HSP90β regulates EAAT2 expression and participates in ischemia-reperfusion injury in rats

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Abstract. Cerebrovascular diseases (CVDs) have become a global public health problem and ischemia-reperfusion injury, the major cause of neurological impairment exacerbation, is closely related to excitotoxicity. The present study aimed to investigate the effects of changes in heat shock protein (HSP)90β expression and verify whether HSP90β regulates EAAT2 expression in a cerebral ischemia-reperfusion injury model. Healthy adult Sprague-Dawley (SD) male rats were used to establish a control group, sham-operated group, middle cerebral artery occlusion (MCAO) group, empty virus group and lentivirus group. A model of cerebral ischemia-reperfusion was established using the MCAO method. Lentivirus construction and injection were used to interfere with the expression of HSP90β. The modified neurological severity score was used to assess neurological deficits. Triphenyltetrazolium chloride staining was used to detect infarct areas. Immunofluorescence was used to detect HSP90β expression localization and the expression levels of HSP90β and EAAT2 were determined using western blotting and reverse transcription-quantitative PCR. An MCAO model was successfully established and it was found that HSP90β, but not HSP90α, was upregulated after MCAO. HSP90β expression coincided with astrocyte markers in the ischemic penumbra area, while no expression was observed in microglia. Inhibition of HSP90β expression improved neurological deficits and alleviated brain injury by increasing EAAT2 expression. These results suggested that HSP90β is involved in the process of cerebral ischemia-reperfusion injury in rats and that inhibition of HSP90β expression increases EAAT2 levels, conferring a neuroprotective effect in MCAO model rats.

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Introduction

Cerebrovascular disease (CVD) has become a global public health problem due to its high morbidity, association with disability and recurrence rates. In China, the incidence of CVD in the population is increasing annually and ischemic stroke is the most common type of CVD; ~70% of patients have ischemic stroke and the recurrence rate is as high as 17.7% (1,2). There is currently a lack of effective treatment methods for ischemic stroke, An effective approach after cerebrovascular embolism in clinical practice is timely thrombolysis or endovascular intervention, intended to restore blood perfusion in the ischemic area; yet at the same time it can induce ischemia-reperfusion injury, which leads to brain damage both in the ischemic core and penumbra area. An ischemic stroke consists of two related pathological injury processes: Primary ischemia-induced brain injury and secondary ischemia reperfusion injury (3). A study has shown that neurons in the ischemic penumbra may undergo apoptosis hours or days after ischemia and alleviating ischemia reperfusion injury is an achievable therapeutic goal in the early intervention of ischemic stroke aimed at limiting the amount of infarction (4). When ischemic stroke occurs, cerebral ischemia and hypoxia cause the release of excessive excitatory amino acids, mainly glutamic acid and aspartic acid, which exert excitotoxic effects on the central nervous system. These excitotoxic effects play important roles in neuronal and blood-brain barrier damage after cerebral ischemia (5,6). Extracellular excitatory amino acids are mainly transported into cells by excitatory amino acid transporters (EAATs) expressed on astrocytes to avoid excessive excitation of neurons. EAAT2 accounts for 80-90% of this activity of extracellular excitatory amino acid uptake (7). Changes in EAAT2 function are closely related to excitotoxicity in the central nervous system. Heat shock protein (HSP) 90 is abundant in cells, mainly in the form of homodimers, including HSP90α and HSP90β. As a molecular chaperone, HSP90 regulates the conformational maturation and functional stability of many signaling proteins in cells, serves important roles in cell growth, differentiation, apoptosis and tumor development and is an important antitumor target (8,9). A study found that HSP90α is not necessary in mammals, but

HSP90β is (10), suggesting that HSP90α and HSP90β have different physiological roles. Significantly increased HSP90 expression levels has been observed in human patients with hippocampal sclerosis and in a mouse model of epilepsy. The increase in the HSP90β expression level was predominant, while the increase in the HSP90α expression level was not significant (11), further supporting the different roles of HSP90α and HSP90β. At present, the role of HSP90β in ischemic brain injury has not been reported. The purpose of the present study was to clarify the changes in HSP90β expression in a rat cerebral ischemia-reperfusion model and the effects of these changes on the expression of EAAT2 and to further explore the potential of HSP90β as a molecular target for reducing brain ischemia-reperfusion injury.

Materials and methods

Laboratory animals. A total of 87 Healthy adult Sprague-Dawley (SD) male rats weighing 280-300 g (6-8 weeks) were purchased from Changsha Tianqin Biological Technology Company [license number: SCXK (Xiang) 2019-0004]. The animals were reared in separate cages with 12-h light/dark cycle at a constant temperature (24±1˚C) and 55±5% humidity with free access to food and water during the experimental period. The experimental groups included the control group, sham-operated group, middle cerebral artery occlusion (MCAO) group, empty virus group and lentivirus group. The rats were anaesthetized with 1% pentobarbital sodium (40 mg/kg) before being sacrificed by cervical dislocation. All efforts were made to minimize the suffering of the rats. All procedures were approved by the Animal Care and Use Committee of Zunyi Medical University, Zunyi, China (approval number ZMU21-2203-487).

Establishment of a cerebral ischemia-reperfusion model in rats through MCAO. The cerebral ischemia-reperfusion model was established through MCAO (12,13). The specific procedure was as follows: The rats were anesthetized by administering an intraperitoneal injection of 1% pentobarbital (40 mg/kg), a small incision in the skin to the right of the neck was created and the carotid sheath was exposed. The common carotid artery and external carotid artery were ligated. A small incision was made at the common carotid artery ~4 mm from the bifurcation of the internal and external carotid arteries and the MCAO suture was inserted into the internal carotid artery. The length of the suture was ~18-20 mm. The suture was fixed onto the internal carotid artery. The incision was sterilized and sutured. After 2 h of embolization, the suture was slowly removed to achieve reperfusion. The sham-operated group underwent the same treatment, but the MCAO suture was only inserted 10 mm into the internal carotid artery. During the entire experimental operation, a 40 W light bulb illuminated the surgical field and an electric blanket was used to keep the rats warm. After the operation, rats were housed in separate cages and they had access to a normal diet and drinking water. Referring to a previous study (14), the modified neurological severity score (mNSS) was determined 24 h after reperfusion and a higher score indicated a more severe neurological deficit.

Reagents, constructs and antibodies. The oligonucleotide sequence for HSP90β microRNA interference (miRNAi) constructs was 5'-AACCGCATCTACCGCATGATT-3'. Antibodies were purchased from Abcam [HSP90β, EAAT2, Bax, Bcl-2 and glial fibrillary acidic protein (GFAP)], ZEN-BIO, Inc. (HSP90β and EAAT2), Proteintech Group, Inc. (β-tubulin and β-actin) and FUJIFILM Wako Pure Chemical Corporation ionized calcium-binding adaptor molecule 1 (IBA1). CoraLite488-conjugated goat anti-rabbit IgG (H+L) and CoraLite594-conjugated goat anti-mouse IgG (H+L) were purchased from Proteintech Group, Inc. Hematoxylin and eosin (H&E) and triphenyltetrazolium chloride (TTC) staining reagents were purchased from Beijing Solarbio Science & Technology Co., Ltd.

Lentivirus construction and injection. HSP90β lentivirus and empty virus were synthesized and constructed by Shanghai Genechem Co., Ltd. HSPβ90 lentivirus decreased expression of HSP90β. The three target sequence of HSPβ90 LV were designed before formal experiments (Table SI). The lentivirus was injected into the right lateral ventricle. Using the fontanel as the origin, a site 1.3 mm horizontally to the right and then 1.5 mm backward was located and a hole was drilled at this site with an electric miniature animal skull drill. Then, a 10 μl microinjector was used to aspirate 10 μl of virus and the needle was slowly inserted vertically 3.8 mm at the drilled hole. The empty virus group was injected with empty virus as a control. Successful infection was confirmed through immunofluorescence and western blotting which screened highest transfection efficiency in decreasing HSP90β (Fig. S1).

TTC staining. Following anesthesia, the rats were quickly decapitated, the brain was removed and the brain tissue was placed in a -20˚C freezer (the olfactory bulb, cerebellum and lower brain stem were removed) for 20 min. The brain tissue was divided into five parts from front to back with a scalpel at the midpoint of the connecting line between the anterior pole and the optic chiasm, the optic chiasm, the infundibular stalk and between the infundibular stalk and the caudal pole of the posterior lobe. The brain slices were placed in a 1% TTC staining solution (37˚C; 30 min) and arranged in order of brain tissue structures from rostral to caudal locations and then images were captured. The infarct volume ratio was calculated using Image-Pro Plus software 6.0 (Media Cybernetics, Inc.) to measure the area as follows: Infarct volume ratio: (normal side volume-infarct side noninfarct volume)/normal side volume

Immunofluorescence staining. Rat brain tissue was perfused with 4% paraformaldehyde overnight at 4˚C, and then rehydrated using a graded ethanol series (70, 80, 90 and 100%) for 1.5 h at room temperature, cleared in xylene twice and embedded in paraffin, then sectioned to a thickness of 5 μm. Sections were dewaxed with xylene, hydrated with a graded series of ethanol solutions and placed in 3% H_2O_2 for 10 min at room temperature to block endogenous peroxidase activity. Antigen retrieval was performed by high-pressure repair for 2 min in 0.01 mol/l citrate (pH=6.0; ~120˚C). Following blocking at room temperature for 30 min using the goat serum (cat no. C0265; Beyotime Institute of Biotechnology) blocking solution, the primary antibody (HSP90β; cat no. R380807;

ZEN-BIO, Inc.; GFAP, cat no. ab4648; Abcam; IBA1; cat no. 019-19741; FUJIFILM Wako Pure Chemical Corporation) was added and then incubated in the refrigerator at 4˚C overnight. After thorough washing, a fluorescent dye-conjugated secondary antibody (cat no. A0507; Beyotime Institute of Biotechnology) was added and incubated for 30 min at room temperature. After adding an anti-fluorescence quencher, sections were mounted and images were captured for observation.

Western blotting. Rats were decapitated quickly following anesthesia and brain tissue was collected. After removing the olfactory bulb, cerebellum and lower brain stem, the brain tissue was quickly placed in a -80˚C freezer for later use. Radioimmunoprecipitation assay (RIPA; cat no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) buffer containing protease inhibitors (PMSF; cat no. P0100; Beijing Solarbio Science & Technology Co., Ltd.) was added to the brain tissue, the tissue was fully disrupted with a homogenizer and centrifuged for 30 min (4˚C; 16,311 x g) and the supernatant was collected for protein quantification. The concentration of the supernatant was estimated using an Instant BCA Protein Assay Kit (cat no. ZJ101; EpiZyme, Inc.). After adding 20 μl of the corresponding sample to SDS-PAGE gels (stacking, 5%; separation, 10%), proteins were electrophoresed and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% non-fat milk powder at room temperature for 1 h, the membrane was transferred into the diluted primary antibody (HSP90β; cat no. ab203085; Abcam; EAAT2; cat no. ab205248; Abcam; HSP90α; cat no. R380809; ZEN-BIO, Inc.; BAX, cat no. ab32503; Bcl-2, Abcam; cat no. ab182858; Abcam; β-actin; cat no. 81115-1-RR; Proteintech Group, Inc.; β-tubulin; cat no: 10094-1-AP; Proteintech Group, Inc.) and placed in a 4˚C refrigerator overnight. After washing, the secondary antibody (HRP-conjugated Affinipure goat anti-rabbit IgG (H+L); cat no. SA00001-2, Proteintech Group, Inc.) was added and incubated for 1 h at room temperature before another washing step. The PVDF membrane was incubated with the luminescent agent (cat no. SQ202, EpiZyme, Inc.) for a sufficient period and then exposed to determine the protein levels. Blots were imaged using ChemicDoc Imaging System (Bio-Rad Laboratories, Inc.). ImageJ v1.52a (National Institutes of Health) was used to assess the greyscale values and SPSS 29.0 (IBM Corp.) was used to analyse the data.

Statistical methods. SPSS 29.0 (IBM Corp.) was used for statistical analyses of experimental data, and the results are presented as the means ± standard deviations (± s). The nonparametric Kruskal-Wallis test was used to analyze the mNSS data. One-way ANOVA and Tukey's post hoc test were used to compare differences between groups. Statistical graphs were plotted using GraphPad Prism 6.0 software (GraphPad; Dotmatics). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Assessment of the MCAO model. The MCAO model was first tested and H&E staining performed to observe whether histopathological changes occurred in rat brain tissue following ischemia and reperfusion. The neurons on the noninfarcted side of the brain tissue were regularly arranged and the nuclei were round, clear and uniform in size. In the ischemic area of the infarcted side, most of the neurons in the brain tissue were irregularly arranged and structurally disordered. The nuclei were not clearly displayed and had various sizes; the nuclei were also darker due to pyknosis (Fig. 1A). The results of TTC staining showed red staining in the control group and the sham-operated group and no infarction was detected. White infarcts were observed in the brain tissue of rats after ischemia-reperfusion for 24 h and the infarct volume ratio was significantly increased compared with the control group and the sham-operated group (Fig. 1B).

Changes in the expression of HSP90β, EAAT2 and HSP90α in the rat MCAO model. According to previous results from our research group, the lowest EAAT2 expression was detected at 24 h of ischemia and reperfusion. Therefore, 24 h of reperfusion was selected as the observation point. Western botting was used to detect the changes in protein expression in the brain tissue of rats from each group. HSP90β, HSP90α and EAAT2 proteins were expressed in the brain tissue of the control group, sham-operated group and MCAO group. The expression level of HSP90β in the MCAO group was increased and the difference was statistically significant compared with those in the control group and the sham-operated group (Fig. 1C). The EAAT2 protein expression level was significantly decreased compared with those in the control group and sham-operated group (Fig. 1E). A significant change in HSP90α expression was not observed (Fig. 1D).

Expression and localization of HSP90β in brain tissue after ischemia-reperfusion in rats. Using immunohistofluorescence, glial cells were simultaneously labeled with the astrocyte marker GFAP and the microglial marker IBA1. The results did not reveal obvious expression of HSP90 in microglia and astrocytes under normal conditions. However, after cerebral ischemia-reperfusion in rats, HSP90β expression coincided with astrocyte markers in the ischemic penumbra area, while no expression was observed in microglia (Fig. 2).

Inhibition of HSP90β expression is neuroprotective in the rat MCAO model. Rats in each group were evaluated by determining the mNSS score to judge the degree of neurological deficiency. The control group and the sham-operated group had no symptoms of neurological deficiency, while the MCAO group, the empty virus group and the lentivirus group had different degrees of symptoms of neurological deficiency. The mNSS score of the lentivirus group was significantly lower than that of the MCAO group and the empty virus group (Fig. 3C; $P<0.05$). TTC staining was performed to analyze the volume of cerebral infarction in each group. Except for the control group with no infarction, the MCAO group, the empty virus group and the HSP90β lentivirus group all had different degrees of infarction. After inhibiting HSP90β expression, the infarct volume ratio was significantly reduced, which was significantly different from the MCAO group and the empty virus group (Fig. 3A and B).

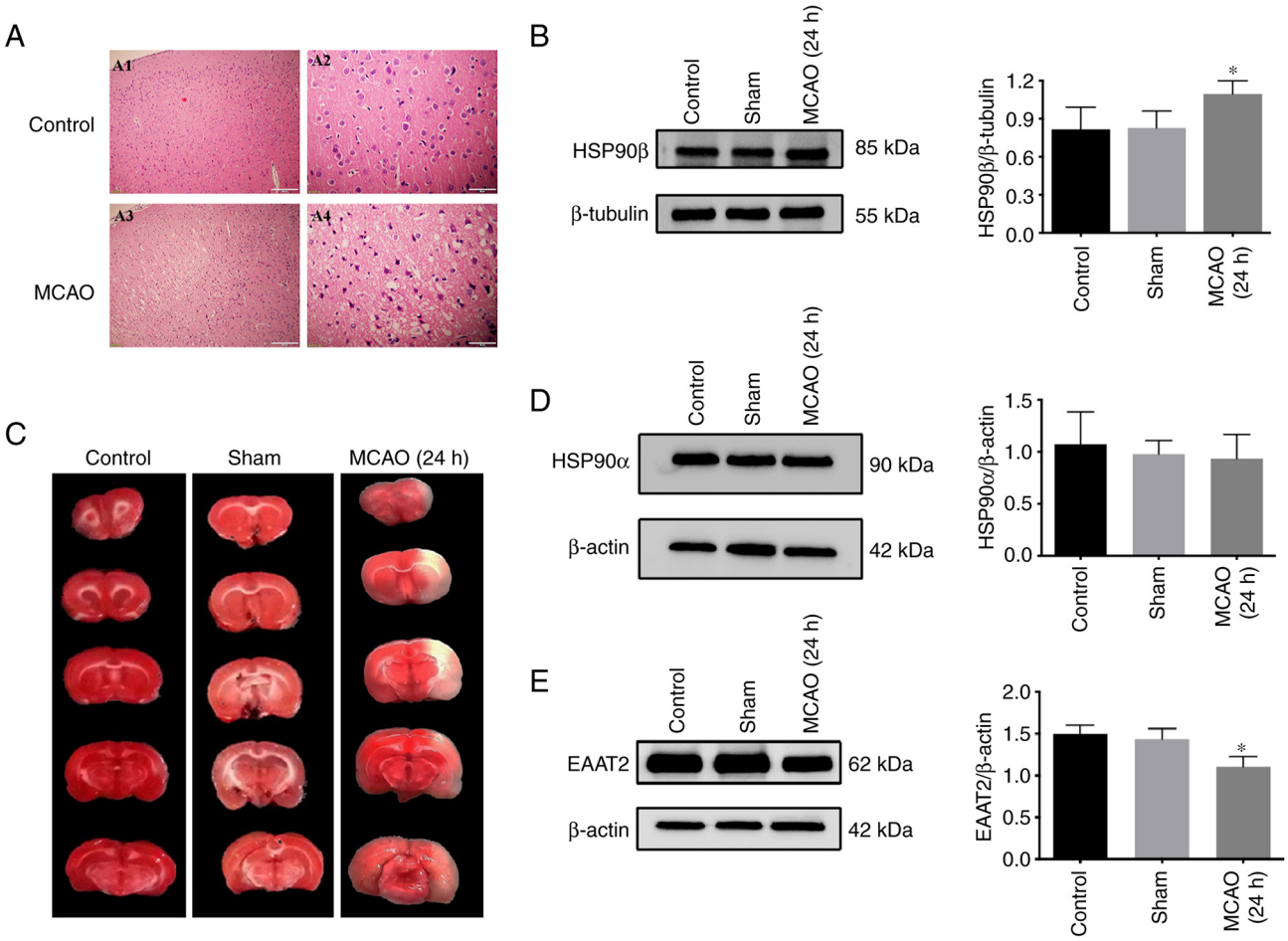

Figure 1. Assessment of the MCAO model and detection of the expression of HSP90α, HSP90β and EAAT2. (A) Hematoxylin and eosin staining suggested pathological damage in the MCAO group, magnification, x10 (A1 and A3) and x40 (A2 and A4). (B) Triphenyltetrazolium chloride staining shows the infarct region (white area). The expression of (C) HSP90α, (D) HSP90β and (E) EAAT2 in each group. Four technical replicates, n=6 in each group, *P<0.05 vs. control group. MCAO, middle cerebral artery occlusion; HSP, heat shock protein; EAAT, excitatory amino acid transporter.

Inhibition of HSP90β expression increases the EAAT2 protein expression level but not the EAAT2 mRNA expression level. After inhibiting the expression of HSP90β, EAAT2 expression was detected using western blotting. The EAAT2 expression level was significantly increased and the HSP90β expression level was significantly decreased (Fig. 4A and B). Additionally, the RT-PCR results showed a decreased HSP90β mRNA expression level, but the expression of EAAT2 mRNA was not affected (Fig. 3D and E). The expression of Bax in the virus intervention group was significantly lower than that in the MCAO group. The expression of Bcl-2 in the virus intervention group was significantly higher than that in the MCAO group (Fig. 4C and D; P<0.05). Based on these results, inhibition of HSP90β exerted neuroprotective effects.

Discussion

Stroke is one of the most common diseases in neurology and is characterized by high morbidity, disability and mortality rates and is the first cause of death and disability among adults in China, seriously threatening human life and health and imposing a huge economic burden on patients, their families and society. Global burden of disease data show that stroke is the number one cause of years of life lost in China (15,16). Effective treatments are lacking for ischemic stroke. The use of thrombolytic drugs and thrombus autolysis can further induce ischemia-reperfusion injury and aggravate cellular dysfunction and structural damage in the ischemic core and surrounding penumbra area, which is the main mechanism by which neurological deficits are aggravated (17-19). Therefore, reducing ischemia-reperfusion injury is one of the key links in the early treatment of ischemic stroke and is a current challenge in the field of neurology. The MCAO model was introduced to simulate the pathological process of ischemia-reperfusion injury, Thus, it is widely used to investigated as an excellent animal model in research field of ischemic stroke.

The relationship between HSP90 and stroke is currently poorly studied. It has been shown that the use of the HSP90 inhibitor 17-allylamino-demethoxygeldanamycin exerts a neuroprotective effect on cultured neurons with oxygen-glucose deprivation and the protective mechanism may be to activate the PI3K/Akt and MAPK cytoprotective pathways, which inhibit the cellular oxidative stress response and produce protective effects (20). Rats are effectively protected against ischemic brain injury after pretreatment with the antioxidant N-acetylcysteine. The mechanism may be attributed to the increased stability of HSP90 binding

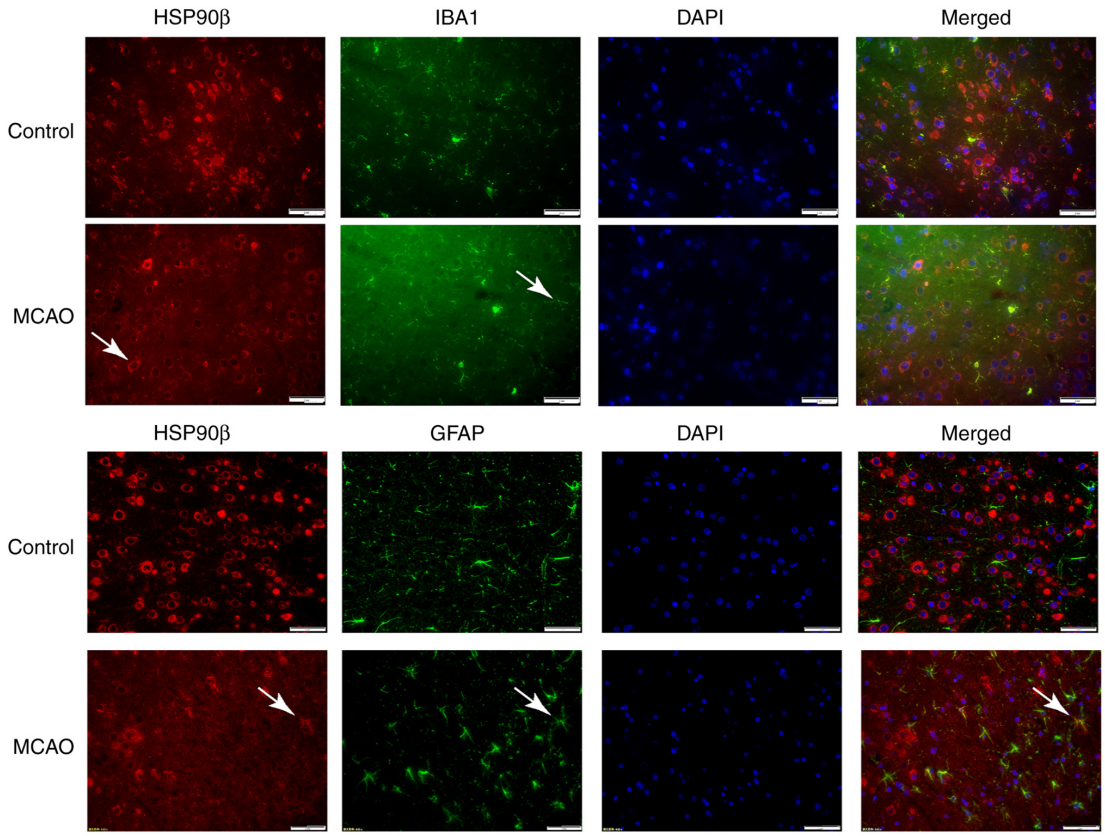

Figure 2. Immunofluorescence detection of the expression of HSP90β in the ischemic penumbra of MCAO rats. Arrows indicate HSP90β-positive cells, GFAP-positive cells, IBA1-positive cells and DAPI-positive cells; magnification, x400, n=6 in each group. HSP, heat shock protein; MCAO, middle cerebral artery occlusion; GFAP, glial fibrillary acidic protein IBA1, ionized calcium-binding adaptor molecule 1; DAPI, 4',6-diamidino-2-phenylindole.

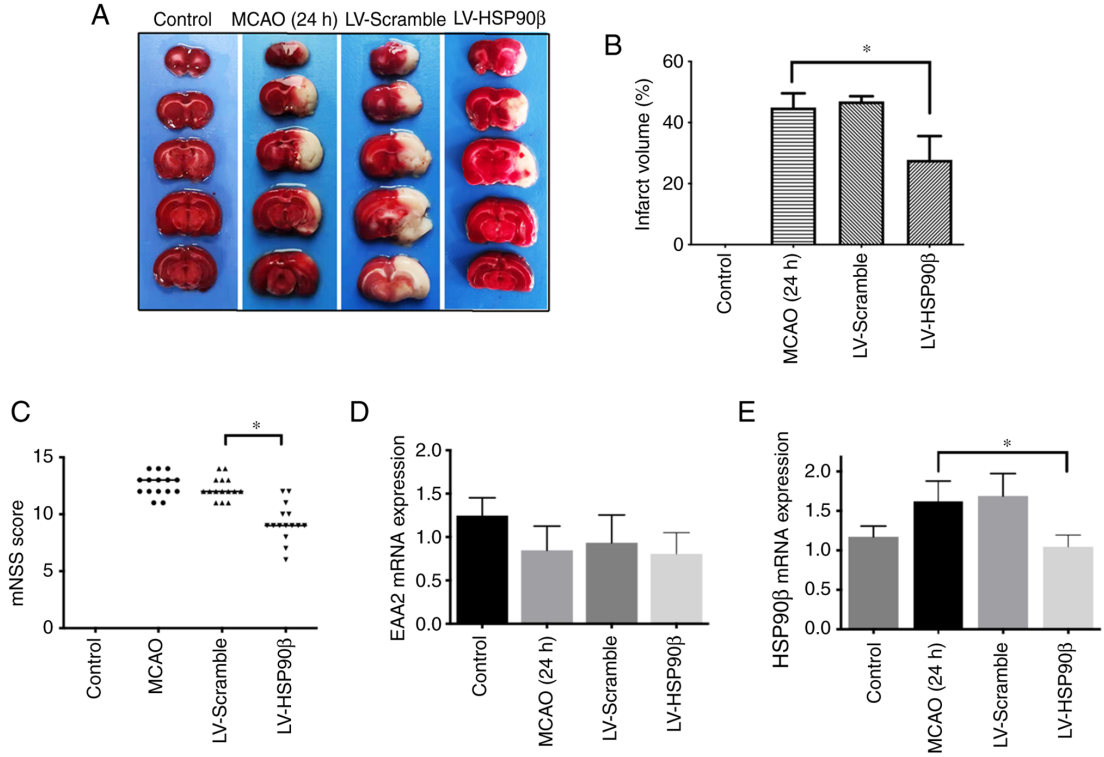

Figure 3. Inhibition of HSP90β expression exhibits a neuroprotective effect in the MCAO model. (A and B) After inhibiting HSP90β expression, the infarct volume ratio was significantly reduced compared with that in the MCAO group; n=6, *P<0.05 vs. control group. (C) The mNSS score of the lentivirus group was significantly lower compared with that of the MCAO group and the empty virus group; n=15, *P<0.05 vs. control group. Reverse transcription-quantitative PCR results showed that inhibition of HSP90β expression (D) did not affect EAAT2 expression but (E) decreased HSP90β mRNA expression; n=5, *P<0.05 vs. MCAO group. HSP, heat shock protein; MCAO, middle cerebral artery occlusion; mNSS, modified neurological severity score.

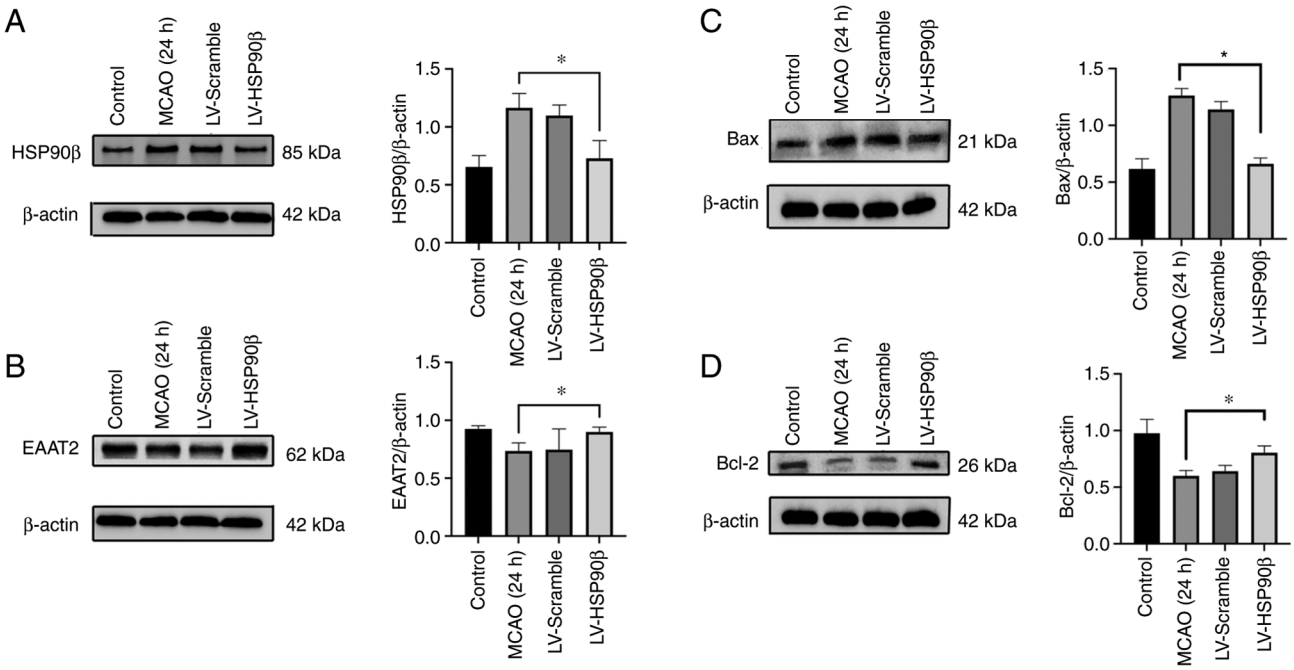

Figure 4. Inhibition of HSP90β expression increased EAAT2 protein expression levels. Compared with the MCAO group, (A) HSP90β expression was significantly decreased and (B) EAAT2 expression was significantly increased; n=6 in each group, *P<0.05 vs. control group. (C) The expression of Bax in the virus intervention group was significantly lower compared with the MCAO group; n=6 in each group, *P<0.05 vs. control group. (D) The expression of Bcl-2 in the virus intervention group was significantly higher than that in the MCAO group; n=6 in each group, *P<0.05 vs. control group. HSP, heat shock protein; EAAT, excitatory amino acid transporter; MCAO, middle cerebral artery occlusion.

to hypoxia-inducible factor 1 alpha, which provides neuroprotection through antioxidant effects (21). The HSP90 inhibitor geldanamycin exerts a dual inhibitory effect on JNK3 and has potent neuroprotective effects (22). Another study found that the HSP90 expression level was elevated after cerebral infarction and that the use of inhibitors reduced the cerebral infarction volume in mice; the mechanism may be related to the inhibition of the inflammatory NF-κB signaling pathway (23,24). Although some of these studies suggested that HSP90 was involved in the process of cerebral ischemic injury, the difference in the expression of HSP90α and HSP90β was not clearly indicated. HSP90β serves an essential role in maintaining cell viability, while HSP90α may mainly serve a role in ensuring cellular adaptability (10,25), suggesting the existence of different physiological functions of HSP90α and HSP90β. The results of the present study further confirmed that the increased expression of HSP90 after cerebral ischemia-reperfusion in rats was mainly attributed to a change in HSP90β expression, while HSP90α expression did not change significantly, suggesting that HSP90β is involved in the process of cerebral ischemia-reperfusion injury. Immunofluorescence staining showed that HSP90β was not significantly expressed in glial cells, including microglia and astrocytes, under normal conditions. However, after ischemia-reperfusion in rat brain, HSP90β expression overlapped with astrocyte markers in the ischemic penumbra area, while no expression was detected in microglia, suggesting that the increased HSP90β was mainly expressed on astrocytes. Astrocytes serve a major role in glutamate uptake from the surrounding neuronal synapsis and its posterior recycling into glutamine, which can then be reused by neurons as a substrate for glutamate synthesis (26). Microglia activation is one of the first events that occurs after an insult such as brain ischemia and some of microglia functions include an increase in phagocytosis rate, release of anti- or pro-inflammatory cytokines, proliferation and migration (27). The different role of astrocyte and microglia in ischemia-reperfusion injury related to HSP90β expression site selectivity.

The main function of EAAT2 is to transport extracellular excitatory amino acids inside cells to avoid excitotoxicity; EAAT2 is mainly expressed on astrocytes (5). Excitatory amino acid release, altered transporter function, receptor expression and the activation of downstream cell death signals caused by excitatory amino acids have become the focus of research on the mechanism of cerebral ischemic injury. EAAT2 dysfunction plays an important role in excitotoxicity and its associated neurological damage and dysregulation at the genetic, epistemic regulatory, transcriptional or translational levels may lead to EAAT2 dysfunction and ultimately cause neuronal cell death (28).

Studies have shown that regulating EAAT2 reduces the extracellular glutamate level, reduces the stimulation of excitatory receptors on the postsynaptic membrane by glutamate, reduces excitatory damage and exerts a neuroprotective effect (29-32). In the MCAO model, HSP90β and EAAT2 were mainly expressed on astrocytes. It was hypothesized that HSP90β may be related to EAAT2. Studies in the field of epilepsy have shown that increased HSP90β expression levels promote the degradation of EAAT2 and EAAT2 degradation is significantly reduced after its expression is inhibited. This mechanism allows the function of EAAT2 to be retained and functional EAAT2 transports extracellular excitatory amino acids, which reduces excitotoxicity (11,33). It has not yet been

determined whether this change occurs in ischemic stroke. The present study showed that after inhibiting the expression of HSP90β with a lentivirus, the mNSS score and the infarct area of the brain tissue in rats decreased, suggesting that inhibiting HSP90β expression has a neuroprotective effect. Western blotting and PCR results showed that inhibiting HSP90β expression increased the expression of EAAT2 protein but not EAAT2 mRNA, indicating that the effect of HSP90β on EAAT2 may be related to its posttranslational regulatory function, consistent with the present study Additionally, after inhibiting the expression of HSP90β, Bax expression was decreased and Bcl-2 expression was increased, indicating that inhibiting the expression of HSP90β reduced the death of neurons. Thus, inhibition of HSP90β exerted neuroprotective effects; HSP90β has the potential to be a molecular target of neuroprotection and inhibition of HSP90β may alleviate cerebral ischemia-reperfusion injury. However, the present study also has some limitations. On the one hand, it did not conduct cell-based experiments to verify that HSP90β promoted EAAT2 degradation or determine the degradation pathway. Sha *et al* (32) verified that HSP90β recruits EAAT2 into the 20S proteasome in human embryonic kidney cells, thereby promoting EAAT2 degradation; thus, this part of the work was not repeated. As the existing inhibitors do not have good selectivity for HSP90α and HSP90β, only a lentivirus was used to inhibit the expression of HSP90β to obtain the results, which is different from clinical applications. It is planned to perform all of these related experiments in the future.

An improved understanding of the exact mechanism of HSP90β involvement in cerebral ischemia-reperfusion injury is important for stroke treatment. It is to be hoped that a novel drug in the field of neuroscience that targets HSP90β will soon be available to provide a new approach for ischemic stroke treatment.

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Availability of data and materials

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

Authors' contributions

PX, JZh and ZX conceived and designed the study. TL, XH, JZe and LZ performed the experiments. TL and ZZ performed statistical analysis. TL and PX wrote the manuscript. JZe, ZX and PX reviewed and edited the manuscript. TL and PX confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures were approved by the Animal Care and Use Committee of Zunyi Medical University, Zunyi, China (approval number ZMU21-2203-487).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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