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Ethanol extract from a Chinese herbal formula, "Zuojin Pill", inhibit the expression of inflammatory mediators in lipopolysaccharide-stimulated RAW 264.7 mouse macrophages

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ABSTRACT

Ethnopharmacological relevance: Zuojin Pill (ZJP), a traditional Chinese medicinal decoction that has been used in treating gastritis, gastric ulcer since 15th century, contains two herbs: Rhizoma Coptidis and Fructus Evodiae in the ratio of 6:1 (w/w). Alkaloids are the main active principles contributing to ZJP's efficacy, but anti-inflammatory mechanism has not been fully clarified.
Aim of the study: The objective of the study is to reveal anti-inflammatory molecular mechanism of ethanol extract from ZJP, which would form an additional proof to the traditional experience of ZJP in clinical administration.
Materials and methods: Seven alkaloids were determined from the ethanol extract of ZJP using High Performance Liquid Chromatography (HPLC) with the gradient mobile phase. The ethanol extract from ZJP were used to evaluate the anti-inflammatory action in murine macrophage cell line RAW 264.7 treated with lipopolysaccharide (LPS). Production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) were measured by the Griess colorimetric method and enzyme-linked immunosorbent assay (ELISA), respectively. Proteome profiler array was analyzed to evaluate 40 cytokines at protein level. In addition, interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) synthesis were analyzed using ELISA to confirm the result of the Proteome profiler array. The gene expression levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α, IL-6, and interleukin 1β (IL-1β) were detected by quantitative real-time reverse-transcription polymerase chain reaction (real-time RT-PCR). Furthermore, the nuclear translocation of the NF-κB p50 and p65 subunits was detected with ELISA.
Results: The secretions of NO, PGE_2 and the mRNA expression of iNOS, COX-2 were significantly inhibited, moreover, the protein and mRNA expressions of IL-6, IL-1β and TNF-α were inhibited by preventing the nuclear translocation of the NF-κB p50 and p65 subunits. The proteome profiler array showed that 15 cytokines and chemokines involved in the inflammatory process were down-regulated by ZJP.
Conclusion: These results suggest that the anti-inflammatory properties of ethanol extract from ZJP might be the results from the inhibition of iNOS, COX-2, IL-6, IL-1β, and TNF-α expression through preventing the nuclear translocation of the NF-κB p50 and p65 subunits in RAW 264.7 cells. In addition, these results provided evidence to understand the therapeutic effects of ZJP on gastritis, gastric ulcer, and other inflammatory diseases in clinic.

1. Introduction

Chinese herbs are commonly used in a combination of two or more herbs, called a formula, to increase or reinforce the effects of each medicinal substance, to accommodate complex clinical situations, etc. (Wu, 2005). Zoujin Pill (ZJP), a typical traditional Chinese medicine (TCM) formula, consists of the Rhizoma Coptidis (*Coptis chinensis* Franch. was used in the study) and Fructus Evodiae (*Evodia rutaecarpa* (Juss.) Benth. was used in the study) in the ratio of 6:1 (w/w). ZJP was first recorded in "Danxi's experiential therapy" for treating gastro-intestinal disorders in the 15th century. It is officially listed in the Chinese Pharmacopoeia as a prescription employed in patients suffering from gastric ulcer, gastroesophageal reflux disease, gastritis, and pyloric obstruction, among other disorders (Pharmacopoeia, 2010). Pharmacological studies have shown that two herbs in ZJP have the antagonistic effects on catecholamine

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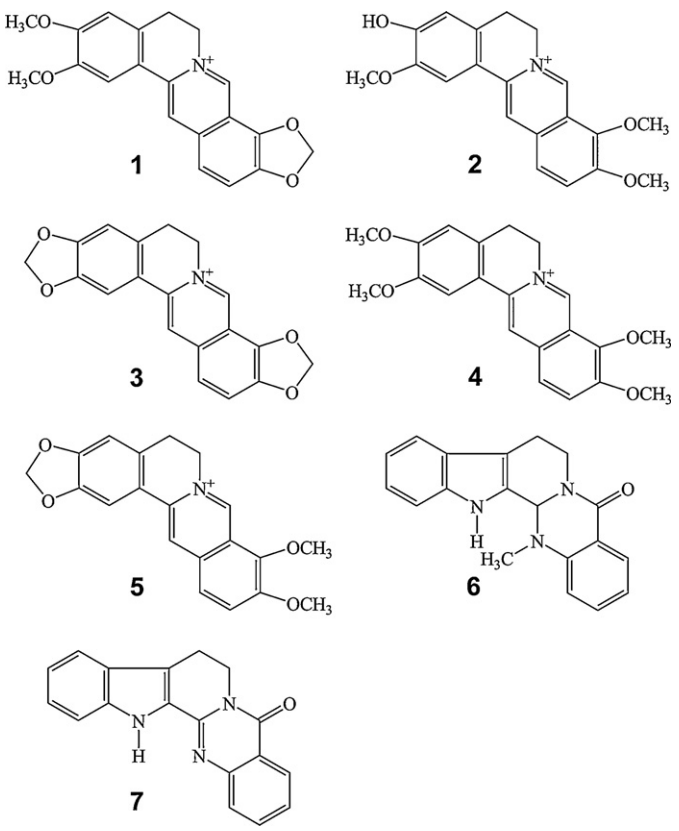

Fig. 1. Chemical structures of 1: epiberberine, 2: jatrorrhizine, 3: coptisine, 4: palmatine, 5: berberine, 6: evodiamine, 7: rutaecarpine.

secretion in bovine adrenal medullary cells (Zhao et al., 2010). Chemical investigations for the ethanol extracts of ZJP have shown 7 kinds of alkaloids (Gao et al., 2010), berberine, palmatine, coptisine, jatrorrhizine, epiberberine in Coptis, evodiamine and rutaecarpine in Evodia, which are the bioactive ingredients with antimicrobial activity (Fan et al., 2008), antiviral activity (Hayashi et al., 2007), anti-inflammatory (Yarosh et al., 2006), anticancer (Zhang et al., 2004), moreover, berberine and evodiamine could increase serotonin transporter expression in the serotonergic neuronal cell line RN46A (Hu et al., 2011). Their molecular structures are given in Fig. 1. Although ZJP could significantly relieve gastric ulcer, gastritis and other disorders in clinic, the anti-inflammatory activity mechanism of ZJP is still not clean.

The inflammation process is crucial to defense against microorganism infection. Key events in the inflammatory process include expression of inflammatory cytokines, chemokines, and other mediators (Baggiolini, 1998). Macrophages activated by interferon-γ (IFN-γ), pro-inflammatory cytokines and LPS, play an important role in inflammatory disease and host defense through the release of factors such as NO, prostaglandin mediators, and cytokines involved in the immune response (Hibbs et al., 1987; Palmer et al., 1988). NO is a major product which is controlled by nitric oxide synthases (NOS), such as iNOS, eNOS and nNOS (Marletta, 1993). Most importantly, iNOS is highly expressed in macrophages, which leads to organ destruction in some inflammatory and autoimmune diseases (Kleinert et al., 2004). PGE_2 is also another important mediator which is produced from arachidonic acid metabolites which are catalyzed by COX-2 in inflammatory responses (Harris et al., 2002).

Nuclear transcription factor kappa-B (NF-κB), a nuclear transcription factor, regulates the expression of various genes, including cytokines, iNOS and COX-2, which play critical roles in apoptosis, various autoimmune diseases, and inflammation (Lawrence et al., 2001). NF-κB exists in most cells as homodimeric or heterodimeric complexes of p50 and p65 subunits and remains inactive in the cytoplasm of cells associated with the NF-κB inhibitory protein (I-κB). NF-κB is activated in response to LPS, which induced NF-κB activation through increasing nuclear p65 protein associated with decreased cytosolic I-κB protein (Baldwin, 1996). The resulting free NF-κB is then translocated into the nucleus, where it binds to κB binding sites in the promoter region of target genes, and induces the transcription of pro-inflammatory mediators, including iNOS, COX-2, TNF-α, IL-1β, and others (Baeuerle and Baltimore, 1996; Lappas et al., 2002). Because of its ubiquitous role in the pathogenesis of inflammatory gene expression, NF-κB is a current target for treating various diseases (Renard and Raes, 1999).

The macrophage cell line (RAW 264.7) used in experiments has been established as a suitable model to investigate compounds interfering with LPS-inducible inflammatory cascades in vitro (Suh et al., 1998; Seo et al., 2001). In this study, the anti-inflammatory effects of the ZJP on the generation of several chemokines, cytokines and enzymes involved in the inflammatory process, such as NO, PGE_2, TNF-α, IL-6, IL-1β, iNOS and COX-2 in LPS-induced RAW 264.7cells were investigated. We also investigated whether the ZJP influences the LPS induced DNA binding activity of NF-κB and the protein level of its subunit, p65 and p50.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium-high glucose (DMEM), 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Lipopolysaccharides from *Escherichia coli* 0111:B4 (LPS) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Prostaglandin E_2 Express EIA Monoclonal Kit was obtained from Cayman Chemical (Ann Arbor, MI, USA). IL-6 Mouse ELISA Kit, IL-1β Mouse ELISA Kit and TNF-α Mouse ELISA Kit were obtained from Invitrogen (Carlsbad, CA, USA). 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate) was purchased from Invitrogen (Carlsbad, CA, USA). BCA Protein Assay Kit was obtained from Pierce (Rockford, IL, USA). Mouse Cytokine Array Panel A Array kit was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Mammalian Cell Lysis Kit and UNIQ-10 column Trizol total RNA extraction kit were bought from Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Improm-II Reverse Transcription System was purchased from Promega Corporation (Madison, WI, USA). FastStart Universal SYBR Green Master (ROX) kit was purchased from Roche (Mannheim, Germany). Nuclear Extract Kit was purchased from Active Motif (Tokyo, Japan). Universal EZ-TFA Transcription Factor Assay and NF-κB Family EZ-TFA Transcription Factor Assay kits were purchased from Millipore (Bedford, MA, USA). N^G-nitro-Larginine methyl ester (L-NAME) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). BAY 11-7082 was purchased from Beyotime Institute of Biotechnology (Haimen, China). Methanol and acetonitrile were of HPLC grade and obtained from Tianjin Concord Technology Co., Ltd. (Tianjin, China). All other chemical agents were of ACS or analytical grade.

2.2. Preparation of the extracts for Chinese herbs

The standards of palmatine, evodiamine, rutaecarpine were purchased from the Chinese National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Berberine was supplied by Sigma–Aldrich (St. Louis, MO, USA). Coptisine and