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Original article

Protective Effect of Salvianolic Acid A on Brain Endothelial Cells after Treatment with Deprivation and Reperfusion of Oxygen-glucose

Si-qi Feng, Jian-liang Geng, Run-bin Sun, Jing-qiu Huang, Zhao-yi Tan, Cai-xia Yan, Ji-ye AA*, Guang-ji Wang

Lab of Metabolomics Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing 210009, China

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ABSTRACT

Objective Salvianolic acid A (SAA) has a significant protective effect on ischemia/reperfusion injury of brain. However, it is not clear for SAA to exert its cerebral protection by targeting at the microvascular endothelial cells of blood brain barrier (BBB). Our previous study demonstrated that SAA could hardly pass through the BBB. This present study was therefore designed to investigate the protective effect of SAA on brain microvascular endothelial cells (BMECs) induced by deprivation and reperfusion with oxygen-glucose. **Methods** Rat BMECs were treated with oxygen glucose deprivation (OGD), followed by reperfusion (OGD/R). Cell viability was assessed by MTT and the content of reactive oxygen species (ROS) in cells after OGD/R in the absence or presence of SAA. GC-MS based metabolomic platform was applied to evaluate the regulation of SAA on the cellular metabolic perturbation induced by OGD/R. **Results** OGD/R significantly increased the production of intracellular reactive oxygen species (ROS), and decreased the activity of cells. SAA significantly reduced ROS and improve the cell viability. Metabolomic study revealed distinct perturbation of metabolic pathways of energy metabolism in the BMEC induced by OGD/R, while SAA significantly regulated the perturbed metabolism involved in energy metabolism pathways, especially for intermediates in TCA cycle. **Conclusion** SAA shows protective effects on BMECs involved in central nervous system.

Key words

metabolomics; oxygen glucose deprivation reperfusion; salvianolic acid A

1. Introduction

Ischemia and blood reperfusion usually cause serious injury of central nervous system including energy depletion, excessive production of reactive oxygen species (ROS), inflammation and so on. ROS is involved in the pathological process of many diseases including central nervous system disease (Li et al, 2013; Yan et al, 2013). Cerebral endothelial cell is an important component of blood-brain barrier (BBB) which limits the transportation of the most polar molecules

* **Corresponding author: AA JY** Tel/Fax: +86-25-8327 1081 E-mail: jiyea@cpu.edu.cn

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due to its unique structure. BBB plays an important biological role in maintenance of vessel permeability and the prevention of formation of blood coagulation and thrombus (Ballabh et al, 2004; Correale and Villa, 2007). The brain microvascular endothelial cells (BMECs) are involved in the process of inflammation and immune response, and neuronal damage during cerebral ischemia/reperfusion (Sandoval and Witt, 2008). In this study, we focus on the injury of BMECs induced by oxygen glucose deprivation (OGD) or OGD followed by reperfusion (OGD/R) and further, the protective effect of Salvianolic acid A (SAA) on OGD/R.

SAA is a main water-soluble phenolic acid with definite anti-oxidative activity in *Salvia miltiorrhiza* (SM, known as *danshen* in Chinese). It was reported that SAA acted as the ROS scavenger primarily targeted at the Nrf2/HO-1 axis (Yang et al, 2012; Zhang et al, 2014) and neutrophil (Lin et al, 1996), which showed significant protective effect on cerebral ischemia/reperfusion damage (Du and Zhang, 1997; Wang et al, 2012). An *in vitro* study demonstrated that SAA played a key role in cerebral protection by inhibiting granulocyte adherencein brain microvascular endothelial cells (Jiang et al, 2011). Our pilot study demonstrated the cerebral protection via its anti- oxidative and metabolic regulation after cerebral ischemia/ reperfusion in a MCAO model, but SAA could not easily passed through the BBB, indicating its protective effect on brain microvascular endothelial cells (BMECs). To examine the protective effect of SAA on microvascular endothelial cells forming BBB, rat BMECs were used as the *in vitro* model in this study, the effect of oxygen glucose deprivation (OGD), followed by reperfusion (OGD/R), and the protective effect of SAA were evaluated based on the physio- and pathological status of the cells and the metabolic patterns of the cells. The endogenous metabolites were profiled and analyzed by using a GC/MS platform of metabolomics (Jonsson et al, 2005).

2. Materials and methods

2.1 Chemicals and reagents

SAA was obtained from Chengdu Must Bio-technology Co., Ltd (purity 98%). Myristic-1,2-$^{13}C_2$ acid, 99 atom % ^{13}C, the stable isotope-labeled internal standard compound (IS), methoxyamine hydrochloride (purity of 98%), alkane solution (C8–C40), and pyridine (≥ 99.8% GC) were purchased from Sigma-Aldrich. *N*-methyl-*N*-trimethylsilyltrifluoro- acetamide (MSTFA) and 1% trimethylchlorosilane (TMCS) were obtained from Pierce Chemical Company (USA). DCFH-DA and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrasodium bromide (MTT) were purchased from Beyotime (China) and Sigma (USA), respectively. Purified water was produced by a Milli-Q system (Millipore, USA).

2.2 Cell culture

RBE4 were purchased from Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin (Invitrogen, USA) at 37 °C with 5% carbon dioxide.

2.3 Oxygen-glucose deprivation and reperfusion

Confluent cells (90%) were washed for three times with PBS (0.2 g/L KCl, 0.22 g/L KH_2PO_4, 8 g/L NaCl and 2.08 g/L Na_2HPO_4:$12H_2O$) and culture medium was replaced by PBS. Then, cell were transferred to a tri-gas incubator (Sanyo, Japan) with 7% CO_2 and 1% O_2 and incubated for 4 h (OGD_4). After oxygen-glucose deprivation (OGD) injury, cells were cultured for 12 h under normal conditions (OGD/R_{12}).

3. Experiment protocols and drug administration

3.1 Determination of intracellular ROS

RBE4 cells were cultured in 12-well plates and treated with 1, 5 or 10 μmol/L SAA. After treatment, the level of intracellular ROS was detected by the conversion of non-fluorescent DCFH-DA to its fluorescent derivative. After washing with cold PBS, the fluorescence intensity was measured at 535 nm with a Synergy-H1 fluorimeter (Bio-Tek Instruments).

3.2 Cell viability analysis

RBE4 cells (2×10^5 cells/mL) were cultured in 96-well plates for MTT assay. Control group was incubated with serum free medium with dimethyl sulfoxide. After OGD/R injury, culture medium was replaced with serum free medium with 20 μL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrasodium bromide (MTT) solution (5 mg/mL in PBS). After 4 h incubation, the medium was discarded and purple formazan product of MTT was dissolved in 150 μL of DMSO. The absorbance was detected at a wavelength of 490 nm.

3.3 Metabolomics analysis

For metabolomics analysis of cells, rBE4 cells were incubated with 1.0 μmol/L SAA when grown to nearly 90% confluency in 6-well plates. Following 4 h OGD and 12 h reperfusion, 100 μL cultured medium was collected in a new eppendorf tube and the remained medium was discarded. The adherent cells were washed with cold PBS twice immediately. Then, 300 μL of ultra pure water was added to each well. The cell cultured plates were stored at –80 °C for quench metabolism until extraction. The extraction process of intracellular metabolites and culture medium was performed as previously described (Cao et al, 2013). The cell lysates (20 μL) was used for the measurement of protein concentration which was calculated for the correction of contents of metabolites in cells.

The cell samples were subjected to three freeze-thaw