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Effects of compounds from Bi-Qi Capsule on the expression of inflammatory mediators in lipopolysaccharide-stimulated RAW 264.7 macrophages

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ABSTRACT

Aim of the study: The Bi-Qi Capsule (Bi-Qi) has been used in clinic as prescribed drug for the treatment of rheumatic arthritis, rheumatoid arthritis and other osteoarticular diseases about 20 years in China. Pharmacological and clinical studies have confirmed the anti-inflammatory and analgesic action of Bi-Qi *in vivo*. However, its anti-inflammatory molecular mechanism is still unclear. The objective of the study is to reveal the anti-inflammatory molecular mechanism of Bi-Qi which would form an additional proof to the traditional experience of Bi-Qi in clinical administration.

Materials and methods: The aqueous extract of Bi-Qi was used to evaluate the anti-inflammatory action in murine macrophage cell line RAW 264.7 treated with lipopolysaccharide (LPS). Cell viability was evaluated by MTT assay. 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. Protein expression levels of cyclooxygenase 2 (COX-2) were monitored by cell-based ELISA. Proteome profiler array was analyzed to evaluate 40 cytokines at protein level. In addition, interleukin 6 (IL-6) and tumor necrosis factor-alpha (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), 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).

Results: Bi-Qi significantly decreased the production of NO, PGE_2, and inhibited the protein expression of COX-2. The Proteome profiler array showed that eight protein cytokines were down-regulated and six protein cytokines were up-regulated by Bi-Qi. Furthermore, the results of TNF-α and IL-6 protein expression analyzed by ELISA were similar to those of Proteome profiler array. The results of real-time RT-PCR demonstrated that iNOS, COX-2, TNF-α, IL-6 and IL-1β gene expression were also significantly reduced by Bi-Qi.

Conclusion: These results suggested that the anti-inflammatory molecular mechanism of Bi-Qi might be the results from modulating the LPS-mediated NO–iNOS pathway, COX-2 pathway via inhibition of iNOS, COX-2, TNF-α, IL-6 and IL-1β expression in activated macrophages. In addition, these results provided evidence to understand the therapeutic effects of Bi-Qi on various inflammatory diseases, e.g. rheumatoid arthritis, rheumatic arthritis and other osteoarticular diseases.

1. Introduction

Bi-Qi Capsule (SFDA approval number: Z10910026), made from Radix et Rhizoma Salviae Miltiorrhizae, Semen Strychni, Radix Glycyrrhizae, Radix Codonopsis, Rhizoma Atractylodis Macrocephalae is a herbal remedy for treating rheumatic arthritis, rheumatoid arthritis, scapulohumeral periarthritis, cervical spondylosis and slipped disk about 20 years in China. The quality criteria of Bi-Qi determined by high performance liquid chromatography (HPLC) shows that the Brucine is (0.54 ± 0.03) g/kg, Strychnine is (0.84±0.05) g/kg and Salvianolic acid B is (6.45±0.07) g/kg (Liu et al., 2009). The aqueous extract of Bi-Qi Capsule used in this paper was prepared as dry powder by lyophilizing the condensed filtrates. The aqueous extract of Bi-Qi Capsule were determined by HPLC and the results showed that there were six mainly compounds, Strychnine, Brucine, Salvianolic acid B, Glycyrrhizin, Liquiritin, Cryptotanshinone in it. Moreover, Strychnine, Brucine, Salvianolic acid B, Glycyrrhizin have been reported that they had the anti-inflammatory effect (Frankstein et al., 1965; Yin et al., 2003; Chen et al., 2006; Schrofelbauer et al., 2009). Pharmacological

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studies demonstrated that Bi-Qi can significantly decrease inflammatory and algesic reaction of inflammatory CIA rats, protect the Osteoarthritis Cartilage and enhance the blood flow (Liu et al., 2006). Pharmacokinetic study confirmed that Bi-Qi could significantly reduce the concentration of the Brucine and Strychnine and prolong the action time of the Brucine and Strychnine in blood, comparing with giving the Brucine or Strychnine, respectively (Gao et al., 2009). The randomized clinical trials (nearly 1000 patients) show that Bi-Qi is a safe and atoxic (especially for neurotoxicity) anti-inflammatory drug than non-steroid anti-inflammatory drug (NSAID), such as Voltaren, Fenbid, Mobic and Difene, which could induce gastrointestinal diseases and destroy cartilage cells for long-term prescription. Moreover, Bi-Qi could significantly relieve rheumatic arthritis, rheumatoid arthritis and scapulohumeral periarthritis. However, the anti-inflammatory activity mechanism of Bi-Qi is still not clean.

The inflammation process is crucial to defense against bacterial infection. Key events in the inflammatory process include expression of inflammatory cytokines, chemokines, and other mediators (Baggiolini, 1998). Macrophages are extraordinarily versatile cells, but it is as sentinels of the immune system that macrophages exploit their full functional repertoire. They detect pathogenic substances through pattern-recognition receptors and subsequently initiate and regulate inflammatory response (Medzhitov and Janeway, 1997) using a wide range of soluble pro-inflammatory mediators. Macrophages are activated by IFN-γ, pro-inflammatory cytokines and LPS. Activated macrophages play an important role in inflammatory diseases via production of cytokines, interleukin-1β (IL-1β), and tumor necrosis factor-alpha (TNF-α), IL-6 (Van Snick, 1990; MacMicking et al., 1997). During inflammatory processes, large amounts of the pro-inflammatory mediators, NO and PGE_2, are generated by inducible iNOS and COX-2, respectively. Therefore, variation in levels of chemokines, cytokines, iNOS and COX-2 can be thought of as a marker of immunomodulation. NO is produced by the oxidation of L-arginine catalyzed by NO synthase (NOS) pathway. In the NOS family, inducible NOS (iNOS) is particularly well known to be involved in the overproduction of NO in cells. PGE_2 is derived from the catalyzation of arachidonic acid by COX-2 pathway. The NO–iNOS pathway and COX-2 pathway are the important pathways which were involved in inflammatory processes.

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 (Suh et al., 1998; Cho et al., 2000; Hinz et al., 2000a,b; Seo et al., 2001) including the COX-2 pathway and NO–iNOS pathway *in vitro*. In this study, the anti-inflammatory effects of Bi-Qi 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 264.7 cells were investigated. The research of anti-inflammatory molecular mechanism will supply an additional proof to the traditional experience of Bi-Qi in clinical administration.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (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. (USA). Prostaglandin E_2 Express EIA Monoclonal Kit was obtained from Cayman Chemical (USA). IL-6 ELISA Kit and TNF-α ELISA Kit were obtained from Invitrogen (USA). Mouse Cytokine Array Panel A Array kit, Cat. No. ARY006, was purchased from R&D Systems, Inc. (USA). UNIQ-10 column Trizol total RNA extraction kit was bought from Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Improm-II Reverse Transcription System was purchased from Promega Corporation (USA). FastStart Universal SYBR Green Master (ROX) kit was purchased from Roche (Germany). Mouse COX-2 antibody was from BD Pharmingen (USA) and Goat Anti-Mouse IgG Peroxidase Conjugate was from Calbiochem (Germany).

2.2. Preparation of Bi-Qi extract

Bi-Qi Capsule was supplied by Tianjin Darentang Jingwanhong Pharmaceutical Co., Ltd. A voucher specimen is kept in our laboratory (Institute of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine, China) for further reference. The aqueous extract of Bi-Qi Capsule was prepared as dry powder by lyophilizing the condensed filtrates. Ultrasonic extraction was used to extract the aqueous components of Bi-Qi Capsule. Briefly, dried medicine powder of Bi-Qi Capsule (10 g) was extracted with 150 ml of deionized water at room temperature for 30 min. The first aqueous extract was filtered and the first filtrate was collected in a separate flask. The remnant was added with 150 ml of fresh deionized water and repeated routines two times. First, second and third filtrates were combined, concentrated and lyophilized by quick freezing to get the aqueous extract powder of Bi-Qi. The yield of the dried extract as the percent weight of the dried Bi-Qi medicine powder was about 34%. The Bi-Qi extract was kept drying preservation at 2–8 °C. The extract was cold-sterilized using a 0.22-μm pore size membrane filter (Millipore, USA) before use.

2.3. Chemicals

Brucine, Strychnine, Salvianolic acid B, Glycyrrhizin, Liquiritin, and Cryptotanshinone were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The structures of standards were listed in Fig. 1. Their purities were above 98% using LC analysis. The HPLC analyses were performed on an Agilent 1100 series HPLC instrument (Agilent, Waldbronn, Germany) composed of a vacuum degasser, a quaternary pump, an autosampler, a column compartment, and a diode array detector (DAD). The chromatographic separation was carried out on a Waters SymmetryShield RP 18 column (5 μm, 3.9 mm × 150 mm) by setting the column temperature at 35 °C. The mobile phase consisted of acetonitrile (A) and water containing 0.1% formic acid (B). A gradient program was used as follows: 4–21% A at 0–20 min, 21–42% A at 20–40 min, and 42–90% A at 40–50 min. The flow rate was kept at 1 ml/min. The detection wavelength was set to monitor at 276 nm. HPLC analysis was performed in triplicate; each with 10 μl of the sample. A typical chromatogram was shown in Fig. 2. The yields of Brucine, Strychnine, Glycyrrhizin, Salvianolic acid B, Liquiritin, and Cryptotanshinone in Bi-Qi dried extract were 0.11 mg/g, 0.66 mg/g, 2.68 mg/g, 4.63 mg/g, 2.61 mg/g and 0.028 mg/g, respectively. The determination of the polysaccharide was undertaken by the method of the Chinese pharmacopoeia 2010 edition. The determination of the Bi-Qi extract was performed in triplicate, and the results showed that the polysaccharide in Bi-Qi extract was 82.71%.

2.4. Cells and cell culture

RAW 264.7 murine macrophages cell line was obtained from Cell Culture Center of Chinese Academy of Medical Sciences (Beijing, China). RAW 264.7 murine macrophages were maintained in DMEM supplemented with 10% heat inactivated fetal bovine serum