Neuroprotective Effects of Alisol A 24-acetate on Cerebral Ischemia-reperfusion Injury by Regulating PI3K/AKT Pathway

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Research Article

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Abstract

Background

Neuroinflammation and apoptosis are involved in the pathogenesis of ischemic stroke. Alisol A 24-acetate (24A) has a strong inhibitory effect on inflammation and cell apoptosis. The neuroprotective effect of 24A in the global cerebral ischemia/ reperfusion (GCI/R) is still unclear.

Methods

GCI/R mice was used to investigated the neuroprotective effect of 24A. Modified neurological deficit scores, Morris Water Maze and object recognition test were used to evaluate behaviors. The metabolism in brain regions was detected by MRS. The changes of microglia, astrocytes and neurons was detected. The inflammation and apoptosis were measured.

Results

The results showed that 24A improved behavioral dysfunction and brain metabolism, alleviate neuroinflammation and apoptosis, inhibited microglia and astrocytes activation, which is associated with the activation of PI3K/AKT pathway.

Conclusions

Taken together, our study demonstrated that 24A could alleviate GCI/R injury through anti-neuroinflammation and anti-apoptosis via regulating the PI3K/AKT pathway.

Introduction

Ischemic stroke results in the damage to brain function of the blood vessel supply area and a series of neurological symptoms, which seriously endangers people's health. Cerebral ischemia-reperfusion (CI/R) injury caused by cerebrovascular recanalization is considered to be a serious problem in treatment of ischemic stroke[1, 2]. CI/R is a complex pathophysiological process involving various mechanisms such as the release of excitatory amino acids, oxidative stress, apoptosis and inflammation. A large amount of evidence indicates that neuroinflammation and apoptosis are involved in the pathogenesis of ischemic stroke. However, numerous neuroprotective drugs have failed to show benefits in the treatment of I/R after ischemic stroke, so there is an urgent need to find new treatments.

Alisol A 24-acetate (24A), belonging to protostane-type tetracyclic triterpenoid, serves as one of the main components in Alisma Orientale (Sam.) Juz[3,4]. Modern pharmacological investigations have demonstrated that 24A is a multi-targeted compound, which regulates the processes of inflammation, oxidative stress, autophagy, and apoptosis. Wu et al. reported that 24A suppressed oxidative stress and stimulated autophagy through the AMPK/mTOR pathway in nonalcoholic steatohepatitis mice[5]. Another *in vitro* study indicated 24A effectively reduced inflammatory factors such as TNF-α, IL-

1β, IL-6, IL-8 in HepG2 Cells[6]. In addition, our previous study has found 24A has anti-apoptotic effect and protects the tight junctions of brain microvascular endothelial cells from damage caused by oxygen and glucose deprivation[7, 8]. But the potential neuroprotective role of 24A on ischemic stroke has yet to be elucidated.

Phosphatidylinositol 3-kinase (PI3K) induces the phosphorylation of protein kinase B (AKT) to responding to extracellular signals for regulating cell survival growth, and angiogenesis. Previous studies have reported PI3K/AKT plays a pivotal role in the defense against various neuronal-damaging insults[9]. In an oxygen-glucose deprivation and reperfusion (OGD/R) model *in vitro*, the expression of PI3K/AKT was inhibited, indicating that I/R injury caused the PI3K/AKT pathway blocked[10]. Neuroinflammation and cells apoptosis can be inhibited through activating PI3K/AKT signaling after cerebral ischemia[11]. Overall, these results indicate that the up-regulation of the PI3K/AKT pathway produces a strong neuroprotective effect by reducing neuroinflammation and apoptosis caused by brain I/R injury. In this study, we evaluated the anti-neuroinflammation and anti-apoptosis effect of 24A with global cerebral ischemia-reperfusion (GCI/R) injury mice and explored its potential mechanism. To verify our hypothesis that the PI3K/AKT signaling pathway was involved in the anti-inflammatory and anti-apoptotic effects of 24A, we used PI3K inhibitor LY294002 for rescue experiment. The results of this study provide scientific basis for clinical application of 24A in the treatment of ischemic brain diseases.

Materials And Methods

Animals

C57BL/6J mice (male, weighing 25-30g, 12 weeks) were purchased from gempharmatech (Jiangsu, China). Mice were kept in standard cages and allowed to eat and drink freely. All experimental procedures were approved by the Ethics Committee of Fujian University of traditional Chinese Medicine and were carried out in strict accordance with the animal care and use guidelines of the National Institutes of Health.

Surgical procedures

The 2-VO method was performed to establish a model of global cerebral ischemia (GCI as previously described[12]. Mice were fasted for 24 h before surgery. 1.5% thiopental sodium was administered to anesthetize mice. Then the surgery was performed to occlude both common carotid arteries for 20 minutes and induce transient global cerebral ischemia-reperfusion injury (GCI/R). The blood flow restore reperfusion after 20 minutes.

Drug management and experimental subgroup

Mice were randomly divided into four groups (n=12 in each group): sham group (Only external carotid artery and vagus nerve were separated without drug intervention). GCI/R group (Reperfusion was performed after 20 minutes of global cerebral ischemia. GCI/R+24A group (24h after reperfusion, 30

mg/kg 24A was given by gavage, once a day for 7 consecutive days). GCI/R+24A+LY group (24h after reperfusion, 30mg/kg 24A was given by gavage for 7 days, and PI3K inhibitor was injected intraperitoneally in the first 3 days).

Neurological deficit score

The modified neurological deficit score (mNSS) was used to evaluate neurological deficit, including tasks of motor, sensory, reflex and balance ability. Neurological defect scores were performed in the first, third, fifth and seventh day after global cerebral ischemia-reperfusion. Scores ranged from 0 to a maximum of 18.

Morris water maze (MWM)

The Morris Water Maze (MWM) is designed to test spatial memory and long-term memory of mice, which includes space exploration experiment and directional navigation experiment. In the space exploration experiment, mice were placed in the water from the first quadrant every day, and the time to find the platform within 90s is the escape latency of the mice in the first quadrant. If the mice could not find the platform within 90s, then the escape latency was recorded as 90s, and guided the mice to the platform and let them stay on the platform for learning for 15s. Each animal was given 4 trials per day to swim to an escape platform. The average escape latency of the four quadrants was recorded as the escape latency of the day. For 4 days, repeat the above steps every day. The directional navigation experiment carried out on the fifth day with the platform removed. The mice were placed in the pool from the opposite side of the original platform quadrant. The times of the mice passed the original platform position and the time in the third quadrant within 90s were recorded.

Novel object recognition test

The recognition memory of each mouse was evaluated by the novel object recognition test. 24 hours before the test, the mice were allowed to explore an empty arena for 5 minutes. Twenty-four hours after habituation, the mice were exposed to the familiar arena with two identical objects placed for 5 minutes. 24 hours after, mice were placed individually in the arena with one familiar object and one novel object. Record the time of mice exploring novel object and familiar object in 5 minutes, and calculate the discrimination index, time ratio according to the formula: discrimination index = novel object exploration times / total exploration times of familiar and novel objects, time ratio = novel object exploration time / total exploration time of familiar and novel objects. Record total exploration time and exploration times at the same time.

MRS

Mice were anesthetized with a mixture of 1.5-2% isoflurane and oxygen. Specific parameters of T2W1 is TE=35 ms, TR=4200 ms, Averages=4, Slice Thickness=0.5 mm, Field of view=20 mm×20 mm. Specific parameters of EPI is TE=25 ms, TR=12000 ms, Averages=2, Slice Thickness=0.5 mm, Field of view=20 mm×20 mm. Finally, MRS scan was performed and T2 was added bilateral hippocampus and cortex were

selected as the region of interest on the transverse, coronal, and sagittal planes of the right, with a size of 1mm × 1mm × 1mm. Specific parameters of MRS is TR = 1500 ms, TE = 144 ms, and number of averages = 256. The post-processing of images and related data were analyzed by using the workstation TOPSPIN (V3.1, Bruker Biospin, Germany) of the MRI instrument. Creatine (Cr) is used as an internal reference. The spectrum peak positions were kept as follow: Cr approximately 3.05 ppm, NAA approximately 2.02 ppm, Glx approximately 2.2-2.4 ppm, MI approximately 3.56 ppm, Taurine approximately 3.4 ppm, CHO approximately 3.2 ppm.

Immunohistochemistry

After rodent anesthesia, 0.9% normal saline was perfused through the heart, then 4% precooled paraformaldehyde was perfused, and then the brain was collected. The brain was fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 4 μm paraffin sections. The immunohistochemical (IHC) process was performed according to the instructions of IHC Kit (KIT-9720, MXB, Biotechnologies, Fujian, China). After dewaxing and rehydration, the sections were immersed in a beaker containing antigen repair solution, heated for 20 minutes, then cooled naturally for 1-2 hours, and washed with PBS for 3 times. Incubated for 15 minutes after dropping 3% H_2O_2 to eliminate the effect of endogenous peroxide, the slices then washed with PBS solution for 3 times. Next, the slides were sealed with blocking buffer at room temperature for 1h, and then incubated with primary antibody (Iba1 1:200, proteintech, Cat No.: 10904-1-AP) (GFAP 1:1000, proteintech, Cat No.:16825-1-AP) (Neun 1:2000, proteintech, Cat No.:26975-1-AP 1:2000) at 4°C overnight and subsequently incubated with secondary antibody. The sections were incubated with DAB-0031 (MXB, biotechnology, Fujian, China) for 1-10 minutes until brown is formed. Images of GFAP, Iba1, Neun deposition in the hippocampus and cortex were captured using an optic microscope (Nikon, Model Eclipse Ci-L, 718345, Japan) and analyzed with an image analysis system (Image-J, version 6.0; Motic China Group Co, Ltd, Xiamen, China).

Golgi staining

According to the instructions of Rapid GolgiStain TM Kit (PK401, FD NeuroTechnologies, Columbia, USA), mixed the staining solution A and B 24 hours in advance. After the mice were anesthetized, their brains were harvested and put into the mixed solution, stored away from light at room temperature for 2 weeks. Two weeks later, brain tissues were transferred to solution C and store at room temperature in the dark for 1 week. The brains were sliced into coronal sections (100μm) by using a vibrating blade microtome (Leica VT1000 S, Leica, Nussloch, German), and sections were mounted on gelatin-coated microscope slides with Solution C. Each mouse was cut into 5 slices and dried in the dark at room temperature. The dyeing solution was prepared by mixing the equal volume of D solution and E solution. After dyeing, slices were dehydrated, transparent and sealed, and photographed under the optical microscope to observe the morphology.

Western Blot

The brain tissue of mice was collected and then the protein was extracted. The protein concentration was determined by BCA protein assay. The protein was separated by 10% SDS-PAGE gels (Bio-Rad Laboratories, Inc. Hercules, CA, USA) and electrophoresis and transferred to polyvinylidene fluoride (PVDF) membrane. PVDF membrane was sealed with 5% milk for 2 hours, incubated with primary antibody IL-1β (1:1000, Abcam, Cat#Ab9722); PI3K (1:1000, Cell Signaling, Cat#4257), AKT (1:1000, Cell Signaling, Cat#4691); p-PI3K (1:1000, Cell Signaling, Cat#17366); p-AKT(1:1000, Cell Signaling, Cat#4060); TNF-α (1:1000, Cell Signaling, Cat#11948); Bcl-2 (1:1000, Proteintech, Cat#26593-1-AP); Bax (1:2000, Proteintech, Cat#50599-2-lg); cle-Caspase-3 (1:1000, Proteintech, Cat#19677-1-AP); GAPDH (1:7000, Proteintech, Cat#60004-1-lg) at 4℃ for 12 hours, then incubated with secondary antibody (1:7000) for 2 hours, and then the membrane was detected with enhanced chemiluminescence kit (Meilunbio, MA0186). The protein bands were imaged with the Bio-Image Analysis system (Bio-Rad Laboratories, Inc.).

Statistical Analysis

Data are presented as means ± SEM and were analyzed by one-way or repeated two-way ANOVA with statistical software IBM Spss Statistics (IBM Spss Statistics for Windows, Version 23.0. Armonk, NY, USA). The pairwise comparison after ANOVA was analyzed by the least significant difference test (LSD-t). p value < 0.05 was defined as being statistically significant.

Results

24A improved the neurological deficit of GCI/R mice

The molecular structure of 24A was showed in the Fig.1A. We scored neurological deficits on the first, third, fifth, seventh day after ischemia-reperfusion. According to the line chart (Fig.1B), it can be found that the neurological deficit scores of all groups have a downward trend. The GCI/R group is significantly higher than the sham group. After 24A intervention, the score decreased, while the use of inhibitor reversed the effect of 24A. These results showed that 24A lessened the neurological deficits, and the effect was reserved by PI3K inhibitor LY294002.

24A improved GCI/R induced spatial memory and learning ability

Water maze is used to test the learning and memory ability of animals. Escape latency and the total distance is the time and distance required for mice to find the target platform. Shorter escape latency and total distance represent better spatial memory and learning ability. Although the escape latency and total distance showed a downward trend in space exploration experiment (Fig.1C-1D). Compared with sham group, GCI/R mice required longer time and exhibited longer path length searching for the platform. After 24A intervention, the escape latency and total distance decreased significantly compared with the GCI/R group, indicating that 24A significantly improved the spatial memory and learning ability of mice. The escape latency of the inhibitor group was longer than that of the sham group and 24A intervention group. Although the difference is not significant enough, it still indicates that the addition of the inhibitor was

unfavorable to the improvement of spatial memory and learning ability of mice. After space exploration experiment, the frequency of mice crossing the target platform and the time spent in the platform were test. Compared with the sham group, the times of crossing the platform (Fig.1E) in the GCI/R group ($p < 0.01$) and inhibitor group ($p < 0.05$) were significantly reduced. After 24A intervention, the times of crossing the platform increased though there is no significant difference ($p > 0.05$). Compared with the GCI/R group, the time spent in the platform (Fig.1F) of the sham group and 24A intervention group was longer ($p < 0.05$). The mice of sham group tended to go to the zone where the platform was located before, while the GCI/R group did not exhibit this preference. 24A treatment increased the proportion of distance and time spent in the platform location, while the effect was reversed by PI3K inhibitor LY294002(Fig.1G).

24A improved the ability to explore novel objects in GCI/R mice

Novel objects recognition test based on the principle that animals have a tendency to explore novel objects (Fig.2A-C). Discrimination index and time ratio represents the frequency and time mice exploring a novel object to that of the familiar object. As shown in Figure 2D, GCI/R group showed lower discrimination index than that in the sham group in ORT 24 hour ($p < 0.05$). 24A treatment significantly increased the discrimination index ($p < 0.05$), while PI3K inhibitor decreased it, demonstrating that 24A improved the ability to explore and distinguish novel objects, and also confirmed its effect on the improvement of long-term memory. Time ratio (Fig.2E) is the exploration time of the novel object to total time. The ratio in GCI/R group is smaller than the sham group in ORT 24 hour ($p < 0.01$). After 24A intervention, the time ratio increased ($p < 0.05$), and the inhibitor reduced it ($p < 0.05$). It means that GCI/R mice spent more time in exploring novel objects after the intervention of 24A, which confirmed that 24A improved the ability to explore novel objects. The exploration trajectory of mice was consistent with the above phenomenon (Fig.2H). However, there was no significant difference in total exploration times and total exploration time between 4 groups (Fig.2F-G).

24A improved the changes of substance metabolism in various brain regions of mice mediated by I/R

In our study, MRS was used to detect the changes of neurometabolites in hippocampus and cortex(Fig.3A-C). In view of Creatine (Cr) is relatively constant and uniform, therefore, it is usually used as an internal reference for measuring the level of other metabolites. In our study, Glx/Cr (Fig.3D) in cortex of GCI/R group increased, while 24A treatment reduced Glx/Cr in cortex ($p < 0.01$). The GCI/R group showed a decrease of GABA/Cr (Fig.3E) in hippocampus ($p < 0.01$)and cortex ($p < 0.01$), while increased after adding 24A ($p < 0.01$). In our study, NAA/Cr (Fig.3F) in hippocampus of GCI/R group decreased significantly ($p < 0.05$), while 24A increased it ($p < 0.01$), but there was no significant change in cortex ($p > 0.05$). MI/Cr (Fig.3G) of hippocampus ($p < 0.01$) and cortex ($p < 0.05$) in the GCI/R group increased, while decreased after intervention of 24A in hippocampus ($p < 0.01$) and cortex ($p < 0.05$). The contents of CHO (Fig.3H) in hippocampus ($p < 0.01$) and cortex ($p < 0.05$) in the GCI/R group increased, and decreased after 24A ($p < 0.01$, $p < 0.05$). I/R injury leads to the increase of taurine in hippocampus and cortex. Our study confirmed that taurine (Fig.3I) in the GCI/R group was significantly higher than that in

the sham group ($p < 0.01$), while 24A intervention reduced it ($p < 0.01$), and the use of inhibitor increased it ($p < 0.01$).

24A reduced GCI/R-induced microglia and astrocyte activation

Iba1 is a positive marker of microglia and GFAP is a positive marker of astrocytes. In the GCI/R group, the number of Iba1 and GFAP in hippocampus (Fig.4A, 4E) and cortex (Fig.4B, 4F) increased, the cell body became larger, indicating that microglia and astrocytes were over activated after GCI/R ($p < 0.01$). The number of microglia and astrocytes in 24A group were markedly decreased ($p < 0.01$). 24A inhibited microglia and astrocytes over activation in hippocampus and cortex. However, this effect can be reversed by PI3K inhibitor ($p < 0.01$), but the Iba1 in cortex showed no significant difference. The number of microglia and astrocytes in hippocampus and cortex were calculated by image J software (Fig.4C-D, 4G-H).

24A attenuated GCI/R induced inflammation and apoptosis

To better understand the anti-inflammatory and anti-apoptotic mechanism of 24A, we detected the expression of proteins related to inflammation and apoptosis with western blot. Respectively, the expression of IL-1β, TNF-α and iNOS in GCI/R group was higher than that in sham group ($p < 0.01$), 24A intervention markedly attenuated over-expression of IL-1ß ($p < 0.01$), TNF-α ($p < 0.05$) and iNOS ($p < 0.01$), while is reserved by the PI3K inhibitor LY294002 (Fig.5A-C). The result showed that 24A reduced inflammation induced by ischemia-reperfusion. The expression of Bax and cleaved-Caspase-3 were significantly increased after GCI/R injury, while Bcl-2 was decreased ($p < 0.01$). Significantly, 24A treatment inhibited the ratio of Bax to Bcl-2 ($p < 0.01$) and the expression of caspase 3 ($p < 0.05$). However, the situation can be reversed by PI3K inhibitor LY294002 (Fig.5D-E). 24A inhibits inflammation and apoptosis after GCI/R via regulation of pro-inflammatory factors, pro-apoptotic and anti-apoptotic proteins. However, LY294002 reversed the effect of 24A.

Therapeutic effect of 24A on GCI/R induced neuronal injury

There were more obvious changes in neurons morphological, for nuclear shrinkage, chromatin deepening, irregular cell morphology and blurred edges in hippocampus and cortex. The addition of 24A improved the destruction of neuron, and the morphology is relatively regular. However, the addition of inhibitor reversed this change (Fig.6A-B).

Therapeutic effect of 24A on GCI/R induced hippocampal and cortical neurons and dendritic spines injury

To further study the destruction of neuronal structure, Golgi staining was used to observe the quantity and arrangement of hippocampal and cortical neurons in each group. As is shown in the figure 6C, the hippocampal neurons were significantly decreased and arranged irregularly in GCI/R group comparing with the sham group. After the intervention of 24A, the number of hippocampal neurons increased, which arranged neatly and regularly, while the addition of PI3K inhibitor weakened the effect of 24A (Fig.6C). Furthermore, we found that the dendritic spines of hippocampal CA1 and DG regions, cortical neurons

(Fig.6D-6F) of the sham group were dense, while in the GCI/R group, the dendritic spines fell off and atrophied, the number of dendritic spines decreased comparing with the sham group ($p < 0.01$), the reduction of dendritic spine density had been found to be associated mainly with brain functional derangements. Although the dendritic spines in the 24A intervention group fell off partially, the number of dendritic spines was higher than that in the GCI/R group ($p < 0.05$). While the PI3K inhibitor decreased the number of dendritic spines ($p < 0.05$).

24A activated PI3K/AKT signaling pathway after GCI/R

To confirm whether PI3K/AKT signaling pathway involved in the protective effect of 24A, we first examined the changes of PI3K/AKT after ischemia-reperfusion and 24A intervention with western blot. The total PI3K, AKT expression was consistent among the four groups. 24A increased PI3K phosphorylation compared with GCI/R group while LY294002 inhibited it (Fig.7A-7B). We found that compared with the sham group, p-PI3K/PI3K and p-AKT/AKT were decreased after GCI/R ($p < 0.01$) and increased after 24A intervention ($p < 0.01$), while PI3K inhibitor reversed the effect of 24A ($p < 0.01$, Fig.7C-7D). PI3K is the upstream of AKT, PI3K/AKT pathway is related to cell survival, growth, which may play an important role in the improvement of cerebral ischemia.

Discussion

Ischemic stroke is the primary cause of death and long-term disability in elderly. Ischemia reperfusion (I/R) is a complex pathological process accompany with ischemic stroke, which exacerbates brain damage, including reactive oxygen species (ROS) outburst, inflammatory mediator overproduction, leukocyte infiltration, Ca^{2+} influx increased, calcium overload and so on, all of which mediated the induction of apoptosis[13]. These factors are considered as significant roles in the neuronal injury of post-ischemic injury[14]. Based on the pathogenesis of I/R, many neuroprotective drugs are designed to protect the brain from reperfusion injury or to inhibit the pathological process. However, there are still few effective treatments for ischemic stroke. The present study has confirmed that Alisol 24 acetate (24A), one of the main active triterpenes of Rhizoma Alismatis[15], significantly attenuated global cerebral ischemia/reperfusion (GCI/R) induced brain injury.

Studies have shown that microglia plays a pro-inflammatory and pro-apoptotic role in the early stage of I/R[16], which regulating immune response and affecting neuronal function. Microglia works in the early stage of ischemia-reperfusion through morphological changes and secretion of inflammatory factors[17]. Global cerebral ischemia is usually followed by inflammation induced by astrocytes in the initial stage of inflammation. In the I/R, the expression of GFAP was up-regulated, which is the characteristic marker of astrocytes, indicating that I/R may be accompanied with reactive astrogliosis. Many studies have revealed that activated astrocytes in ischemia are potentially harmful because they release chemokines and promote the levels of NOS and neurotoxic NO, thereby aggravating ischemic brain diseases[18]. Furthermore, hippocampus and cortex are vulnerable to microglial activation and reactive astrocytes in GCI/R[19]. In our study, we found that the activation of microglia and

astrocytes in hippocampus and cortex of GCI/R group increased, and the morphology changed significantly, which confirmed that hippocampus and cortex may be damaged by GCI/R injury. While the 24A intervention down regulated the activation of microglia and astrocytes, indicating that 24A may effectively suppressed inflammation in the process of GCI/R injury. IL-1β and TNF-α play the primary role in inflammation responding to transient I/R, and the expression of iNOS is up-regulated during I/R, which lead to ischemic neural damage[20]. Further, the expression of IL-1β, TNF-α and iNOS are suppressed by 24A treatment. Generally speaking, inflammatory process continued during I/R and have a vital role in the disease outcome. Our results are consistent with the study that TNF-α and IL-1β were markedly inhibited by 24A[6]. It seems that 24A downregulate inflammatory factors can be a great candidate in investigations of novel therapeutic targets for GCI/R injury.

The anti-inflammatory effect of 24A has been proved. Interestingly, inhibition of neuroinflammation may reduce neuronal apoptosis[21]. Apoptosis is crucial to neuronal death after cerebral I/R injury, which is considered the prominent form of neuronal death in the penumbra. The expression of apoptosis-related genes up-regulated in cerebral I/R. Bcl-2 is a key anti-apoptotic factor and Bax is a pro-apoptotic factor. Various reports suggest that Bcl-2 expression is decreased while Bax is increased after reperfusion, and inhibition of Bcl-2 reduces apoptosis and neuro-inflammation after I/R[22]. Caspase and Bcl family play the equally important role in regulation of apoptosis. Activation of caspase-3 is an important feature of apoptosis following I/R, which increases in hippocampus in I/R[23]. Our previous findings indicated that 24A showed anti-apoptotic effect on OGD-induced BMECs[8]. The vitro experiments showed that 24A down regulated the proportion of Bax/Bcl-2, indicating that 24A as a anti-neuroinflammatory drug effectively ameliorated apoptosis. These findings are consistent with previous cellular studies [7,8] that 24A exerts anti-apoptotic effect by regulating the expression of Bax/Bcl-2 in vivo experiments.

Ischemic stroke contributes to the development of neurological deficit and cognitive decline. Neurological deficit scores and MWM are often used as indicators to evaluate the neurological function of rodent models. We found that 24A significantly improved the neurological function and the spatial learning and memory during GCI/R injury. In order to further investigate the internal mechanism of neuronal changes during GCI/R injury, we observed the synaptic morphology of hippocampus and cortex. Synaptic plasticity is the cellular physiological basis of learning and memory[24]. Dendritic spines are small processes of dendrites, and their dynamic changes are the important form of synaptic remodeling[25, 26]. It has been reported that cerebral ischemia affects the synaptic structure and the density of dendritic spines, and has a continuous decreasing trend with the extension of ischemia time. The present study found that the branches and density of dendritic spines increased after 24A intervention. It showed that 24A can effectively promote the remodeling of hippocampal and cortical dendritic spine structure and function, so as to promote the recovery of neurological function after CI/R. The ability of 24A regulating neuroprotection makes it as a promising and effective drug for improving neurological function after I/R.

MRS is a novel technique used to detect brain metabolites in vivo. Ischemia induced neurochemical and metabolic changes in the brain, including creatine (Cr), lactic acid, N-acetylaspartate (NAA), γ-aminobutyric acid (GABA), myoinositol (MI) and glutamate (Glu), glutamine alterations. Myoinositol (MI)

is a marker of glial hyperplasia, and it increases in some nervous system diseases. Choline (CHO) increased during cerebral ischemia. Amino acid neurotransmitters are the most abundant neurotransmitters in the central nervous system. In the pathophysiological process, its concentration will change obviously. During early stage of cerebral ischemia, neuronal damage may be induced by excitatory neurotoxicity due to the over secretion of neurochemicals[27]. Glx is a complex of glutamate and glutamine, which is an excitatory neurotransmitter and participates in many important metabolic pathways. Under ischemic conditions, its peak becomes larger. While GABA is an inhibitory neurotransmitter, which plays a key role in motor learning and mediates the recovery of brain injury, but it decreases after stroke. N-acetylaspartate (NAA), which is considered a predominantly neuronal marker, reflecting the functional state of neurons. The inhibition of neuronal metabolism leads to the significant decrease of NAA/Cr during cerebral ischemia indicating the development of cognitive deficits. Our study found that NAA decreased after I/R, which is consistent with the existing studies[28]. Reduction of NAA is often regarded as an indicator of neuron loss[29]. Recent MRS studies have been proposed to explain the susceptibility of hippocampal neurons to metabolic stress and delayed neuronal death, such as glutamate excitotoxicity, changes in lipid metabolism and production of free radicals, energy metabolism and local blood flow dysfunction, and induce apoptosis[30,31]. This explains why ischemia causes an increase in Glx and subsequently mediates neuronal death. Our experiments have shown that 24A regulated these substances, which provides a research basis for its application in the protection of brain injury after cerebral I/R. Furthermore, the application of MRS provides a scientific basis for the monitoring of cognitive function caused by neurotransmitter changes after cerebral ischemia.

Inflammation and apoptosis caused by ischemia-reperfusion injury have a serious impact on neurons, and ultimately damage cognitive function. In our study, we found that the neuroprotective effect of 24A is weaken when inhibiting the PI3K-AKT signaling pathway on the GCI/R mice. In order to verified that this protective effect is mediated via PI3K/AKT pathway, we detected behavioral performance, MRS, inflammation and apoptosis related factors. We found that 24A activated PI3K/AKT signaling pathway. Recent reports have highlighted the critical role of PI3K/AKT signaling pathway in promoting cell survival. It has been reported that Bax and Bcl-2 are downstream proteins in the PI3K/AKT signaling pathway that regulate the activation of caspase[32]. Phosphorylated AKT increases the expression of the anti-apoptotic protein Bcl-2, and mediates inhibition of pro-apoptotic protein. Recently, we found that the expression of PI3K and AKT were increased by 24A treatment. So we speculate that 24A may works through PI3K/AKT signaling. As expected, we found that inhibition of PI3K/AKT pathway attenuated the protective effect of anti-inflammatory and anti-apoptotic by treatment of 24A. Besides that, the neurological deficit worsened and the behavioral performance deteriorated due to the use of LY294002. It further indicated that PI3K/AKT signaling pathway is involved in the protective effect of 24A in GCI/R.

Conclusion

Taken together, our study firstly found that 24A has the effect of anti-inflammatory, anti-apoptotic and improving neurological deficit via PI3K/AKT signaling pathway in GCI/R, which provides new ideas and inspiration for the treatment of cerebral ischemia-reperfusion injury.

Abbreviations

I/R: Ischemia Reperfusion; GCI/R: Global Cerebral Ischemia Reperfusion;

24A: Alisol A 24-acetate; MRS: Magnetic resonance spectroscopy; Cr: creatine;NAA: N-acetylaspartate; GABA: γ-aminobutyric acid; Glu: glutamate; MI: Myoinositol; CHO: Choline; ROS: Reactive Oxygen Species; Glx: complex of glutamate and glutamine.

Declarations

Authors' contributions

XX and TJ designed the research project, conducted the analysis, interpreted the data and wrote the manuscript. LT and LH contributed to animals work, manuscript writing and figure layout. WW and ZY contributed to analysis of multiplex sandwich Elisa. ZT and LW supervised the research project, interpreted the data and contributed to writing of the manuscript. All authors critically reviewed and approved the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All experimental procedures were approved by the Ethics Committee of Fujian University of traditional Chinese Medicine and were carried out in strict accordance with the animal care and use guidelines of the National Institutes of Health.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

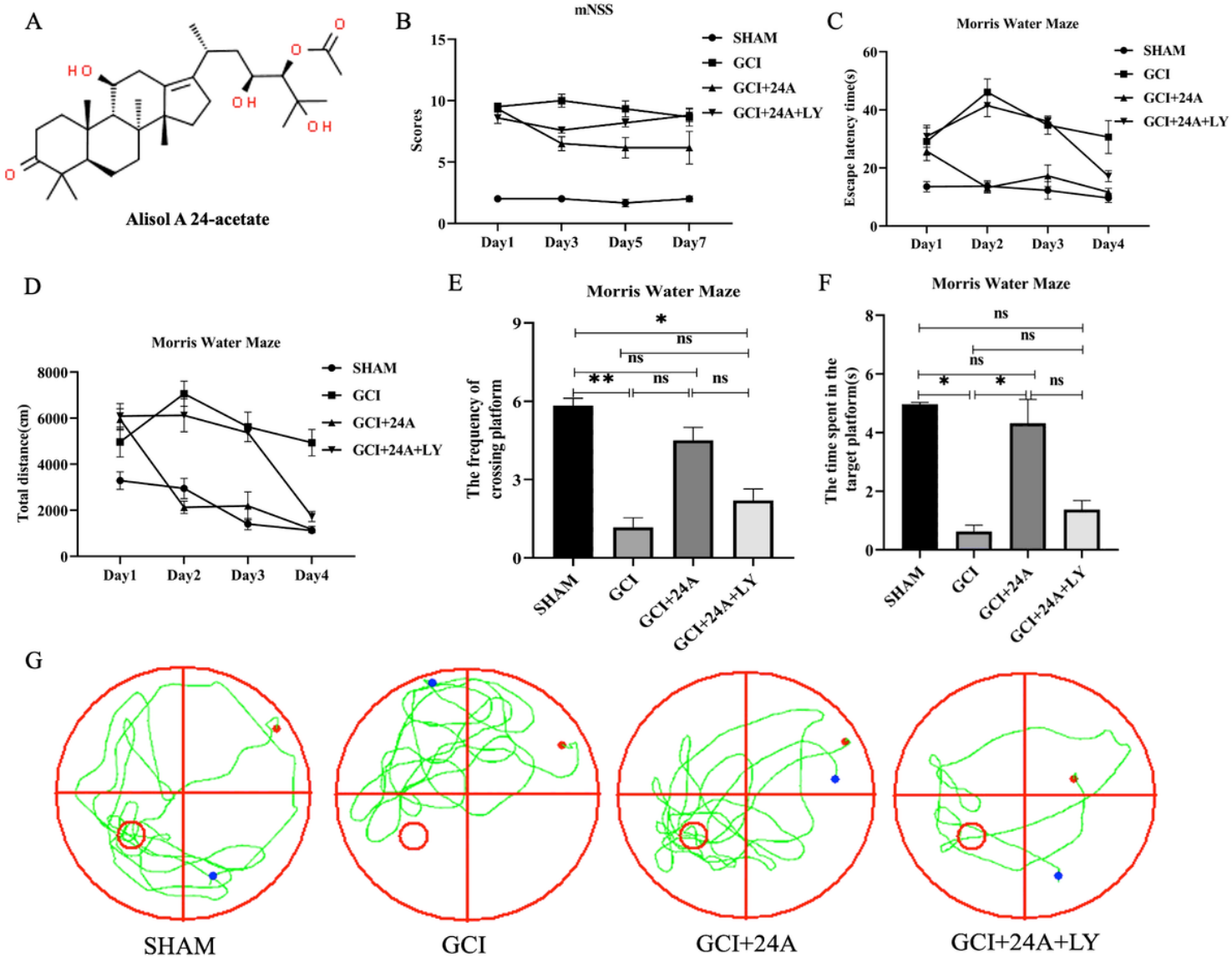

Figure 1

24A improved the neurological deficit of GCI/R mice. A The molecular structure of 24A. B The neurological deficit scores evaluated in the first, third, fifth and seventh day. C-D The time and distance to find the platform in space exploration experiment of MWM. E-F The frequency of crossing the target platform and the time spent in the target platform in the directional navigation experiment of MWM. G Representative trajectories in the directional navigation experiment of MWM. (** $p < 0.01$, * $p < 0.05$, ns means no significant differences n = 12).

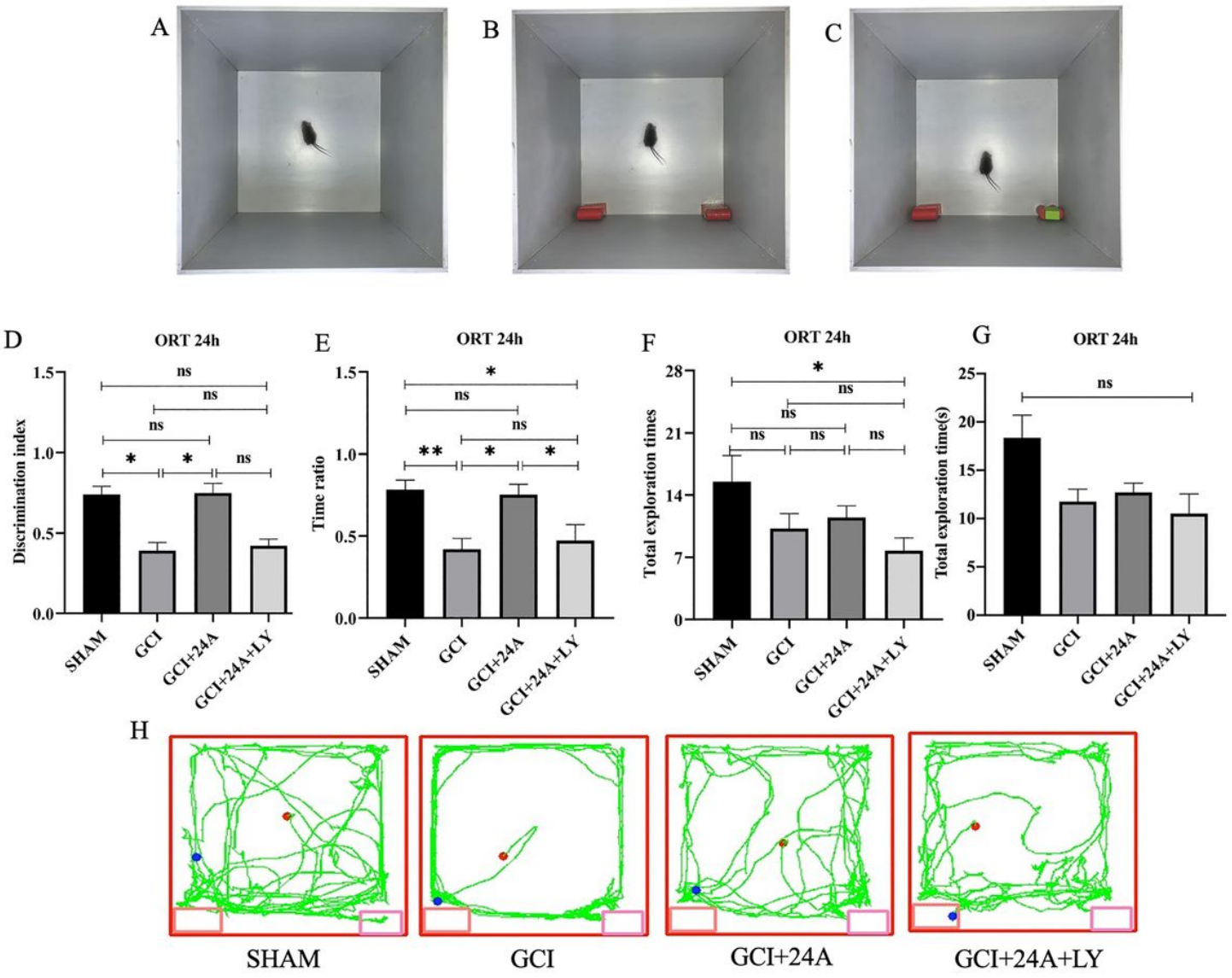

Figure 2

24A improved the ability to explore novel objects in GCI/R mice. A-C Schematic diagram novel object recognition test. D-E Differences of discrimination index, time ratio, total exploration time and total exploration times among each group were analyzed. (discrimination index = novel object exploration times / total exploration times of familiar and novel objects), (time ratio = novel object exploration time / total exploration time of familiar and novel objects). F-G Total exploration times and total exploration time in ORT 24h. H Representative trajectory diagram in ORT 24h test. (**p < 0.01, *p < 0.05, ns means no significant differences n = 12)).

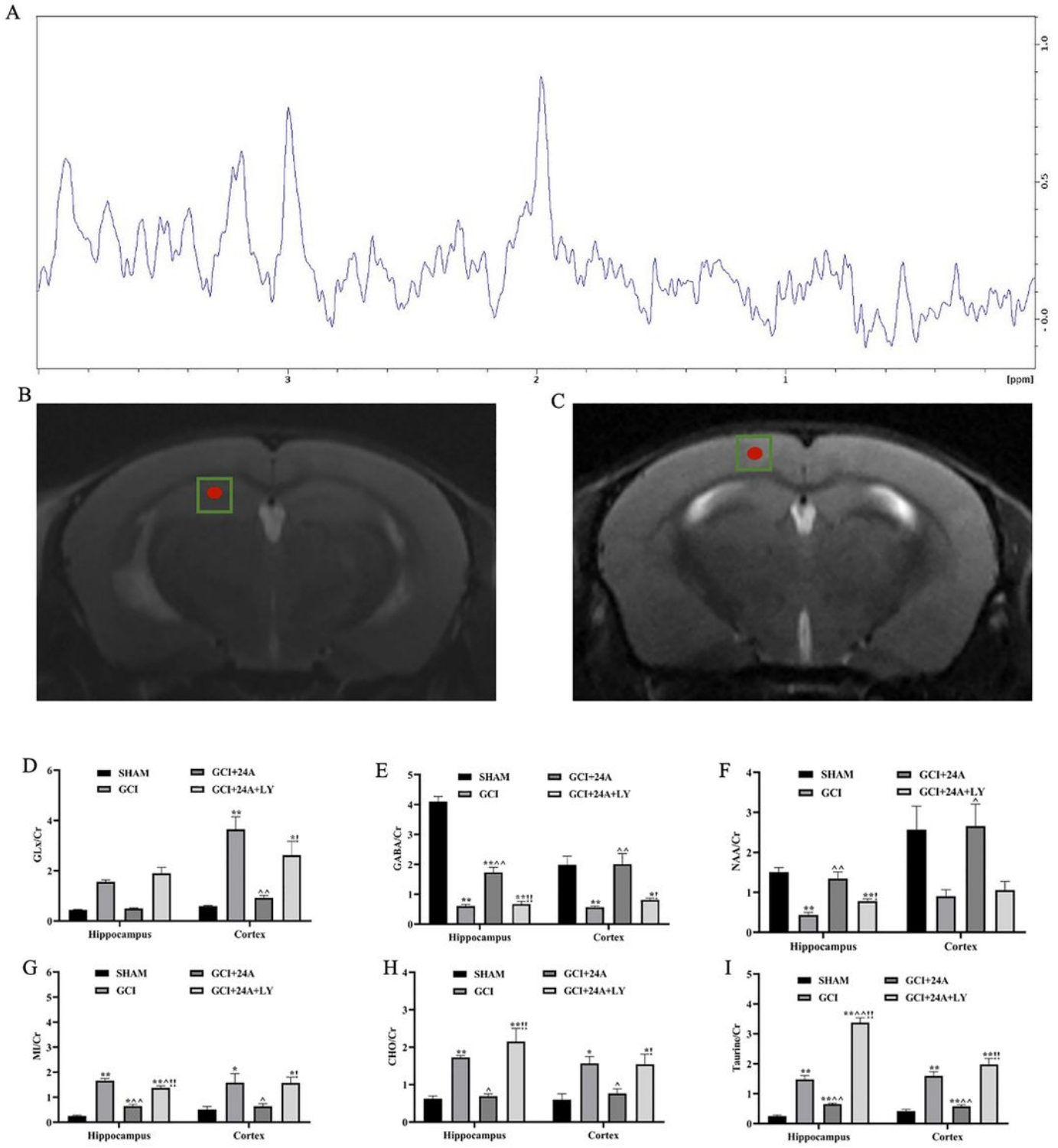

Figure 3

24A improved the metabolism in different brain regions of GCI/R mice .The substance metabolism in hippocampus and cortex was detected by MRS. A Typical spectra recorded from the hippocampus and cortex of mouse. B-D Spectra in hippocampus and cortex were acquired at 9.4 T. E-J The changes of the metabolites Glx, GABA, NAA, MI, CHO and Taurine in hippocampus and cortex. *p < 0.05, **p < 0.01,

compared with the sham group; ^p < 0.05, ^^p < 0.01, compared with the GCI group!p < 0.05, !!p < 0.01, compared with the 24A intervention group.

Figure 4

24A reduced GCI/R induced microglia and astrocyte activation. A-B Immunohistochemical staining for Iba1 in hippocampus and cortex (100×, 400× magnification). C-D Quantitative analysis of microglia in hippocampus and cortex with Image J software. E-F Immunohistochemical staining for GFAP in hippocampus and cortex (100×, 400× magnification). G-H Quantitative analysis of astrocytes in hippocampus and cortex with Image J software. (**p < 0.01, *p < 0.05, ns means no significant differences, n = 3).

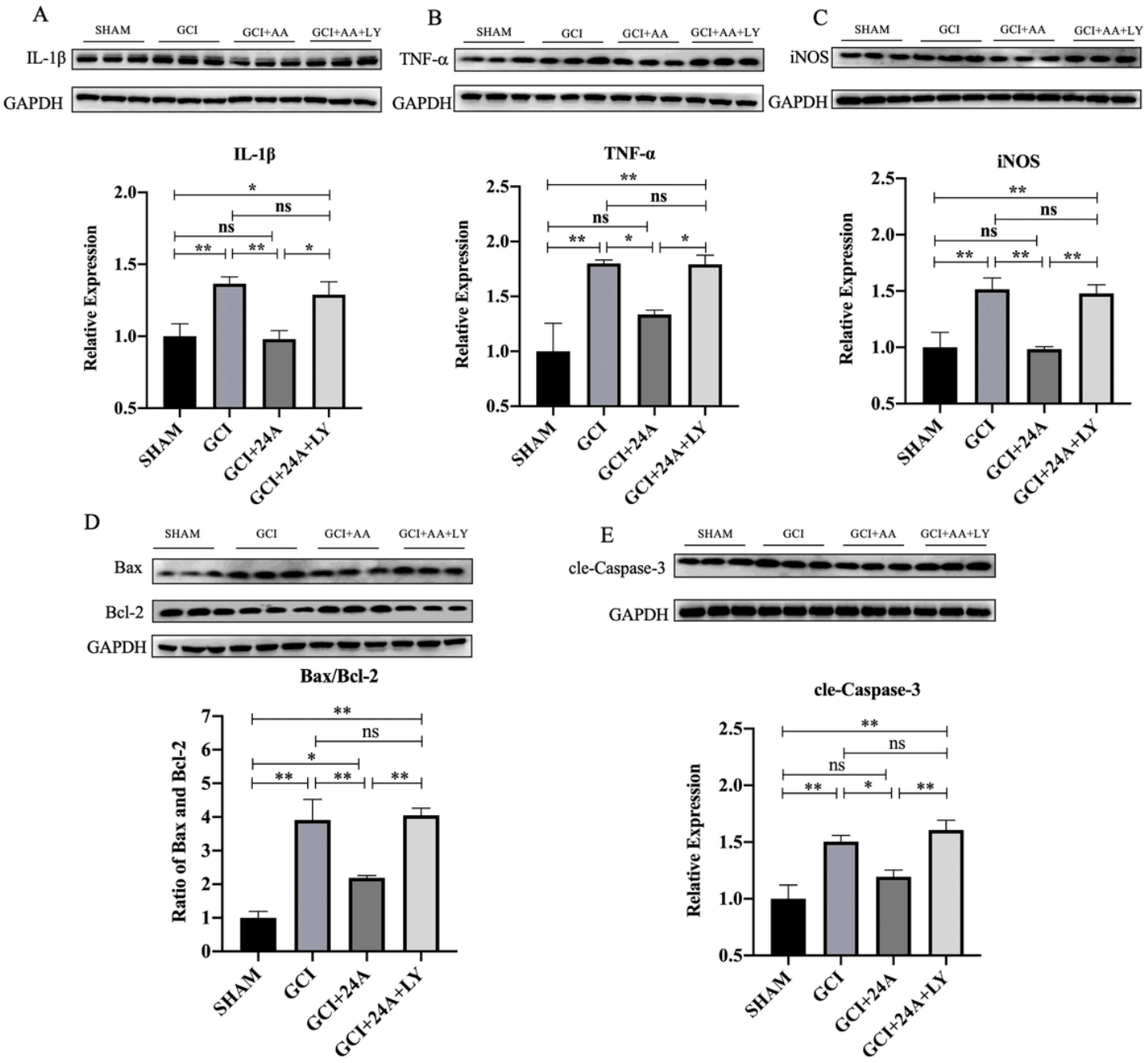

Figure 5

24A attenuated GCI/R induced inflammation and apoptosis. A-E Representative western-blot images and quantitative analysis of IL-1β, TNF-α, iNOS, Bax/Bcl-2, cle-Caspase 3. (** $p < 0.01$, * $p < 0.05$, ns means no significant differences n = 3).

Figure 6

Therapeutic effect of 24A on GCI/R induced apoptotic neuronal death. A-B Immunohistochemical staining for neurons in hippocampus and cortex (10×, 400× magnification). CThe quantity and arrangement of neurons in hippocampus and cortex were observed by Golgi staining (40× magnification). D, F The density of dendritic spines of hippocampal and cortical neurons were observed by Golgi staining (1000×magnification). E, G Differences of the density of dendritic spines in hippocampal and cortical neurons among each group were analyzed. (**p < 0.01, *p < 0.05, ns means no significant differences n = 3).

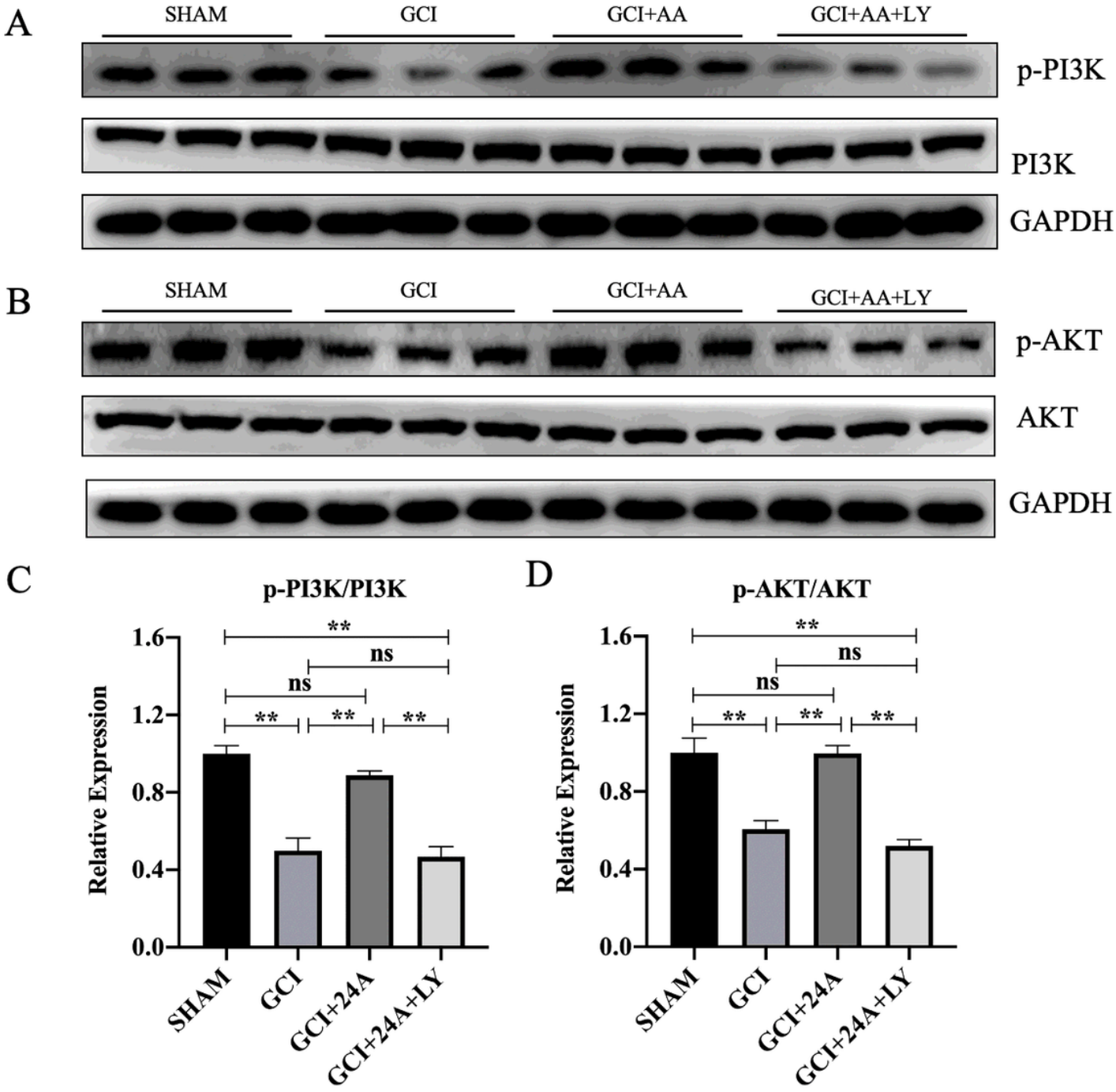

Figure 7

24A activated PI3K/AKT pathway in GCI/R. A-BThe expression of p-PI3K, PI3K, p-AKT, AKT were detected by western blot with GAPDH as loading control. C-D The ratio of p-PI3K to PI3K and p-AKT to AKT among different groups were analyzed. (** $p < 0.01$, * $p < 0.05$, ns means no significant differences among each group, n = 3).