

Original Article

Effects of PI3K/Akt signaling pathway on serum C-reactive protein, serum amyloid A and cognitive dysfunction in mice with Alzheimer's disease

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Abstract: Objective: To investigate the regulatory mechanism of PI3K/Akt signaling pathway on serum C-reactive protein (CRP), serum amyloid A (SAA) and cognitive dysfunction in mice with Alzheimer's disease (AD). Methods: Ten of 40 healthy male C57BL/6 mice were randomly selected as a normal group, and the remaining 30 mice were used to construct AD model by treating them with D-galactose (120 mg/kg) combined with aluminum chloride (10 mg/kg). The model mice were divided into the model group (model mice), IGF-I group (model mice with lateral ventricle injection of PI3K/AKT agonist IGF-I), wortmannin group (model mice with lateral ventricle injection of PI3K/AKT inhibitor wortmannin), with 10 mice in each group. The learning and memory ability of mice was detected by the jumping platform test and Morris water maze test. The level of reactive oxygen species (ROS) in brain tissue was detected by chemical fluorescence method, the level of superoxide dismutase (SOD) in brain tissue was detected by WST-1 method, the levels of serum CRP and SAA were measured by ELISA, and the expression of AKT, PI3K, COX-2, iNOS mRNA and protein of mice in each group was detected by qRT-PCR and western bolt. Results: Compared with the normal group, the expression of PI3K, AKT mRNA and protein in model group, IGF-I group, and wortmannin group was significantly lower, the learning and memory ability was significantly worse, the levels of ROS, SOD, CRP and SAA were higher, the expression of COX-2, iNOS mRNA and protein was also increased (all $P < 0.05$). Compared with the model group, the above indexes of the wortmannin group showed the same trend (all $P < 0.05$), while the IGF-I group showed the opposite trend (all $P < 0.05$). Conclusion: Activation of PI3K/Akt signaling pathway can decrease the level of serum CRP and SAA and improve cognitive dysfunction in AD mice.

Keywords: PI3K/Akt signaling pathway, Alzheimer's disease, C-reactive protein, amyloid A, cognitive dysfunction

Introduction

Alzheimer's disease (AD) is the most common age-related neurodegenerative disease, characterized by progressive learning and memory impairment [1]. AD is the most common form of dementia, and there is currently no effective treatment to improve the symptoms [2]. The neuropathological markers of AD include senile plaques, neurofibrillary tangles, synaptic defects, and cerebral neurodegeneration. The typical pathological change in the diagnosis of AD is the increase of neurofibrillar plaques in the brain [3, 4]. The aggregation of these insoluble proteins consists of hyperphosphorylated tau, abnormal amyloid-beta ($A\beta$) isoforms, and

some metals such as Fe, Zn, and Cu [5]. Oxidative stress can aggravate the progress of AD [6, 7]. The brain is an active tissue that relies on oxidative phosphorylation to provide biological energy [8]. This oxidative phosphorylation occurs in the mitochondria. This process produces by-products of destructive oxygen free radical. Therefore, reactive oxygen species (ROS) is produced in the oxidative metabolism of mitochondria and in the cellular response to pathogens. Pathogens act as signal molecules and regulate many physiological processes, including differentiation, proliferation, apoptosis, and cell migration [9, 10]. Thus, ROS is considered to be a key determinant of brain health. Eukaryotes have an evolutionary

defense system against this destructive ROS. The main members of the system are superoxide dismutases (SODs), a family of enzymes that efficiently convert superoxides into hydrogen peroxide with low reactivity [11]. Loss of SODs activity may lead to many pathological phenotypes, including neurodegenerative diseases, such as AD in metabolically active tissues [12, 13].

C-reactive protein (CRP) is a relatively stable marker of peripheral inflammation that has been used as a marker of low-grade inflammation and moderately elevated in acute myocardial infarction, coronary artery disease, metabolic syndrome, neurodegenerative diseases, and hypertension [14, 15]. Since several cardiovascular diseases have been shown to be risk factors for dementia, many studies have investigated whether there is a relationship between low-grade peripheral inflammation and AD [16, 17]. Serum amyloid A (SAA) is a polymorphic protein that regulates human lipid and protein metabolism, which is synthesized by liver cells. The formation of SAA may be caused by inflammatory stimulation, infection, and autoimmune injury. These are closely related to the occurrence and progress of the ischemic cerebrovascular disease, but there are few studies related to SAA and Parkinson's disease (PD) [18, 19].

PIP3 is a key second messenger of PI3K, which mediates the signal transduction of receptor tyrosine kinase to AKT kinase. High levels of PIP3 on the plasma membrane stimulate proteins containing PH domains to phosphorylation, such as AKT, and participate in cell survival and metabolism of intracellular target proteins, thereby protecting cells [20]. Therefore, AKT activation may play a therapeutic role in neurodegenerative diseases. PI3K/AKT signaling dysfunction leads to a decrease in neuronal activity [21]. Activated PI3K/AKT phosphorylates various biological substrates, including GSK-3 β . It has been shown that GSK-3 β phosphorylates tau. PI3K/AKT signaling dysfunction leads to overactivation of GSK-3 β , leading to hyperphosphorylation of tau, which is an important event in the pathogenesis of AD [22]. Downregulation of AKT corresponding to elevated GSK-3 β activity may be associated with the pathogenesis of brain dysfunction in AD, and PI3K/AKT signaling is attenuated in the brains of AD patients [23]. Inducible cyclooxy-

genase (COX-2) is an important enzyme in a cellular inflammatory response, and the expression of COX-2 is significantly increased in cerebral ischemia, epilepsy, edema, and AD. Inducible nitric oxide synthase (iNOS) is a kind of gas vector of intracellular signaling transmission, and NO has the function of second messenger and neurotransmitter, but the high concentration of NO has neurotoxic effects, which leads to neuronal necrosis, and the expression of iNOS is increased in AD patients [24].

In this study, we established an AD mouse model in C57BL/6 mice, and treated mice with PI3K/AKT signaling pathway inhibitor wortmannin and agonist IGF-I. The learning and memory ability of mice, levels of ROS and SOD in brain tissue, levels of serum CRP and SAA, and the expression of COX-2, iNOS mRNA and protein in each group were measured. The aim of this study was to investigate whether PI3K/AKT signaling pathway can regulate the serum CRP, SAA and cognitive dysfunction in AD mice.

Materials and methods

Experimental subjects, grouping and processing

Forty healthy clean-grade male C57BL/6 mice, aged 2 weeks and weighing 35 ± 5 g, were used in this study. 10 mice were randomly selected as the normal group, and the remaining 30 mice were used to construct the AD model. The AD mouse model was constructed with the administration of 120 mg/kg D-galactose combined with 10 mg/kg aluminum chloride in mouse [25]. After 90 days of continuous administration, the model was successfully constructed and the successful rate of modeling was 100%. After the model was constructed, the mice were divided into four groups: the normal group (normal mouse), model group (model mice), IGF-I group (model mice with lateral ventricle injection of PI3K/AKT agonist insulin-like growth factor-I (IGF-I)), wortmannin group (model mice with lateral ventricle injection of PI3K/AKT inhibitor wortmannin), with 10 mice in each group. After the behavioral test, blood was taken from the eyeball and the specimen of brain tissue was taken. The brain tissues and venous blood collected from five mice were used for detection in each group, and the brain tissues were stored in liquid nitrogen for later use. This study was

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Table 1. qRT-PCR primer sequence

Gene	Primer sequence
PI3K	F: 5'-GAAGTTGCTTACCCAGTGCC-3'
	R: 5'-GATAGCCGTTCTTTTCATTGG-3'
AKT	F: 5'-ACTCATTCCAGACCCACGAC-3'
	R: 5'-AGCCCGAAGTCCGTTATCTT-3'
COX-2	F: 5'-GGAGAGACTATCAAGATAGT-3'
	R: 5'-ATGGTCAGTAGACTTTTACA-3'
iNOS	F: 5'-GAGCGAGTTGTGGATTGTC-3'
	R: 5'-CTCCTTTGAGCCCTTTGT-3'
β -actin	F: 5'-TCTGCTCCTCCTGTTCTA-3'
	R: 5'-TGTCATATTTCTCGTGGTTC-3'

approved by the Animal Care and Use Committee of The Second Affiliated Hospital of Qiqihar Medical University.

qRT-PCR

Total RNA was extracted with Trizol (article number: 16096020, Thermo Fisher Scientific, New York, USA; article number: B1802, Harbin Xinhai Genetic Testing Co., Ltd., China). The cDNA was synthesized by reverse transcription using Taq Man MicroRNA Assays Reverse Transcription Primer (Thermo Scientific, USA). SYBR® PremixExTaqTMII Kit (Xingzhi Biotechnology Co., Ltd., China) was used for quantitative fluorescence PCR (QF-PCR) detection. The following agents were added in sequence: 25 μ L of SYBR® PremixExTaqTMII (2X), 2 μ L of PCR upstream and downstream primers, 1 μ L of ROX Reference Dye (50X), 4 μ L of DNA template, and 16 μ L of ddH₂O. QF-PCR was performed in ABI PRISM® 7300 (type: Prism® 7300, Shanghai Kunke Instrument Equipment Co., Ltd., China). The reaction conditions were: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 15 s, annealing at 60°C for 30 s. After 32 cycles, elongation was conducted at 72°C for 1 min. β -actin was used as an internal reference and $2^{-\Delta\Delta Ct}$ was used for the calculation of relative expression of each target gene. Primers are shown in **Table 1**.

Western blot

Total protein was extracted using RIPA buffer (R0010, Solarbio) containing PMSF. BCA kit (Thermo, Inc., USA) was used to measure the protein concentration. The sample was mixed with the loading buffer followed by incubating in boiling water for 10 min. A total of 30 μ g of protein sample was loaded, and electrophoresis

was conducted at 80 V of constant pressure for 2 h. The protein was transferred to a PVDF membrane (ISEQ00010, Millipore, Billerica, MA, USA) at a voltage of 110 V for 2 h. The PVDF membrane was blocked with 5% skim milk powder at 4°C for 2 h followed by washing with TBST for once. The primary rabbit anti-mouse antibodies PI3K (ab151549, 1:1,000, Abcam, UK), AKT (ab179463, 1:10,000, Abcam, UK), COX-2 (ab179800, 1:1,000, Abcam, UK), iNOS (ab213987, 1:1,000, Abcam, UK), β -Actin (ab8226, 1:2,000, Abcam, UK) were added onto the membranes, and incubated overnight at 4°C. The membranes were washed with BST for 10 min, thrice, and incubated with HRP-labeled goat anti-rabbit IgG antibody (Beijing Zhongshan Biotechnology Co., Ltd., diluted with 1:5,000) at room temperature for 2 h. After rinsing with TBST, the sample was placed on a clean glass plate and developed by the ECL fluorescence detection kit (article number: BB-3501, Ameshame, UK). The Bio-Rad image analysis system (BIO-RAD, USA) was used for imaging, and the images were analyzed by ImageJ software. The relative protein content was expressed by the gray value of the corresponding protein band/the gray value of the β -Actin protein band.

Jumping platform test

WX-2 type mouse electro-optic stimulation conditional reflex jumping platform was used in this study (purchased from the Institute of Materia Medica, Chinese Academy of Medical Sciences). Mice were put in turn facing the pool wall, the bottom was covered with copper grids, the alternating voltage was 36 V, and a platform with a height and a diameter of 4.5 cm was placed in the left front corner of each reaction box. After 5 days of feeding, the mice were put into the reaction box and adapted to it for 5 min, then the box was electrified and the time taken for the mice to jump on the platform and stabilize for 10 s after electrical stimulation was recorded. For each mouse, the test was repeated 3 times, and the reaction time and the number of errors were recorded.

Morris water maze test

The water maze is a circular pool (150 cm in diameter and 60 cm in height), with a temperature control of 20°C-25°C. The circular pool was divided into four quadrants of the lower right, upper right, lower left, and upper left

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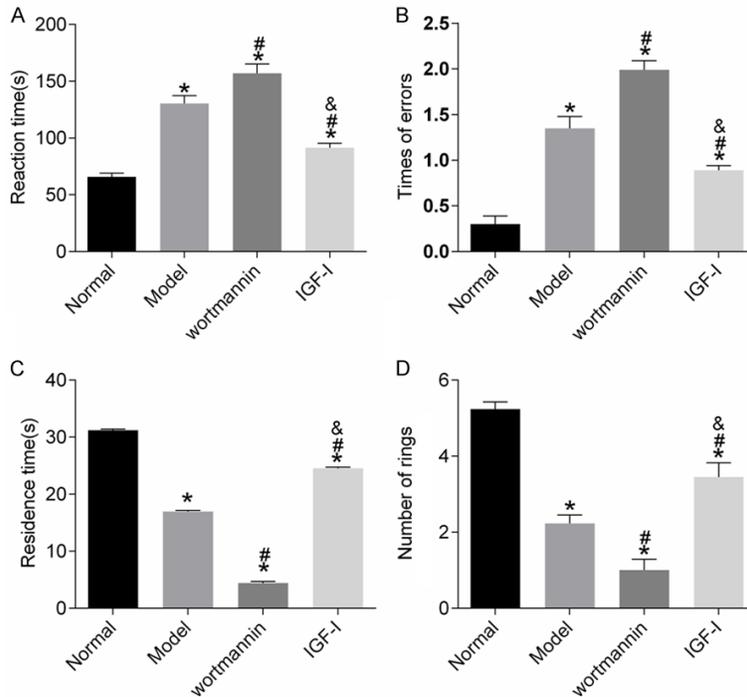


Figure 1. Learning and memory ability of mice in each group. A. The reaction time in jumping platform test; B. The times of errors in jumping platform test; C. The residence time in water maze test; D. The number of rings in water maze test. Compared with normal group, * $P < 0.05$; compared with model group, # $P < 0.05$; compared with wortmannin group, & $P < 0.05$.

and a platform was installed in the lower right quadrant. After 7 days of training, the mice were put into water in turn, and the time of searching for the platform within 2 min (escape latency) was recorded. If the platform was not found within 2 min, the mice were guided to the platform by the experimenter and stay for 10 s, and the escape latency was recorded as 2 min. On the 5th day, the platform in the water was removed and the space exploration experiment was carried out. The entry point of mice into the water was fixed, and the time of mice stayed in the original platform quadrant (target quadrant) and the number of times the mice traveled through the original platform position (number of crossing platform) were recorded within 60 seconds.

Determination of ROS and SOD content in brain tissue

The levels of ROS and SOD in brain tissue were detected by chemical fluorescence method (E004-1-1, Nanjing Jiancheng Chemical Industrial Co., Ltd.), and WST-1 method (A001-3-

2, Nanjing Jiancheng Chemical Industrial Co., Ltd.), respectively. The brain tissue was immediately put into the pre-cooled PBS, and the tissue mass was cut into small pieces of about 1 mm³ with ophthalmic scissors, rinsed in PBS, digested with pancreatin, digested with a constant temperature water bath at 37°C for 30 min. The digestion was stopped with PBS, then filtered with nylon mesh (300 meshes), and centrifuged at 1,000 rpm for 10 min. The supernatant was discarded, and the cells were resuspended for detection. The operation was carried out by following the kit instructions.

ELISA

Venous blood was taken, kept in standing for 1 h, and centrifuged at 3,000 rpm for 10 min. The supernatant was

taken and stored at -20°C. The levels of serum CRP and SAA in brain tissue were measured according to the instructions of ELISA kits. (The serial number of CRP detection kit was: 69-21206; SAA: 69-20249, Wuhan Merck, China).

Statistical analysis

All data were analyzed using SPSS21.0 statistical software. The measurement data were expressed as mean \pm standard deviation. One-way ANOVA was used for comparisons between multiple groups. Tukey's post-hoc test was used for pair-wise comparisons of the mean values between multiple groups. $P < 0.05$ indicated that the differences were statistically significant.

Results

Learning and memory ability of mice in each group

The results of jumping platform test (**Figure 1A** and **1B**) showed that compared with the normal

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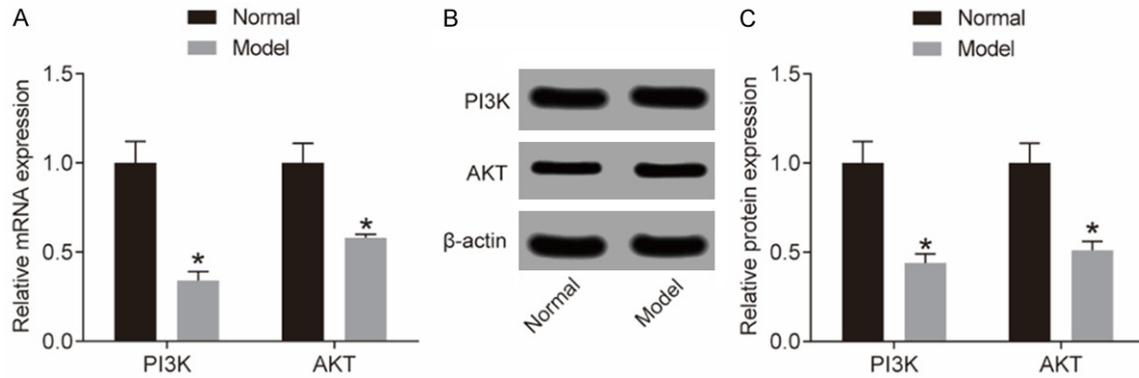


Figure 2. Expression of PI3K and AKT in brain tissue of mice in the normal and model group. A. PI3K and AKT levels in brain tissue of mice. B. PI3K and AKT protein bands in brain tissue of mice. C. PI3K and AKT protein levels in brain tissue of mice. Compared with normal group, * $P < 0.05$.

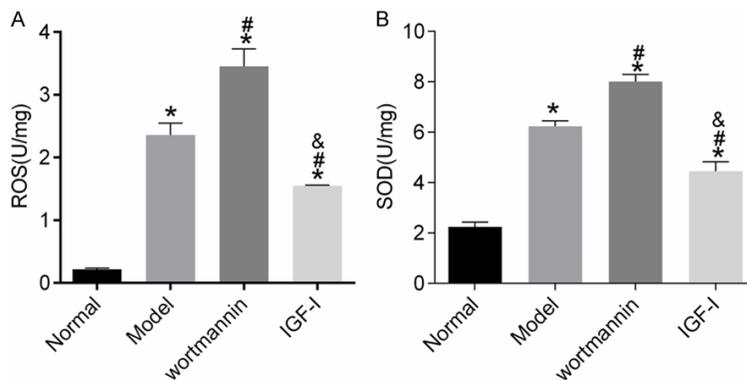


Figure 3. Determination of ROS and SOD levels in brain tissue. A. ROS level in brain tissue of mice. B. SOD level in brain tissue of mice. Compared with normal group, * $P < 0.05$; compared with model group, # $P < 0.05$; compared with wortmannin group, & $P < 0.05$. ROS, reactive oxygen species; SOD, superoxide dismutase.

group, the reaction time of the other groups was significantly longer and the number of errors was significantly higher (all $P < 0.05$). Compared with the model group, the reaction time of the IGF-I group was significantly shorter, the number of errors was significantly less (both $P < 0.05$), while the wortmannin group showed an opposite result (both $P < 0.05$).

The results of water maze test (**Figure 1C** and **1D**) showed that compared with normal group, the latency of the other groups was significantly shorter, and the number of crossing platform was significantly less (all $P < 0.05$). Compared with the model group, the latency of the IGF-I group was significantly longer, and the number of crossing platform was significantly higher (both $P < 0.05$).

Decreased expression of PI3K and AKT in brain tissue of mice in the model group

The results in **Figure 2** showed that compared with the normal group, the expression of PI3K, AKT mRNA and protein were significantly lower in the model group (all $P < 0.05$). The PI3K/AKT signaling pathway was inhibited in AD.

Determination of ROS and SOD levels in brain tissue

The results of ROS and SOD levels in brain tissue of mice in each group (**Figure 3**) showed that compared with the normal group, the levels of ROS and SOD in the brain tissue of the other groups were significantly higher (all $P < 0.05$).

Compared with the model group, the levels of ROS and SOD in the brain tissue of the IGF-I group were significantly less (both $P < 0.05$), while the wortmannin group showed opposite results (both $P < 0.05$).

Levels of serum CRP and SAA of mice in each group

The levels of serum CRP and SAA of mice in each group were detected by ELISA. The results (**Figure 4**) showed that compared with the normal group, the levels of serum CRP and SAA in the other groups were significantly higher (all $P < 0.05$). Compared with the model group, the levels of serum CRP and SAA in IGF-I group

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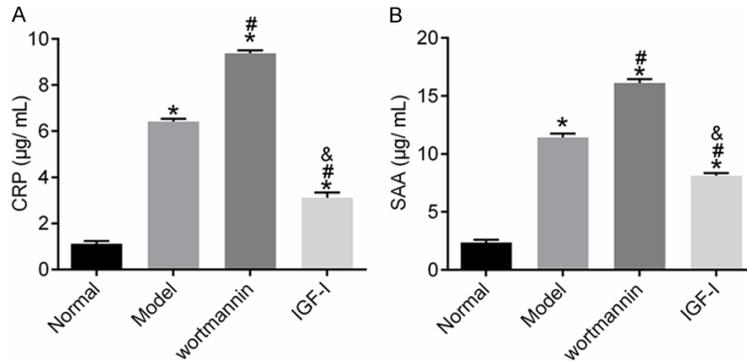


Figure 4. Levels of serum CRP and SAA of mice in each group. A. Serum CRP level in mice. B. Serum SAA level in mice. Compared with normal group, * $P < 0.05$; compared with model group, # $P < 0.05$; compared with wortmannin group, & $P < 0.05$. CRP, C-reactive protein; SAA, serum amyloid A.

were significantly lower (both $P < 0.05$), while the wortmannin group showed opposite results (both $P < 0.05$).

Expression of PI3K, AKT, COX-2, iNOS mRNA and protein in brain tissue of mice in each group

The expressions of PI3K, AKT, COX-2, iNOS mRNA and protein in brain tissue of mice were detected by qRT-PCR and WB. The results (Figure 5) showed that compared with the normal group, the expression of PI3K, AKT mRNA and protein in other groups was significantly lower, while the expression of COX-2, iNOS mRNA and protein was significantly higher (all $P < 0.05$). Compared with the model group, the expression of PI3K, AKT mRNA and protein in IGF-1 group was significantly higher, the expression of COX-2, iNOS mRNA and protein was significantly lower (all $P < 0.05$), while the wortmannin group showed opposite results (all $P < 0.05$).

Discussion

Cognitive impairment and dementia are the main causes of disability and death in the elderly. AD is the most common cause of dementia in the world, which is a progressive neurodegenerative disease and increases exponential with age [26]. More than 35 million people worldwide suffer from dementia, and the prevalence is expected to double every 20 years. This disease is becoming a major public health problem and socio-economic burden, with an estimated global prevalence of about 107 million in 2050 [27]. The clinical manifes-

tations of AD patients may be quite heterogeneous, and there is a lack of data that can effectively subdivide AD. With the gradual loss of cognition and function, dementia leads to other important challenges, including mental symptoms such as anxiety, depression, hallucinations, delusions; behavioral problems such as agitation, aggression, hypersexuality, sleep and diet issues, and motor symptoms [28]. AD may require individualization and specific treatment, so it is important to understand the

pathogenesis of AD, and a clear molecular network of AD pathogenesis can play a key role in the prevention and treatment of cognitive decline and AD.

One of the important characteristics of AD is the oxidative damage of neuronal lipids and proteins, and it is clear that oxidative stress has an important relationship with AD. In the animal model of AD, ROS is the key factor affecting brain health, and reducing ROS levels can effectively treat AD [29]. High concentrations of metal ions are found in amyloid plaques, which are markers of AD and bind to amyloid-beta ($A\beta$) peptides. In particular, the copper ions bound to $A\beta$ have antioxidant reduction capacity, and reductants (such as ascorbic acid) exist to produce ROS [30]. Clinical findings suggest that up-regulation of antioxidant enzymes such as SOD may be effective in the early progression of AD [31]. In our mouse model of AD, ROS was significantly up-regulated, which was consistent with the above reports.

The PI3K pathway is known to regulate metabolism, cell growth and cell survival. Some studies have shown that psychotropic drugs such as antidepressants can exert their pharmacological effects by activating some signaling pathways, including PI3K pathway, and these drugs can up-regulate the expression of some neurotrophic factors [32]. Akt is one of the key molecules for downstream activation of the PI3K signaling pathway, and its dysregulation plays an important role in the pathogenesis of many diseases. This kinase has been

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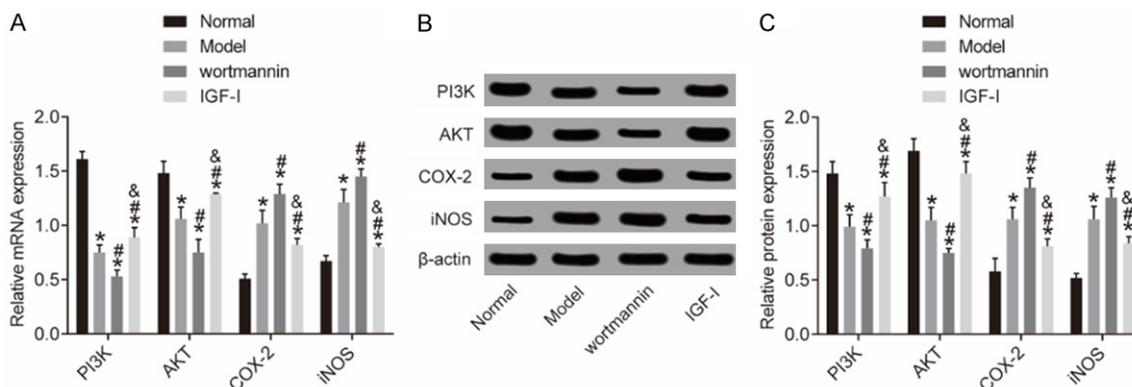


Figure 5. Expression of PI3K, AKT, COX-2, iNOS mRNA and protein in brain tissue of mice in each group. A. PI3K, AKT, COX-2, iNOS mRNA levels in brain tissue of mice. B. Protein band map of PI3K, AKT, COX-2, iNOS in brain tissue of mice. C. PI3K, AKT, COX-2, iNOS protein levels in brain tissue of mice. Compared with normal group, * $P < 0.05$; compared with model group, # $P < 0.05$; compared with wortmannin group, & $P < 0.05$.

shown to play multiple roles in cell regulation. It is widely expressed and its activity is very important for the regulation of translation initiation [33]. In this study, we treated mice with the PI3K/AKT signaling pathway inhibitor wortmannin and the activator IGF-I, and found that after activation of PI3K/AKT signaling pathway, the learning and memory ability of AD mice was significantly improved, the levels of ROS, SOD in brain tissue were significantly decreased, the levels of serum CRP and SAA were significantly decreased, and the expression of COX-2, iNOS mRNA and protein was significantly decreased. The results showed that the activation of PI3K/AKT signaling pathway could improve brain injury in AD mice and reduce the levels of serum CRP and SAA, and improve cognitive dysfunction, while the effect of inhibiting PI3K/AKT signaling pathway was opposite.

In this study, we demonstrated the regulation of PI3K/AKT signaling pathway on serum CRP, SAA and cognitive dysfunction in AD mice, and further elucidated the pathogenesis of AD and laid a theoretical basis for the clinical treatment of AD. In order to further confirm the above results, we need to further supplement the clinical data. However, the relationship between PI3K/AKT signaling pathway and AD has not been fully explained so far, and the upstream molecular mechanism of the PI3K/AKT signaling pathway needs to be further explored. The molecular mechanism of the relationship between PI3K/AKT signaling pathway and AD is still unclear.

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Disclosure of conflict of interest

None.

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