



The role of histone deacetylase inhibitors in regulation of Akt/GSK-3 β signaling pathway in mice following transient focal cerebral ischemia¹

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Abstract

Purpose: To investigate whether the neuroprotective effect of TSA on cerebral ischemia reperfusion injury is mediated by the activation of Akt/GSK-3 β signaling pathway.

Methods: Mice were randomly divided into four groups (n=15): sham group (S); ischemia reperfusion group (IR); ischemia reperfusion and pretreated with TSA group (IR+T); ischemia reperfusion and pretreated with TSA and LY294002 group (IR+T+L). The model of cerebral ischemia reperfusion was established by 1h of MCAO following 24h of reperfusion. TSA (5mg/kg) was intraperitoneally given for 3 days before MCAO, Akt inhibitor, LY294002 (15 nmol/kg) was injected by tail vein 30 min before the MCAO.

Results: TSA significantly increased the expression of p-Akt, p-GSK-3 β proteins and the levels of SOD, Bcl-2, reduced the infarct volume and the levels of MDA, ROS, TNF- α , IL-1 β , Bax, Caspase-3, TUNEL and attenuated neurological deficit in mice with transient MCAO, LY294002 weakened such effect of TSA dramatically.

Conclusions: TSA could significantly decrease the neurological deficit and reduce the cerebral infarct volume, oxidative stress, inflammation, as well as apoptosis during cerebral ischemia reperfusion injury, which was achieved by activation of the Akt/GSK-3 β signaling pathway.

Key words: Histone Deacetylases. Glycogen Synthase Kinase 3 beta. Brain Ischemia. Reperfusion Injury. Mice.

■ Introduction

Stroke, one of the serious diseases that threaten human health, is a pathological condition due to the occlusion of blood vessels which providing oxygen and essential nutrients to the brain, and it is characterized with high incidence, high morbidity and high mortality¹. In all cases, stroke eventually leads to nerve cells dysfunction or death. The severity of brain damage is closely related with the length of ischemic time and the degree of ischemia². Current clinical treatment for ischemic stroke is regaining blood supplies to the ischemic brain tissue, but more and more clinical cases show that this treatment may alleviate the situation of cerebral ischemia in a short time, but it is very limit for improvement of neurological function, and even aggravate the injury, this phenomenon is called cerebral ischemia reperfusion injury (CIRI)³.

Recent studies have shown that the gene expression in ischemic stroke is abnormal, and acetylation is one of important mechanisms for gene transcription, regulation and expression, which mainly consists of histone acetylase (HAT) and histone deacetylase (HDAC)^{4,5}. HDAC plays a significant part in epigenetic related signaling pathways which can participate in various life activities by regulating the balance of acetylation and deacetylation by histone and non-histones, including cell growth, differentiation, death, as well as the interactions and inflammations between cells and interstitial⁶.

Akt/GSK-3 β pathway is an important signaling pathway in reperfusion injury salvage kinase (RISK). This pathway is considered to be a key to regulating cell survival, proliferation and growth⁷. Akt/GSK-3 β pathway is also plays an important role in many diseases processes⁸. Studies have shown Akt/GSK-3 β pathway is involved in oxidative stress, apoptosis and

inflammatory reactions, and it is also closely related with ischemia reperfusion injury^{9,10}.

Studies have shown that the HDAC inhibits transcription by making it difficult for gene promoters to get close to the transcriptional regulatory component, which leads to brain damage. Histone deacetylase inhibitors (HDACI) can inhibit HDAC activity and produce brain protection^{11,12}. However, it is unclear whether HDACI has a protective effect on the Akt/GSK-3 β signaling pathway. This study was designed to investigate the role of HDACI in the regulation of Akt/GSK-3 β pathway in the cerebral ischemia reperfusion injury.

■ Methods

This study was approved by the ethics committee of the Renmin Hospital, Wuhan University, China. All animal procedures received the approval of the Wuhan University Animal Care and Use Committee and followed the Principles of Laboratory Animal Care and are in accordance with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health.

Male Balb/c mice (10–12 weeks old) were maintained on sterile, standard laboratory chow and water *ad libitum* in individual ventilated cages under specific pathogen-free (SPF) conditions, with a maintained temperature of 22°C, relative humidity of 50 \pm 15%, and a 12-hour light/12-hour dark cycle, in the animal facility of the Experimental Research Centre of Wuhan University.

Experimental protocols

Sixty mice were randomly divided into four groups: (1) sham operated mice (S); (2) mice subjected to MCAO (IR); (3) mice subjected to MCAO, pretreated with TSA (IR+T); (4) mice subjected to MCAO, pretreated with TSA and LY294002 (IR+T+L). Mice in each group except

that in the sham group underwent 1h of MCAO following 24h of reperfusion. TSA (5mg/kg) was intraperitoneally given for 3 days before MCAO, Akt inhibitor, LY294002 (15 nmol/kg) was injected by tail vein 30min before the MCAO.

Model of focal cerebral ischemia

Mice were anesthetized with 2% isoflurane in 70% N₂O and balance O₂ by facemask. Cerebral ischemia was induced by 1h of MCAO with a silicone-coated 6-0 monofilament followed by 24h of reperfusion. Sham-operated mice underwent an identical procedure, without inserting sutures. Rectal temperature was maintained at 37 \pm 0.5 $^{\circ}$ C with a heating pad. Heart rate, oxygen saturation, and respiratory rate were monitored continuously. Animals with no observable deficits immediately after ischemia, those that died before 24h were excluded from analysis.

The histopathology of brain tissue

After 24h of reperfusion, mice were anesthetized with isoflurane and executed, and their brains were fixed with 4% formaldehyde solution for 24h, embedded in paraffin and cut into 4 μ m pieces by microtome, and stained with haematoxylin and eosin stain (H&E). The HE staining was quantified under a BX51 microscope (\times 400, Olympus Inc, Japan).

TTC staining

After 24h of reperfusion, mice were killed on ice and their brains were removed and frozen at -20 $^{\circ}$ C for 10min, then sectioned coronally with a rodent brain slicer matrix (Zivic Instruments, Pittsburgh, PA). Sections were incubated in 2% 2,3,5-triphenyletrazolium chloride (TTC) (Sigma Inc, USA). After the staining, the infarct tissue was white and the normal tissue was red.

Determination of oxidative stress

MDA level and SOD activity were detected using commercial kits (Jiancheng Bioengineering, China). Tissue homogenate was prepared according to the user manual. Briefly, brain tissue was homogenized in 10vol (w/v) ice-cold 0.1MPBS. Then the homogenate was centrifuged at 4000 \times g for 10min and the supernatant was used for MDA level and SOD activity.

Mitochondria-generated ROS (Jiancheng Bioengineering, China): protein were incubated in reaction buffer, Malate and glutamate were added as substrates. After 40min incubation at 37 $^{\circ}$ C, the formation of the oxidized fluorescent product dichloro-fluorescein was monitored with excitation at 488nm and emission at 525nm. The results were expressed as arbitrary fluorescence units per mg protein (U/mg).

ELISA to detect TNF- α , IL-1 β

Brain samples were pulverized using a porcelain mortar and pestle under liquid nitrogen. Then the tissues were incubated in a lysis buffer for 1h at 4 $^{\circ}$ C. After 10 min centrifugation at 10000g, the supernatant was collected for ELISA. The levels of TNF- α and IL-1 β were measured using an ELISA kit following the manufacturer's specification (Boster Biotech Inc. China). Each tested sample was normalized by its protein concentration in mg level.

Immunohistochemical staining of Bax, Bcl-2 and Caspase-3

Sections were dewaxed, rehydrated and then incubated in PBS for 10min to block endogenous peroxidase activity. Sections were subjected to antigen retrieval by boiling sections in 10mM sodium citrate buffer in a microwave for 20min. After cooling, sections

were blocked in normal goat serum (1:10, ZSGB Biotech Inc. China) for 10min, then incubated overnight at 4°C with rabbit anti- Bax, Bcl-2, Caspase-3 polyclonal antibodies(1:100, ZSGB Biotech Inc. China). After being washed with PBS, sections were incubated for 10min with biotinylated goat anti-rabbit IgG (1:10, ZSGB Biotech Inc China). Staining was visualized using diaminobenzidine as a substrate. The number of positive cells was quantified under a BX51 microscope ($\times 400$, Olympus Inc, Japan).

TUNEL staining for cell apoptosis

Paraffin embedded sections were dewaxed and rehydrated, then incubated in 20 μ L/mL proteinase K for 15 min. TUNEL was accomplished using an in situ cell death detection kit (Roche Inc, USA). After immersion in equilibration buffer for 10 min, sections were incubated with TdT and dUTP-digoxigenin, then washed before incubation in anti-digoxigenin-peroxidase solution, and colored with diaminobenzidine-H₂O₂ solution. The number of TUNEL-positive cells was quantified under the BX51 microscope ($\times 400$, Olympus Inc, Japan).

Western blot analysis

Brain tissue was sampled and homogenized with 1000 μ l lysis buffer. The homogenates was centrifuged at 12000g at 4°C for 15min; equivalent amountsof total protein extracts were loaded into each lane and separated by 10% SDS gels, then transferred onto PVDF membrane. The membrane was blocked with 5% BSA for 1h and incubated with the following primary rabbit monoclonal

antibodies: Akt, p-Akt, GSK-3 β and p-GSK-3 β (1:1000, Cell Signaling Technology, USA) diluted in 5% w/v BSA, and overnight at 4°C. The membrane was incubated with fluorescent tags Goat anti-Rabbit polyclonal IgG (1:10000, LI-COR Inc, USA) for 1h followed by additional washing. GAPDH was chosen as a loading control to further assure the same volume for all the samples.

Statistical analysis

Data was presented as mean \pm SD and analyzed using SPSS17.0 software. The differences associated with main sources of variation were tested using one-way analysis of variance (ANOVA). When the F statistic was significant for ANOVA comparisons, the differences between individual means were tested for significance using Bonferroni tests. $P < 0.05$ was considered statistically significant.

■ Results

The histopathology of brain tissue

Microscopically, we found that when compared with S group, the neurons in IR group were irregular, with uneven distribution of cytoplasm and vacuolization, and the nucleation was dissolved or disappeared. In IR+T group, the neurons were relatively orderly, with complete cytoplasm, abundant cytoplasm, and a relatively clear structure. But in IR+T+L group, the protective role of TSA to maintain the normal morphological characteristics was weakened (Figure 1).

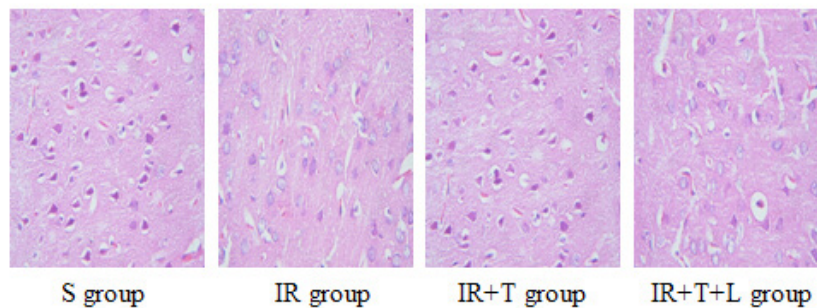


Figure 1 - The cerebral histopathology haematoxylin and eosin staining. Microscopic observation showed that the cortical neurons were arranged in neat rows with abundant cytoplasm, and the nuclei were round and basophilic in S group. The structures of the cortical neurons were damaged, the cytoplasm was light red with uneven distribution and vacuoles, nuclei were condensed in IR group. In IR+T group, however, the cell structure was normal. Most of the neurons had complete membrane integrity and the nuclei were clear. In IR+T+L group, it seems almost like IR group.

S group=sham group, IR group=cerebral ischemia reperfusion group, IR+T group= cerebral ischemia reperfusion+TSA (HDAC inhibitor) group, IR+T+L group= cerebral ischemia reperfusion group+TSA+LY294002 (Akt inhibitor) group.

TTC staining

Mice in the sham group had little infarction as expected, while other groups had different extent of cerebral infarction. TSA in IR+T group significantly decreased the

infarction volume when compared with IR group ($P<0.05$). But in the IR+T+L group by joint application of TSA and LY294002, could attenuated such neuroprotective effects of TSA significantly when compared with IR+T group ($P<0.05$) (Figure 2).

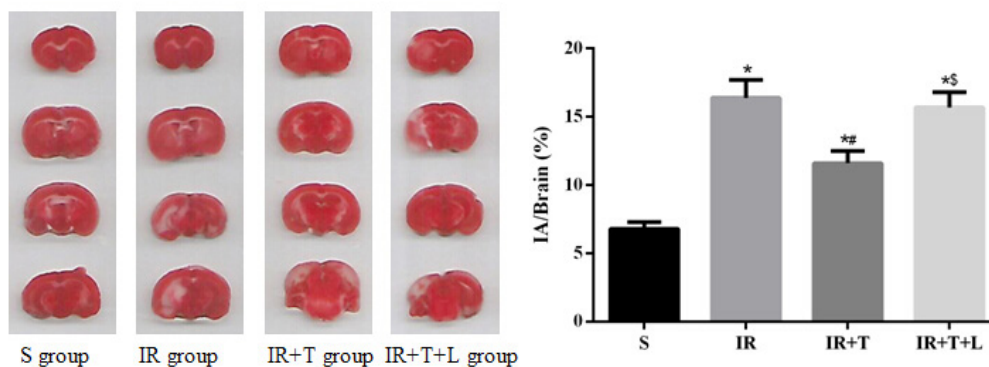


Figure 2 - The cerebral TTC staining. Mice in the sham group had little infarction as expected, while other groups had different extent of cerebral infarction. TSA in IR+T group significantly decreased the infarction volume when compared with IR group ($P<0.05$). But in IR+T+L group by joint application of TSA and LY294002, could attenuated such neuroprotective effects of TSA significantly when compared with IR+ T group ($P<0.05$).

* $P<0.05$, vs. S group; # $P<0.05$, vs. IR group; § $P<0.05$, vs. IR+T group.

Determination of oxidative stress

Compared with S group, MDA and ROS increased, while SOD decreased in IR group ($P<0.05$). TSA in IR+T group significantly decreased the expression of MDA and ROS,

and increased SOD expression as compared with IR group ($P<0.05$). However, in IR+T+L group using TSA and LY294002 together, could decrease such antioxidant stress effects of TSA to a certain degree when compared with IR+T group ($P<0.05$) (Figure 3).

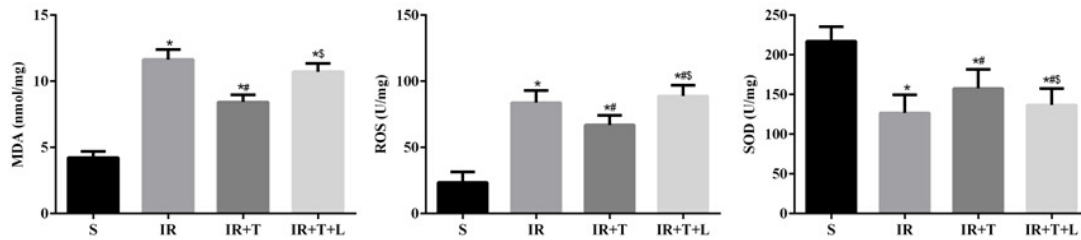


Figure 3 - Expression of oxidative stress in the brains (MDA, ROS, SOD). MDA, ROS, SOD are the important indicators of oxidative stress. In the research, it found that MDA and ROS expressions were significantly increased while the expressions of SOD were significantly decreased in IR group ($P<0.05$). TSA in IR+T group significantly decreased the expression of MDA and ROS and increased SOD expression when compared with IR group ($P<0.05$). However, in IR+T+L group, using TSA and LY294002 together, could decrease such antioxidant stress effects of TSA to a certain degree when compared with IR+T group ($P<0.05$).

* $P<0.05$, vs. S group; ** $P<0.05$, vs. IR group; [§] $P<0.05$, vs. IR+T group.

Expression of TNF- α , IL-1 β in the brains

When compared with S group, the levels of TNF- α , IL-1 β in the brain tissue were increased in IR group ($P<0.05$). Compared with IR group, the levels of TNF- α , IL-1 β in IR+T

group were significantly lower when TSA was used ($P<0.05$). While when TSA and LY294002 were jointed, the anti-inflammatory effect of TSA were abolished in IR+T+L group ($P<0.05$) (Figure 4).

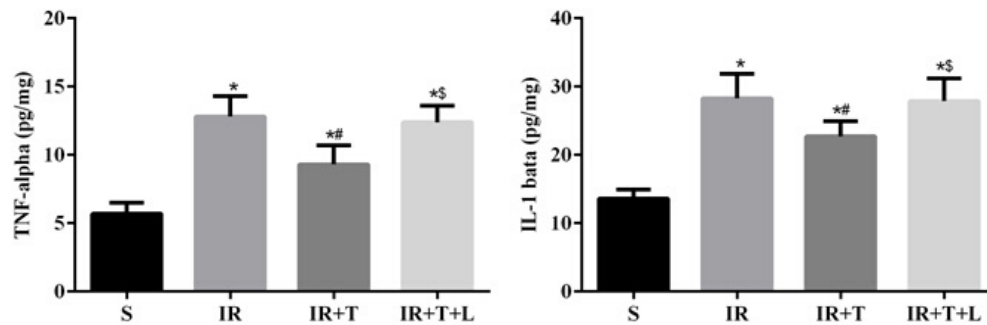


Figure 4 - Expression of inflammatory cytokines in the brains (TNF- α , IL-1 β). TNF- α , IL-1 β are the important indicators of inflammatory cytokines. In the research, it found that TNF- α , IL-1 β expressions were significantly increased in IR group ($P<0.05$). In contrast, TSA reversed these changes in IR+T group ($P<0.05$). In IR+T+L group, the anti-inflammatory effect of TSA were abolished ($P<0.05$).

* $P<0.05$, vs. S group; ** $P<0.05$, vs. IR group; [§] $P<0.05$, vs. IR+T group.

Expression of immunohistochemical in the brains (Bax, Bcl-2, Caspase-3)

In this research, we found that Bax and Caspase-3 expressions were significantly increased while the expression of Bcl-2 was significantly decreased in IR group ($P<0.05$).

TSA in IR+T group significantly decreased Bax and Caspase-3 expressions and increased the expression of Bcl-2 as compared with IR group ($P<0.05$). But in IR+T+L group, the anti-apoptotic effects of TSA was decreased when compared with IR+T group ($P<0.05$) (Figure 5).

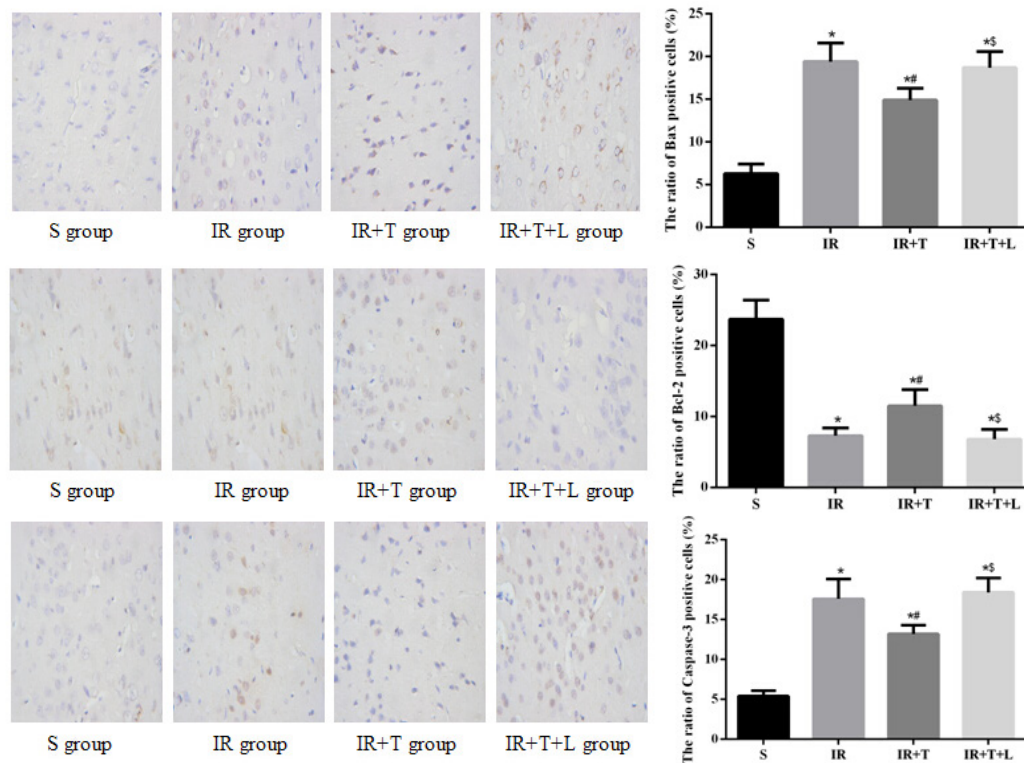


Figure 5 - Expression of immunohistochemical in the brains (Bax, Bcl-2, Caspase-3). Bax, Bcl-2 are belong to Bcl-2 gene family, which is an essential factors for cell death, caspase-3 is the important indicators of apoptosis response. In the research, it found that the expression of Bax, Caspase-3 were significantly increased while the expression of Bcl-2 was significantly decreased in IR group ($P<0.05$). In contrast, TSA reversed these changes in protein expression in IR+T group ($P<0.05$). In IR+T+L group, the consequence was no better than IR group ($P<0.05$).

* $P<0.05$, vs. S group; # $P<0.05$, vs. IR group; [§] $P<0.05$, vs. IR+T group.

Expression of apoptosis in the brains (TUNEL)

Compared with S group, the number of TUNEL positive cells in the brain tissue were increased in IR group ($P<0.05$). Compared with

IR group, TUNEL in IR+T group were significantly lower ($P<0.05$). While when LY294002 was used, the anti-apoptotic effect of TSA were abolished in IR+T+L group ($P<0.05$) (Figure 6).

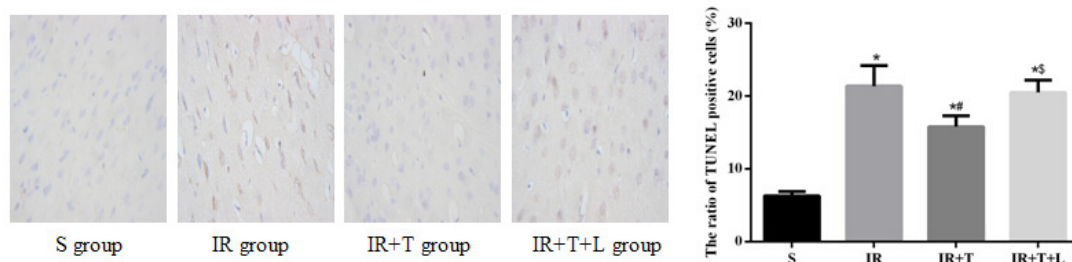


Figure 6 - Expression of apoptosis in the brains (TUNEL). The number of TUNEL positive cells in the brain tissue were increased in IR group when compared with S group ($P<0.05$). Compared with IR group, TUNEL in IR+T group were significantly lower when TSA was used ($P<0.05$). While when TSA and LY294002 were joint used, the anti-apoptotic effect of TSA in IR+T group were abolished when compared to the IR+T+L group ($P<0.05$).

* $P<0.05$, vs. S group; # $P<0.05$, vs. IR group; [§] $P<0.05$, vs. IR+T group.

Western blot for cerebral p-Akt(Akt) and p-GSK-3 β (GSK-3 β) expression

Compared with S group, the levels of p-Akt and p-GSK-3 β in IR group were decreased ($P<0.05$). TSA treatment in IR+T group could significantly increase the expression of p-Akt and p-GSK-3 β when compared with IR group ($P<0.05$). Whereas, IR+T+L group could significantly decrease the expression of p-Akt and p-GSK-3 β when compared with IR+T group ($P<0.05$) (Figure 7).

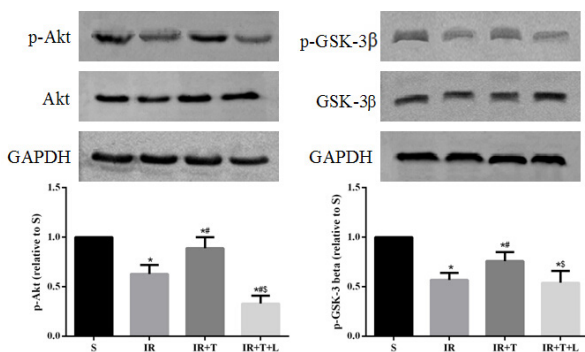


Figure 7 - Western blot for the expression of Akt and GSK-3 β in the brains. Cerebral Akt, p-Akt, GSK-3 β , p-GSK-3 β expression were examined by western blot. As shown, there were no noticeable differences in the expression of total Akt and GSK-3 β in four groups. The p-Akt and p-GSK-3 β level was markedly decreased in IR group ($P<0.05$), TSA increased the level of p-Akt and p-GSK-3 β in IR+T group ($P<0.05$). However, in IR+T+L group using TSA and LY294002 together, the expression of p-Akt and p-GSK-3 β protein decreased when compared with IR+T group ($P<0.05$).

* $P<0.05$, vs. S group; ** $P<0.05$, vs. IR group; [§] $P<0.05$, vs. IR+T group.

■ Discussion

The results of this study showed that TSA preconditioning can increase the expression of p-Akt, p-GSK-3 β and the neuroprotection induced by TSA pretreatment can be blocked by LY294002, which indicating that the Akt/GSK-3 β pathways involved in cerebral ischemia

reperfusion injury, and the neuroprotective effect may be partly due to reducing oxidative stress, inflammation and apoptosis. It is proved that the connection between Akt/GSK-3 β pathways and TSA in neuroprotective effect against cerebral ischemia reperfusion injury.

There are many hypotheses about the pathogenesis of ischemic stroke, including oxidative stress, inflammatory response, immune regulation, excitatory amino acid toxicity, calcium overload, apoptosis, blood-brain barrier damage and neurovascular cell interactions^{13,14}. There are also many cross-effects among this different hypotheses, which makes the pathogenesis of ischemic stroke more complex¹⁵. In those hypotheses, oxidative stress and inflammatory response are the most classic, which mainly refers to that a large number of oxidized and inflammatory mediators released after cerebral ischemia reperfusion, leading directly to the functional impairment, affecting the proliferation and apoptosis of neurons, aggravating cerebral ischemia reperfusion injury^{16,17}.

Recent studies have shown that the gene expression in ischemic stroke is abnormal, whereas HDACI can regulate the expression of genes related to neurological function through acetylation/deacetylation by histone and nonhistone, reducing the injury, promoting the revascularization of ischemic area and promoting the neuronal plasticity and functional recovery^{18,19}. As a result, HDACI plays a key role in regulating neurological function and attracts a wide range of concerns as a potential drug target. Studies have shown that HDACI can inhibit the catalytic activity of HDAC, change the chromosome structure, activate the gene transcription, correct the chromosomes from "closed" to "open" state, play a neuroprotective role, including: inhibition of neuronal apoptosis, regulation of microglia/macrophage polarization, reduction of inflammatory response, promotion of nerve regeneration^{5,20}.

HDACI not only interacts with histones that regulate gene transcription, but also with non-histones that regulate cell stability. Studies have shown that HDACI can produce crosstalk in kinase signaling cascades in an animal model of ischemic stroke^{21,22}. Our results also confirm that HDACI has a protective effect in cerebral ischemia reperfusion, which may be associated with activation of Akt/GSK-3 β pathway.

GSK-3 β is highly expressed in the brain, lungs and kidneys of the human body. Evidences have shown that GSK-3 β has a close relationship with oxidative stress, cell apoptosis and inflammatory reaction. It can reduce the release of inflammatory factors and oxygen free radicals by regulating the mitochondrial permeability transition pore (mPTP) so as to produce a protective effect^{23,24}. Meanwhile, GSK-3 β is also been proved to have a role in the innumerable downstream effects of Akt, suggesting that Akt/GSK-3 β is a very important signaling pathway that has a crucial effect on the progression of many diseases^{25,26}. Therefore, the researchers speculated that by regulating the expression of Akt/GSK-3 β , may have a positive impact on reperfusion injury, which has important clinical value. Our results confirm that after exposure to HDACI, Akt/GSK-3 β signaling pathway is activated, which reduces the expression of oxidative stress, apoptosis factor and TUNEL, beneficial to the brain protection. When Akt inhibitor is added, the signal pathway is inhibited and the protective effects induced by TSA is crippled.

It is now generally accepted that neuroinflammation mediated by activated microglia and infiltrating leukocytes, including monocytes/macrophages has a prominent role in ischemia induced brain injury^{27,28}. Inflammatory cytokines (TNF- α and IL-1 β), reactive oxygen species (ROS) are overexpressed to induce the inflammatory response. After stroke, many of the leukocytes including monocytes and macrophages move from the

blood vessels and accumulate in the infarct zone to contribute to neuroinflammation and neurodegeneration²⁹⁻³¹. Our results showed that TSA treatment significantly reduced the expression of TNF- α , IL-1 β and the ROS following MCAO. When Akt inhibitor is added, the signal pathway is inhibited and the protective effects is abolished.

■ Conclusion

HDAC inhibitor TSA plays a protective role on cerebral ischemia reperfusion injury by activating the Akt/GSK-3 β pathway, inhibiting the activation of this pathway alleviates the protective effects.

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