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Original Paper

Icariside II, a Phosphodiesterase-5 Inhibitor, Attenuates Beta-Amyloid-Induced Cognitive Deficits via BDNF/TrkB/CREB Signaling

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Key Words
Icariside II • Alzheimer's disease • Brain-derived neurotrophic factortyrosine receptor kinase • Tyrosine receptor kinase B • cAMP response element binding

Abstract
Background/Aims: Icariside II (ICS II) is an active component from *Epimedium brevicornum*, a Chinese medicine extensively used in China. Our previous study has proved that ICS II protects against learning and memory impairments and neuronal apoptosis in the hippocampus induced by beta-amyloid$_{25-35}$ (Aβ_{25-35}) in rats. However, its in-depth underlying mechanisms remain still unclear. Hence this study was designed to explore the potential underlying mechanisms of ICS II by experiments with an *in vivo* model of Aβ_{25-35}-induced cognitive deficits in rats combined with a neuronal-like PC12 cells injury *in vitro* model. ***Methods:*** The cognitive deficits was measured using Morris water maze test, and apoptosis, intracellular reactive oxygen species (ROS) and mitochondrial ROS levels were detected by TUNEL, DCFH-DA and Mito-SOX staining, respectively. Expression of Bcl-2, Bax, brain derived neurotrophic factor (BDNF), tyrosine receptor kinase B (TrkB), and cAMP response element binding (p-CREB) and active-Caspase 3 levels were evaluated by Western blot. ***Results:*** It was found that ICS II, a phosphodiesterase-5 inhibitor, significantly attenuated cognitive deficits caused by Aβ_{25-35} injection in rats, and ICS II not only significantly enhanced the expression of BDNF and TrkB, but also activated CREB. Furthermore, ICS II also significantly abrogated Aβ_{25-35}-induced PC12 cell injury, and inhibited Aβ_{25-35}-induced intracellular reactive oxygen species (ROS) overproduction, as well as mitochondrial ROS levels. In addition, ICS II up-regulated the expressions of BDNF and TrkB consistent with the findings *in vivo*. ANA-12, a TrkB inhibitor,

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blocked the neuroprotective effect of ICS II on $A\beta_{25-35}$-induced neuronal injury. ***Conclusion:*** ICS II mitigates $A\beta_{25-35}$-induced cognitive deficits and neuronal cell injury by upregulating the BDNF/TrkB/CREB signaling, suggesting that ICS II can be used as a potential therapeutic agent for dementia, such as Alzheimer's disease.

Introduction

Alzheimer's disease (AD), the most common type of dementia, is a neurodegenerative disorder characterized by senile plaques (SP) formed by beta- amyloid (Aβ) deposits outside the neurons and neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein intra neurons [1]. These typical pathological features of AD result in gradual cognitive dysfunction, including spatial learning and memory impairment [2]. However, the exact pathogenesis of AD remains not entirely clear, and to date, all therapeutic drugs for AD have limited efficacy, including acetylcholinesterase (AChE) inhibitors such as tacrine and donepezil, and N-methyl-D-aspartate (NMDA) receptor antagonist such as memantine. In addition, these drugs have side effects and toxicity [3]. Thus, it is urgent to develop new and alternative AD therapeutic drugs.

Phosphodiesterase 5 (PDE 5), a PDE enzyme that specifically hydrolyzes cyclic guanosine monophosphate (cGMP), plays a significant part in the mediation of memory [4]. Recently, evidences have indicated that PDE 5 inhibitors (PDE 5-Is) exert positive effects on the different AD models. Sildenafil, a typical PDE 5 inhibitor, may improve memory function via reduction of Aβ levels and inhibition of neuroinflammatory responses in amyloid precursor protein (APP)/presenilin 1(PS1) transgenic mice [5]. Sildenafil also alleviate memory deficits via counteraction of apoptosis by modulating key pro- and anti-apoptotic molecules and the APP pathway in mouse model of aging [6]. Consequently, inhibiting PDE 5 is increasingly considered as a novel strategy for AD therapy. It is well known that many compounds extracted from plants are of biological activities with abundant resources, which may be used to explore promising PDE 5 inhibitor for the intervention and treatment of AD.

Plants of the genus *Epimedium* (Herba *Epimedii*) is a Chinese medicine traditionally utilized for treating dementia [7]. Icariin (ICA) and icariside II (ICS II) are two flavonoids extracted from Herba *Epimedii* with similar structures. A series of studies have demonstrated that ICA improves cognitive impairments trough different mechanisms in diverse animal models with AD [8-10]. It is also reported that ICA is a cGMP-specific PDE 5 inhibitor for the treatment of erectile dysfunction (ED) through stimulation of nitric oxide (NO)/cGMP signaling pathway with selective inhibitory activity on PDE 5 in the penile cavernosum [11-13]. Our previous study has shown that ICA improves learning and memory ability in APP/PS1 transgenic mice by stimulating NO/cGMP signaling [14]. ICS II, a primary active metabolite of plant-derived ICA *in vivo*, is short of a glucose moiety at C-7 and is more bioavailable than ICA [15, 16]. Hence, it is worthy to further investigate the beneficial effect and the underlying mechanisms by which ICS II elevated cognitive function, which may reveal new clues for potential application of ICS II. Therefore, in this study the spatial learning and memory impairment model *in vivo*, and neuronal-like PC12 cells injury *in vitro* were induced by $A\beta_{25-35}$ to evaluate the effect of ICS II on learning and memory impairment, and its the possible mechanisms were explored.

Materials and Methods

Materials

Icariside II (purity ≥ 98% by HPLC) was purchased from Nanjing Zelang Medical Technology Corporation Ltd (Nanjing, China), and sildenafil was purchased from Targetmol, $A\beta_{25-35}$ (#A4559), 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT, #M2128) were purchased from Sigma-Aldrich, Fetal bovine serum (FBS, #10099-141) were purchased from Gibco, Dulbecco's modified Eagle's medium (DMEM, #SH30243.01) and DCFH-DA (#CA1410) was purchased from Suolaibao. Mito-SOX Red (#M36008) and Mito-Tracker green probe (#M7514) were purchased from Invitrogen (Eugene, OR, USA). One step TUNEL cell apoptosis detection kit (green fluorescence), PDE5 ELISA kit and rat lactate dehydrogenase (LDH) ELISA Kit were purchased from Shanghai Jiang Lai Biotechnology (Shanghai, China), Reactive oxygen species assay kit were purchased from solarbio (Beijing, China). Anti-β-actin (#AA128) and anti-GAPDH (#HC301-01) were purchased from TransGen Biotech (Beijing, China). Anti-TrkB (#ab187041), anti-CREB (#9197) and anti-p-CREB (#9198) were purchased from Cell Signaling Technology (Boston, USA). Anti-BDNF (#ab203573), anti-Bax (#ab7977) and anti-Bcl2 (#ab7973) were purchased from Abcam (Cambridge, UK). Anti-Caspase-3 (19677-1-AP) was purchased from Proteintech Group (Beijing, China).

Experimental animals

Healthy male-specific pathogen free Sprague-Dawley (SD) rats (weighing from 220 to 250 g) were obtained from Animal Center of the Third Military Medical University (Chongqing, China) (Certificate No. SCXK 2012-0005). The animals were maintained on a 12 h light/dark cycle in a stable temperature (23 ± 1 °C) with humidity (relative, 60%)-controlled rooms and allowed free access to food and water. The experiments were carried out with the approval of the Animal Experimentation Ethics Committee of the Zunyi Medical University. All possible efforts were made to minimize the number of animals used and their suffering in the present study.

Surgery

Seventy rats were randomly divided into five groups, which were sham, Aβ, Aβ+ ICS II 3, 10 mg/kg and sildenafil 3 mg/kg groups, respectively. $A\beta_{25-35}$ from Sigma-Aldrich (St. Louis, MO, USA) was dissolved in sterilized normal saline (NS) at the concentration of 2 μg/μL, and incubated at 37 °C for 7 days before injection [17]. The animals were anaesthetized by intraperitoneal injection with 2% sodium pentobarbital at the dose of 60 mg/kg, and then were placed in a stereotaxic device (SR-6N, Narishige, Japan). The aggregated $A\beta_{25-35}$ was injected into rat hippocampi according to the established approach [18]. In brief, a midline incision was made on the head skin of rats after routinely sterilizing, exposing the periosteum, then 5 μL microsyringe was implanted into bilateral CA1 subregion, which the coordinates were as follows: 3.3 mm posterior to bregma, 2 mm lateral to sagittal suture, 3 mm beneath the surface of brain. The rats in sham group were injected with sterilized normal saline instead. Rats were injected with 5 μL $A\beta_{25-35}$ or 5 μL sterilized NS in each CA1 subregion at the rate of 1 μL/min. The needle was left for 5 min after each injection. Then stitch the wound and feed in the corresponding cage.

Administration of drugs

ICS II and sildenafil were dissolved in NS and ultra-sonicated for 20 min. From second days after surgery, rats in Aβ + ICS II 3, 10 mg/kg and sildenafil 3 mg/kg groups were intragastrically administered with corresponding doses once a day, while rats in the sham and $A\beta_{25-35}$ groups were administered with volume-matched NS for 15 days, instead.

Behavioral examination

Spatial learning and memory abilities of rats were evaluated using Morris water maze (MWM) task according to the procedure as previously reported [19, 20]. Trials were carried out during day 11 to day 15 after $A\beta_{25-35}$ injection. In brief, the apparatus which MWM test used was made up of a circular basin with 120 cm in diameter and 50 cm in height, which was filled with water with 30 cm in depth and 23 ± 1 °C. All experiment animals were trained to find a hidden platform with 10 cm diameter and 1 cm below the water. The position of the platform remained unchanged for all training session. The pool was automatically divided into four quadrants and the quadrant was altered every day. It was required that rats were faced

towards the pool wall before being liberated. The test program consisted of two steps. The first step was the place navigation test twice daily (in a.m. and p.m., respectively). Days 1 to 4, both the escape latency that the time rats reached the hidden platform in 120 s and the swimming speed was recorded. Upon reaching the platform, the rats were allowed to rest there for 15 s. If the rat failed to reach the platform within 120 s, it was manually directed to the platform and also allowed to stay there for 15 s. In this case, its escape latency was still marked as 120 s. The secondary step was a probe trial test on the 5th day. This test was performed without the hidden platform, in order to evaluate the final memory strength. Time percentage in the target quadrant (%) and target quadrant frequency for the rat to remain at the former location of the platform in the primary test were recorded. Performance in all trials was automatically recorded and assessed by TopScan-Topview Behavior Analyzing System (TopScan Version 3.00).

Enzyme-linked immunosorbent assay (ELISA)

PDE 5 and cGMP contents of hippocampi were determined by ELISA. In brief, six rats were randomly extracted in each group. After being anaesthetized and sacrificed, right hippocampus was collected for ELISA assay, which was performed according to our previous report [14]. PDE 5 and cGMP were quantified in these samples according to the PDE 5 ELISA kit and cGMP ELISA kit, respectively.

Cell culture

The highly differentiated rat pheochromocytoma line PC12, a clonal cell line derived from a rat adrenal medulla tumour, provided by American Type Culture Collection (Rockville, MD, USA). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, and maintained at 37 °C and 5% CO_2 in a humidified atmosphere. The cells were grown 80 % confluence at 37 °C about 24 h in complete medium. Then, the cells were used for treatment.

Cell viability assessment by MTT assay

ICS II and sildenafil were dissolved in dimethylsulfoxide (DMSO) at 10 mM as stock solution and diluted in culture medium respectively. The final concentration of DMSO in the media was less than 0.01 %. $A\beta_{25-35}$ was diluted to 1 mM with sterilized saline water and then incubated at 37°C for seven days before use [21]. PC12 cells (1×10^5cells/well in 96-well plates) were cultured with complete DMEM 24 h for stabilization at 37 °C. Cell viability following treatment with $A\beta_{25-35}$, ICS II or sildenafil for 48 h was detected by MTT assay. The TrkB inhibitor ANA-12 (10 μM) was added to the particular cell cultures 1 h before ICS II treatment. After 48 h, 5 mg/ml MTT was added to each well for 4 h at 37°C. The dark-blue formazan crystals formed in intact cells were dissolved in DMSO and their absorbance was measured at 570 nm with a microplate reader (Thermo, US). Results were expressed as the percentage of MTT reduction relative to the absorbance of the control cells. Additionally, cellular morphologic changes were observed using phase contrast microscopy.

Determination of lactate dehydrogenase release

The level of lactate dehydrogenase release was measured by LDH ELISA Kit. PC12 cells were treated as mentioned above, the supernatant was collected form each well according to the specifications provided by the supplier. Each sample was measured in 450 nm of wavelength by Microplate Reader.

Determination of PDE 5 level in vitro

In brief, PC12 cells were treated as described above. Cells were lysed in the lysis buffer and centrifuged for 20 min (12000 × g, at 4°C). The supernatant was stored at -80°C for subsequent measurement. The levels of PDE 5 were quantified by the PDE 5 ELISA kit according to the manufacturer's indications.

Intracellular reactive oxygen species (ROS) levels assay and mitochondrial ROS analysis

The fluorescent probe DCFH-DA was used to detect intracellular ROS. PC12 cells were treated as mentioned above, and then cells were washed twice with PBS and added DMEM containing 10 μM DCFH-DA, followed by incubation at 37°C for 20 min in dark. Thereafter, the cells were harvested and analyzed by flow cytometry (Navios, Beckman Coulter, US). Moreover, Mito-SOX Red, a fluorescent indicator for mitochondrial superoxide, was used to evaluate mitochondrial ROS [22]. Following treatment, both 5 μM Mito-SOX Red and 200 nM Mito-Tracker green probe were added to the cells followed by incubation at 37 °C in dark for 30

min. Afterwards, the cells were washed with balanced salt solution and imaged by fluorescence microscopy (Olympus IX73; Olympus, Tokyo, Japan; 2009) with excitation/emission (510/580 nm) filters.

TUNEL staining assay

Apoptotic cells were detected by TUNEL staining using the one step TUNEL cell apoptosis detection kit (green fluorescence). Briefly, PC12 cells were treated as described above. TUNEL staining was performed according to the manufacturer's specification and then observed under a fluorescence microscope (Olympus IX73; Olympus, Tokyo, Japan) with excitation/emission (450/515 nm) filters.

Western blot analysis

Expression of Bax, Bcl-2, pro- and cleaved-caspase-3, BDNF, TrkB, p-CREB, CREB, and β-actin were all analyzed by Western blot. Three rats randomly extracted from each group were sacrificed and the left hippocampal tissues were dissected, and cells were uniformized in suitable lysis buffer. The other steps were performed according to our previous report [23]. The corresponding proteins were probed with a primary antibody against Bax (1:1000), Bcl-2 (1:500), pro- and cleaved-caspase-3 (1:2000), BDNF (1:1000), TrkB (1:500), p-CREB (1:1000), CREB (1:1000) and β-actin (1:5000) at 4°C overnight, respectively.

Data analysis

All data were expressed by the mean ± the standard error of the mean (SEM). Repeated measures ANOVA process in SPSS 17.0 was applied to analyze the comparisons among different groups and different measurement times in the MWM results. When repeated measures were unnecessary, one-way ANOVA was used. When ANOVA test results for all data were significant, post-hoc least significant difference (LSD) was used to determine the individual differences. When a value of $P < 0.05$ was considered statistically significant.

Results

ICS II ameliorated cognitive deficits induced by $A\beta_{25-35}$ in rats

To investigate the effect of ICS II on cognitive deficits produced by $A\beta_{25-35}$ injection, the spatial learning and memory ability of rats were tested using MWM test. The *P* value of Mauchly's Test of Sphericity in latency was 0.011, suggesting that sphericity assumption was not accepted.Then the Greenhouse–Geisser correction was used. The main effect of "latency between days" was significant [F (2.443, 97.727) = 72.161, *P*< 0.001], and the main effect of "latency between groups" was significant [F (4, 40) = 3.910, *P* =0.009], but the interaction of "days × groups" was no significance [F (9.773, 97.727) = 0.937, *P* =0.502]. There was significance between Aβ group and other four groups till day 4; however, no significance among sham, two ICS II-treated and sildenafil groups (Fig. 1). All the rats showed gradual decreases in escape latency to reach the hidden platform during the 4-day acquisition trials in the water maze test. However, rats with $A\beta_{25-35}$ injection slowed down the learning capability. No significant differences in escape latency were detected in the first three days, while marked differences were found on the 4th day. Rats in the $A\beta_{25-35}$ group exhibited significantly longer escape latency than sham group, whereas animals in the ICS II (10 mg/kg) and sildenafil group demonstrated significantly decreased escape latency relative to the $A\beta_{25-35}$ group (Fig. 1A). Notable differences in time percentage [F (4, 40) = 2.768, *P* =0.04] and the target quadrant frequency [F (4, 40) = 3.024, *P* =0.029]in the spatial probe test were detected on the 5th day. The target quadrant time and target quadrant frequency of $A\beta_{25-35}$-injected rats were significantly reduced compared with those of the sham group, indicative of cognitive deficits. However, administration of ICS II (10 mg/kg) dramatically increased the time percentage and the target quadrant frequency compared with rats injected with $A\beta_{25-35}$ alone (Fig. 1C and D). The same effect was observed in sildenafil group (Fig. 1C and D). To note, swimming speed [F (4, 40) = 0.223, *P* =0.924] showed no significant differences between groups (Fig. 1B), suggesting that Aβ and ICS II did not affect the motor function of

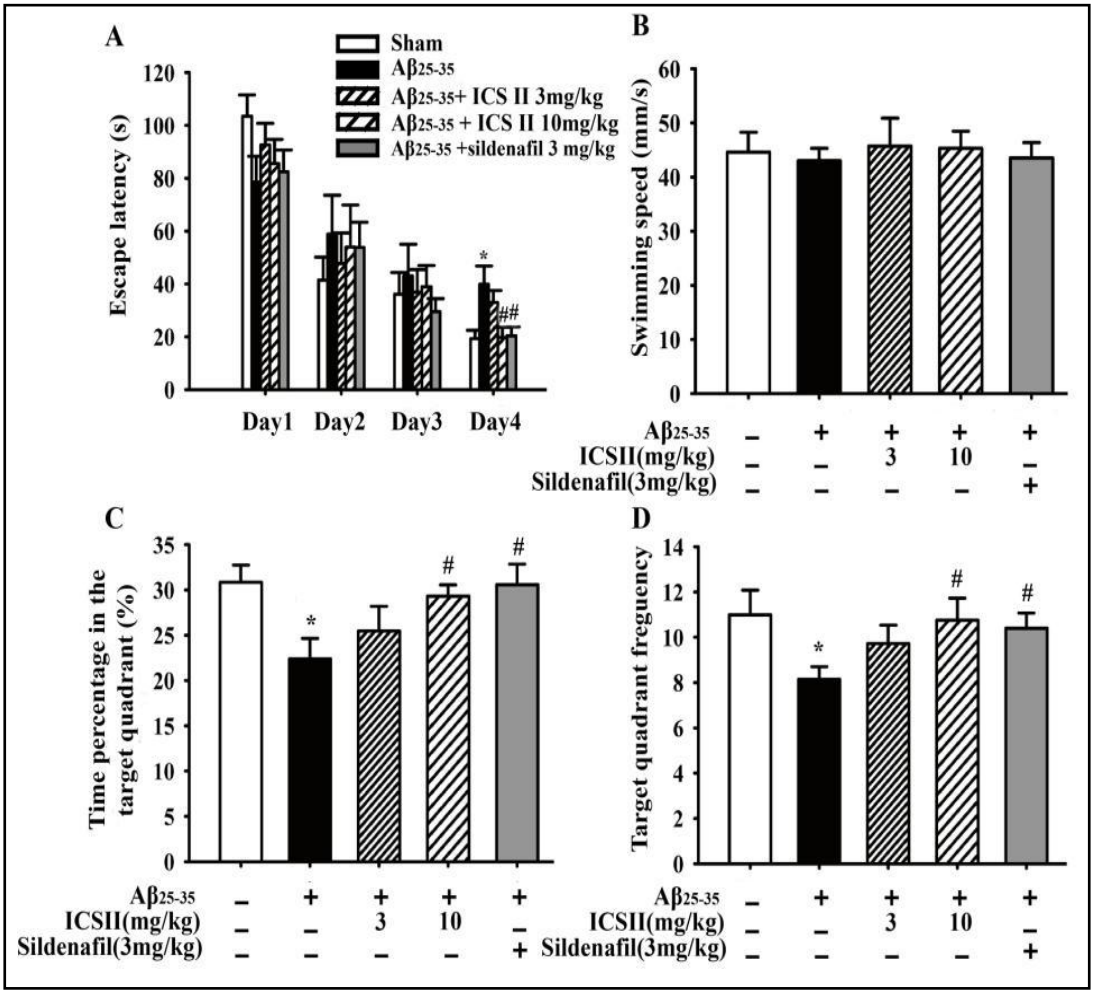

Fig. 1. ICS II ameliorates $A\beta_{25-35}$-induced cognitive deficits in rats. The hippocampus of rats injected with $A\beta_{25-35}$ or NS, followed by administration of ICS II and sildenafil for 15 days. Rats were subjected to a 5-day test of MWM from the 10th day after surgery. (A) In the navigation test, the escape latency of each group from day 1 to day 4; (B) Swimming speed of each group in the target quadrant on the 5th day; (C) In the spatial probe test, the percentage of time in the target quadrant on the 5th day; (D) In the spatial probe test, the frequency in the target quadrant on the 5th day. Data are presented as mean ± SEM, n = 8-12. $^{*}P<0.05$ vs sham; $^{\#}P<0.05$ vs Aβ alone.

rats. These results demonstrated that ICS II (10 mg/kg) and sildenafil may enhance spatial cognition function, whereas no difference was found in the low dose of ICS II (3 mg/kg).

ICS II decreased the level of PDE 5 and cGMP in hippocampus of $A\beta_{25-35}$-induced rats

PDE 5 and cGMP levels in hippocampus were detected by ELISA Kit (Fig. 2). The effect of ICS II on PDE 5 and cGMP level was statistically significant [$F(4, 20) = 13.271$, $P < 0.01$; $F(4, 20) = 24.791$, $P < 0.01$]. Compared with sham group rats, Aβ group rats had higher PDE 5 protein level, while cGMP level was lower in hippocampus. In contrast, ICS II (10 mg/kg)-treated rats showed a decrease in PDE 5 level and an increase in cGMP level compared to those of Aβ group. A similar effect was also observed in the sildenafil-treated group.

Effect of ICS II on the protein expression of BDNF and TrkB and the activation of CREB in $A\beta_{25-35}$-induced rat hippocampi

The expression of hippocampal BDNF, TrkB and CREB phosphorylation (p-CREB) level was determined by Western blot (Fig. 3). Compared with sham group, the expressions of

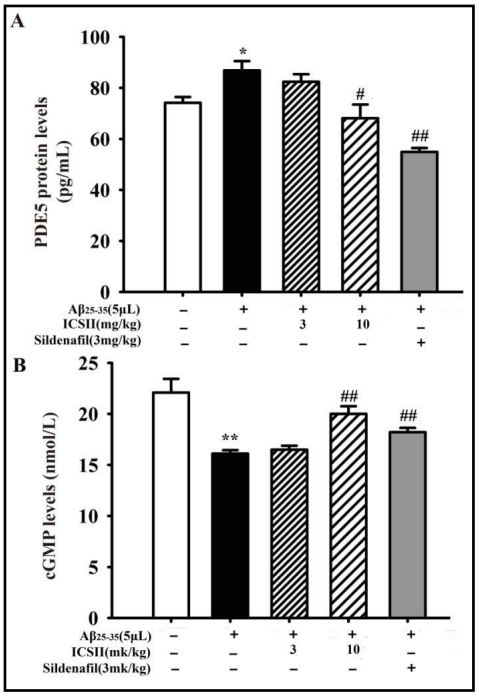

Fig. 2. Effect of ICS II on the levels of PDE 5 and cGMP in hippocampus. (A) PDE 5 protein levels in hippocampus. (B) cGMP level in hippocampus. Data are presented as mean ± SEM (n=5). $^{*}P<0.05$, $^{**}P<0.01$ vs sham; $^{\#}P< 0.05$, $^{\#\#}P<0.01$ vs Aβ alone.

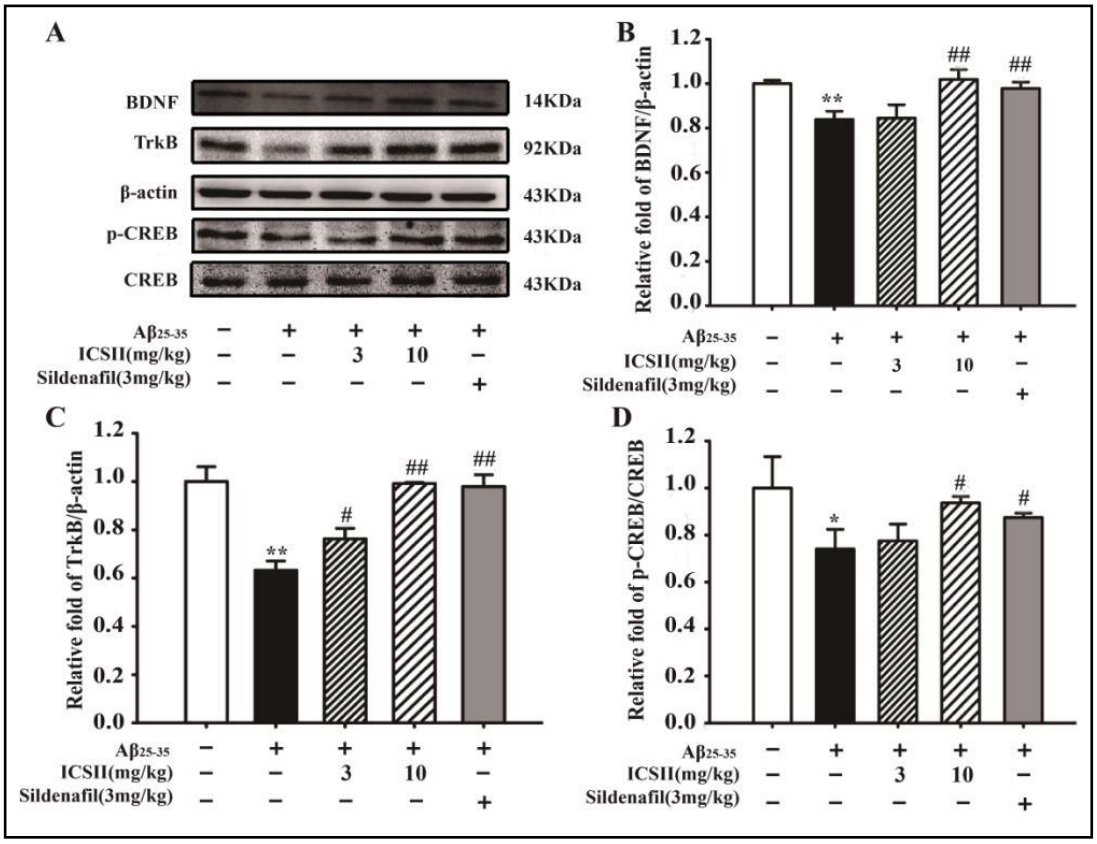

Fig. 3. ICS II increased the BDNF and TrkB protein expression and also enhanced the p-CREB level in the hippocampi of $Aβ_{25-35}$-induced rats. (A) Representative bands of BDNF, TrkB and p-CREB in the hippocampus of different groups. (B) Quantitation of BDNF protein; (C) Quantitation of TrkB protein; (D) Quantitation of p-CREB protein. Relative optical density was normalized to β-actin and CREB, respectively. Data are given as mean ± SEM (n = 3). $^{**}P<0.01$ vs sham; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs Aβ alone.

BDNF, TrkB and the p-CREB were lower than those in Aβ group [F (4, 10) = 15.746, $P < 0.01$; F (4, 10) = 57.143, $P < 0.01$; F (4, 10) = 7.653, $P < 0.05$, respectively]. However, administration of ICS II at the dose of 10 mg/kg increased the expression of BDNF and TrkB and the level of p-CREB, which was similar to sildenafil treatment. It was found that, ICS II treatment at the dose of 3 mg/kg also displayed an upregulated effect on the expression of TrkB protein.

ICS II mitigated $A\beta_{25-35}$-induced PC12 cells death

Since Aβ-induced apoptosis in PC12 cells was widely recognized as a reliable cellular toxicity model for mimicking AD *in vitro*. The cell viability of the $A\beta_{25-35}$-induced PC12 cells was determined by MTT assay. The results showed that treatment with $A\beta_{25-35}$ at different concentrations (0.5, 1, 2.5, 5, 10, 20, 40 μM) for 12, 24, 48, 72 h decreased cell viability in a dose- and time-dependent manner. Considering exposure of PC12 cells to 20 μM $A\beta_{25-35}$ for 48 h significantly decreased the viability to approximately 50 % [F (7, 16) =50.638, $P < 0.01$] (Fig. 4A), such concentration and incubation time were adopted in the following experiments.

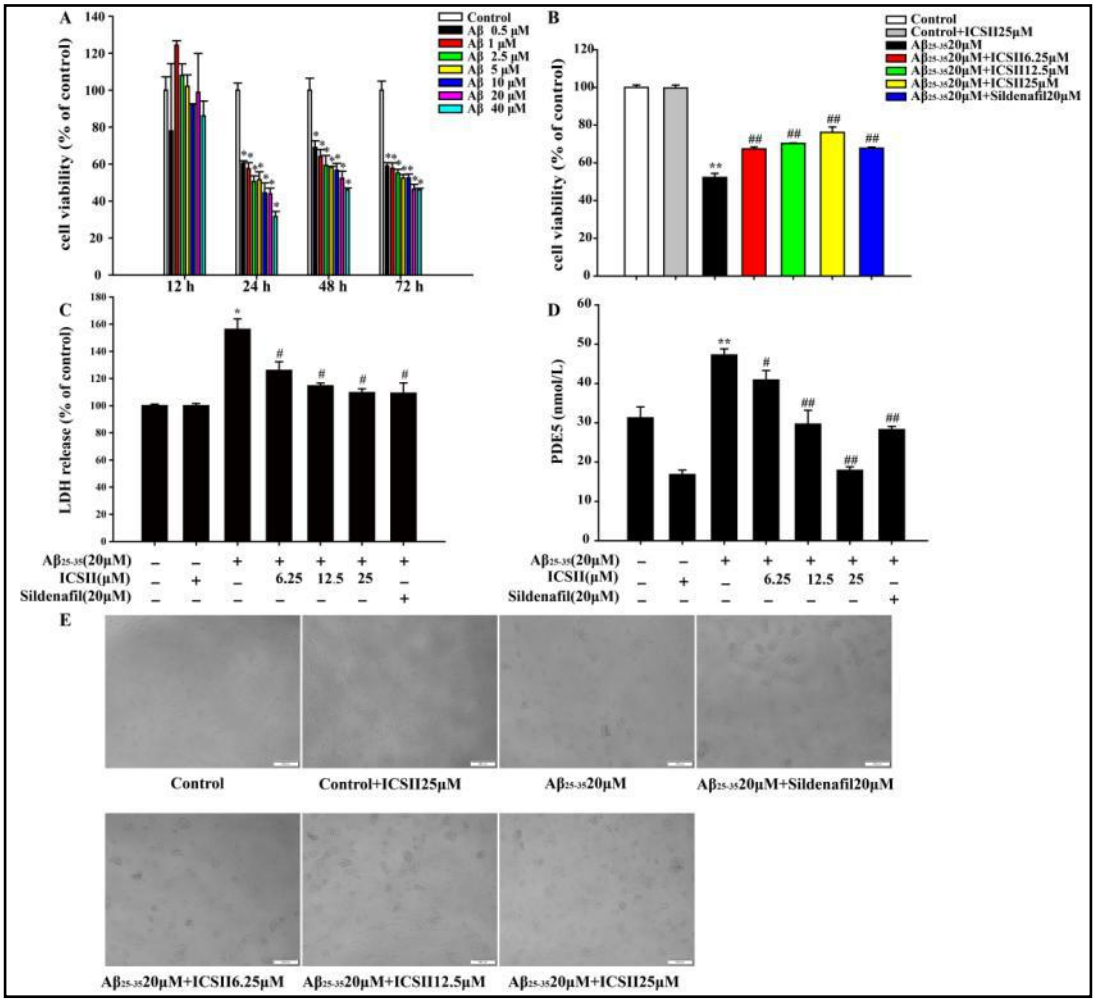

Fig. 4. Effect of ICS II on $A\beta_{25-35}$-induced injury in PC12 cells. (A) PC12 cells were treated with various concentrations of $A\beta_{25-35}$ (0.5, 1, 2.5, 10, 20 and 40 μM) for 12, 24, 48 and 72 h. (B) PC12 cells were treated with different concentrations of ICS II (6.25, 12.5, 25 μM) and 20 μM $A\beta_{25-35}$ for 48 h, and cell viability was determined by MTT assay. (C) LDH release from PC12 cells was determined by a LDH release assay. (D) PDE 5 levels from PC12 cells were examined by ELISA. (E) The effect of ICS II on $A\beta_{25-35}$-induced morphological changes in PC12 cells (200 × magnification). Data were presented as mean ± S.D. of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ vs untreated control cells; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs $A\beta_{25-35}$ alone.

It is found that 6.25, 12.5 and 25 μM ICS II or 20 μM sildenafil significantly attenuated $A\beta_{25-35}$-induced decrease of metabolically active cells in a concentration-dependent manner [$F(6, 14) = 337.868$, $P < 0.01$] (Fig. 4B). In parallel, in order to determine the effect of ICS II on $A\beta_{25-35}$-induced cytotoxicity, LDH release was also measured. The results showed that $A\beta_{25-35}$ significantly increased LDH release, indicating that $A\beta_{25-35}$ was toxic to PC12 cells. In contrast, ICS II decreased the LDH release in a concentration-dependent manner [$F(6, 14) = 45.807$, $P < 0.01$] (Fig. 4C). Moreover, ICS II or sildenafil also decreased the level of PDE5 compared with $A\beta_{25-35}$ alone [$F(6, 14) = 83.003$, $P < 0.05$] (Fig. 4D). In addition, the beneficial effect of ICS II was also confirmed by morphological changes. These results indicated that $A\beta_{25-35}$-induced cells lost cell viability and normal morphological characteristics with the plaque formation. Whereas ICS II or sildenafil increased the number of cells and reduced the plaque compared with $A\beta_{25-35}$ alone (Fig. 4E).

ICS II inhibited $A\beta_{25-35}$-induced apoptosis in PC12 cells

Cells were exposed to $A\beta_{25-35}$ for 48 h.The treatment of $A\beta_{25-35}$ reduced living cells with the number of TUNEL positive cells being significantly increased number of TUNEL positive cells was reversed by treatment with ICS II or sildenafil, suggesting that ICS II inhibited $A\beta_{25-35}$-induced apoptosis in PC12 cells (Fig. 5).

ICS II attenuated $A\beta_{25-35}$-induced ROS generation

$A\beta_{25-35}$-induced cytotoxicity is known to be mediated mainly by oxidative stress, hence we determined the generation of intracellular and mitochondrial ROS. Firstly, effect of ICS II on the level of ROS was examined by flow cytometry analysis. The results indicated that $A\beta_{25-35}$ resulted in an increase in level of ROS increase, whereas this effect was reversed by

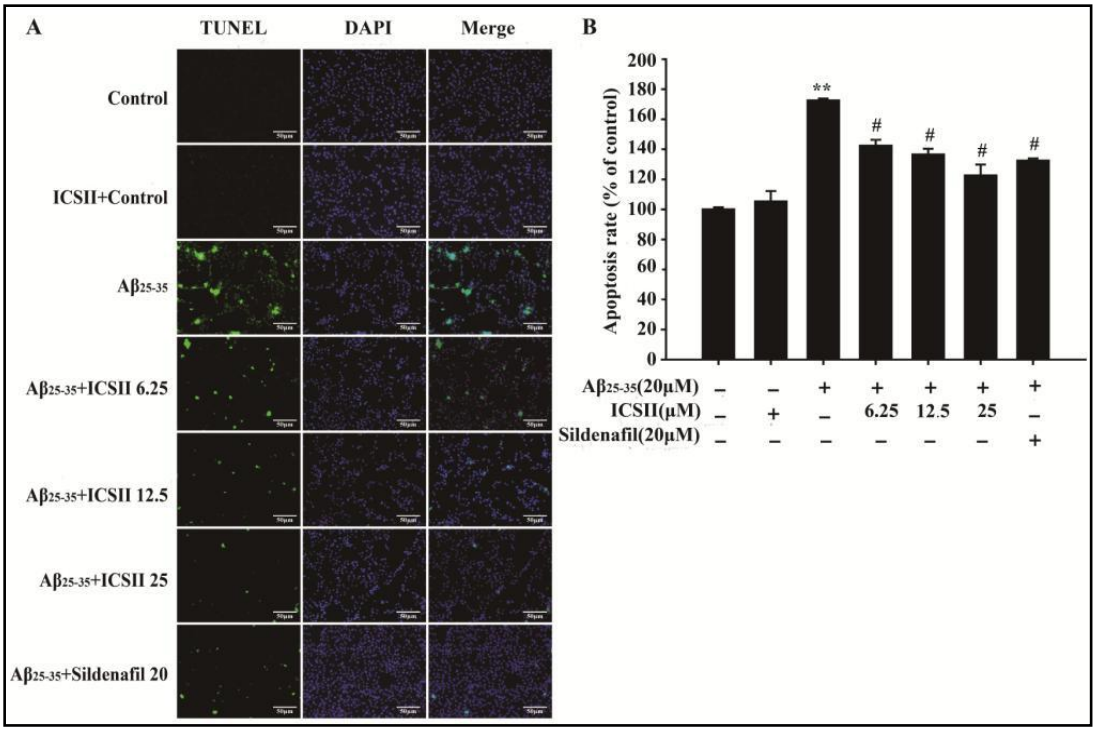

Fig. 5. Effect of ICS II on $A\beta_{25-35}$-induced apoptosis in PC12 cells. Cells were treated with different concentrations of ICS II (6.25, 12.5, 25 μM) and 20 μM $A\beta_{25-35}$ for 48 h. Apoptosis was detected by staining with TUNEL and visualized by fluorescence microscopy. (A) Fluorescence microscope observation of fluorescence intensity (200 × magnification). (B) The mean fluorescence intensity was measured by Image Pro Plus software. The result shown in B was presented as the mean ± S. D. of three independent experiments. $^{**}P<0.01$ vs untreated control cells; $^{\#}P<0.05$ vs $A\beta_{25-35}$ alone.

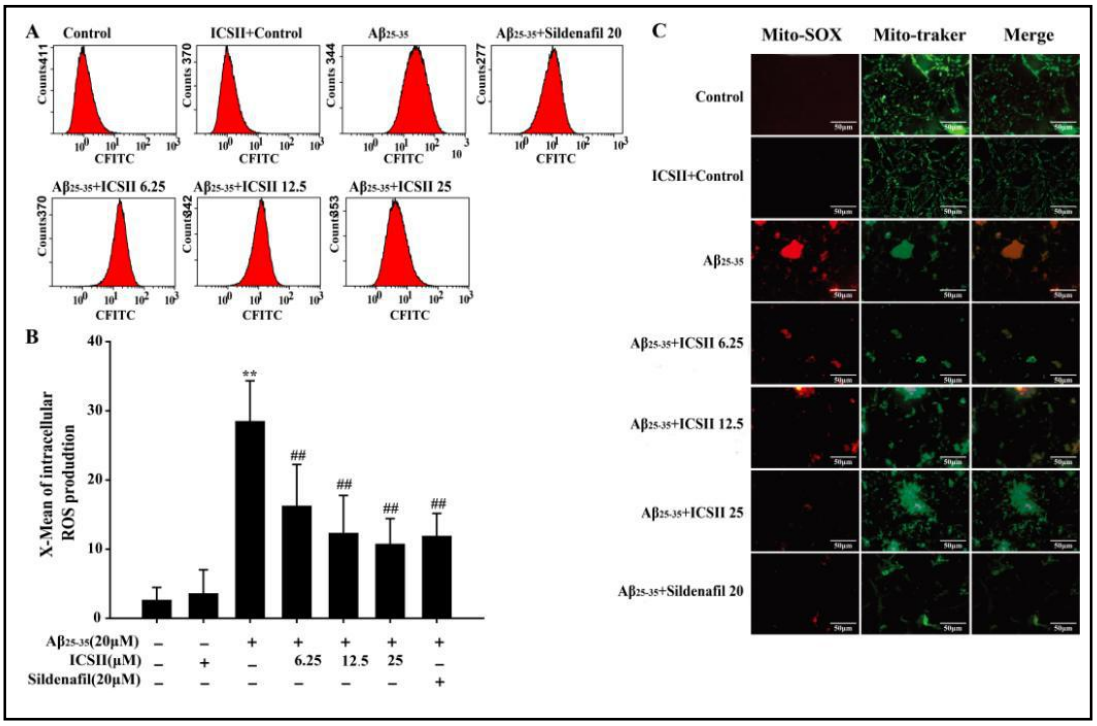

Fig. 6. Effect of ICS II on intracellular and mitochondrial ROS level. (A) Intracellular ROS level was determined with DCFH-DA dye by FCM. (B) The X-mean was measured. (C) Mitochondrial ROS level was determined with Mito-SOX Red dye (200×magnification). The result shown in B awas presented as the mean ± S.D. of three independent experiments. $^{**}P$<0.01 vs untreated control cells; $^{\#\#}P$<0.01 vs $A\beta_{25-35}$ alone.

ICS II or sildenafil [F (4, 10) =10.080, $P < 0.001$] (Fig. 6A, B). Mitochondrial ROS was detected using Mito-SOX, which is a cationic probe that distributes to the mitochondrial matrix and specifically detects superoxide anion. The results showed that $A\beta_{25-35}$ significantly increased the generation of mitochondrial superoxide, while this effect was reversed by ICS II or sildenafil (Fig. 6C). These findings demonstrated that ICS II could reduce both the intracellular and mitochondrial ROS level.

ICS II mitigated $A\beta_{25-35}$-induced apoptosis in PC12 cells by regulating BDNF/TrkB/CREB signal pathway

$A\beta_{25-35}$ significantly decreased the level of pro-caspase-3 and Bcl-2 expression, while, increased the level of active-caspase-3 and Bax expressions. However, ICS II or sildenafil reversed those changes [F (4, 10)=10.080, $P < 0.01$; F (4, 10)=7.865, $P < 0.05$; F (4, 10)=7.010, $P < 0.01$; F (4, 10)=6.639, $P < 0.05$; F (4, 10)=7.622, $P < 0.05$] (Fig. 7). Furthermore, to determine whether BDNF/TrkB/CREB signal pathway was involved, expressions of BDNF, TrkB, CREB and p-CREB were examined. The results showed that the expression of BDNF, TrkB and p-CREB level were obviously improved by ICS II compared with $A\beta_{25-35}$ alone [F (4, 15) =6.072, P<0.05; F (4, 10) =13.310, $P < 0.01$; F (4, 20) =2.724, $P < 0.05$] (Fig. 8), consistent with the results *in vivo*. To further determine the role of ICS II in BDNF/TrkB/CREB signal pathway, ANA-12, a TrkB inhibitor, was used. The results showed that ANA-12 significantly decreased cell viability compared with control or $A\beta_{25-35}$ alone [F (9, 20) =585.335, P<0.05]. In addition, ANA-12 also abolished the beneficial effect of ICS II on $A\beta_{25-35}$-induced cell viability. (Fig. 8E). Parallel analysis of LDH release showed ANA-12 significantly increased LDH release compared with control or $A\beta_{25-35}$ alone [F (9, 20) =83.879, P<0.05]. Whereas, ANA-12 also reversed the decrease in the LDH release treated with ICS II (Fig. 8F). These findings further suggested that BDNF/TrkB/CREB signal pathway plays a role in the effect of ICS II against $A\beta_{25-35}$-induced cell injury.

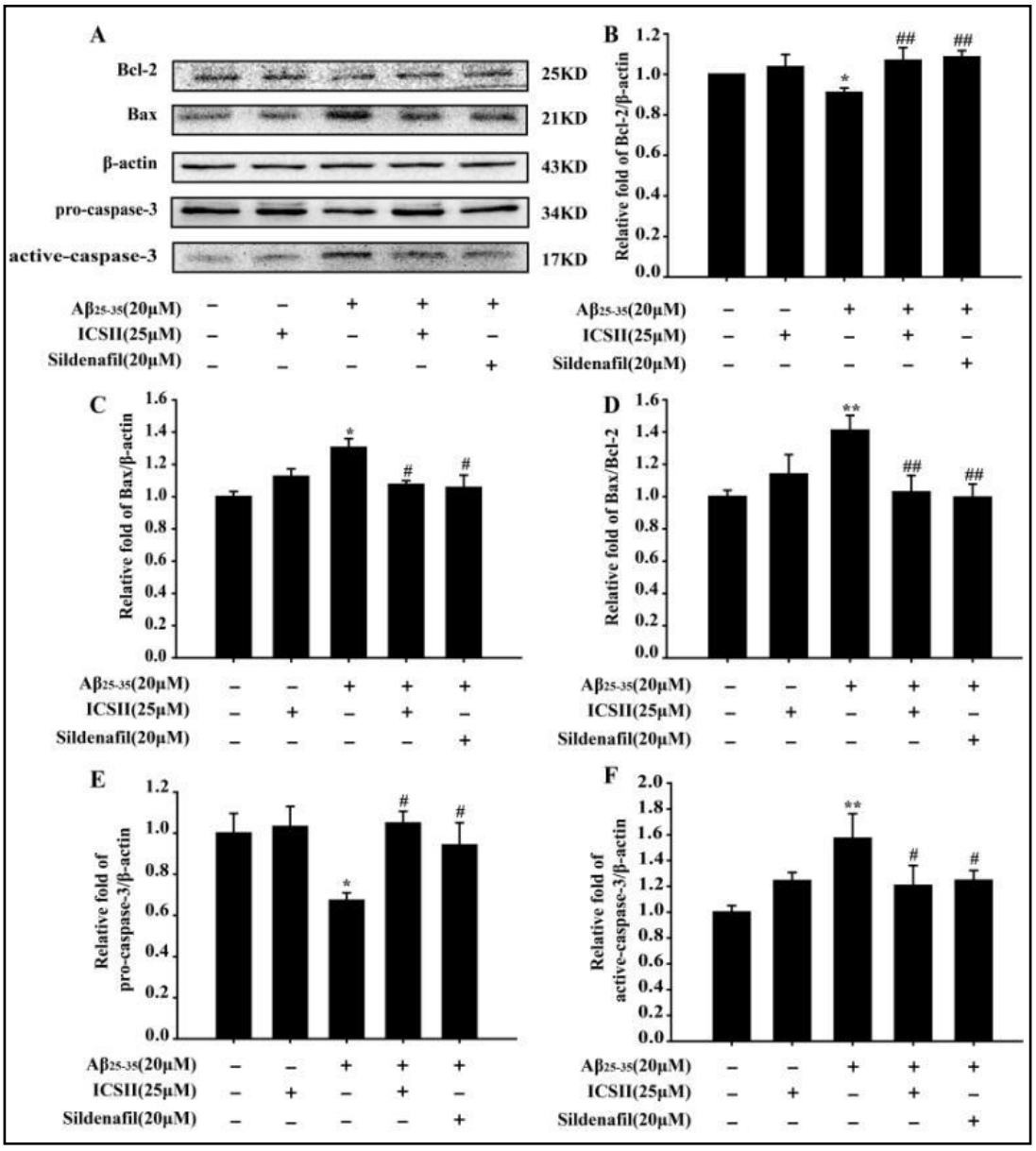

Fig. 7. Effects of ICS II on the expression of Bax, Bcl2 and the level pro-caspase-3, active-caspase-3. (A) A representative Western blot was shown for Bcl-2, Bax, pro-caspase-3, active-caspase-3 protein. (B) Quantitation of Bcl-2 protein. (C) Quantitation of Bax protein. (D) Quantitation of Bax/Bcl-2 protein. (E) Quantitation of pro-caspase-3 protein. (F) Quantitation of active-caspase-3 protein. Data were presented as the mean ± SD of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ vs untreated control cells; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs $A\beta_{25-35}$ alone.

Discussion

The main findings of the present study are as follows: (i) ICS II exerted beneficial effects on $A\beta_{25-35}$-induced cognitive deficits and neuronal cell injury; (ii) ICS II attenuated excessive intracellular and mitochondrial ROS production and mitochondrial dysfunction; (iii) ICS II inhibited PDE5 protein level and increased cGMP levels; (iv) ICS II activated the BDNF/TrkB/CREB signaling pathway, and the effect could be abolished by ANA-12, a TrkB inhibitor.

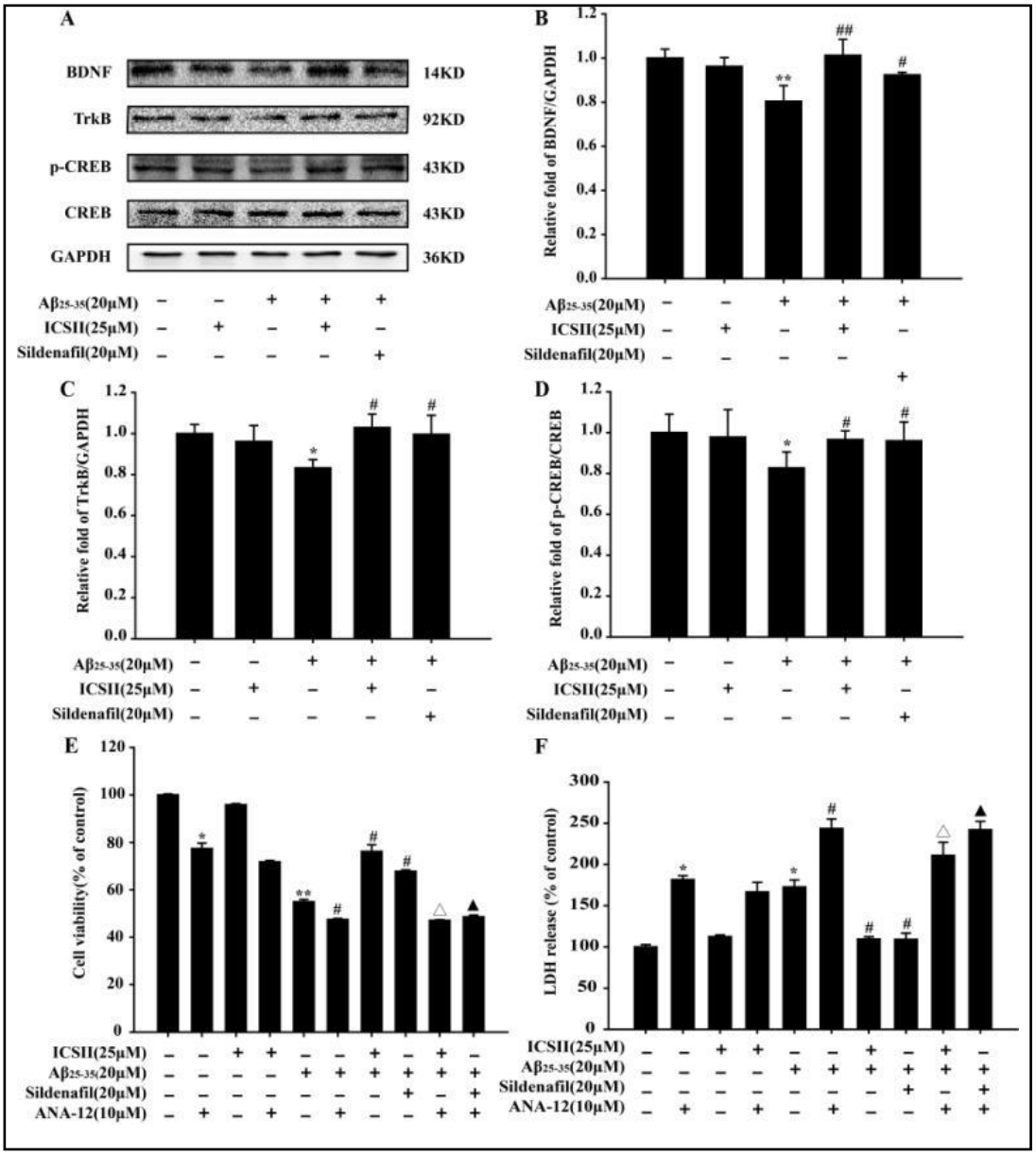

Fig. 8. Effect of ICS II on the expression of BDNF, TrkB, and p-CREB level. (A) A representative Western blot was shown for BDNF, TrkB, and p-CREB/CREB protein. (B) Quantitation of BDNF protein. (C) Quantitation of TrkB protein. (D) Quantitation of p-CREB/CREB protein. The TrkB inhibitor ANA-12 (10 μM) was added to the particular cell cultures 1 h before ICS II treatment. (E) Cell viability was analysed using the MTT assay. (F) Cytotoxicity was analysed by the LDH release assay. Data were presented as the mean ± SD of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ vs untreated control cells; $^{\#}P<0.05$ vs $A\beta_{25-35}$ alone; $^{\Delta}P<0.05$ vs $A\beta_{25-35}$ + ICS II group; $^{\blacktriangle}P<0.05$ vs $A\beta_{25-35}$ + sildenafil group.

In our previous study, we demonstrated that ICS II exerted neuroprotective effect and ameliorated $A\beta_{25-35}$-induced cognitive impairment. Thus, it is reasonable to hypothesize that neurotrophic factors and its signal pathway may be related with elevated cognitive function following ICS II treatment. Although our preliminary study showed that ICS II protects against $A\beta_{25-35}$- induced learning and memory impairments and neuronal apoptosis in rat hippocampus [24], its in-depth underlying mechanisms have not yet been elucidated. The present study demonstrated that ICS II treatment attenuated the learning and memory impairment, which further confirmed our previous findings [24]. Notably, it is reported that

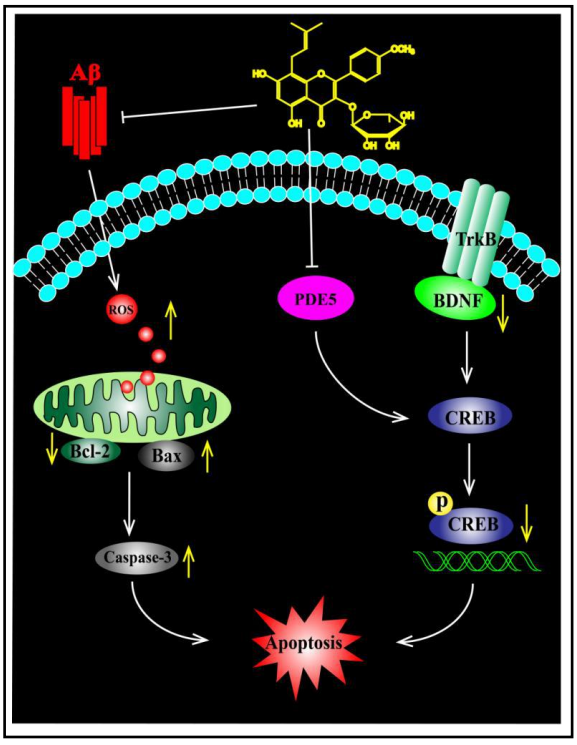

Fig. 9. Schematic presentation of a proposed mechanism for the protective role of ICS II against $A\beta_{25-35}$-induced cells death in PC12 cells. $A\beta_{25-35}$ can increase oxidative stress and apoptosis, and accumulated intracellular ROS trigger mitochondrial dysfunction, reducing the content of PDE 5, and down- regulate BDNF/TrkB/CREB signal pathway. ICS II, a PDE5 inhibitor, attenuates ROS production and apoptosis induced by $A\beta_{25-35}$ via BDNF/TrkB/CREB signal pathways.

BDNF protects neurons from neuronal apoptosis subjected to Aβ [25]. Neurotrophins, in particular BDNF, are crucial in long-term potentiation and synaptic plasticity, which play critical roles in memory formation and retention. It is reported that the levels of BDNF and its corresponding high-affinity receptor TrkB were decreased in AD. And changes in both BDNF and TrkB contribute to the loss of synaptic plasticity. In other words, BDNF and TrkB can protect against memory disability and regulate neurogenesis in the hippocampus of AD [26]. Our study showed that there was a decrease in the expression of both BDNF and TrkB in Aβ-treated group, while ICS II treatment enhanced their levels. The present study suggested that it is possible that ICS II inhibits hippocampal neuronal apoptosis by increasing BDNF level *via* BDNF/ TrkB signaling. Notably, the nuclear transcription factor CREB is activated by phosphorylation at the site of serine133, which is the major physiological parameter of AD and its activation is essential for the formation and storage of memory [27, 28]. And the activation of BDNF/TrkB signaling leads to the phosphorylation of CREB. Therefore, CREB and p-CREB levels under the treatment with ICS II were tested in hippocampi of Aβ rats. Our results suggested that the level of p-CREB in Aβ group was lower than that in sham group, and ICS II significantly increased the phosphorylation of CREB, while the total protein was not altered. It is indicated that ICS II inhibits hippocampal neuronal apoptosis by activating CREB through BDNF/TrkB signaling. The present study indicated that ICS II exerts a neuroprotective effect by increasing the expression of BDNF, which, in turn, induces TrkB advance, then increases the phosphorylation of CREB in rat hippocampi subjected to Aβ. Interestingly, PDE5, which plays an important role in synaptic plasticity and memory [29], regulates the signal transduction pathway, ultimately by activating CREB to promote gene transcription. It is also considered as a strategy for treatment of neurodegenerative diseases, such as AD. The present study also suggested that ICS II might exhibit PDE5 inhibitor and activate the CREB signaling pathway *via* increased cGMP levels. Next, to further test our hypothesis as mentioned above, the effect of ICS II on $A\beta_{25-35}$-induced PC12 cell death was evaluated *in vitro*. As it is expected, ICS II mitigated Aβ-induced neuronal death through inhibiting PDE5 and regulating Caspase 3, consistent with previous findings *in vivo* [5]. Furthermore, several studies revealed that oxidative stress is the redox state resulting from an imbalance between the generation and detoxification of ROS, and it is known to play an important role in the

pathogenesis of AD [30]. Aggravation of Aβ promotes ROS overproduction and excessive ROS accelerates the neurotoxicity of Aβ in turn [31]. Additionally, mitochondria are the major source of ROS and excessive ROS produced in mitochondria lead to dysfunction of mitochondria, thereby resulting in apoptosis or even neuronal cell death [32]. Notably, our findings demonstrated that $A\beta_{25-35}$ increased intracellular and mitochondrial ROS in neuronal cells, whereas treatment with ICS II or sildenafil significantly suppressed these abnormal changes in neuronal cells, suggesting that ICS II mitigated $A\beta_{25-35}$-induced neuronal cell death by inhibiting both in the intracellular and intramitochondrial ROS overproduction. Consistent with the results *in vivo*, ICS II not only elevated the expressions of BDNF and TrkB, but also activated CREB. In view of PC12 cells stably over-expressing TrkB[33], we tried to knockdown the expression of TrkB by TrkB siRNA, but we were able to knockdown the gene expression to only approximately 50%. Hence we applied TrkB inhibitor instead of TrkB siRNA to further explore whether or not BDNF/TrkB/CREB signaling pathway was involved in the effect of ICS II. Interestingly, ANA-12, a TrkB inhibitor, blocked the neuroprotective effect of ICS II on $A\beta_{25-35}$-induced neuronal injury, which further confirmed that BDNF/TrkB/CREB signaling pathway plays a direct role in the beneficial effect of ICS II on $A\beta_{25-35}$-induced cognitive deficits and apoptosis. Our result also suggest that an interaction exists between overproduction of ROS, mitochondrial dysfunction and apoptosis (Fig. 9).

Conclusion

Collectively, the present study found that ICS II, a PDE 5 inhibitor, mitigates $A\beta_{25-35}$-induced cognitive deficits and neuronal cell death, at least in part, by upregulating of the BDNF/TrkB/CREB signaling. This indicates that ICS II can be used as a potential therapeutic agent for dementia, such as AD.

Disclosure Statement

The authors have no conflicts of interest to disclose.

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