Original Paper

Salvianolic Acid B Ameliorates Cognitive Deficits Through IGF-1/Akt Pathway in Rats with Vascular Dementia

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Key words

Salvianolic acid B • Vascular dementia • Cognitive impairment • IGF-1 • Akt

Abstract

BackgroundAims: Salvianolic acid B (SalB) is a natural polyphenolic compound enriched in *Salvia miltiorrhiza* Bunge. Our study was designed to explore the role of Sal B on cognitive impairment in vascular dementia (VD) model rats, as well as its possible molecular mechanisms.

Methods: Rats were randomly divided into four groups (n = 15 for each group): Control group, Sal B group (normal Sprague Dawley rats treated with Sal B), VD group and VD + Sal B group. The VD group rats were established by permanent bilateral common carotid artery occlusion (BCCAO). Animals in the Control and Sal B group received the same operation without bilateral common carotid arteries occlusion. The animals in Sal B group and VD + Sal B group received Sal B (20 mg/kg) orally once a day for consecutive 6 weeks. We investigated the effects of SalB on BCCAO-induced cognitive deficits rats models via the Morris water maze experiment. To explore the mechanisms of Sal B on cognitive function, we detected the expression of IGF-1, Akt and p-Akt, and the rate of cell apoptosis in CA1 region. **Results:** Our results observed that hippocampal IGF-1 was decreased in VD model rats, while SalB reversed the alteration of IGF-1 levels. The expression of hippocampal Akt showed no significant difference between control and VD group, however, p-Akt level was significantly decreased in VD group. After 6 weeks of SalB treatment, p-Akt level was significantly increased. A large number of apoptotic neurons were found in VD model rats, while SalB prevented apoptosis of hippocampal neurons in CA1 region in VD model rats. **Conclusion:** SalB significantly ameliorated cognitive deficits in BCCAO-induced VD model rats. The potential mechanism underlying the protective effects may be mediated through IGF-1/Akt pathway.

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Introduction

Vascular dementia (VD), the second most common type of dementia after Alzheimer's disease in humans globally, is characterized by a progressive decline in cognitive function [1, 2]. VD is a clinical mental disorder caused by cardiovascular pathological changes and cerebrovascular disease, which can lead to ischemic, ischemic-hypoxic or hemorrhagic brain tissue lesions [3, 4]. VD in turn increases the risk of recurrent cerebrovascular disease, dependent living and death. With the increasing age of the population and improved survival rates from cerebrovascular diseases, VD may affect more individuals in the future. The increasing incidence of VD impose a heavy social and economic burden on individuals, families, communities and countries. Up to now, there is no effective treatment for VD and the exact pathogenesis of VD are still elusive. In consequence, the effective treatment for VD is urgently needed.

Salvianolic acid B (SalB) is a natural polyphenolic compound enriched in *Salvia miltiorrhiza* Bunge [5]. SalB has a variety of pharmacological effects for its anti-inflammatory and anti-oxidative activities [6]. A large number of research have been used SalB to treat cardiovascular diseases [7, 8]. Recently, SalB is demonstrated effective in diabetic rats, obesity for its role of regulating glucose and lipid metabolism [9, 10]. SalB is also widely used in diseases of nervous system. For instance, SalB could attenuated the cognitive decline in mice with traumatic brain injury and A β 25–35 peptide-induced Alzheimer's disease [11–13]. However, no research have been focused on the cognitive enhancing effects of SalB in VD. If SalB ameliorates cognitive deficits caused by chronic cerebral hypoperfusion, it would become a candidate drug of enhancing cognitive function for treating VD.

Although the precise mechanisms underlying VD is unknown, chronic cerebral hypoperfusion is regarded as a main underlying pathogenesis for VD. Bilateral common carotid artery occlusion (BCCAO), a suitable model for exploring the mechanisms of VD, can mimic chronic cerebral hypoperfusion in rats [14]. In the present study, we investigated the effects of SalB on BCCAO-induced cognitive deficits rats models via the Morris water maze experiment. Research [15] have reported downregulated expression of IGF-1 and p-Akt were related to the cognitive deficits in VD. Therefore, whether SalB affects IGF-1/Akt signal pathway in VD model rats is interesting. The purpose of our research was to investigate whether SalB could ameliorate memory deficits in VD rats and, if so, What are the potential mechanisms.

Materials and Methods

Animals and Model induction

Adult healthy male Sprague-Dawley (SD) rats (aged 2 months, weighed 200–240 g) were acquired from the Experimental Animal Center of Hebei Medical University. All rats were raised in a house with a 12 hour light-dark cycle and 22–25°C temperature. All rats housed 2 or 3 per cage and had access to food and water ad libitum. The experimental protocol was performed in compliance with the regulations approved by the Committee on the Ethics of Animal Experiments of the Hebei Medical University.

The vascular dementia models were made via permanent bilateral common carotid artery occlusion (BCCAO). Rats were anesthetized with 10 % chloral hydrate (35 mg/100 g) by intraperitoneal injection. The skin of the rat was incised along the midline of the neck region and the bilateral common carotid arteries were exposed. Then the bilateral common carotid arteries were tied with silk sutures. The wound was sutured and animals were returned to the cage for recovery from the anesthesia. Rats in control group were received the same operation without bilateral common carotid arteries occlusion.

Animal groups and drug application

Sal B was obtained from phytomarker Ltd. (Tianjin, China), and its purity was detected to be more than 98% by high-performance liquid chromatography. Rats were randomly divided into four groups (n = 15 for each group): Control group, Sal B group (normal Sprague Dawley rats administered with Sal B), VD group

and VD + Sal B group. The VD group rats were established by BCCAO. Animals in the Control and Sal B group were received the same operation without bilateral common carotid arteries occlusion. The animals in Sal B group and VD + Sal B group were received Sal B (20 mg/kg) orally once a day for consecutive 6 weeks, while the rats in Control group and VD group administered with saline orally.

Morris water maze (MWM) test

The Morris water maze test was performed to evaluate spatial learning and memory performance of rats. Tests were carried out four times per day for 5 consecutive days with an interval between sessions of 40-60min. The Morris water maze consists of a large black cylindrical pool (200 cm in diameter and 60 cm in height) filled with 22±2°C nontoxic black water (45 cm in depth). The pool was divided into 4 quadrants and a removable circular hidden platform (10 cm in diameter and 2 cm below the surface of the water) was placed in the center of the target quadrant. In the 5 days of navigation test, rats were released facing the pool wall in one of the four different start positions and allowed to swim freely to the hidden platform. The sequences of four different start positions list in Table 1. The time of each rat taken to climb onto the hidden platform was recorded as the escape latency. If a rat escape onto the platform within 120 s, it was allowed to stay there for 20 s. If a rat could not reach the hidden platform within 120 s, it was guided to the platform and stay there for 20 s, and the escape latency was recorded as 120 s. On day 6, the probe trial was tested by removing the platform and a rat was allowed to swim freely to find the missing platform for 120 s. The performance of rats were recorded and analyzed by a video tracking system. The times across the platform and escape latency were recorded.

Table 1. The sequences of four different start positions

Day	Trial 1	Trial 2	Trial 3	Trial 4
1	N	E	SE	NW
2	SE	N	NW	E
3	NW	SE	E	N
4	E	NW	N	SE
5	N	SE	E	NW
6 (Probe)	NE			

ELISA Analysis

After MWM test, 5 rats of each group were sacrificed and hippocampal homogenates were prepared. The expression of IGF-1, Akt and p-Akt were tested by specific ELISA kit followed the manufacturer's instructions (Cusabio Biotech, China).

Western Blotting Analysis

The expression of IGF-1, Akt and p-Akt were tested by Western blotting analysis. Rats of each group were anesthetized and the brain was obtain, then the hippocampus were rapidly dissected on the ice. The hippocampus was homogenized in RIPA assay lysis buffer. The proteins were subsequently separated by electrophoresis on 10% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. The membrane was blocked with 5% dried, defatted milk in TBS buffer for 2 h at room temperature and then incubated with primary antibodies anti Akt (1:1000, Cell Signaling, USA), p-Akt (Ser473) (1:1000, Cell Signaling, USA), IGF1 (1:1000, Cell Signaling, USA), and β-actin (1:2000, Cell Signaling, USA) at 4° C overnight. After several washes, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membranes were detected by a chemiluminescent system. The protein bands were measured by image analysis software. All relative optical densities were normalized to β-actin.

Semi-quantitative reverse transcription PCR (semi-RT-PCR) assay

Qiagen Rneasy mini kit (74014) was used to isolate total RNA from the hippocampus tissue. Then, primescript RT kit (Takara, Japan) and PCR ExTaq (Takara) were used to perform reverse transcription PCR according to the manufacturer's protocol. The primer sequences were 5'-TGTGATCTGAGGAGGCTGGA-3' and 5'-GCAAAGGATCTTGCGGTGAC-3' for IGF-1 (216 bp), and 5'-GGTCAGAAGGATTCTCTATGTG-3' and 5'-ATTGCCAATGGTGACCTG-3' for β-actin (599bp). The amplified PCR products were then resolved by electrophoresis on a 1.5% agarose gel and analyzed by Quantity one software (Bio-Rad). The expression levels of IGF-1 mRNA were normalized to β-actin.

Hippocampal morphology observation

Rats were anesthetized with 10 % chloral hydrate (35 mg/100 g) by intraperitoneal injection. After Perfusion the brain tissue was obtained, then fixed by 4% polyformaldehyde solution for 24 hours. Dehydrated by gradient ethanol, be transparent by dimethylbenzene, waxed and embedded, then serially sectioned coronally. HE staining was performed to observe the morphologic changes in hippocampal neurons.

TUNEL staining

The apoptosis of neurons in hippocampal CA1 region from the four groups were tested by TUNEL staining. The brain tissue was fixed in 4% PFA and embedded into paraffin wax. In Situ Cell Death Detection Kit (Roche, Switzerland) was applied to test the apoptosis followed the manufacturer's instructions. Three sections were used for each group, and five fields were randomly selected from each section ($\times 400$). Images were measured using Image-Pro Plus 6.0 software (Olympus Co., Tokyo, Japan). The apoptotic rate was calculated. Apoptotic rate = (number of apoptotic cells/total number of cells) $\times 100\%$.

Statistical analysis

Data are expressed as the mean $\pm$ SD. Statistical analysis was performed using SPSS 17.0 software. In the MWM test, escape latency was analyzed by two-way repeated measures ANOVA followed by LSD post hoc test. All other data were analyzed by one-way ANOVA. Statistically significant was set at $p < 0.05$.

Results

Sal B ameliorated cognitive deficits in VD model rats

We evaluated the cognitive function in different groups by the MWM experiment to determine whether Sal B ameliorates cognitive deficits (Fig. 1). There were no significant differences in cognitive function between the Control group and the Sal B group, demonstrating that Sal B did not influence cognitive function in normal animals. The escape latency was significantly increased in VD model rats compared to Control group. However, the escape latency was significantly decreased after 6 weeks of Sal B treatment (Fig. 1A). In the probe test, the times across the platform was significantly decreased in the VD group compared to the Control group (Fig. 1B). The times across the platform was significantly increased

Fig. 1. Results of MWM experiment. (A) The escape latency was significantly increased in VD model rats compared to Control group. However, the escape latency was significantly decreased after 6 weeks of Sal B treatment. (B) The times across the platform was significantly decreased in the VD group compared to the Control group, the times across the platform was significantly increased in the VD + Sal B group compared to the VD group. There were no significant differences in cognitive function between the Control group and the Sal B group. * $p < 0.05$ vs. Control group. # $p < 0.05$ vs. VD group.

Fig. 2. Quantification of hippocampal IGF-1, Akt and p-Akt protein levels tested by ELISA. (A) IGF-1. (B) Akt. (C) p-Akt. The expression of IGF-1 and p-Akt in the VD group were significantly decreased than the Control group. However, the expression of IGF-1 and p-Akt were significantly increased in the VD + Sal B group. There were no significant differences between the Control group and the Sal B group. The expression of Akt showed no significant difference among different groups. * $p < 0.05$ vs. Control group. # $p < 0.05$ vs. VD group.

Fig. 3. Western blotting analysis of IGF-1 expression. (A) IGF-1 and β -actin protein bands. (B) The relative protein expression of IGF-1. The expression of hippocampal IGF-1 in the VD group was significantly decreased than the Control group. However, the expression of hippocampal IGF-1 was significantly increased in the VD + Sal B group. * $p < 0.05$ vs. Control group. # $p < 0.05$ vs. VD group.

in the VD + Sal B group compared to the VD group.

Sal B increased IGF-1 expression in VD model rats

Western blotting and ELISA results showed that the expression of hippocampal IGF-1 in the VD group was significantly decreased than the Control group. However, the expression of hippocampal IGF-1 was significantly increased in VD + Sal B group (Fig. 2A and Fig. 3). RT-PCR results showed that the mRNA expression of hippocampal IGF-1 in the VD group was significantly decreased than the Control group. However, the mRNA expression of hippocampal IGF-1 was significantly increased in VD + Sal

Fig. 4. RT-PCR analysis of IGF-1 expression. (A) IGF-1 and β -actin mRNA bands. (B) The relative mRNA expression of IGF-1. The expression of hippocampal IGF-1 in the VD group was significantly decreased than the Control group. However, the expression of hippocampal IGF-1 was significantly increased in the VD + Sal B group. * $p < 0.05$ vs. Control group. # $p < 0.05$ vs. VD group.

Fig. 5. Western blotting analysis of Akt expression. (A) Akt and β -actin protein bands. (B) The relative protein expression of Akt. The expression of hippocampal Akt showed no significant difference among different groups. * $p < 0.05$ vs. Control group. # $p < 0.05$ vs. VD group.

Fig. 6. Western blotting analysis of p-Akt expression. (A) p-Akt and β -actin protein bands. (B) The relative protein expression of p-Akt. The expression of p-Akt was significantly decreased in VD group compared to Control group. After 6 weeks of SalB treatment, p-Akt level was significantly increased. * $p < 0.05$ vs. Control group. # $p < 0.05$ vs. VD group.

B group (Fig. 4). There were no significant differences between the Control group and the Sal B group. Our results suggested that BCCAO inhibited the expression of hippocampal IGF-1, while SalB reversed the alteration of IGF-1 levels.

Sal B increased p-Akt expression in VD model rats

Western blotting and ELISA results revealed that the expression of hippocampal Akt showed no significant difference among different groups (Fig. 2B and Fig. 5). BCCAO

Fig. 7. Morphologic changes in hippocampal neurons. (A) Control group, (B) Sal B group, (C) VD group, (D) VD + Sal B group. In Control and Sal B group the neurons in CA1 region were tightly aligned, cytoplasm and nuclei were clear. In VD group the neurons in CA1 region were disorganized and loose, the cells became smaller, appeared chromatic agglutination and karyopyknosis. In VD + Sal B group, the normal neurons were more than VD group, and chromatin is richer than VD group, cell arrangement were tighter than VD group.

Fig. 8. Sal B prevented apoptosis of hippocampal neurons in CA1 region in VD model rats. (A) A few apoptotic neurons were found in Control group. (B) A few apoptotic neurons were found in Sal B group. (C) Significant increase of apoptotic neurons were found in VD group. (D) The apoptotic neurons were decreased in VD + Sal B group. (E) Quantification of the apoptotic neurons. * $p < 0.05$ vs. Control group. # $p < 0.05$ vs. VD group.

and SalB had no obvious effect on Akt level. However, p-Akt level was significantly decreased in VD group compared to Control group. After 6 weeks of SalB treatment, p-Akt level was significantly increased (Fig. 2C and Fig. 6). Our results suggested that SalB treatment improves cognitive impairment in VD rats partly through phosphorylation of Akt rather than by regulating its Akt levels.

Morphologic changes in hippocampal neurons

In Control and Sal B group the neurons in CA1 region were tightly aligned, cytoplasm and nuclei were clear. In VD group the neurons in CA1 region were disorganized and loose, the cells became smaller, appeared

chromatic agglutination and karyopyknosis. In VD + Sal B group, the normal neurons were more than VD group, and chromatin is richer than VD group, cell arrangement were tighter than VD group. See (Fig. 7)

Sal B prevented apoptosis of hippocampal neurons in CA1 region in VD model rats

The rate of cell apoptosis in CA1 region was remarkably increased in the VD group than in the Control group ($p < 0.05$). The rate of cell apoptosis in the hippocampal CA1 region was significantly decreased in the VD + Sal B group than in the VD group ($p < 0.05$). There were no significant differences in apoptotic rate between the Control group and the Sal B group ($p > 0.05$). See (Fig. 8). Our results suggested that the SalB treatment efficiently decreased cell apoptosis in the hippocampal CA1 region.

Discussion

SalB, a natural polyphenolic compound enriched in *Salvia miltiorrhiza* Bunge, possess a variety of pharmacological effects, for instance, anti-inflammatory, anti-oxidative, glucose regulated and neuroprotective activities [10, 16, 17]. Although a large number of research have demonstrated the close relationship between Sal B and cognitive ability, the underlying mechanisms remain elusive. Meanwhile, no research have been focused on the cognitive enhancing effects of SalB in VD. Therefore, we investigated the effects of Sal B on cognitive deficits in BCCAO-induced VD model rats. Our result showed that VD rats accompanied by cognitive decline while SalB ameliorates the memory impairments of VD model rats. SalB ameliorates cognitive deficits caused by chronic cerebral hypoperfusion, it would become a candidate neuroprotective drug of enhancing cognitive function for treating VD.

Insulin-like growth factor-1 (IGF-1), a small single-chain polypeptide (7.5 kDa), is widely expressed in several parts of the central nervous system, such as the cerebral cortex, cerebellum, hypothalamus and hippocampus [18]. IGF-1 is a multifunctional polypeptide essential for neuroprotection, normal growth and development [19]. IGF-1 exerts its roles on brain development through control of neurotrophic responses and cell signalling [20]. IGF-1 could decrease the glycogen synthase kinase 3 and the A β level in the brain [21, 22], and the reduction of IGF-1 is associated with A β deposit and cognitive impairment [23]. Several prospective studies demonstrated a close correlation between serum IGF-I levels and cognitive status in the elderly [24, 25]. All the research mentioned above demonstrated the important role of IGF-1 in maintaining cognitive function. There also some researches focus the neuroprotective effects of IGF-1 on treating cerebral ischemic brain injury [26, 27]. Gong et al. [15] reported IGF-1/IGF-1R signaling system may involved in the onset and development of VD. Therefore, whether SalB affects IGF-1 expression in VD model rats is interesting. To investigate the mechanisms underlying the cognitive enhancing effects of SalB in BCCAO-induced VD model rats, we examined the expression of IGF-1. Our results observed that hippocampal IGF-1 was decreased in VD model rats, while SalB reversed the alteration of IGF-1 levels. Our research suggest that SalB improved the cognitive performance in BCCAO-induced VD model rats partly through enhancing hippocampal IGF-1 expression.

Akt, a downstream target of phosphatidylinositol 3 kinase (PI3K), is a serine /threonine kinase taken part in cell survival [28]. Activated Akt promotes cell survival and inhibits apoptosis by phosphorylating its down-stream proteins, such as caspase-9, Bcl-2-associated death protein (Bad), glycogen synthase kinase 3 β (GSK3 β) [29-31]. IGF-1 binding to its receptor may activate one of the two major intracellular signaling pathways, PI3K/Akt and mitogen activated protein kinase (MAPK) pathways [32]. The neuroprotective effects of IGF-1 is mainly activated by the PI3K/Akt pathway [33]. Research have reported downregulated expression of IGF-1 and p-Akt were related to the cognitive deficits in VD [15]. Improvement of Akt expression is an attractive neural protection goal for the Akt pathway modulates cell survival after stroke [34]. To investigate the mechanisms underlying the cognitive enhancing effects of SalB in VD model rats, we examined the expression of Akt and p-Akt as well. In

our study, the expression of hippocampal Akt showed no significant difference between control and VD group, however, p-Akt level was significantly decreased in VD group. After 6 weeks of SalB treatment, p-Akt level was significantly increased. SalB improves cognitive impairment in VD rats partly through phosphorylation of Akt rather than by regulating its protein levels. Our results suggest that Akt pathway plays an important role in the SalB-induced improvement of the cognitive performance.

Apoptosis is an important type of active cell death and an important way for pyramid neuron loss, which has been specifically clarified previously. It is characterized by cytoplasmic condensation, pyknotic nuclei and DNA fragmentation, which can be labeled by TUNEL staining of single or double strands break in DNA. Research have reported chronic cerebral hypoperfusion could promote apoptosis [35]. Our results observed that a large number of apoptotic neurons in VD model rats, while SalB prevented apoptosis of hippocampal neurons in CA1 region in VD model rats. It has been shown that IGF-1 increased cell viability while decreasing apoptosis [36]. Our research suggest that SalB inhibited apoptosis of hippocampal neurons in VD model rats maybe through enhancing hippocampal IGF-1 expression.

Conclusion

Taken together, in our present study, we found that the cognitive performance, associated with the expression of IGF-1 and p-Akt were significantly increased in the SalB-treated VD rats than those in the VD model rats. SalB also prevented apoptosis of hippocampal neurons in CA1 region in VD model rats. Therefore, we speculated that SalB has the beneficial neuroprotective effect to VD. This study could provide valuable experimental basis for VD clinical treatment. The neuroprotective effects of SalB may be associated with IGF-1/Akt pathway, but the molecular mechanisms remain to be further explored, for example, whether it related to neurogenesis, neuroinflammation and synaptic plasticity etc.

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Disclosure Statement

We declare that we have no conflicts of interest in the authorship or publication of this contribution.

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