Neuroprotective Capabilities of Tanshinone IIA against Cerebral Ischemia/Reperfusion Injury via Anti-apoptotic Pathway in Rats

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Received August 13, 2011; accepted November 14, 2011; published online November 17, 2011

Danshen, derived from the dried root or rhizome of Salviae miltiorrhizae Bge., has Tanshinone IIA (TSA) as one of its active ingredients. Recent reports have shown that TSA can inhibit the apoptosis induced by serum withdrawal or ethanol in cultured PC12 cells. However, whether TSA has any neuroprotective effect remains unknown. In this study, we investigated the effects of TSA on cerebral apoptosis induced by middle cerebral artery occlusion (MCAO) in which cerebral ischemia had been induced 2 h earlier. Twenty-four hours after reperfusion, the rats were assessed for infarct volume etc. Intraperitoneal administration of 25 and 40 mg/kg TSA 10 min after MCAO significantly diminished infarct volume and brain water content and improved neurological deficits in a dose-dependent manner. The 25 mg/kg dosage was more effective. Treatment with 25 mg/kg TSA significantly improved symptoms and reduce infarct volume at different points in time, of which 10 min after MCAO was the most significant. Nissl-staining and HE-staining of the 25 mg/kg TSA group were more appreciable in terms of improvement relative to the vehicle group in the infarct core. TSA of dosage 25 mg/kg significantly decreased the expression of cleaved caspase-3 protein and increased the expression of B-cell lymphoma 2 (bcl-2) protein in the ischemic cortex. Fewer terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL)-positive cells were found in the penumbra of the treated group, but they were significantly more common in the vehicle group. We here conclude that the neuroprotective effects of TSA against focal cerebral ischemic/reperfusion injury are likely to be related to the attenuation of apoptosis.

Key words Tanshinone IIA; cerebral; apoptosis; ischemia; reperfusion

Stroke remains a leading cause of death and adult disability worldwide. Ischemic stroke accounts for approximately 80% of all strokes. Reperfusion of the occluded vessels is the standard treatment for acute ischemic stroke. The extent of brain damage is determined by the severity of primary injury and the intensity of secondary injury cascades that contribute to delayed cellular destruction. Apoptosis and oxidative stress have been found to play an important role in the pathogenesis of cerebral injury secondary to ischemia/reperfusion (I/R). The striking relationship between apoptosis and brain I/R injury has stimulated considerable interest in the development of anti-apoptosis therapies.

Danshen, a very important component of Chinese medicine derived from the dried root or rhizome of Salviae miltiorrhizae Bge. (SM), has been commonly used in China for the treatment of cardiovascular and cerebrovascular disease. Tanshinone IIA (TSA), a derivative of phenanthrenequinone, is a major active component of danshen (Fig. 1). Researchers have reported that TSA can inhibit serum-withdrawal- and ethanol-induced apoptosis in cultured PC12 cells, and other studies have demonstrated that TSA has protective effects against focal cerebral I/R injury. However, whether TSA has a neuroprotective effect against apoptosis remains unknown. Here, we studied the effects of TSA on cerebral apoptosis induced by middle cerebral artery occlusion (MCAO).

MATERIALS AND METHODS

Chemicals TSA was purchased from Sciphar Biotechnology Co. (Shaanxi, China). TSA was isolated from the roots of SM, based on the method described previously.

Briefly, the methylene chloride fraction of methanol extract of SM was subjected to column chromatography over a silicon gel and recrystallized. The purity of TSA (99%) was determined by high performance liquid chromatography (HPLC) according to the method provided for TSA assays in ‘Chinese Pharmacopoeia.’ TSA was dissolved in phosphate buffered saline (PBS) including 1% dimethyl sulfoxide (DMSO), 2,3,5-Triphenyltetrasodium chloride (TTC) was purchased from Sigma-Aldrich Co. (U.S.A).

Animals Adult male Sprague-Dawleys weighing 250±20 g were purchased from Chongqing Medical University. The protocol was approved by the institutional animal care and use committee and the local experimental ethics committee. All rats were allowed free access to food and water before surgery under optimal conditions (12/12 h light/dark with humidity 60±5%, 22±3°C).

Rat Model of Transient Focal Cerebral Ischemia Transient cerebral ischemia was induced by MCAO in rats as

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into a sham group, vehicle group, and 25 mg/kg TSA group (PBS including 1% DMSO) randomly divided into 5 groups (n=16 for each group), a sham group, vehicle group, 10 mg/kg TSA group, 25 mg/kg TSA group, and 40 mg/kg TSA group. TSA was injected intraperitoneally 10 min after MCAO. The vehicle, 1 mL/kg PBS including 1% DMSO was also given at this time. Experiment 2, therapeutic time window screening: 96 rats were divided into 5 groups (n=16 for each group). A dose of 25 mg/kg TSA or 1 mL/kg PBS including 1% DMSO was given 3 days before MCAO (once a day for 2 consecutive days with the last injection 2 h before MCAO), 10 min after MCAO, 0 h after reperfusion, and 2 h after reperfusion. Experiment 3, exploration of possible mechanisms: Here, 36 rats were divided into a sham group, vehicle group, and 25 mg/kg TSA group (n=12 for each group). A dose of 25 mg/kg TSA or 1 mL/kg PBS including 1% DMSO was given 10 min after MCAO.

Assessment of Neurological Deficits A neurological test was carried out by one examiner blinded to the experimental groups after 2 h of MCAO according to the method described by Longa et al.\textsuperscript{17,18} Neurological findings were scored on a five-point scale: 0, no neurological deficits; 1, failure to extend right forepaw fully; 2, circling to the right; 3, falling to the right; 4, inability to walk spontaneously and depressed levels of consciousness.

Measurement of Brain Water Content Samples from all five groups in experiment 1 (n=6—8 for each group) were used for assessment. The rats were killed with chloral hydrate anesthetize, and the brain was rapidly removed and dissected. Brain samples from the ischemic hemisphere were immediately weighed on an electronic balance and then dried in an oven at 100°C for 24 h to obtain the dry weight. Brain water content was calculated as follows: (wet weight−dry weight)/wet weight×100%.

Measurement of Infarct Volume in the Brain The other samples in experiment 1 (n=5—8 for each group) and samples in experiment 2 (n=6—8 for each group) were used for further analysis. At 24 h after reperfusion, rats were killed and their brains quickly removed and frozen at −20°C for 15 min. Coronal brain sections (2 mm thick) were stained with 2% TTC at 37°C for 20 min followed by fixation with 4% paraformaldehyde. The staining images were recorded using a digital camera (Canon Oxsus 950IS) and then quantified using an Image J (ver 1.37c, NIH). To compensate for the effect of brain edema, the corrected volume was calculated using the following equation: Percentage hemisphere lesion volume (%HLV)={([total infarct volume−(left hemisphere volume−right hemisphere volume)]/right hemisphere volume)×100%}. Infarct volume measurements were carried out by an investigator blinded to the treatment groups.

Histological Assessment Samples from three groups in experiment 3 (n=5—6 for each group) were used for experimentation. The rats were killed with chloral hydrate 24 h after reperfusion, and their brains were perfused transcardially with 0.9% sodium chloride followed by 4% paraformaldehyde. Following decapitation, brains were removed and embedded in paraffin. Coronal sections were taken at 1.20 mm anterior to the bregma and 3.6 mm posterior to the bregma. Five micrometers of the coronal sections were cut and stained with 0.1% cresyl violet (Nissl staining) or hematoxylin–eosin (HE staining) and prepared for subsequent microscopic mounting. Intact neurons were counted in ten high power fields under light microscope (×400).\textsuperscript{19}

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Biotin Nick-End Labeling (TUNEL) Staining The samples used for histological assessment were fixed immediately after reperfusion (neurological score 0) to the bregma and 3.6 mm posterior to the bregma. Five micrometers of the coronal sections were cut and stained with 0.1% cresyl violet (Nissl staining) or hematoxylin–eosin (HE staining) and prepared for subsequent microscopic mounting. Intact neurons were counted in ten high power fields under light microscope (×400).\textsuperscript{19}

Fig. 2. Effects of TSA on Neurological Deficit and Water Content 24 h after Focal Cerebral Ischemia in Rats

(A) The bar graph shows the neurological scores of animals (measured 2 h after MCAO). Mean±S.E.M. *p=0.05 vs. sham; **p<0.05 vs. vehicle, n=12—16. (B) The water content of ipsilateral hemispheres was significantly reduced in 25 mg/kg and 40 mg/kg TSA groups vs. vehicle group (*p<0.05), but no significant difference was found between the 10 mg/kg TSA group and vehicle group (p>0.05). No difference was found in contralateral hemispheres (p>0.05) (n=6—8 for each group).
Assessment were also used for TUNEL staining. TUNEL staining was performed according to the manufacturer’s instructions (Roche Molecular Biochemicals, Inc., Mannheim, Germany). Briefly, the tissue sections were treated with Proteinase K (20 μg/mL for 15 min) and 0.3% H₂O₂ for 30 min. Then the sections were incubated with terminal deoxyribonucleotidyl transferase (TdT) enzyme at 37°C for 1 h and further incubated with peroxidase-conjugated antibody for 30 min at 37°C. Sections were allowed to react with 3,3'-diaminobenzidine tetrahydrochloride solution for 10 min at room temperature. TUNEL-positive cells in the penumbra of brain were counted in ten high power fields under a light microscope (×400).

**Western Blot Analysis** The other samples from the three groups in experiment 3 (n=6 for each group) were used for Western blot analysis. Brains were quickly removed after 24 h of reperfusion. The ischemic cortex was harvested for the assay of protein expressions. The tissues were lysed in ice-cold lysis buffer (Tris-(hydroxymethyl)-aminomethane 50 mmol/L, pH 7.5, NaCl 150 mmol/L, 1% Triton X-100, edetic acid 1 mmol/L, phenylmethylsulfonyl fluoride 1 mol/L, and aprotinin 5 mg/L), centrifuged at 12000×g at 4°C for 20 min.

Fig. 3. Dose-Dependent and Time-Dependent Neuroprotection by TSA on Infarction at 24 h after Focal Cerebral Ischemia in Rats

(A, B) The infarct volumes were significantly decreased in 25 mg/kg and 40 mg/kg TSA groups relative to the vehicle group (*p<0.05), but no significant difference was found between the vehicle group and 10 mg/kg TSA group (p>0.05). Twenty-five milligrams per kilogram of TSA, however, showed a decrease in infarct volume relative to other doses (‘p<0.05 vs. 40 mg/kg TSA). (C) Infarct volume was significantly attenuated at 24 h with 25 mg/kg TSA treatment at any point of time (‘p<0.01 vs. sham, *p<0.05 vs. vehicle), of which 10 min after MCAO was the most significant (‘p<0.05, one-way ANOVA (n=6−8 for each group)).
The supernatants were then collected as total cellular proteins by electrophoresis through a 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). They were then transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline and incubated with primary antibody (anti-B-cell lymphoma 2 (bcl-2), 1:500, Santa Cruz; anti-cleaved caspase-3, 1:500, Cell Signaling Technology; anti-β-actin, 1:1000, Santa Cruz) overnight at 4°C. After four washes with TBS, membranes were incubated with the secondary antibodies (goat anti-rabbit, 1:1000) for 1 h at room temperature. Blots were subjected to gel formatter (BIO-RAD) and quantified by Quantity One analysis. β-Actin was used as an internal loading control.

Statistical Analysis All data were expressed as mean±S.E.M. The differences between various groups were analyzed by one-way analysis of variance (ANOVA) followed by multiple comparison tests as post hoc comparison. The neurological deficit scores were analyzed by Mann–Whitney U test. A level of p<0.05 was considered to be statistically significant and statistical software package SPSS17.0 was used.

RESULTS

Effect of TSA on Brain Water Content As shown in Fig. 2B, in the sham group, water content was 78.31±0.07%; and in the 25 mg/kg and 40 mg/kg TSA groups, brain water content was reduced (80.09±0.25%, 82.49±0.18%) relative to the vehicle group (83.84±0.79%, p<0.05); but no significant decrease was observed in the 10 mg/kg TSA group (83.04±0.49%, p>0.05) and no differences were found across contralateral hemispheres (p>0.05).

Effects of TSA on Infarction and Neurological Deficit To determine the neuroprotective effect of TSA against I/R injury, we measured the infarct volume and the neurological score with and without administration of TSA. No infarction was observed in the sham group, but extensive lesions developed in both the striatum and lateral cortex in the vehicle group. As shown in Figs. 2A, 3A, and 3B, relative to the vehicle group; neurological scores and cerebral infarct volumes were significantly decreased after treatment with 25 mg/kg and 40 mg/kg TSA (p<0.05), and no significant reduction was found in the treatment with 10 mg/kg TSA (p>0.05). The dose of 25 mg/kg TSA showed better protective effects than the other doses with regard to cerebral injury after MCAO (p<0.05). As shown in Fig. 3C, 25 mg/kg TSA reduced infarct size relative to the vehicle group at all points in time (p<0.05). Of these, 10 min after MCAO was most significant (p<0.05). We chose to administer 25 mg/kg TSA 10 min after MCAO in subsequent experiments.
Effects of TSA on Morphology and Nissl Staining  
HE-staining and Nissl-staining were used to determine the morphological changes in the infarct core. Figures 4A—C depict HE-staining and Nissl-staining of injured cerebral hemispheres 24h after reperfusion; neurons in the cortex showed no morphological changes in the sham group. In the vehicle group, there were many atrophic neurons, showing shrunken cytoplasm and damaged nuclei. Administration of 25 mg/kg TSA reduced the number of degenerated neurons and significantly increased the number of intact neurons ($p<0.05$).

Effects of TSA on in Situ Labeling of DNA Fragmentation  
Cerebral apoptosis was determined by terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining. Results are shown in Figs. 5A, B. TUNEL-positive cells containing apoptotic bodies and darkly stained cells were considered apoptotic. The sham group did not show any obviously TUNEL-positive cells, and the cells treated with 25 mg/kg TSA 10 min after MCAO showed a significantly reduced number of TUNEL-positive cells in the penumbra of brain relative to the vehicle group ($p<0.05$).

Effects of TSA on Expression of Cleaved Caspase-3 and Bcl-2  
The neuroprotective effects of TSA against transient cerebral I/R-induced apoptosis were investigated by analyzing the expressions of cleaved caspase-3 and bcl-2 in the ischemic cortex. Western blot analysis (Figs. 6A, B) of brain samples showed that the expression level of cleaved caspase-3 was increased in the vehicle group at 24h after focal cerebral ischemia but significantly decreased in the 25 mg/kg TSA group relative to the vehicle group ($p<0.05$). The expression level of bcl-2 was significantly increased in the 25 mg/kg TSA treated group in the brain sample after 24h relative to the vehicle group ($p<0.05$).
DISCUSSION

MCAO is a generally accepted model of cerebral ischemia. In this study, we have shown that TSA can reduce brain infarct volume and water content, and improve neurological outcomes differently at different dosages and at different administration times after focal cerebral I/R injury. TSA was here found to provide neuronal protection through anti-apoptotic mechanisms.

Recent studies have demonstrated that TSA reduces cerebral I/R injury. Liu et al. and Wang et al. reported that, relative to the vehicle group, TSA (20 mg/kg) dramatically lessened neurological deficit scores, brain water content, and infarct area as determined 24 h after ischemic injury; 30 mg/kg TSA was found to be most significant. In the present study, we chose a concentration between 20 mg/kg and 30 mg/kg and a markedly higher concentration 40 mg/kg. Our results show that TSA can improve neurological deficits and decrease brain infarct volume and water content in a dose- and time-dependent manner. We here provide evidence that 25 mg/kg is the optimal concentration. The reason for this, however, requires further exploration. Some studies have shown that pretreatment with some drugs and post-treatment with others can be effective. The present study provides evidence that TSA has a wide therapeutic time window and that 10 min after surgery is the ideal time for TSA administration.

It is now well known that apoptosis during I/R injury plays a major role in brain injury associated with stroke. Previous studies have demonstrated that TSA can protect the brain from I/R injury. However, whether TSA has a neuroprotective effect against apoptosis remains unknown. The present study investigated the neuroprotective effects of TSA. Our results showed a lower number of TUNEL-positive cells in the penumbra of the treated group, significantly more than in the vehicle group. To clarify the neuroprotective mechanism of TSA, we investigated the expressions of key apoptosis-related molecules and found that cleaved caspase-3 was increased in the vehicle group 24 h after focal cerebral ischemia but was significantly decreased in the 25 mg/kg TSA treatment group relative to the vehicle group. Bel-2, however, was found to be significantly increased in the 25 mg/kg TSA treatment group after 24 h relative to the vehicle group. The results of the present study suggest that TSA provides neuronal protection, at least partly by anti-apoptotic mechanisms.

Our results indicate that TSA improves outcomes after focal cerebral ischemia and that these effects appear to be related to anti-apoptotic action by TSA.

Acknowledgments This work was supported by Grants from National Nature Science Foundation of China (No. 30900457 and No. 81070917).

REFERENCES


