changes in AD, and been suggested as essential contributors of the etiology of neurodegenerative disorders (Fumagalli et al., 2006). Therefore, BDNF is a critical molecule in dementia and neurodegenerative diseases (Schindowski et al., 2008). BDNF exerts its pro-survival effects by binding TrkB and activating two key signaling pathways. In one pathway, the binding of BDNF to TrkB induces PI3K activity. In the other pathway, TrkB-induced ERK activation results in enhanced Rsk2 or Msks kinase activities (Sossin and Barker, 2007).

In the present study, we have not only shown that D-gal led to lower-expression of BDNF and TrkB in hippocampus, but for the first time, demonstrated that ICA protected the brain disorder induced by D-gal, as evidenced by the Morris water maze test, morphology observation, and the hippocampus specific expressions of BDNF and TrkB. The efficacy of ICA in improving learning and memory deficits by D-gal differed from other approved AD drugs such as donepezil, which is primarily effective against scopolamine-induced deficits, with small effects on working memory and spatial mapping (Lindner et al., 2006), and no report on D-gal model. Thus this study provides mechanistic basis for ICA in the protection against neurodegenerative disease, such as AD.

In conclusion, this study demonstrated that D-gal-induced learning and memory deficits are associated with hippocampus neurodegeneration and reduction of BDNF and TrkB, which can be attenuated by ICA.

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