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Dangguishaoyao-San attenuates LPS-induced neuroinflammation *via* the TLRs/NF-κB signaling pathway

Rui-Rui Ding[a,b,1], Wang Chen[a,b,1], Cong-Ying Guo[a,b], Wei-Tao Liao[a,b], Xia Yang[e], Feng-Er Liao[c], Jing-Ming Lin[d], Han-Fang Mei[e,**], Yu Zeng[a,b,*]

[a] State Administration of TCM, PR China
[b] Key Laboratory of Digital Quality Evaluation of Chinese Materia Medica, College of Traditional Chinese Medicine, Guangdong Pharmaceutical University, Guangzhou, Guangdong 510006, PR China
[c] The First Affiliated Hospital of Guangdong Pharmaceutical University, Guangzhou, Guangdong, PR China
[d] Zhu Jiang Hospital of Southern Medical University, Guangzhou, Guangdong, PR China
[e] Department of Biochemistry and Molecular Biology, Guangdong Pharmaceutical University, Guangzhou, Guangdong, PR China

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ABSTRACT

Introduction: Dangguishaoyao-San (DSS) is composed of six traditional Chinese medicines, including Angelica sinensis, Paeoniae radix, Rhizoma Ligusticum, Poria cocos, Rhizoma Atractylodis Macrocephalae, and Rhizoma Alismatis. DSS has been reported to be effective in alleviating the symptoms of Alzheimer's disease (AD). The aim of this study was to investigate the mechanism of action of DSS *in vitro* using lipopolysaccharide (LPS)-stimulated BV-2 microglia cells.

Materials and methods: BV-2 cells were pretreated with 0.58–1.16 mg/mL of DSS for 2 h and then treated with 1 μg/mL LPS for 24 h. Cell viability was determined by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The protein expression levels were measured by Western blots. Inflammatory factors were detected by enzyme-linked immunosorbent assays (ELISAs). The mRNA levels of inflammatory factors were analyzed by quantitative real-time PCR (qRT-PCR).

Results: DSS treatment at concentrations of 0.58–1.16 mg/mL resulted in no significant cytotoxicity. DSS attenuated the release of pro-inflammatory factors, such as interleukin-1β (IL-1β), iNOS and tumor necrosis factor-α (TNF-α) in LPS-induced BV-2 cells. DSS attenuated the mRNA expression of pro-inflammatory cytokines, TLR2, and TLR4 and decreased TLR4 and TLR protein levels as well as the phosphorylation of IκB in LPS-induced BV-2 cells. DSS also down-regulated the nuclear translocation of p65.

Conclusion: This study demonstrated that DSS has a protective effect on neuroinflammation in LPS-induced BV-2 microglia cells through the TLRs/NF-κB signaling pathway.

1. Introduction

Alzheimer's disease (AD), a progressive neurodegenerative disorder, is the most common type of dementia, with millions of sufferers worldwide. Its prevalence is expected to continue to gradually increase as life expectancy increases [1]. It is estimated that more than 115 million people worldwide will have AD by 2050 [2]. Diffused brain atrophy, neurofibrillary tangles, amyloid plaques, the deposition of amyloid fibrils, and extensive neuronal loss serve as pathological hallmarks of AD and activate microglia cells. Activated microglia cells release pro-inflammatory factors such as interleukin-1β (IL-1β), inducible NO synthase (iNOS), and tumor necrosis factor-α (TNF-α), and these factors lead to further microglia activation [3,4]. Currently, no available drugs exist that cure AD or slow its progression; therefore,

Abbreviations: DSS, Dangguishaoyao-San; AD, Alzheimer's disease; LPS, lipopolysaccharide; Aβ, amyloid β; NF-κB, nuclear factor κB; IκB, inhibitor of NF-κB; TLR-2, toll-like receptor-2; TLR-4, toll-like receptor-4; TNF-α, tumor necrosis factor-α; iNOS, inducible NO synthase; IL-1β, interleukin-1β; DAPI, 2-(4-amidinophenyl)-6-indolecarbamidinedihydrochloride; PBS, phosphate buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

* Corresponding author at: State Administration of TCM, P.R.China; Key Laboratory of Digital Quality Evaluation of Chinese Materia Medica, College of Traditional Chinese Medicine, Guangdong Pharmaceutical University, No. 280 East Road, Outer Ring, Guangzhou Higher Education Mega Center, Guangzhou, Guangdong 510006, PR China.
** Corresponding author at: Guangdong Key Laboratory of Pharmaceutical Bioactive Substances, Guangdong Pharmaceutical University, No. 280 Wai Huan Dong Lu, Guangzhou, Guangdong 510006, PR China.
E-mail addresses: linjm1231@163.com (J.-M. Lin), 123031306@qq.com (H.-F. Mei), zengcomsic@yeah.net (Y. Zeng).
[1] These authors contributed equally to this work.

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identification of such new drugs is a highly valuable avenue of research. Many studies have demonstrated that stimulated microglia cells produce a variety of neurotoxic molecules [5]. Drugs that inhibit microglia activation may therefore be useful for the treatment of neurodegenerative diseases.

Lipopolysaccharide (LPS), a bacterial endotoxin, causes neuroinflammation and is commonly used to model the pro-inflammatory and neurotoxic activation of microglia [6–8]. Amyloid-β (Aβ) peptides are the core components of amyloid plaques, which can trigger microglia activation [9,10]. Previous studies have suggested that Aβ-activated microglia induce the expression of pro-inflammatory molecules, such as TNF-α and IL-1β [11]. Similar to Aβ, LPS also stimulates the expression of pro-inflammatory factors. Determination of the molecular mechanism responsible for microglia activation could provide novel opportunities to inhibit inflammatory responses. Immortalized BV-2 cells were derived from infected J2 mouse primary cultured microglia transfected with the Blasi v-raf/v-myc cancer gene in 1990. These cells have been highly purified and retain the morphology, phenotype, and functional characteristics of primary cultured microglia, making them relatively easy to cultivate. BV-2 microglia cells are commonly used to study various neurodegenerative disorders such as AD and Parkinson's disease (PD) [12,13]. Therefore, we stimulated murine BV-2 microglia cells with LPS to investigate the effect of DSS *in vitro*.

DSS is a traditional Chinese medicine that is primarily used for gynecological disorders and is currently widely used for dementia. Several previous studies have suggested that DSS could improve cognitive impairment in AD [14,15,16], but the specific mechanisms by which DSS acts on AD *in vivo* and *in vitro* are unknown. We therefore conducted a study on BV-2 microglia cells to determine whether DSS protects against LPS-induced neuroinflammation through the TLRs/NF-κB signaling pathway.

2. Materials and methods

2.1. Materials

BV-2 microglia cells were purchased from the Shanghai Institutes for Biological Sciences (Shanghai, China). LPS was obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-TLR4, Anti-TLR2, anti-NF-κB p65, anti-IκBα, anti-phospho-IκBα (phospho S32 + S36), and anti-NF-κB p65 (phospho S276) antibodies were obtained from Abcam (UK). Anti-IL-1β, anti-IL-6, β-actin and HRP-Goat anti-Rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA, USA). Alexa Fluor 488-conjugated secondary goat anti-rabbit antibody IgG (H + L) was purchased from the Beyotime Institute of Biotechnology (Shanghai, China). All other reagents were obtained from Sigma-Aldrich.

2.2. Cell culture

The immortalized BV-2 murine microglia cell line was grown in Dulbecco's modified Eagle's medium (DMEM) (Gebico, Thermo Fisher Scientific, MA, USA) with 10% (v/v) fetal bovine serum (Gebico) and 1% (v/v) penicillin-streptomycin antibiotic (Gebico). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. 3-(4,5-Dimethylthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The effect of DSS on BV-2 microglia cells was determined by an MTT assay. After treatment with DSS for 2–24 h and/or LPS (1 μg/mL, 24 h), cells were incubated with MTT (5.0 mg/mL) for 4 h at 37 °C. Formazan crystals in each well were then dissolved in dimethyl sulfoxide (DMSO), and the absorbance was measured at 595 nm with a microplate reader (Thermo MK3, USA).

2.4. ELISA assay

BV-2 microglia cells were pretreated with DSS for 2 h and then stimulated with LPS (1 μg/mL) for 24 h. The culture supernatants were measured using the mouse IL-1β ELISA Ready-set-Go 2 × 96 test (eBioscience, USA), the mouse TNF-α ELISA Ready-set-Go 2 × 96 test (eBioscience, USA), and the mouse iNOS ELISA Kit (Bangjing, China) according to the manufacturer's instructions. The absorbance was measured at 595 nm with a microplate reader (Thermo MK3, USA).

2.5. Real-time quantitative PCR analysis

The pretreated BV-2 microglia cells were lysed, and total RNA was extracted using the TRIGene reagent (GenStar, China). Reverse transcription was performed using the Goscript Reverse Transcription System (Promega, USA) according to the manufacturer's instructions. Real-time quantitative PCR was performed on the Applied Biosystems Step One Plus Real-time PCR System (ABI). The primer sequences used for *IL-1β*, *IL-6*, *TNF-α*, *iNOS*, *TLR-2*, *TLR-4* were as follows: *IL-1β* forward 5′-TACAGGCTCCGAGATGAACAAC-3′; *IL-1β* reverse 5′-TTTGAGGCCCAAGGCCACAG-3′; *IL-6* forward 5′-CTCTGGTCTTCTGGAGTACCATAG-3′; *IL-6* reverse 5′−CCTTCTGTGACTCCAGCTTATC-3′; *TNF-α* forward 5′-TCCCAAATGGCCTCCCTCTC-3′; *TNF-α* reverse 5′-TCGGCTGGCACCACTAGTTG-3′; *iNOS* forward 5′-AACATCAGGTCGGCCATCAC-3′; *iNOS* reverse 5′-AGCCTAGGTCGATGCACAAC-3′; *TLR-2* forward 5′-TCCGCGACATCCATCACCTG-3′; *TLR-2* reverse 5′-AGAACCGAGCCTCGGAATGC-3′; *TLR-4* forward 5′-AAGTGGCCCTACCAAGTCTC-3′ *TLR-4* reverse 5′-ATGGCACCATTGAAGCTGAG-3′; *Mouse actin* forward 5′-AACAGTCCGCCTAGAAGCAC-3′; *Mouse actin* reverse 5′-CGTTGACATCCGTAAAGACC-3′.

2.6. Western blot analysis

Pretreated BV-2 cells were washed in ice-cold PBS and then lysed with RIPA buffer containing protease and phosphatase inhibitors (Roche Applied Science, Mannheim, Germany). The BCA protein assay kit was used to determine protein concentrations. A total of 50–100 μg of the lysates was separated by SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked with 5% bovine serum albumin (BSA)in TBST for 1 h and probed with antibodies(1:1000) against the target proteins overnight at 4 °C and incubated with an anti-rabbit secondary antibody conjugated to HRP(1:2000) for 1 h. The protein bands were visualized with an ECL kit (Thermo Fisher Scientific, Waltham, MA, USA), and the images were captured on an Amersham Imager 600 (General Electric Company, CT, USA).The primary antibodies were purchased from Abcam (UK) and Cell Signaling Technology (Danvers, MA, USA), and the secondary antibody was obtained from the Beyotime Institute of Biotechnology (Shanghai, China). The blot intensities were quantified with ImageJ.

2.7. Immunofluorescence

BV-2 cells were grown in culture dishes. After pretreatment, the cells were washed three times with PBS, fixed with 4% paraformaldehyde for 15 min, and washed with PBS followed by permeabilization with 1% TritonX-100 for 30 min at room temperature. Then, the cells were incubated with anti-NF-κB p65 primary antibody (1:200) in 5% bovine serum albumin (BSA) and 0.1% TritonX-100 overnight at 4 °C. The cells were then treated with Alexa Fluor 488-Conjugate secondary antibody (1:1000) with 5% BSA for 1 h at room temperature and washed three times with PBS. Finally, the cells were incubated with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, 1 mg/mL) for 5 min. The cells were observed with laser scanning confocal microscopy (LSM510, Zeiss).