Supporting Information

Salviaolate Protects Rat Brain from Ischemia-Reperfusion Injury through Inhibition of NADPH Oxidase

Zheng Lou1*, Kai-Di Ren1*, Bin Tan1, Jing-Jie Peng1,2, Xian Ren1, Zhong-Bao Yang1, Bin Liu1, Jie Yang3, Qi-Lin Ma4, Xiu-Ju Luo2, Jun Peng1

*These authors contributed equally to this work.

Affiliations

1Department of Pharmacology, School of Pharmaceutical Sciences, Central South University, Changsha, China
2Department of Laboratory Medicine, Xiangya School of Medicine, Central South University, Changsha, China
3Department of Neurology, Xiangya Hospital, Central South University, Changsha, China
4Department of Cardiovascular Medicine, Xiangya Hospital, Central South University, Changsha, China
Correspondence

Professor Dr. Jun Peng

Department of Pharmacology
School of Pharmaceutical Sciences
Central South University
Changsha 410078
China
junpeng@csu.edu.cn

Dr. Xiu-Ju Luo

Department of Laboratory Medicine
Xiangya School of Medicine
Central South University
Changsha 410013
China
xjluo22@csu.edu.cn
**Cell experiment**

To verify the beneficial effect of salvialiate against I/R injury in an *in vivo* model, an additional set of experiments were performed in a nerve cell model of H/R injury *in vitro*. NG108-15 cells were randomly divided into five groups (six individual experiments per group): the control group, without any treatment; the H/R group, cells were subjected to 5 h of hypoxia followed by 20 h of reoxygenation; and the salvialiate (low dose, medium dose, or high dose) plus H/R groups, salvialiate (3, 10, or 30 mg/L) was added to the culture medium before the cells were subjected to H/R. At the end of the experiments, the culture mediums or cells were collected for analysis of cellular apoptosis as well as LDH release.

**Rat model of cerebral ischemia/reperfusion injury**

The rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). The left common carotid artery was exposed and clipped with an artery clamp. The external carotid artery was isolated and ligatured. A nylon suture with a blunted tip (0.40 mm diameter) was introduced through a small incision in the external carotid artery and then into the internal carotid artery. The middle cerebral artery was occluded by a nylon suture in the position 18-20 mm distal from a carotid bifurcation. The nylon suture was left in place for 2 h and then withdrawn for reperfusion for 24 h. The rats were returned to their cage with free access to water and food after the incision was closed. Animals from the sham group underwent the same procedure except that the occluding filament was inserted only 7 mm above the carotid bifurcation.
Measurement of infarct volume

The infarct volume was evaluated by 2,3,5-triphenyltetrazolium chloride (TTC) staining. After a neurological function evaluation, the rats were sacrificed under the condition of anesthesia. Brains were rapidly removed and were sliced into 2-mm thick coronal sections with the aid of a brain matrix. Sections were stained with 2% TTC for 10 min at 37°C, followed by overnight immersion in 4% paraformaldehyde, then scanned into a computer, and measured with the imaging analysis software. The presence or absence of infarction was determined by examining the TTC stain. The infarct volume (in cm³) for each section was equal to the infarct area (in cm²) multiplied by the section thickness (0.2 or 0.3 cm). The total infarct volume for each brain was then calculated by summing up the infarct volume of all sections. To minimize the effect of edema on the accuracy of the infarct volume measurement, the final infarct volume was corrected by the following equation: corrected infarct volume = total infarct volume × (left hemisphere volume/right hemisphere volume). Here, right hemisphere refers to the no ischemic hemisphere of the brain, while the left hemisphere refers to the ischemic contralateral side (see definition in Fig. 2B).

Measurement of cellular apoptosis

Cellular apoptosis in the brain tissue from the different groups was analyzed by a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. The procedure was performed according to the manufacturer’s instructions. TUNEL reaction mixture without terminal transferase served as a negative control.
The brain slides were examined microscopically at 200× magnification and photographed by a high-resolution digital camera. Two distinct patterns of TUNEL staining cells were observed. Some were densely stained and displayed clear apoptotic characteristics while others were slightly stained and were considered to be necrotic cells. Only the former were counted as TUNEL-positive cells. Twenty random high-power fields in the ischemic boundary area from each brain sample were chosen and blindly quantified. The total number of positively stained cells was counted and expressed as cell numbers per mm² of brain tissue.

Hoechst staining was used to evaluate H/R-induced NG108-15 cell apoptosis. The procedure for Hoechst staining was performed following the manufacturer’s instruction. Briefly, the NG108-15 cells were fixed for 15 min in 4% paraformaldehyde, washed in PBS, air-dried, and incubated at room temperature for 5 min with 1 g/mL of Hoechst 33258, a bisbenzimide cell-permeant dye that emits a bright blue fluorescence on binding to DNA. Stained cells were washed twice with PBS and imaged under a fluorescent microscope (excitation, 350 nm; emission, 460 nm). Twenty random high-power fields from each sample were chosen and blindly quantified. The number of apoptosis cells was presented as the percentage of the total cells.

**Measurement of activities of SOD and catalase**

Total SOD activities were measured following the manufacturer’s instructions. Briefly, 20 μl of cell lysates were incubated with 18 μl of WST-1 and 20 μl of enzyme
working solution at 37°C for 20 min. The change of absorbance at 450 nm was monitored by a spectrophotometer. One unit of SOD is defined as the amount of the enzyme in 20 μl of sample solution that inhibits the reduction reaction of WST-1 with superoxide anion by 50%.

For the measurement of catalase activity, the reaction mixture was incubated for 2 min at 37°C and the absorbance was monitored at 240 nm. The change in absorbance with time was proportional to the breakdown of H₂O₂. The catalase activity was expressed as U/g of protein, and 1 U of enzyme activity was defined as the amount of enzyme required to degrade 1 µmol of H₂O₂ in a second per g of protein.

**Determination of the anti-superoxide formation activity in brain tissue**

The superoxide anion (O₂⁻) level in the brain tissue was reflected indirectly by measuring the anti-O₂⁻ formation activity following the instructions provided by the O₂⁻ assay kit supplier. Briefly, the formation of O₂⁻ was based on the xanthine-xanthine oxidase reaction system. The final step was terminated by adding Griess reagent and the absorbance was determined at 550 nm with a spectrophotometer.
**Fig. S1** Effect of salvialolate on the activities of SOD, catalase, and anti-superoxide formation following cerebral I/R. **A** SOD activity in the brain tissue from each group. **B** Catalase activity in the brain tissue from each group. **C** The anti-superoxide formation activity in each group. All values are expressed as means ± S.E.M.; n = 6 in each group.

I/R: ischemia/reperfusion; +salviaolate: I/R + salviaolate; +MLB: I/R + magnesium lithospermate B; +edaravone: I/R + edaravone; +vehicle: I/R + vehicle of salviaolate (0.9% NaCl); **p < 0.01 vs. sham; ++ p < 0.01 vs. I/R.
**Fig. S2** Effect of salviaolate on hypoxia/reoxygenation-induced NG108-15 cells injury. **A** Representative image of Hoechst staining from each group. The apoptotic cells are indicated by arrows. **B** Percentage of apoptotic cells per total number of NG108-15 cells in each group. **C** LDH release (indication for cellular damage) from NG108-15 cells in each group. All values are expressed as means ± S.E.M.; n = 6 in each group.

H/R: hypoxia/reoxygenation; +salviaolate (L, M, or H): H/R + salviaolate (3, 10, or 30 mg/L); **p < 0.01 vs. control; +p < 0.05, ++p < 0.01 vs. H/R.