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Danshensu attenuates scopolamine and amyloid-β-induced cognitive impairments through the activation of PKA-CREB signaling in mice

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ABSTRACT

Alzheimer's disease (AD) is an important chronic neurodegenerative disorder and is mainly associated with cognitive dysfunction. At present, bioactive compounds from traditional medicinal plants have received much attention for the enhancement of cognitive function. Danshensu, a phenolic acid isolated from herbal medicines, has various pharmacological activities in the central nervous system, including anxiolytic-like and neuroprotective properties. The present study aimed to investigate the ameliorating effects of danshensu on scopolamine- and amyloid-β (Aβ) protein-induced cognitive impairments in mice. Danshensu (3 and 10 mg/kg, p.o.) effectively ameliorated scopolamine-induced cognitive dysfunction in mice, as measured in passive avoidance and Y-maze tasks. In a mechanistic study, danshensu inhibited monoamine oxidase A (MAO-A) activity but not MAO-B. Additionally, danshensu treatment increased the dopamine level and the phosphorylation levels of protein kinase A (PKA) and cAMP response element binding protein (CREB), in the cortex of the brain. Furthermore, the ameliorating effect of danshensu against scopolamine-induced cognitive impairment was fully blocked by H89, a PKA inhibitor. Finally, danshensu also ameliorated Aβ-induced cognitive impairments in an animal model of AD. The results revealed that danshensu treatment significantly improved scopolamine and Aβ-induced cognitive impairments in mice by facilitation of dopamine signaling cascade such as PKA and CREB due to MAO-A inhibition. Thus, danshensu could be used as a promising therapeutic agent for preventing and treating AD.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and is characterized by cognitive impairments and psychiatric symptoms. Growing evidence has implicated that neurofibrillary tangles of misfolded tau protein and amyloid plaques of amyloid-β (Aβ) protein are observed in the brain of AD patients, which may lead to the death of neurons, particularly cholinergic and monoaminergic neurons (Hardy and Selkoe, 2002; Wenk, 2003). Medications, such as cholinesterase inhibitors (ChEIs) and memantine, *N*-methyl-D-aspartate receptor antagonist, that are currently approved for the treatment of mild to moderate AD have been shown to improve cognition, daily activity,

Abbreviations: 6-OHDA, 6-hydroxydopamine; Aβ, amyloid beta; AChE, Acetylcholinesterase; AD, Alzheimer's disease; AGE, advanced glycation end products; ChEI, cholinesterase inhibitor; CREB, cAMP response element-binding protein; DA, dopamine; DNZ, donepezil; MAO, monoamine oxidase; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A

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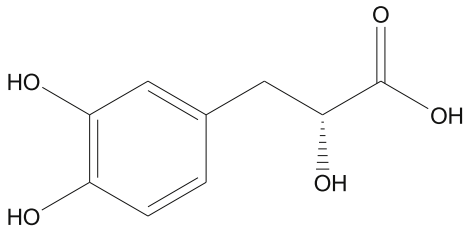

Danshensu

Fig. 1. The chemical structure of danshensu (salvianic acid A).

and function (Terry and Buccafusco, 2003). However, ChEIs, the most prescribed drugs for AD treatment, are generally well-tolerated but exhibit adverse effects, including diarrhea, headache, and vomiting, which are related to cholinergic stimulation in the brain and peripheral tissues (Thompson et al., 2004). Many kinds of drugs, such as anti-amyloid, anti-tau, anti-inflammatory, and neurotransmitter-based drugs, have been attempted for treating AD, but most of these drugs have failed in clinical trials; there have been no new drug approvals for treatment of AD since 2003 (Cummings et al., 2018). Accordingly, novel therapeutic strategies that target modulation of the monoaminergic system via the inhibition of monoamine oxidase (MAO) has been attempted to develop an effective medication for AD (Anand et al., 2014; Cai, 2014).

Danshensu (3-(3,4-dihydroxyphenyl)-(2*R*)-lactic acid, salvianic acid A; Fig. 1) has been reported to be a phenolic compound in several herbal materials, such as *Salvia miltiorrhiza* and *Prunella vulgaris* var. *lilacina* (Kwon et al., 2014; Zhou et al., 2005). We recently reported that danshensu exhibits an anxiolytic-like effect in mice via activation of the dopamine (DA) D1 receptor by the inhibition of MAO-A (Kwon et al., 2014). Along with affecting monoamine metabolism, MAO inhibitors are known to exhibit a neuroprotective effect (Al-Nuaimi et al., 2012; Trillo et al., 2013). Danshensu has also been shown to protect against Aβ-induced cytotoxicity by improving cell viability, inhibiting Ca^{2+}-intake, and reducing lactate dehydrogenase release (Zhou et al., 2011). Danshensu attenuated 6-hydroxydopamine (6-OHDA)-induced cytotoxicity and the production of reactive oxygen species in PC12 cells and effectively reduced 6-OHDA-induced dopaminergic neuronal loss in zebrafish (Chong et al., 2013). Danshensu also exhibited neuroprotective activity against ischemia-reperfusion injury in rats (Guo et al., 2015; Xu et al., 2017). In addition, danshensu is known to ameliorate cognitive impairment in streptozotocin-induced diabetic mice by attenuating neuroinflammation (Wang et al., 2012). Thus, previous studies suggest that danshensu may exhibit a therapeutic effect against cognitive impairment in AD.

Here, we investigated the effects of danshensu on scopolamine and Aβ-induced cognitive impairments in mice. Moreover, we examined whether the memory-ameliorating effects of danshensu were associated with dopaminergic neurotransmission.

2. Materials and methods

2.1. Animals

All experiments with mice were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Kyung Hee University (KHP-2014-02-03). Male ICR mice (5 weeks old; 23–25 g body weight) were purchased from Orient Co. Ltd., a branch of Charles River Laboratories (Gyeonggi-do, Korea). After delivery, five mice were housed in each cage for an additional week, and these mice were provided with food and water *ad libitum*. The cages were kept in a room with a 12 h light/dark cycle (light on 07:30–19:30) at constant temperature (23 ± 2 °C) and humidity (60 ± 10%). Animal maintenance and treatment were carried out in accordance with the Animal Care and Use Guidelines issued by Kyung Hee University and Kangwon National University, Republic of Korea.

2.2. Materials

Danshensu was donated by one of the authors (D.S. Jang), and the purity was greater than 99%, as previous report (Kwon et al., 2014). Donepezil hydrochloride (DNZ), (−)-scopolamine hydrobromide, clorgyline, H89, and human amyloid-$\beta_{1\text{-}42}$ fragment ($A\beta_{1-42}$) were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies against protein kinase A (PKA) and cAMP response element-binding protein (CREB) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against phosphorylated (Ser/Thr) PKA (p-PKA) and phosphorylated CREB (p-CREB) were purchased from Cell Signaling (Danvers, MA). All other materials were of the highest grades available and were obtained from normal commercial sources. Danshensu, donepezil, H89 and scopolamine were dissolved in 0.9% saline solution.

2.3. Scopolamine or $A\beta_{1-42}$ protein injection and drugs administration

Cognitive impairment was established using scopolamine or $A\beta_{1-42}$ protein injection as in a previous study with slight modification (Park et al., 2012). In the case of the scopolamine-injected amnesic model, danshensu (1, 3 or 10 mg/kg, p.o.), donepezil (DNZ, 5 mg/kg, p.o.), H89 for the antagonism test (0.25 mg/kg, i.p.), clorgyline (10 mg/kg, p.o.) or the same amount of vehicle solution was administered to the mice 1 h before the behavioral tests or the sacrifice for sampling. And then, the mice were treated with scopolamine hydrobromide (1 mg/kg, i.p.) 30 min prior to the behavioral tests or the sacrifice for sampling. The dose of H89 is a sub-effective dose with no effect on memory performance based on our previous report (Lee et al., 2015).

In the case of the Aβ-induced amnesic model, $A\beta_{1-42}$ protein was dissolved in sterile saline (1 μg/μL) and incubated for 24 h at 37 °C to cause aggregation. After anesthesia with Zoletil 50® (10 mg/kg, i.m.), the mice were injected with aggregated $A\beta_{1-42}$ protein or vehicle (3 μL/3 min) into the right lateral ventricle at stereotaxic coordinates (AP, -0.2 mm; ML, +1.0 mm; DV, - 2.5 mm) taken from the atlas of the mouse brain (Paxinos and Franklin, 2004). After 5 min, the needle was removed using three intermediate steps with 1 min interstep delays to minimize backflow, and the mice were kept in a warm incubator (32–33 °C) until they awoke. After $A\beta_{1-42}$ protein aggregates injection, danshensu (1, 3, or 10 mg/kg), donepezil (5 mg/kg), clorgyline (10 mg/kg, p.o.) or the same amount of vehicle solution was orally administered to the mice at the same time (10:00–12:00 a.m.) once a day for 7 days. Donepezil, an acetylcholinesterase inhibitor, or clorgyline, a MAO-A inhibitor, was used as a positive control for the behavioral tests or the molecular tests, respectively.

2.4. Behavioral tests

2.4.1. Step-through passive avoidance task

A step-through passive avoidance task was conducted in two identical light and dark Plexiglas square boxes (20 cm × 20 cm × 20 cm), as previously described (Park et al., 2012). The light compartment contained a 50 W bulb, and its floor was composed of 2 mm steel rods spaced 1 cm apart. The floor of the non-illuminated, dark compartment also consisted of 2 mm steel rods spaced 1 cm apart. Briefly, the animals underwent two separate trials: an acquisition trial and a retention trial 24 h later. For the acquisition trial (AT), each mouse was initially placed in the light compartment, and 10 s later, the door (5 cm × 5 cm) between the two compartments was opened. When a mouse entered the dark compartment, the door automatically closed, and an electrical foot shock (0.5 mA, 3 s) was delivered through the grid floor. If the mouse did not enter the dark compartment within 120 s, the mouse was gently

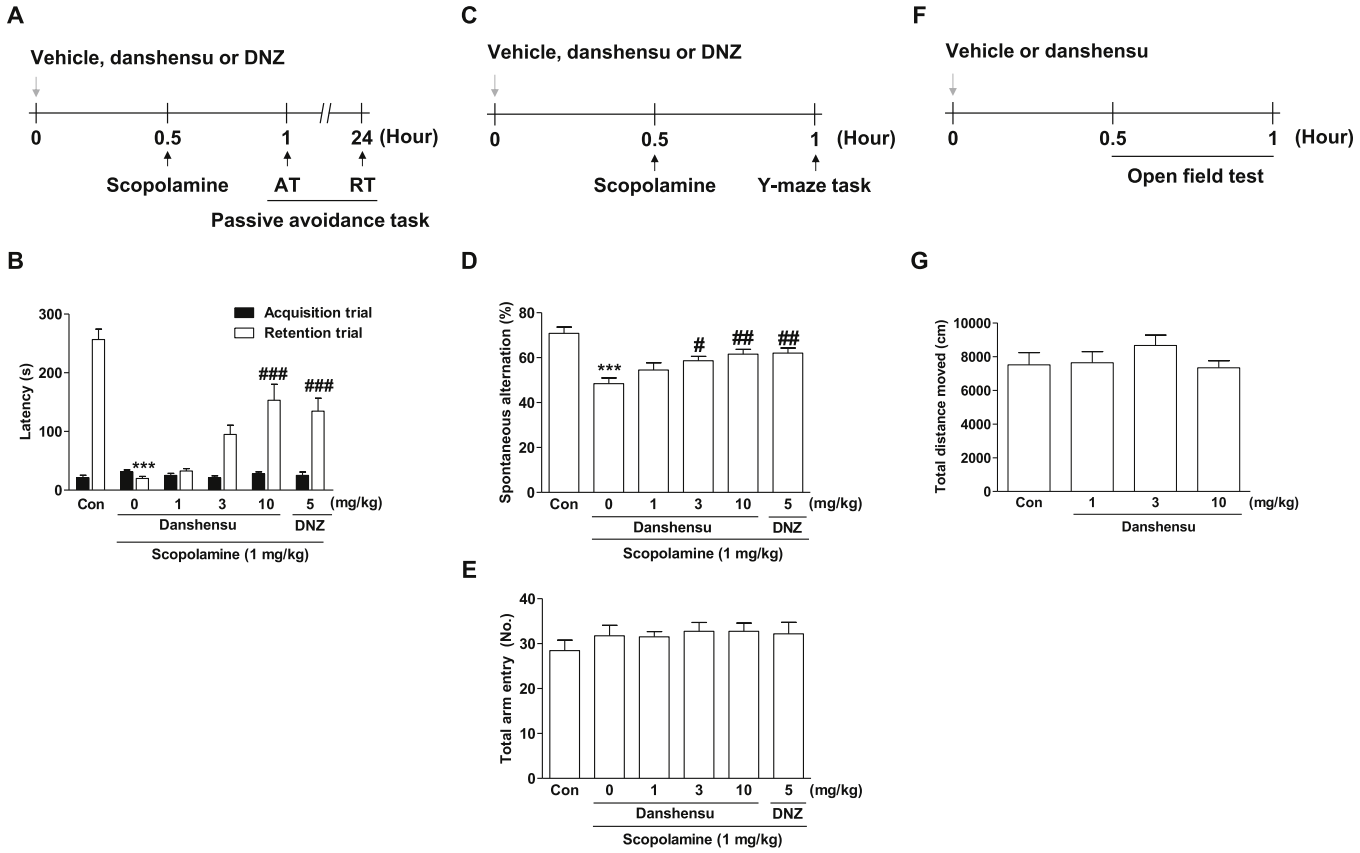

Fig. 2. Danshensu ameliorates scopolamine-induced learning and memory deficits in mice. A. Time schedule for passive avoidance task. B. Effect of danshensu on scopolamine-induced memory impairment in the step-through passive avoidance task (n = 8–10/group). C. Time schedule for Y-maze test. D, E. Effect of danshensu on scopolamine-induced memory impairment in the Y-maze test (n = 10–12/group). Spontaneous alternation behavior (D) and the number of arm entries (E) were recorded during an 8 min session. F. Time schedule for open field test. G. Effect of danshensu on locomotor activity in the open field test (n = 12/group). The exploratory behaviors of the mice in the open field test were observed for 30 min. Con, vehicle-treated control; DNZ, donepezil. Data represent the mean ± S.E.M (n = 8–12/group). $^{***}P < 0.001$ vs. vehicle-treated controls; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. scopolamine only treatment.

forced into the dark compartment. In this case, the acquisition trial was assigned a latency of 120 s (ceiling score). The retention trial (RT) was conducted 24 h after the acquisition trial (Fig. 2A). The mouse was again placed in the light compartment, and the time required to enter the dark compartment (latency) was recorded for each mouse. If the mouse did not enter the dark compartment within 300 s, we concluded that the mouse remembered the acquisition trial, and a latency of 300 s was assigned.

2.4.2. Y-maze task

The Y-maze test was conducted in a three-arm maze with angles of 120° between the arms; the arms were 40 cm long and 3 cm wide with walls that were each 12 cm high. The maze floor and walls were constructed from dark opaque polyvinyl plastic as previously described (Chong et al., 2013). The mice were initially placed within one arm, and the sequence and number of arm entries were recorded manually for each mouse over an 8 min period. The percentage of triads in which all three arms were represented, i.e., ABC, CAB, or BCA but not BAB, was recorded as an 'alternation' to estimate short-term memory. The arms were cleaned with 70% ethanol between each test to remove odors and residues. The alternation score (%) for each mouse was defined as the ratio of the actual number of alternations to the possible number (defined as the total number of arm entries minus two) multiplied by 100 as shown in the following equation: % Alternation = (Number of alternations)/(Total arm entries - 2) × 100. The number of arm entries was used as an indicator of locomotor activity. Danshensu (1, 3 or 10 mg/kg, p.o.), donepezil (5 mg/kg, p.o.) or the same amount of vehicle solution was administered to the mice 1 h before the test. Scopolamine was administered 30 min before the test to induce memory impairment (Fig. 2C).

2.4.3. Open-field test

To determine the effects of danshensu on the horizontal locomotor activity, an open-field test was performed as previously described (Park et al., 2015). The test was carried out in clear black Plexiglas boxes (41.5 cm × 41.5 cm × 41.5 cm) equipped with the video-based Ethovision System (Noldus, Wageningen, Netherlands). Thirty minutes after the treatment of danshensu (1, 3, or 10 mg/kg, p.o.) or same volume of vehicle solution, the mouse were placed in the center of the apparatus and locomotor behaviors were recorded for 30 min using video-tracking system (Fig. 2F). Horizontal locomotor activity was expressed as total ambulatory distance. The test box was cleaned with 70% ethanol between each test.

2.5. Acetylcholinesterase inhibition assay

Acetylcholinesterase (AChE) inhibitory assay was performed using acetylthiocholine iodide as a synthetic substrate in a colorimetric assay, as previously described (Ellman et al., 1961). AChE from *Electrophorus electricus* (electric eel) was used as the enzyme source for the assay. Each drug was initially dissolved in DMSO and diluted to several concentrations immediately before use. An aliquot of each diluted drug solution was then mixed with 640 μL of phosphate buffer (0.1 M, pH 8.0), 25 μL of buffered Ellman's reagent (10 mM 5,5-dithiobis(2-nitrobenzoic acid), 15 mM sodium bicarbonate) and the enzyme source (100 μL); the mixture was then preincubated at room temperature for

10 min. Ten minutes after the addition of 5 μL of an acetylthiocholine iodide solution (75 mM), the absorbance was measured at 410 nm using a UV spectrophotometer (OPTIZEN 2120UV, Mecasys Co., Ltd., Korea). The concentration of drug required to inhibit AChE activity by 50% (IC_{50}) was calculated using an enzyme inhibition dose-response curve.

2.6. Measurement of cortical dopamine level

Danshensu (3 or 10 mg/kg, p.o.), clorgyline (10 mg/kg, p.o.) or the same amount of vehicle solution was administered to the mice 1 h before the sacrifice. Clorgyline, an MAO-A inhibitor, was used a positive control. The level of dopamine in the cortex was measured using Dopamine Research ELISA kits (Labor Diagnostika Nord GmbH & Co. KG, Nordhorn, Germany) according to the manufacturer's instructions. After every reaction was terminated with stop solution, the result was determined using a spectrophotometer at a wavelength of 450 nm.

2.7. Western blotting

After the drug treatment, the mice were sacrificed by decapitation, and the brain was immediately removed. Isolated cortical tissues were homogenized in ice-chilled Tris-HCl buffer (20 mM, pH 7.4) containing protease and phosphatase inhibitors. The tissue lysate was centrifuged at 12,000 rpm at 4 °C for 20 min. The supernatant was quantified by the Bradford method using the Pierce BCA Protein Assay kit (Thermo Scientific, PA), and 15 μg of protein was subjected to SDS-PAGE (8% gel) under reducing conditions. Western blot analysis was conducted as described elsewhere (Park et al., 2012). Proteins were transferred to a PVDF membrane in transfer buffer and further separated at 100 V for 2 h at 4 °C. The membrane was incubated for 2 h with blocking solution (5% skim milk) at 4 °C and the incubated overnight with a primary antibody (p-PKA, 1:1000; PKA, 1:1000; p-CREB, 1:1000; CREB, 1:3000). The membrane was then washed twice with Tween 20/Tris-buffered saline (TTBS), incubated with a horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature, washed three times with TTBS, and developed using enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL). The immunoblots were imaged using a bio-imaging program on a LAS-4000 mini imager (Fujifilm Lifescience USA, Stamford, CT) and analyzed using Multi Gauge version 3.2 (Fujifilm Holdings Corporation, Tokyo, Japan). The phosphorylation level was determined by calculating the ratios of phosphorylated protein to total protein on the same membranes.

2.8. Statistics

The results of studies were expressed as means ± S.E.M. Data from behavioral tasks and Western blot analysis were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* analysis for multiple comparisons. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Danshensu attenuates scopolamine-induced cognitive impairments

To explore whether danshensu affects scopolamine-induced long-term fear-based learning and memory deficits, we conducted the step-through passive avoidance task after the treatment with danshensu (1, 3, or 10 mg/kg) and donepezil (DNZ, 5 mg/kg) (Fig. 2A). One-way ANOVA showed significant changes in the latency of the retention trial of the passive avoidance task ($F_{5, 50} = 21.03$, $P < 0.001$). The latency was significantly decreased by the acute administration of scopolamine (1 mg/kg) compared to that of the vehicle-treated controls, and this change was blocked by the single administration of danshensu or donepezil ($P < 0.001$, Fig. 2B). However, no significant difference was observed in the latency of the acquisition trial ($F_{5, 50} = 0.732$, $P = 0.293$). Next, to determine the effect of danshensu on the scopolamine-induced working memory impairment, we performed the Y-maze task (Fig. 2C). One-way ANOVA revealed significant differences in the spontaneous alternation behavior ($F_{5, 65} = 8.782$, $P < 0.001$, Fig. 2D). The percentage of spontaneous alternation was significantly reduced after acute treatment with scopolamine (1 mg/kg) compared to that in vehicle-treated controls ($P < 0.001$). Furthermore, these behavioral alternations were significantly reversed by the single administration of danshensu (3 mg/kg, $P < 0.05$; 10 mg/kg, $P < 0.01$) or donepezil (5 mg/kg, $P < 0.01$). However, the number of total arm entries was unchanged by any treatment ($F_{5, 65} = 0.562$, $P = 0.728$, Fig. 2E). In addition, we tested whether danshensu alters the general locomotion behavior using the open-field test (Fig. 2F). In line with our previous study (Kwon et al., 2014), one-way ANOVA revealed that the treatment of danshensu did not cause any change on the total distance moved compared to vehicle-treated controls ($F_{3, 44} = 0.943$, $P = 0.428$, Fig. 2G), which indicating that danshensu unchanged the motor function. Collectively, these results indicate that single orally administration of danshensu attenuates the scopolamine-induced learning and memory deficits in mice.

3.2. Danshensu increases dopamine level and activates the PKA and CREB signaling cascade in the cortex

Scopolamine is well known as an anticholinergic drug which leads to cognitive impairments. AChE inhibitors such as donepezil prevented the progression of scopolamine-induced memory impairment in mice (Shin et al., 2018). Thus, we compared the effects of danshensu and donepezil on AChE activity in vitro study. As shown in Fig. 3A, donepezil inhibited AChE activity in a concentration-dependent manner (donepezil IC_{50}, 0.037 μg/mL), but danshensu failed to inhibit it (danshensu IC_{50}, > 1000 μg/mL), suggesting that the memory-ameliorating effect of danshensu is not related to cholinergic neurotransmission.

We have previously reported that danshensu selectively inhibits MAO-A but not MAO-B and that it exhibits anxiolytic effects through the activation of dopamine D1 receptor (Kwon et al., 2014). Therefore, we wanted to measure the dopamine level in the cerebral cortex after single oral administration of danshensu (3 and 10 mg/kg) or clorgyline (10 mg/kg), a selective MAO-A inhibitor as a positive control, by ELISA method. In line with our previous study, the cortical dopamine level was significantly increased by single administration of danshensu (10 mg/kg, $P < 0.05$) and clorgyline ($P < 0.01$) compared to vehicle-treated controls in normal naïve mice (one-way ANOVA, $F_{3, 24} = 6.711$, $P = 0.002$, Fig. 3B). Furthermore, the administration of danshensu or clorgyline also significantly increased the cortical dopamine level when compared to vehicle-treated control group ($P < 0.001$) or scopolamine-only treatment group ($P < 0.05$) in the scopolamine-induced amnesic mice (one-way ANOVA, $F_{4, 24} = 8.580$, $P = 0.0003$, Fig. 3C).

It was reported that the activation of dopamine D1 receptor modulates the downstream signaling pathway, such as PKA-CREB, which plays an important role in the improvement of learning and memory deficits (Chartoff et al., 2003; Vitolo et al., 2002; Wu et al., 2013). Thus, we next performed Western blot analysis to determine whether danshensu interacts with PKA and CREB signaling molecules in the cerebral cortex of mice. Similar to the findings of behavioral studies, the administration of danshensu (10 mg/kg) significantly increased the phosphorylation levels of PKA (one-way ANOVA, p-PKA/PKA ratio, $F_{2, 9} = 9.373$, $P < 0.01$, Fig. 3D and E) and CREB (one-way ANOVA, p-CREB/CREB ratio, $F_{2, 9} = 14.85$, $P < 0.01$, Fig. 3D and F) compared to vehicle-treated controls in the cerebral cortex of normal naïve mice. Moreover, the administration of danshensu or clorgyline also significantly increased the phosphorylation levels of PKA (one-way ANOVA, p-PKA/PKA ratio, $F_{4, 15} = 5.139$, $P = 0.008$,Fig. 3G and H) and CREB (one-way ANOVA, p-CREB/CREB ratio, $F_{4, 15} = 6.314$, $P = 0.004$, Fig. 3G and I) when compared to scopolamine-only treated group in the cerebral cortex of scopolamine-induced amnesic mice. These results demonstrate that danshensu promotes PKA and CREB

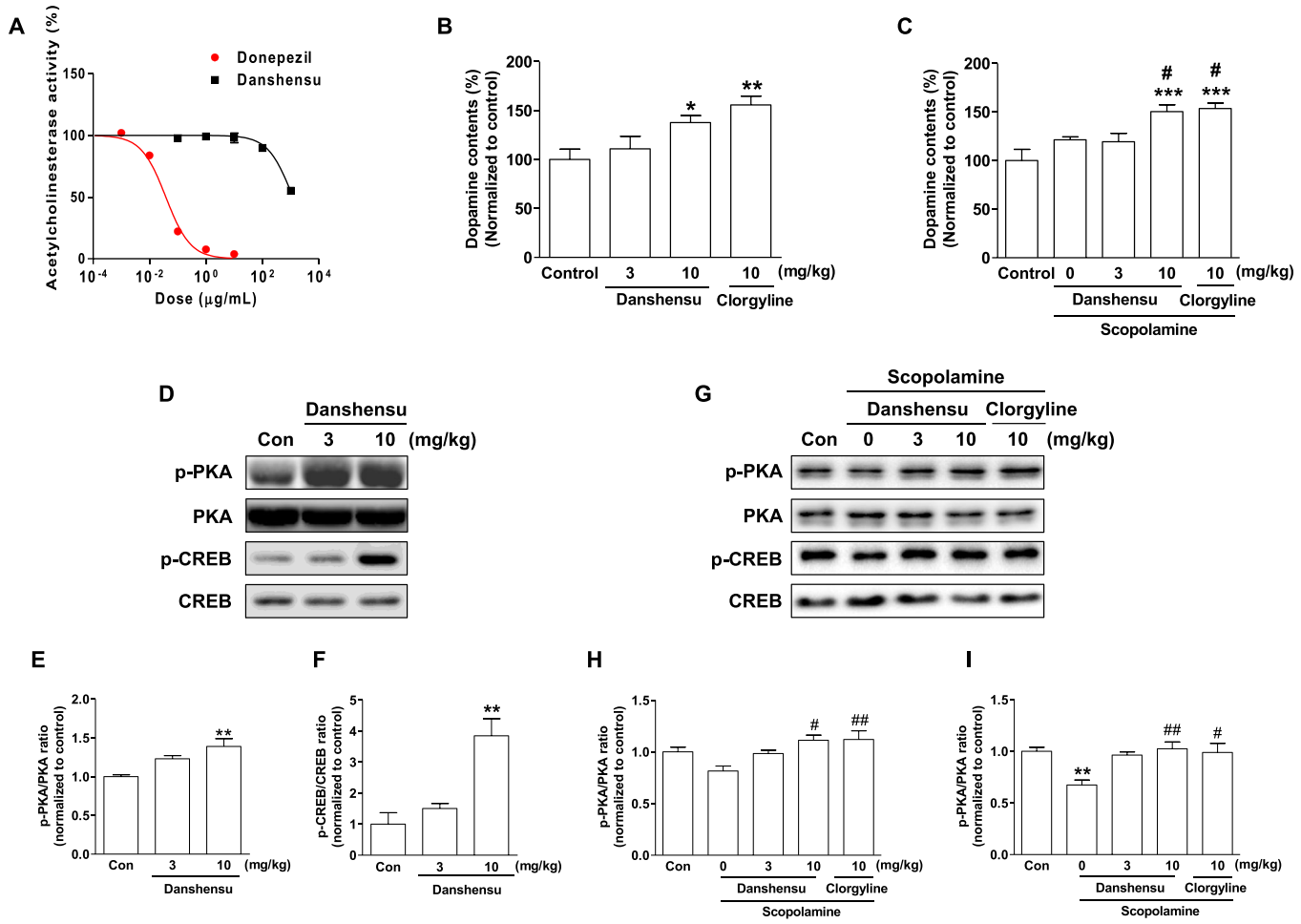

Fig. 3. Danshensu increases dopamine level and phosphorylation of PKA and CREB in the cerebral cortex. A. Effect of danshensu on acetylcholinesterase activity. B, C. Effects of danshensu and clorgyline (a MAO-A inhibitor) on dopamine level in the cerebral cortex of normal naïve mice and scopolamine-induced amnesic mice (n = 5–8/group). D-I. The immunoreactivity and quantitative analysis of PKA, phosphorylated PKA (p-PKA), CREB, and CREB (p-CREB) after danshensu treatment in the cerebral cortex of normal naïve mice and scopolamine-induced amnesic mice (n = 3–4/group). Con, control. Data represent the mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle-treated controls; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. scopolamine only treatment.

activation, which may further affect cognitive function.

3.3. Memory-ameliorating effect of danshensu on the scopolamine-induced learning and memory deficits is blocked by H89, a PKA inhibitor

To directly confirm whether danshensu's ameliorating effect of scopolamine-induced memory deficits is related to PKA, the antagonism experiments were performed using the PKA inhibitor H89 in the step-through passive avoidance task (Fig. 4). One-way ANOVA revealed significant changes in the latency of the retention trial of the passive avoidance task ($F_{4,\ 43} = 95.51$, $P < 0.001$). Similar with Fig. 2B, administration of danshensu (10 mg/kg, p.o.) significantly attenuated the scopolamine-induced cognitive impairment ($P < 0.001$, vs. scopolamine-only). As expected, this effect was blocked to the level of the scopolamine-treated group by co-administration of H89 ($P < 0.001$, scopolamine + danshensu vs. scopolamine + danshensu + H89). In addition, no significant difference was observed in the latency of the acquisition trial among all groups (one-way ANOVA, $F_{4,\ 43} = 0.350$, $P = 0.842$). This result indicates that the memory improvement of danshensu is mainly associated with the activation of PKA.

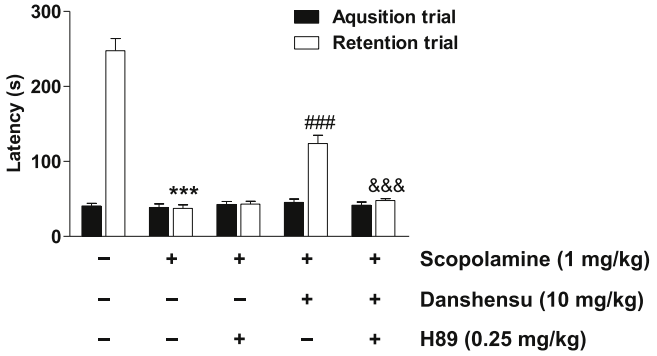

Fig. 4. Sub-effective dose of H89 blocks the ameliorating effect of danshensu against scopolamine-induced memory impairment in the step-through passive avoidance task. Con, control. Data represent the means ± S.E.M. (n = 9–10/group). $^{***}P < 0.001$ vs. vehicle-treated controls; $^{\#\#\#}P < 0.001$ vs. scopolamine only treatment; $^{\&\&\&}P < 0.001$ vs. scopolamine + danshensu treatment.

3.4. Danshensu ameliorates $A\beta_{1\text{-}42}$ protein-induced cognitive impairment in the step-through passive avoidance task

After the injection of $A\beta_{1\text{-}42}$ protein aggregates (3 μg/head, i.c.v), the mice were orally administered with danshensu (1, 3, and 10 mg/kg), donepezil (5 mg/kg), and same amount of saline vehicle for 7days (Fig. 5A). One hour after the final administration, the step-through passive avoidance task was conducted to confirm whether danshensu exerts ameliorating effects against $A\beta_{1\text{-}42}$ protein-induced cognitive

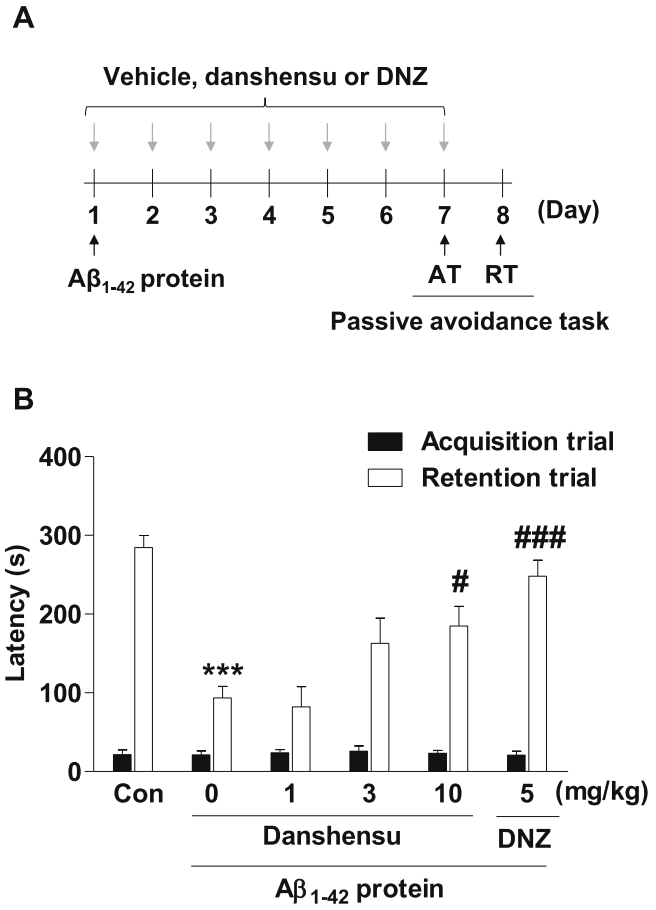

Fig. 5. Danshensu ameliorates $A\beta_{1-42}$ protein-induced memory deficit. A. After $A\beta_{1-42}$ protein aggregates injection into the right lateral ventricle under anesthesia, danshensu (1, 3, or 10 mg/kg), donepezil (5 mg/kg) or same amount of vehicle solution was orally administered to the mice once a day for 7 days. On the 7th day, the step-trough passive avoidance task was performed (n = 8–10/group). B. Effect of danshensu on $A\beta_{1-42}$ protein-induced memory impairment in the step-through passive avoidance task. AT, acquisition trial; RT, retention trial; Con, control; DNZ, donepezil. Data represent the mean ± S.E.M. $^{***}P < 0.001$ vs. vehicle-treated controls; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. $A\beta_{1-42}$ only treatment.

impairment in an animal model of AD. As shown in Fig. 5B, $A\beta_{1-42}$ protein-induced cognitive impairment ($P < 0.001$ vs control) was significantly attenuated by the repeated administration of danshensu ($P < 0.05$) or donepezil ($P < 0.001$) in the retention trial (one-way ANOVA, $F_{5,\ 45} = 12.19$, $P < 0.001$). However, there were no significant differences in the step-through latency in the acquisition trials among all groups (one-way ANOVA, $F_{5,\ 45} = 0.134$, $P = 0.984$).

We also tested whether repeated administration of danshensu (10 mg/kg) for 7 days increases dopamine level and activates PKA-CREB signaling in the cerebral cortex of $A\beta_{1-42}$ protein-induced amnesic mice (Fig. 6A). Similar to the above findings, the repeated administration of danshensu significantly increased the cortical dopamine level compared with vehicle-treated controls ($P < 0.05$) and $A\beta_{1-42}$ protein-only treatment group ($P < 0.05$) (one-way ANOVA, $F_{3,\ 21} = 25.24$, $P < 0.001$, Fig. 6B). Furthermore, danshensu treatment significantly increased the phosphorylation levels of PKA (one-way ANOVA, p-PKA/PKA ratio, $F_{3,\ 19} = 5.073$, $P = 0.0095$, Fig. 6C) and CREB (one-way ANOVA, p-CREB/CREB ratio, $F_{3,\ 19} = 4.489$, $P = 0.017$, Fig. 6D) compared to $A\beta_{1-42}$ protein-only treatment group. In addition, clorgyline significantly also increased dopamine level and the phosphorylation of PKA, but not CREB. These results suggest that danshensu would be effective in the treatment of Aβ-induced cognitive dysfunctions through the activation of PKA-CREB signaling cascade.

4. Discussion

AD is the most common cause of dementia and leads to a gradual deterioration of memory, language, problem-solving and other cognitive functions. A number of studies have focused on herbal medicines to discover new agents against AD due to adverse effects of synthetic drug treatments. For example, donepezil, an acetylcholinesterase inhibitor, is the most commonly prescribed medication for AD. However, donepezil produces various adverse effects during the dose-escalation phase of therapy (Gauthier, 2001). Recently, we isolated a water-soluble compound, danshensu, with anxiolytic-like effects from *P. vulgaris* var. *lilacina*, a safe herbal medicine, by activity-guided fractionation. Oral administration of danshensu (3 or 10 mg/kg) mediates the anxiolytic-like activities, as shown in the elevated plus-maze or hole-board tests (Kwon et al., 2014). Therefore, we are interested in the application of danshensu as one of cognitive enhancers with several beneficial effects, including anxiolytic, neuroprotective and antioxidative activities (Chong et al., 2013; Kwon et al., 2014).

Several reports showed that danshensu, one of active phenolic acids from *P. vulgaris* var. *lilacina*, may be a potential ingredient in both traditional medicine and the food industry (Zhao et al., 2008). We examined the possible actions of danshensu against scopolamine- and Aβ protein-induced cognitive impairment in mice. In current research fields, the mouse model of scopolamine-induced cognitive impairment has been widely used to screen therapeutic agents against cognitive dysfunction by learning acquisition and short-term memory processes. This model also offers a simple and easy way for testing the cognitive enhancement potential of new drugs (Klinkenberg and Blokland, 2010). In the present study, scopolamine-induced cognitive impairment was significantly reversed by the oral administration of danshensu, as observed in the passive avoidance and Y-maze tasks. In the passive avoidance task, the scopolamine-induced mice pretreated with danshensu exhibited a similar effect at the concentration of 10 mg/kg than the mice treated with the standard compound, donepezil (5 mg/kg), on the step-through latency of the retention trial. Danshensu also enhanced cognitive performance in a manner similar to that of donepezil, as measured via the spontaneous alternation behavior in the Y-maze test. In regards to Aβ protein-induced cognitive impairment, the latency was significantly increased by the repeated administration of danshensu (10 mg/kg for 7 days). Excitingly, we observed that danshensu ameliorates scopolamine and $A\beta_{1-42}$ protein-induced cognitive dysfunction, suggesting that danshensu would be a useful therapeutic agent against cognitive impairment.

Further, danshensu increased the level of dopamine in the cerebral cortex. It is well known that cholinergic agonists against AD may increase the extracellular levels of dopamine, suggesting that an increased dopamine level may be associated with the therapeutic effect (Preda et al., 2008). In addition, MAO isoenzymes such as MAO-A and MAO-B inactivate various catecholamine neurotransmitters, including dopamine, adrenaline, and serotonin. Hence, the inhibition of these enzymes has been considered an effective therapeutic target in neurological disorders (Borroni et al., 2017; Nave et al., 2017; Youdim et al., 2006). Previous studies reported that danshensu has the potential to inhibit both MAO-A and MAO-B activities. Danshensu activated dopamine D1 receptors by inhibiting MAO-A in a chemical assay as well as down-regulated the activation of NF-κB by inhibiting MAO-B activity in A549 and NSCLC cells (Kwon et al., 2014; Son et al., 2016). In addition, in the prefrontal cortex, D1-like receptors are highly involved in the regulation of attention, cognitive and emotional processes by stimulating the cAMP-PKA-CREB signaling pathway (Olianas et al., 2013). In particular, stimulation of D1 receptors induces the phosphorylation of Ser897 on the NR1 subunit by PKA, and this phosphorylation event is essential for the D1 receptor-mediated phosphorylation of CREB (Dudman et al., 2003). In a mechanistic study, we found that danshensu

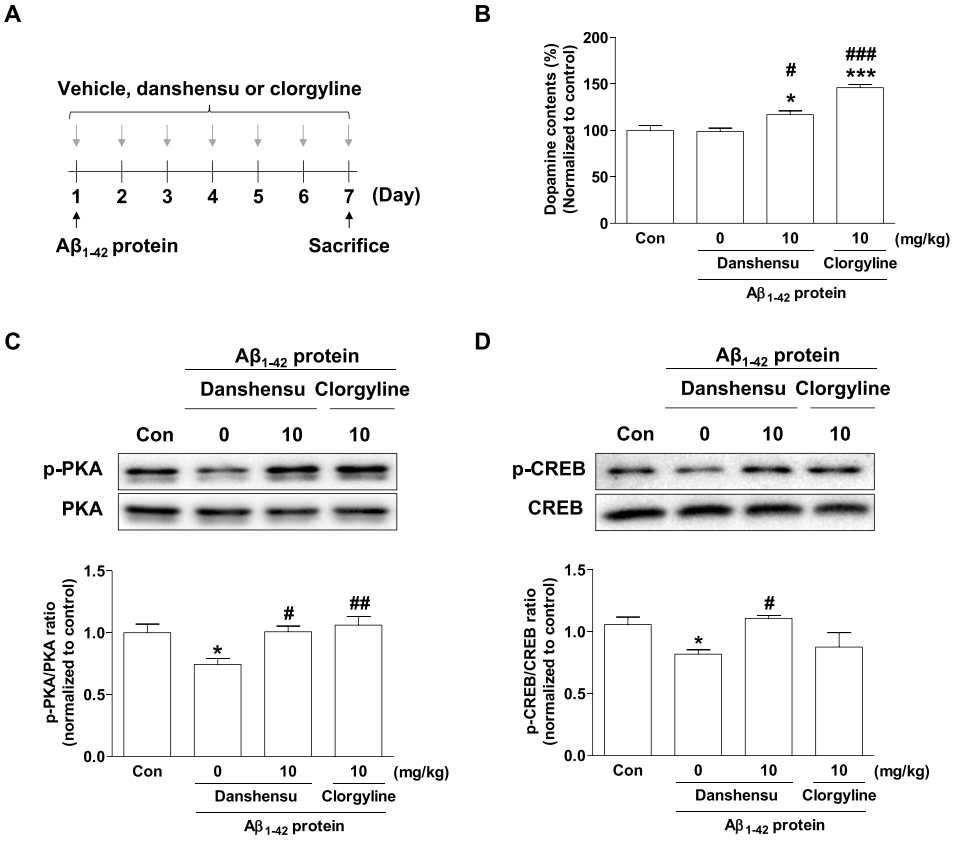

Fig. 6. Danshensu increases dopamine level and phosphorylation of PKA and CREB in the cerebral cortex of $A\beta_{1-42}$-induced amnesic mice. A. Time schedule for $A\beta_{1-42}$ and drug injection. B. Effects of danshensu and clorgyline (a MAO-A inhibitor) on dopamine level in the cerebral cortex of $A\beta_{1-42}$-induced amnesic mice (n = 6–7/group). C, D. The immunoreactivity and quantitative analysis of PKA, phosphorylated PKA (p-PKA), CREB, and CREB (p-CREB) after danshensu treatment in the cerebral cortex of $A\beta_{1-42}$-induced amnesic mice (n = 5–6/group). Con, control. Data represent the mean ± S.E.M. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. vehicle-treated controls; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. $A\beta_{1-42}$ only treatment.

increased the activation of PKA-CREB in the cortex. Therefore, we can speculate that danshensu increases the dopamine level by inhibiting MAO and that danshensu consequently activates the cAMP-PKA-CREB signaling cascade in the cerebral cortex.

Many researchers also suggested a possible role of cAMP-PKA-CREB in memory processing (Hernandez and Abel, 2011; Kandel, 2012; Mackiewicz et al., 2008). Wu et al. (2013) found that tetramethylpyrazine isolated from a Chinese herb, *Ligusticum wallichii*, showed a protective effect against scopolamine-induced memory impairments in rats by upregulating the cAMP/PKA/CREB signaling pathway (Wu et al., 2013). Therefore, it is likely that danshensu activates the PKA-CREB pathway, which induces memory formation and consolidation. Numerous bioactive compounds have been reported to enhance spatial memory by increasing the phosphorylation of the CREB/brain-derived neurotrophic factor pathway in the hippocampus (Lee et al., 2012; Scott Bitner, 2012). In these situations, CREB plays a major role in the activation of signaling cascades required for long-lasting activity-dependent synaptic plasticity and neuroprotective responses (Lee et al., 2009; Lonze and Ginty, 2002). PKA is an important upstream kinase that phosphorylates CREB at Ser133, accordingly facilitating CREB binding to various promoters (Kandel, 2012). In this study, we found that the memory improving effect of danshensu was completely blocked by the PKA inhibitor H89, indicating that their effects may be closely associated with the activation of PKA-CREB signaling cascade.

An earlier study reported that danshensu treatment significantly attenuated 6-OHDA-induced neurotoxicity and the production of reactive oxygen species in PC12 cells (Cui et al., 2016). In addition, danshensu increases the expression of heme oxygenase-1 to suppress 6-OHDA-induced oxidative damage via the phosphoinositide 3-kinase (PI3K)/Akt/Nrf2 signaling pathway. Moreover, danshensu reduced 6-OHDA-induced dopaminergic neuronal loss in zebrafish (Chong et al., 2013). Danshensu has also shown a neuroprotective effect against cerebral ischemic/reperfusion injury in rats through the activation of the PI3K/Akt signaling pathway (Guo et al., 2015). Wang et al. (2012) investigated the effect of danshensu on the cognitive dysfunction in streptozotocin-induced diabetic mice. In their study, danshensu effectively reduced the mean escape latency and increased the percentage of time spent in the target quadrant, as measured in the Morris water maze test. Further, danshensu partly blocked the expression of advanced glycation end products (AGEs) by activating the receptor of AGEs, p-p38, and cyclooxygenase-2, and the nuclear factor-κB and attenuated the increase in interleukin-6, tumor necrosis factor-α, and prostaglandin E2 (Wang et al., 2012). Considering these reports and our results together, danshensu may be a potent neuroprotective agent that enhances cognitive performance.

5. Conclusion

In summary, the findings of this study strongly suggest that danshensu may ameliorate cognitive impairment via MAO-A inhibition and PKA-CREB activation. Accordingly, the present study demonstrates that danshensu would be useful as a new supplement against cognitive dysfunction.

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Author's contributions

The study was conceived and designed by J.H.R. and S.J.P. Behavioral studies were conducted by K.S., H.J.B., and H.P.

Immunoblotting assays and dopamine analysis were performed by S.K., D.H.K., S.K., and J.W.C. Danshensu sample was prepared by D.S.J. The manuscript was written by K.S., J.H.R., and S.J.P.

Conflicts of interest

The authors declare that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.104537.

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