Journal of Animal and Veterinary Advances 13 (11): 664-667, 2014
ISSN: 1680-5593

Salvianolate Inhibites Upregulation of TLR4 in Cultured Rat's Microglia after Oxygen-Glucose Deprivation/Refusion Injury

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Abstract: To evaluate the effects of salvianolate, an aqueous extract of Salviae miltiorrhizae on the expression of Toll-Like Receptor 4 (TLR4) in microglia after Oxygen-Glucose Deprivation (OGD) followed by refusion using Real Time fluorescence quantitative PCR (RT-qPCR). The expression of TLR4 in primary cultured rat's microglia was examined after OGD/Refusion injury, the interfering effect of salvianolate on TLR4 expression was investigated under the same condition. Experimental groups; control group 1: the rat's microglia cells were maintained in cell culture medium (DMEM/F12 supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) normal oxygen supply. Model group 2: cultured microglia cells were deprived of oxygen-glucose for 4 h and then refused for 12 h. Salvianolate group 3: microglia were incubated with salvianolate dissolved in glucose-free DMEM (10 mg L^{-1}) during OGD then maintained in the normal cell culture medium containing same concentration of salvianolate during refusion. The expressions of TLR4 in microglia cells in different experimental groups were tested by RT-qPCR. The expression level of TLR4 mRNA in model group was higher than that in control group also higher than that in salvianolate group with statistical significance ($p<0.05$) however there was no significant difference between the expression in control group and salvianolate group ($p>0.05$). OGD/Refusion injury activated the TLR4 signaling pathway in cultured microglia. The endogenous mediators might act as the ligands to up-regulate TLR4 and salvianolate significantly inhibited the activity. These results highlighted the importance of salvianolate as a potential therapeutic target in treating cerebral ischemia.

Key words: *Salviae miltiorrhizae*, salvianolate, microglia, oxygen-glucose deprivation, TLR4, RT-QPCR

INTRODUCTION

Ischemic stroke is a clinical syndrome characterized by local brain tissue ischemic necrosis or softening which is caused by disturbance in the blood supply to the brain either is blockage (cerebral arterial thrombosis, arterial embolism) or a hemorrhage both can case local ischemia-hypoxia (Ginsberg and Busto, 1989). This is one of the major diseases which threats human health with high morbidity, high mortality, high disability rate and high recurrence rate. Recent reports (Gachet, 2012; Boscia *et al*., 2013) suggest that activation of microglia cells may arise following local cerebral hypoxia-ischemia they increase the secretion of cytokines and up-regulate genes expression in order to clear the metabolites and swallow the tissue fragments surrounding the lesion. Microglia is not only a sensitive indicator of central nervous system lesions but also a key factor in many neurotoxicity and degenerative diseases (Mika and Prochnow, 2012). Excessive activation of microglia cells produces more neurotoxic cytokines or proteins which increased neuronal damage, inflammatory receptor plays an important role in this process. Salvia miltiorrhiza, a traditional Chinese medicine which improves patient's blood circulation (Zhou *et al*., 2005) is often used in the clinical treatment of coronary heart disease, angina, ischemic stroke and other diseases, Salvianolate is the main active ingredients of Salvia miltiorrhiza. TLR4 is an important immune molecule in the brain (Arumugam *et al*., 2009) however, it is still unclear the effect of OGD/Refusion injury on TLR4 pathway in microglia cells. What also unclear is the interfering effects of Salvianolate. In this study, primary cultured microglia cells have been maintained in an OGD/Refusion environment, the effect of OGD/Refusion injury on TLR4 expression and the interfering effect of salvianolate have been investigated in order to unveil the possible mechanism of salvianolate in the stroke patients.

MATERIALS AND METHODS

Materials: Rat microglia cells were purchased from ScienCell, USA, DMEM/F12 cell culture medium was obtained from GIBCO, USA, high-purity total RNA

Extraction kit was purchased from Generay Company, Reverse Transcription kit-Revert Aid First Strand cDNA synthesis kit was purchased from Fermentas Company, qPCR reagents IQ SYBR Green Supermix was purchased from Bio-Rad Company. Salvianolate purchased from Sichuan Xin Fu Kang Pharmaceutical Co., Ltd.

OGD/Refusion Model preparation and experimental groups: Well maintained rat microglia cells were used in the experiments, the cell concentration was adjusted to 1×10^{9} L^{-1} seeded into 24 well culture plate each well contained 1 mL. When the microglia cells grown to 80% confluence, washed the cells twice with PBS then replaced the normal culture medium with sugar-free DMEM. Oxygen-deprivation condition was simulated in the anaerobic box, the culture plates were placed in the box then supplied the gas mixture containing 95% N_2 and 5% CO_2 for 20 min (flow rate 1.5 L min^{-1}), clipped the inlet and outlet of the box, cultured the plates in 37°C for 4 h, later changed the culture medium with normal medium and incubated the plates under oxygen condition for 12 h.

Experimental groups; control group 1: the rat microglia cells were cultured in normal culture medium (DMEM/F12 medium containing 10% FBS) without oxygen deprivation. Model group 2: microglia cells were deprived of oxygen and glucose for 4 h then refused for 12 h. Salvianolate group 3: Microglia cells were incubated with salvianolate dissolved in glucose-free DMEM (final concentration of 10 mg L^{-1}) during OGD then maintained in the normal culture medium containing same concentration of salvianolate during refusion.

RT-qPCR detection of TLR4 mRNA expression: Trizol Method was used to extract total cellular RNA from different samples, UV spectrophotometer was used to assess the nuclei acid purity with the A260/A280 ratios, the result was 1.8-2.0 which confirming RNA purity meets the requirements for RT-PCR reactions. Using total RNA as a template, Oligo-d (T) 18 as primer to reverse transcriptase first strand cDNA, specific primers were used to amplify TLR4 and β-actin individually. Homo TLR4 forward, 5'-GCACTGTTCTTCTCCTGCC-3'; Homo TLR4 reverse: 5'-GTTTCCTGTCAGTATCAAG-3'; PCR product size: 250 bp. Homo β-actin forward: 5'-TGTGCTA TGTTGCCCTAGACT-3'; Homo β-actin reverse: 5'-TCGT ACTCCTGCTTGCTGAT-3' (Table 1) PCR product size: 442 bp. CFX connect Real-Time PCR System was used to analysis quantitative PCR, PCR conditions were 94°C for 5 min, 95°C denaturation 30 sec, 54°C annealing 30 sec, 72°C extension 30 sec, TLR4 35 cycles, β-actin 30 cycles, extension at 72°C for 7 min. The 1.5% agarose gel electrophoresis used to test PCR products, quantity one software was used to analysis the gray values of TLR4 and β-actin PCR products, the ratio of TLR4 mRNA expression to β-actin was measured, experiments were repeated 3 times.

Table 1: RT-QPCR primer sequences

Genes	Primers sequence
TLR4 forward	5'-GCACTGTTCTTCTCCTGCC-3'
TLR4 reverse	5'-GTTTCCTGTCAGTATCAAG-3'
β-actin forward	5'-TGTGCTATGTTGCCCTAGACT-3'
β-actin reverse	5'-TCGTACTCCTGCTTGCTGAT-3'

Statistical analyses: Experimental data was analyzed and processed using SPSS 13.0 Statistical Software. t-test was used to compare the groups differences. The $p<0.05$ indicated that there was a significant difference.

RESULTS

The ct value of *TLR4* gene RT-qPCR amplification curve (Fig. 1) was between 20-35 which indicating the normal gene amplification. *TLR4* gene RT-qPCR melting curve (Fig. 2) showed a steep peak indicating that the product was specific.

The quantitative PCR results were calculated and statistically analysed using BIO-RAD CFX Manager

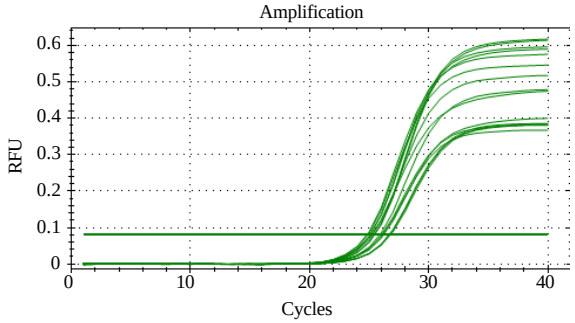

Fig. 1: *TLR4* gene RT-qPCR amplification curves

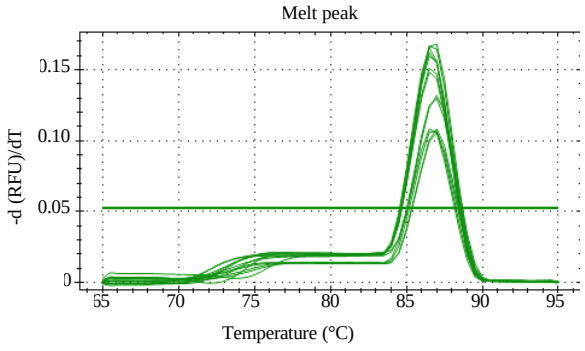

Fig. 2: *TLR4* gene RT-qPCR dissolution curves

Table 2: Comparison of the relative expression of TLR4 mRNA in each group

Groups	Relative expression of TLR4 mRNA
Control	1.00±0.27[a]
Model	1.59±0.16
Salvianolate	1.08±0.24[ab]

[a]p<0.05 vs. model group, [b]p>0.05 vs. control group

Analysis Software, experiment was repeated three times, the average values of TLR4 mRNA expression were used in statistics. RT-qPCR results showed that TLR4 mRNA expression level in the model group was relative higher than the expression in control group, the difference was statistically significant ($p<0.05$). The expression of TLR4 mRNA in model group was also higher than the expression level in salvianolate group, the difference was statistically significant ($p<0.05$). There was no statistically significant difference between TLR4 mRNA expression in control group and salvianolate group ($p>0.05$) (Table 2).

DISCUSSION

TLRs are an important group of protein molecules which are involved in non-specific immunity (Hamanaka and Hara, 2011) and function as a bridge to connect non-specific and specific immunity. TLR4 is one of the most important immune molecules in the brain. TLR4 can be activated by its ligands and produce a cascade in signal pathway, prompting the release of pro-inflammatory cytokines and subsequent immune response. All the toll-like receptors are homologous type-transmembrane protein can be divided into the outer membrane, cytoplasmic and transmembrane region of three parts. Currently, mainly two TLR4 signalling pathways have been proven: MyD88-dependent signalling pathway and MyD88-independent pathway (Fang and Hu, 2011). Long term of evolution gives the natural plants power to grow and form various molecules structures with biologically activity which become the material basis for pharmacodynamics. Salvianolate, an effective ingredient of salvia is derived from natural plants and plays an important role in improving blood circulation in humans. A growing number of clinical studies approved that (Yagi and Takeo, 2003; Sze *et al*., 2005) salvianolate can benefit microcirculation, improve antithrombotic effects, anti-inflammatory, antioxidant, alleviate ischemia-reperfusion injury, scavenge free radicals, inhibit the release of endothelin, block calcium channels and so on. It is characteristics of multi-targets and multi-link function in the pharmacological activities and widely used in clinical treating coronary heart disease, angina, stroke and other diseases in China and has a significant beneficial effect in therapy. The current study investigated the effects of OGD/Refusion injury on TLR4 signal pathway and the interfering effect of salvianolate using RT-qPCR. The results showed that the expression of TLR4 mRNA was higher in model group than that in control group with statistically significant difference which indicated that TLR4 signal pathway could be activated by OGD/Refusion injury and intracellular mediators which induced by the injury might serve as ligands to active TLR4. Another results showed that TLR4 mRNA expression level in model group was higher than that in salvianolate group with statistically significant difference. Therefore, researcher believed that the interfering effects of salvianolate significantly inhibited the expression of TLR4 mRNA which means salvianolate inhibited the TLR4 expression at transcriptional level.

CONCLUSION

In general, OGD/Refusion injury activated the microglial TLR4 signal pathway also induced the activation of microglia to fulfill the role of immune defender in cerebral ischemia. However, excessive activation of microglia cells caused severe brain tissue damages. Salvianolate significantly inhibited the activity of TLR4 pathway in microglia cells which might suggested that salvianolate, the active ingredient of salvia salvianolate, have certain therapeutic effect on cerebral ischemia.

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