Calycosin improves cognitive function in a transgenic mouse model of Alzheimer’s disease by activating the protein kinase C pathway

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Abstract

The major pathological changes in Alzheimer’s disease are beta amyloid deposits and cognitive impairment. Calycosin is a typical phytoestrogen derived from radix astragali that binds to estrogen receptors to produce estrogen-like effects. Radix astragali Calycosin has been shown to relieve cognitive impairment induced by diabetes mellitus, suggesting calycosin may improve the cognitive function of Alzheimer’s disease patients. The protein kinase C pathway is upstream of the mitogen-activated protein kinase pathway and exerts a neuroprotective effect by regulating Alzheimer’s disease-related beta amyloid degradation. We hypothesized that calycosin improves the cognitive function of a transgenic mouse model of Alzheimer’s disease by activating the protein kinase C pathway. Various doses of calycosin (10, 20 and 40 mg/kg) were intraperitoneally injected into APP/PS1 transgenic mice that model Alzheimer’s disease. Calycosin diminished hippocampal beta amyloid, Tau protein, interleukin-1beta, tumor necrosis factor-alpha, acetylcholinesterase and malondialdehyde levels in a dose-dependent manner, and increased acetylcholine and glutathione activities. The administration of a protein kinase C inhibitor, calphostin C, abolished the neuroprotective effects of calycosin including improving cognitive ability, and anti-oxidative and anti-inflammatory effects. Our data demonstrated that calycosin mitigated oxidative stress and inflammatory responses in the hippocampus of Alzheimer’s disease model mice by activating the protein kinase C pathway, and thereby improving cognitive function.

Key Words: nerve regeneration; neurodegeneration; Alzheimer’s disease; calycosin; hippocampus; oxidative stress; inflammation; mice; protein kinase C; calphostin C; glutathione; malondialdehyde; neural regeneration
Introduction

Alzheimer's disease (AD) is the most common type of senile dementia, and is one of the greatest burdens of healthcare in developed countries. Its main pathological features include profuse and widespread extracellular amyloid plaques as well as intraneuronal fibrotic tangles (Sperling et al., 2014; Ramezani et al., 2016; Zhang et al., 2016). However, the underlying cause of these pathologies remains uncertain, and there is no effective disease modifying treatment. In recent years, the main focus of research on novel pharmacotherapies of AD has been the amyloidogenic hypothesis, which posits that the accumulation of amyloid beta (Aβ) peptide is responsible for cognitive impairment and neuronal death (Nasca-Labouze et al., 2015; De Strooper and Karran, 2016; Scheltens et al., 2016). Thus, there has been an effort towards the pharmacological attenuation of Aβ production by inhibiting β and γ secretase enzymes, and via immunotherapy targeting existing cerebral Aβ plaques. However, to date, these approaches have proven only modestly effective (Lahiri et al., 2014; Roher et al., 2014).

Radix astragali is a traditional Chinese herbal medicine that possesses various biological functions. Three groups of active constituents have been identified in astragalus extracts: flavonoids (which impart a yellow color to the root slice), saponins (a common constituent of plants in this family), and polysaccharides (long-chain polysaccharides with potential medicinal benefit mediated by effects on leukocytes) (Cai et al., 2016; Shahzad et al., 2016). Astragaloside IV, calycosin, and formononetin are the three main bioactive compounds that are implicated in the pharmacological activities and therapeutic efficacy of radix astragali (Zhang et al., 2016). Indeed, radix astragali is widely used in traditional Chinese medicine, with indications for the treatment of diabetes, cardiovascular diseases, nephropathy, cancer and neuropathy (Fu et al., 2014a). The main source of radix astragali in China is the dried root of Astragalus membranaceus var. mongholicus (Bge.) Hsiao, and both cultivated and wild plants are used clinically (Li et al., 2015).

Calycosin is a typical phytoestrogen that binds to estrogen receptors to produce estrogen-like effects. It is also reported to have antioxidant, anti-osteoporosis, anti-tumor and immunomodulating activities (Zhang et al., 2015). Recently, Wang and Zhao (2016) reported that calycosin had beneficial effects on the amelioration, prevention and treatment of diabetes-associated cognitive deficits, through its effects on oxidative stress, synaptic function and the PI3K/Akt/GSK-3β pathway, all of which have likewise been implicated in AD pathology. In another study, treatment with calycosin-7-O-β-D-glucoside significantly reduced infarct volume, histological damage and blood-brain barrier defects in a rat middle cerebral artery occlusion model (Fu et al., 2014b). Furthermore, calycosin-7-O-β-D-glucoside treatment inhibited the expression of matrix metalloproteinases (MMPs), and stabilized the expression of cav-1 and tight junction proteins in microvessels isolated from the ischemic rat cortex (Fu et al., 2014b). The putative mechanism is unknown, but calycosin-7-O-β-D-glucoside was reported to scavenge nitric oxide radicals, inhibit MMP-2 and MMP-9 activity, and attenuate death of brain microvascular endothelial cells (Fu et al., 2014b).

Given this background, it seems appropriate to test the effects of calycosin in a model of AD. To study the pathobiology and possible pharmacological targets of AD, various transgenic mouse models have been developed, which emulate specific aspects of AD pathology in the human brain. The double mutant APP/PS1 mouse has become a well-established model for preclinical AD research, manifesting a broad range of behavioral and pathological abnormalities, notably increased brain levels of Aβ, cerebellar dysfunction, impairment of motor coordination and deficits in learning and memory, as well as increased markers of oxidative stress, inflammation, and neuronal apoptosis, all of which clearly mimic the condition of clinical AD (Gao et al., 2015; Toba et al., 2016). Thus, this study investigated the effects of calycosin treatment on the development of pathologies in the APP/PS1 transgenic mouse model of AD.

Materials and Methods

Animals

Sixty male APP/PS1 transgenic mice aged 7–8 months old and weighing 26–28 g (Nanjing Biomedical Research Institute, Nanjing, China, license No. SYXK (Su) 2010003) were used as the experimental group, and male C57BL/6 mice (n = 36) of the same age (Animal Center, Jilin University, China) were used as controls.

The study protocol was approved by the Animal Experiment Committee of Jilin University of China (approval number: 2014–014). The experimental procedure followed the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1986).

Drug treatment

Mice were randomized into 8 groups (n = 12 per group) as follows.

Group I: No treatment was given to C57BL/6 male mice, and they were assessed for behavioral parameters from day 64 onwards.

Group II: Dimethyl sulfoxide (0.5%; 10 mL/kg, intraperitoneally) was administered to C57BL/6 mice from day 1 to day 70.

Group III: Calycosin (40 mg/kg, intraperitoneally, daily, Sigma-Aldrich, St. Louis, MO, USA) in dimethyl sulfoxide as described for group II above, was administered to C57BL/6 mice according to a previous study (Cheng et al., 2015).

Group IV: No treatment was given to APP/PS1 transgenic mice until day 70.

Groups V, VI and VII: APP/PS1 mice received daily injections of calycosin at one of three different doses (10, 20 and 40 mg/kg) intraperitoneally, from day 1 to day 70 with behavioral testing initiated on day 64.

Group VIII: APP/PS1 mice were administered calycosin (40 mg/kg, intraperitoneally) from day 1 until day 70, and received additional treatment with calphostin C one hour
before calycosin treatment from day 64 to day 70. Calphostin C (0.1 μg in 3 mL 0.5% dimethyl sulfoxide per mouse, Sigma-Aldrich), a selective protein kinase C (PKC) inhibitor, was administered into cerebral ventricles at the following coordinates: −2.5 mm dorsal/ventral, −1.0 mm lateral, and −0.5 mm anterior/posterior from bregma), while under anesthesia (Galeotti and Ghezardini, 2011).

**Behavioral assessment**

**Morris water maze test**

The Morris water maze test was performed to evaluate learning and memory abilities in mice. The Morris water maze consisted of a circular water tank (180 cm in diameter, 70 cm in height) filled with water at 22 ± 1°C. The pool was divided into four equal quadrants labeled north, west, south and east. A colorless escape platform (10 cm in diameter) was submerged 2 cm below the surface in the east quadrant, which was designated the target quadrant. We recorded escape latency time from day 64 to day 67 of the study. On day 68, we recorded the escape latency time, time spent in the target quadrant and number of crossings in the platform area, according to a previously defined procedure (Kummer et al., 2015).

**Passive avoidance test**

The passive avoidance test was performed to evaluate learning and memory abilities in mice. The experimental box used for the passive avoidance test consisted of two identical 25 × 40 × 25 cm³ compartments with a metal grid floor. The compartments were separated by a guillotine door. One of the compartments was white and illuminated, while the other was black and un-illuminated. Mice were trained in the passive avoidance apparatus on day 69, and 24 hours after the acquisition conditioning session (day 70). We tested passive avoidance as the retention latency in accordance with previously published methods (Liu et al., 2014).

**Collection of samples and biochemical measurement**

Mice were decapitated 60 minutes after the final behavioral tests. Brains were carefully removed, and hippocampi were immediately dissected on a cold plate, weighed, and quickly homogenized in ice-cold 0.9% saline. The homogenate was centrifuged at 3,000 r/min for 10 minutes at 4°C. The supernatants were collected and stored at 4°C for assays of amyloid beta, tau protein, acetylcholine (ACE), acetylcholinesterase (AchE) activity, interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNF-α), glutathione (GSH) and malondialdehyde (MDA) measurements, all according to the manufacturer’s instructions for the respective enzyme linked immunosorbent assay kits (Abcam, Hong Kong, China).

**Statistical analysis**

The results were expressed as the mean ± SD. All data were analyzed using SPSS 20.0 software (IBM, Armonk, NY, USA). The data were statistically analyzed using one-way analysis of variance followed by Tukey’s multiple range test. A value of \( P < 0.05 \) was considered statistically significant.

**Results**

**Effect of calycosin on cognitive function of APP/PS1 mice**

**Morris water maze**

The control mice had a significant reduction in escape latency time on day 67 compared with day 64 of the study, which suggests normal spatial learning capacity. However, the APP/PS1 mice had a significantly higher escape latency time on day 67 compared with control mice, suggesting impaired learning. The daily administration of calycosin significantly and dose-dependently reduced the escape latency time of APP/PS1 mice on day 67, which was significantly abolished by co-treatment with calphostin C (Figure 1A). Furthermore, on day 68 of the study, control mice spent a significantly longer time in the target quadrant compared with the other quadrants, which suggest normal memory. APP/PS1 mice showed a significant reduction in the time spent in the target quadrant on day 68 and performed fewer crossings in the platform area compared with control mice, indicating impaired memory. The daily administration of calycosin significantly and dose-dependently increased the time spent in the target quadrant on day 68 and the number of crossings in the platform area of APP/PS1 mice, which were significantly abolished by treatment with calphostin C (Figure 1A–C).

**Passive avoidance test**

All mice showed a normal mean initial latency on day 32, but significant changes were observed in retention latency on day 33, as assessed in the passive avoidance test. APP/PS1 mice had a significant decrease in retention latency on day 70 of the study compared with control mice, which suggests impaired cognitive function. The daily administration of calycosin significantly and dose-dependently increased the retention latency of APP/PS1 mice, which was significantly abolished by co-treatment with calphostin C (Figure 2).

**Effect of calycosin on the levels of hippocampal amyloid beta, tau protein, inflammation, and oxidative stress in APP/PS1 mice**

APP/PS1 mice showed a significant increase in hippocampal amyloid beta, tau protein, AChE activity, ACh, TNF-α, IL-1β and MDA along with a reduction in GSH levels. The daily administration of calycosin significantly and dose-dependently decreased the hippocampal amyloid beta, tau protein, TNF-α, IL-1β, AChE and MDA levels, but increased ACh and GSH levels in APP/PS1 mice (\( P < 0.05 \) or \( P < 0.01 \)). These beneficial effects of calycosin were significantly abolished by the treatment of calphostin C (Figures 3–6).

**Discussion**

APP/PS1 mice in this study had significantly impaired learning and memory characterized by increased levels of hippocampal amyloid beta, tau protein, pro-inflammatory mediators (IL-1β and TNF-α), and oxidative stress (increased TBARS and decreased GSH) compared with age-matched C57BL/6 mice. The daily administration of calycosin at three different doses significantly and dose-dependently corrected...
the impaired spatial learning and memory, and normalized the levels of hippocampal amyloid beta, tau protein, inflammation and oxidative stress in APP/PS1 animals. These beneficial effects of calycosin in APP/PS1 animals were significantly abolished by the co-administration of a selective inhibitor of PKC, calphostin C, suggesting the neuroprotective effects of calycosin were dependent on activation of the PKC pathway.

APP/PS1 mice are a widely used transgenic model of AD to research the mechanistic processes involved in AD. It was reported that 39 species of Aβ, of both murine and human origin, were detectable in APP/PS1 mouse brain, demonstrating a higher abundance than other widely used transgenic models of AD such as Tg2576 (Allue et al., 2016). This supports the use of APP/PS1 to study disease-altering treatments for AD. The amyloid-beta hypothesis remains the most widely accepted notion to explain the induction of impaired learning, memory and the cardinal neuropathological features of AD. A vast array of possible pathways has been reported for the induction of AD by Aβ. Bian and colleagues recently reported evidence for increased intracerebral levels of Tau and phosphorylated Tau, along with increased neuronal death, and altered morphology in the hippocampus of APP/PS1 mice, which were associated with learning and memory deficits (Bian et al., 2016). This suggests that tau-pathology is initiated by Aβ. Higher levels of pro-inflammatory cytokines and inducible nitric oxide synthase along with reduced anti-inflammatory cytokines and arginase-1 have been found in the brain of APP/PS1 animals, suggesting the activation of pro-inflammatory pathways (Wan et al., 2016). In addition, Tian and colleagues observed a reduction of superoxide dismutase activity along with increased levels of MDA in APP/PS1 mice compared with wild-type C57 mice, which suggests higher levels of oxidative stress (Tian et al., 2016). Thus, our results with APP/PS1 mice are consistent with previously published reports.

We found that the daily administration of calycosin to APP/PS1 mice corrected their impaired learning and memory, and reduced the hippocampal levels of Aβ, tau protein, and oxidative stress markers and inflammatory cytokine levels. Among the *radix astragali* constituents, calycosin, a typical phytoestrogen, induces estrogen-like effects. In addition, calycosin was reported to have antioxidant, anti-osteoporosis, anti-tumor and immunomodulating activities (Zhang et al., 2015). The potential pharmacological properties of calycosin for the treatment of tumors, inflammation, stroke, and cardiovascular diseases have gained increasing attention in recent years (Wang et al., 2014; Cheng et al., 2015; Su et al., 2016; Zhao et al., 2016). Indeed, the potential use of calycosin to treat diseases is attributed to its isoflavonoid and phytoestrogenic properties. The beneficial effects of calycosin most likely result from its interaction with endoplasmic reticulum receptors on the cell membrane, and modulation of the mitogen-activated protein kinase signaling pathway (Gao et al., 2014).

Wang and Zhao (2016) reported that calycosin ameliorated and prevented diabetes-associated cognitive deficits, oxidative stress, and tau phosphorylation (Wang and Zhao, 2016); however, no other study has reported the effects of calycosin in learning, memory and dementia models, even though *radix astragali* is considered a possible treatment for vascular dementia (Man et al., 2012). A mixture of *radix astragali* with other herbs (Buyuan Congnao decoction) has been used to improve learning and memory in a rat model of AD, while reducing hippocampal Aβ accumulation (Chen et al., 2012), and remains the only study of the potential utility of calycosin, an important constituent of *radix astragali*, to rescue behavioral and neurochemical pathology in a transgenic model of AD.

As noted above, the glycoside of calycosin was reported to significantly reduce infarct volume, histological damage and blood-brain-barrier permeability in a middle cerebral artery occlusion stroke model, and the glycoside remarkably inhibited the expression and activities of MMPs, nitric oxide and stabilized the expression of cavi-1 and tight junction proteins in microvessels isolated from ischemic rat cortex (Fu et al., 2014b). Calycosin treatment decreased the expression of synapsin and post-synaptic density protein, as well as brain-derived neurotrophic factor, in diabetic rats (Wang and Zhao, 2016). Calycosin was also reported to downregulate the expression levels of NFATc1 and c-Fos by suppressing the activation of nuclear factor-kB and mitogen-activated protein kinases (Quan et al., 2015) and to reduce neutrophil infiltration and myeloperoxidase levels in myocardial infarction (Cheng et al., 2015). These studies indicate the anti-inflammatory potential of calycosin.

The proinflammatory cytokines, TNF-α and IL-1β, are thought to be involved in the progression of AD pathology (Quintanilla et al., 2012). The present study showed that calycosin normalized the levels of TNF-α and IL-1β in the hippocampus of AD model mice, suggesting the rescue of functions by attenuating inflammatory signaling. Free radicals are the products of normal cellular metabolism and are involved in the development of AD (Quintanilla et al., 2012). The increased production of free radicals and decreased endogenous antioxidant systems may accelerate membrane phospholipid breakdown, leading to lipid peroxidation and cellular dysfunction. In the present study, increased MDA and decreased GSH levels were found in the hippocampus of APP/PS1 mice and these were reversed by calycosin to potentially rescue lipid peroxidation in the AD model hippocampus. Similarly, calycosin treatment was reported to decrease MDA levels, and increase SOD levels and GSH-Px activity in the hippocampus of diabetic animals (Wang and Zhao, 2016). The glycoside of calycosin significantly increased the activities of antioxidant enzymes, scavenged reactive oxygen species and reduced MDA production during thiocacetamide-induced oxidative stress in BRL-3A cells (Jian et al., 2015), suggesting free radical scavenging and anti-oxidative stress effects of calycosin. Here, we showed that prolonged treatment with calycosin rescued spatial memory and normalized a whole range of neuropathological markers in the APP/PS1 mouse model of AD. PKC, a family of protein kinase enzymes involved in controlling the function of other
Figure 1 Effect of calycosin on learning and memory abilities of APP/PS1 mice assessed by the Morris water maze.
(A) Escape latency time during an acquisition trial in the Morris water maze. (B) Mean time spent in the target quadrant on day 68 during a retrieval trial in the Morris water maze. Results are expressed as the mean ± SD (n = 12; one-way analysis of variance followed by Tukey’s multiple range test). *P < 0.05, **P < 0.01, vs. group IV; #P < 0.05, ##P < 0.01, vs. group VII; †P < 0.01, vs. groups I, II and III. Group I: Untreated C57BL/6 mice; group II: C57BL/6 with daily vehicle; group III: C57BL/6 mice treated with calycosin 50 mg/kg; group IV: APP/PS1 mice; groups V, VI, VII: APP/PS1 mice treated with calycosin 10, 20 and 30 mg/kg daily, respectively; group VIII: calycosin 40 mg/kg + calphostin C (a selective protein kinase C inhibitor, 0.1 μg in 3 μL 0.5% dimethyl sulfoxide per mouse). S: Second.

Figure 2 Effect of calycosin on the cognitive function of APP/PS1 mice assessed by the passive avoidance test.
Results are expressed as the mean ± SD (n = 12; one-way analysis of variance followed by Tukey’s multiple range test). *P < 0.05, **P < 0.01, vs. group IV; #P < 0.01, vs. group VII. Group I: Untreated C57BL/6 mice; group II: C57BL/6 with daily vehicle; group III: C57BL/6 mice treated with calycosin 50 mg/kg; group IV: APP/PS1 mice; groups V, VI, VII: APP/PS1 mice treated with calycosin 10, 20 and 30 mg/kg daily, respectively; group VIII: calycosin 40 mg/kg + calphostin C (a selective protein kinase C inhibitor, 0.1 μg in 3 μL 0.5% dimethyl sulfoxide per mouse). S: Second.

Figure 3 Effect of calycosin on the levels of hippocampal amyloid beta (Aβ) and tau protein levels assessed by enzyme linked immunosorbent assay.

(A) Aβ levels and (B) tau protein levels. Results are expressed as the mean ± SD (n = 12; one-way analysis of variance followed by Tukey’s multiple range test). *P < 0.05, **P < 0.01, vs. group IV; #P < 0.05, ##P < 0.01, vs. group VII; †P < 0.01, vs. groups I, II and III. Group I: Untreated C57BL/6 mice; group II: C57BL/6 with daily vehicle; group III: C57BL/6 mice treated with calycosin 50 mg/kg; group IV: APP/PS1 mice; groups V, VI, VII: APP/PS1 mice treated with calycosin 10, 20 and 30 mg/kg daily, respectively; group VIII: calycosin 40 mg/kg + calphostin C (a selective protein kinase C inhibitor, 0.1 μg in 3 μL 0.5% dimethyl sulfoxide per mouse).
Figure 4 Effect of calycosin on the hippocampal cholinergic dysfunction in APP/PS1 mice assessed by enzyme linked immunosorbent assay. (A) Levels of hippocampal acetylcholine and (B) acetylcholinesterase activity. Results are expressed as the mean ± SD (n = 12; one-way analysis of variance followed by Tukey's multiple range test). *P < 0.05, **P < 0.01, vs. group IV; #P < 0.05, ##P < 0.01, vs. group VII. Group I: Untreated C57BL/6 mice; group II: C57BL/6 with daily vehicle; group III: C57BL/6 mice treated with calycosin 50 mg/kg; group IV: APP/PS1 mice; groups V, VI, VII: APP/PS1 mice treated with calycosin 10, 20 and 30 mg/kg daily, respectively; group VIII: calycosin 40 mg/kg + calphostin C (a selective protein kinase C inhibitor, 0.1 μg in 3 μL 0.5% dimethyl sulfoxide per mouse).

Figure 5 Effect of calycosin on hippocampal inflammatory factor in APP/PS1 mice assessed by enzyme linked immunosorbent assay. (A) Levels of interleukin-1 (IL-1) protein and (B) levels of tumor necrosis factor-alpha (TNF-α) protein. Results are expressed as the mean ± SD (n = 12; one-way analysis of variance followed by Tukey's multiple range test). *P < 0.05, **P < 0.01, vs. group IV; ##P < 0.01, vs. group VII. Group I: Untreated C57BL/6 mice; group II: C57BL/6 with daily vehicle; group III: C57BL/6 mice treated with calycosin 50 mg/kg; group IV: APP/PS1 mice; groups V, VI, VII: APP/PS1 mice treated with calycosin 10, 20 and 30 mg/kg daily, respectively; group VIII: calycosin 40 mg/kg + calphostin C (a selective protein kinase C inhibitor, 0.1 μg in 3 μL 0.5% dimethyl sulfoxide per mouse).

Figure 6 Effect of calycosin on hippocampal oxidative stress in APP/PS1 mice assessed by enzyme linked immunosorbent assay. (A) Levels of hippocampal malondialdehyde (MDA) and (B) levels of hippocampal glutathione (GSH). Results are expressed as the mean ± SD (n = 12; one-way analysis of variance followed by Tukey's multiple range test). **P < 0.01, vs. group IV; #P < 0.05, vs. group VII. Group I: Untreated C57BL/6 mice; group II: C57BL/6 with daily vehicle; group III: C57BL/6 mice treated with calycosin 50 mg/kg; group IV: APP/PS1 mice; groups V, VI, VII: APP/PS1 mice treated with calycosin 10, 20 and 30 mg/kg daily, respectively; group VIII: calycosin 40 mg/kg + calphostin C (a selective protein kinase C inhibitor, 0.1 μg in 3 μL 0.5% dimethyl sulfoxide per mouse).
mechanisms, which might be involved in the beneficial effects of calycosin in AD.

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