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Hydroxysafflor yellow A suppresses inflammatory responses of BV2 microglia after oxygen–glucose deprivation

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HIGHLIGHTS

- HSYA protected BV2 microglia against OGD-induced cell injury.
- HSYA suppressed inflammatory responses in BV2 microglia induced by OGD.
- HSYA inhibited NF-κB signaling pathway and phosphorylation of p38.

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ABSTRACT

Inflammation is a pivotal pathological progress in the development of ischemic stroke. Modulating inflammatory cytokines released by microglia is thought to be a potential strategy for the treatment of ischemic stroke. Hydroxy-safflor yellow A (HSYA), a chemical component of the safflower yellow pigments, was reported to protect against brain injury in experimental stroke through anti-inflammation. However, the direct effect of HSYA on microglia following ischemia is unknown. This study confirmed whether HSYA could suppress inflammatory responses of BV2 microglia after oxygen glucose deprivation (OGD). BV2 microglia viability after OGD with or without HSYA was measured by MTT assay, PI/Annexin staining and LDH assay. Pro-inflammatory cytokines including 1L-1β, TNF-α, iNOS, COX-2, MCP-1 were determined by RT-PCR and western blotting. Activity of NF-κB and MAPK pathway were detected by western blotting. The results demonstrated that HSYA improved the viability of BV2 cells 12 h after OGD with the profound dosage at 100 mg/L by MTT assay. This observation was also confirmed by PI/Annexin staining and LDH assay. HSYA decreased the mRNA level of 1L-1β, TNF-α, iNOS, COX-2, MCP-1 and protein level of iNOS, COX-2 in BV2 microglia 12 h after OGD. OGD enhanced the phosphorylation of p38 and nuclear translocation of p65 in BV2 microglia, which was partially reserved by HSYA. Our results suggested that HSYA suppressed inflammatory responses in BV2 microglia induced by OGD, which is probably associated with the inhibition of the NF-κB signaling pathway and phosphorylation of p38.

1. Introduction

Ischemic stroke is one of the leading causes of morbidity and mortality, which brings a great burden to the family and society. It is widely accepted that inflammation plays a critical role in the progression of ischemic stroke [3,10]. Once ischemic stroke occurs, inflammatory response is initiated and characterized by up-regulated pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6 [9]. These pro-inflammatory cytokines play a critical role in neuronal dysfunction and neuronal death in ischemic stroke [5]. Thus, modulating these inflammatory cytokines or their regulatory pathways is a potential strategy for the treatment of ischemic stroke.

Abbreviations: CNS, central nervous system; ERK1/2, extracellular signal-regulated kinases; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSYA, hydroxy-safflor yellow A; JNK, c-Jun N-terminal kinases; IL-1β, interleukin-1β; LDH, Lactate dehydrogenase; MAPK, mitogen-activated protein kinase; OGD, oxygen glucose deprivation; PI, propidium iodide; TNF-α, tumor necrosis factor–α.

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Hydroxy-safflor yellow A (HSYA) is the main effective chemical component of the safflower yellow pigments [12]. It has been demonstrated to have several pharmacological effects, such as anti-thrombosis [13], anti-platelet aggregation [17] and cardioprotective effects [7]. Previous studies have demonstrated HSYA has promising neuroprotection under several ischemic models *in vivo* and in *vitro* [22] through a variety of mechanisms including antioxidant [14] and anti-excitatory effects [15]. But HSYA's anti-inflamatory effects may be its most potent protective mechanism [16]. Although some work has been done to explore the anti-inflammatory function of HSYA in cerebral ischemic models, few studies examined the anti-inflammatory effect of HSYA in microglia directly.

Microglia, the resident macrophages of the central nervous system (CNS), are now recognized as the prime cells in the immune defense and inflammatory response [5]. In physiological condition, resting microglia plays an important role in homeostasis within the CNS and to support neuronal cell function [2]. Upon activation, microglia undergo morphologic alterations, changing from resting microglia into activated microglia. Activated microglia have the capability to produce pro-inflammatory mediators and neurotoxic compounds such as IL-1β, IL-6, and tumor necrosis factor (TNF)-α, reactive oxygen species, nitric oxide, and prostaglandin E2 [8], which is considered to be an important determinant of the neuronal death in cerebral ischemia.

The present study was conducted to demonstrate the protective effect of HSYA on BV2 microglia after OGD. Furthermore, we investigated the anti-inflammatory action of HSYA in BV2 microglia and its underlying mechanisms.

2. Methods

2.1. BV2 microglia culture and treatments

The BV2 microglia were obtained from American Type Culture Collection and cultured in dulbecco's modified eagle medium supplemented with 10% fetal bovine serum. OGD model was made as described previously [18]. BV2 microglia were switched from a normal feeding medium to an oxygen-depleted, glucose-free medium, then incubated in a hypoxia chamber for 15 min with 5% CO_2/95% N_2. Valves were closed, and chambers were incubated at 37 °C for 3 h. At the end of OGD, cells were returned to the normal feeding medium and incubated under normal conditions at 37 °C for 24 h for the later experiments. BV2 microglia were treated with the indicated concentrations of HSYA (Pharmaceutical Co. Ltd. Yongning, China) or saline as vehicle immediately after OGD. The experimental design is shown in Fig. 1A.

2.2. Cell viability and death

BV2 microglia viability was evaluated by MTT assay and LDH assay. After various treatments, cell medium in 96-well plates was replaced with 0.5 mg/ml MTT in fresh medium for 4 h. The supernatant was removed and 100 μl dimethyl sulfoxide was added. The optical density was assessed at 570 nm using an ELISA plate reader (TECAN, Switzerland). Cell survival rates were expressed as percentages of the value of normal cells. The amount of LDH released by cells was determined by a LDH assay kit according to manufacture's protocol (Nanjing Institute of Jianchen Biological Engineering, China). The results were calculated by the following formula: LDH cytotoxicity = (sample OD − blank OD)/(standard solution OD − blank standard solution OD) × 2000. Apoptosis was determined by Annexin V-FITC binding and propidium iodide (PI) staining by using apoptosis detection kit (KeyGen Biotech, Nanjing, China) according to the manufacture's instructions. After treatment, the cells stained with 0.5 ml binding buffer containing 5 μl Annexin V and 5 μl PI for 15 min at 37 °C in the dark. The apoptotic rate was analyzed by flow cytometry. PI negative and Annexin V-FITC positive cells were defined as apoptotic cells.

2.3. Real-time PCR

Real-time PCR was performed as described previously [20]. Briefly, the total RNA of the cells was isolated by a Trizol commercial kit (Invitrogen, USA). The cDNA was synthesized using a reverse transcriptase kit (Takara, Dalian, China). Quantitative PCR (ABI 7500, USA) was performed by using SYBR green kit (Takara, Dalian, China). The relative abundance of mRNA was calculated after normalization to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA. The primer sequences (Invitrogen, USA) are as follow:

TNF-α: F: CAAGGGACA AGGCTGCCCCG, R: GCAGGGGCTCTTGACGGCAG;
iNOS: F: CAGCTGGGCTGTACAAACCTT, R: CATTGGAAGTGAAGCGTTTCG;
IL-1β: F: AAGCCTCGTGCTGTCGGACC, R:TGAGGCCCAAGGCCACAGGT;
MCP-1: F: CCAGCACCAGCACCAGCCAA, R: TGGATGCTCCAGCCGGCAAC;
COX-2: F: GATGACTGCCCAACTCCC, R: AACCCAGGTCCTCGCTTA;
GAPDH: F: GCCAAGGCTGTGGGCAAGGT, R: TCTCCAGGCGGCACGTCAGA;

The GenBank accession numbers of the primers are TNF-α: NM_013693,iNOS: NM_010927,IL-1β: NM_008361,MCP-1: NM_011333,COX-2: NM_011198,GAPDH: NM_008084,species: Mus musculus (house mouse).

2.4. Western blotting

Western blot was performed as described previously [19]. Cytoplasmic and nuclear proteins were prepared using nuclear and cytoplasmic protein extraction kit (Beyotime Institute of Biotechnology, China) according to the manufacture's instructions. Equal amounts of protein in each sample was separated by sodium dodecyl sulfate-PAGE and blotted onto polyvinylidene fluoride membranes. Membranes were incubated at 4 °C overnight with primary antibodies against COX-2 (1:300, Santa Cruz Biotechnology, USA), iNOS (1:300, Santa Cruz Biotechnology, USA), NF-κB p65 (1:1000, Cell Signaling, USA), IκBα (1:1500, Cell Signaling, USA), phospho-SAPK/JNK, phospho-p38, phospho-p44/42 (ERK-1/2), SAPK/JNK, p38, p44/42 (ERK-1/2), His (1:1000, Cell Signaling, USA), and GAPDH (1:2000, Bioworld, USA). Nuclear proteins were detected with primary antibodies p65 (1:1000, Cell Signaling, USA). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies were then used and the reaction was visualized using enhanced chemiluminescence detection reagents (Bioworld, USA) and exposed to a film. The intensity of blots was quantified by Image-J software. Total SAPK/JNK, p38, p44/42 (ERK-1/2), His and GAPDH were used as loading controls.

2.5. Statistical analysis

The results were expressed as mean ± SEM and analyzed by SPSS 13.0 software (SPSS, Chicago, IL, USA). The data were statistically assessed by Student's *t*-test (two-tailed) or one-way ANOVA followed by Tukey *post hoc* test. $P < 0.05$ was considered as statistically difference.