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Insight into the molecular mechanism of a herbal injection by integrating network pharmacology and in vitro

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ABSTRACT

Chinese medical herbs could treat complex diseases through the synergistic effect of multi-components, multi-targets and multi-channels. However, it was difficult to systematically investigate the pharmacological mechanisms of action due to the complex chemical composition and the lack of an effective research approach. Fortunately, network pharmacology as an integrated approach was proposed to systematically investigate and explain the underlying molecular mechanisms of Chinese medical herbs. Reduning injection (RDN) is one of the herbal injections for treatment of upper respiratory tract infections (URTIs). Previous studies revealed the molecular mechanism of RDN on URTIs through network pharmacology. In this work, the mechanism of RDN was verified by enzyme linked immunosorbent assay (ELISA), Western Blot, immunofluorescence assay and electrophoretic mobility shift assay (EMSA) in lipopolysaccharide (LPS)-induced RAW264.7 cells and enzyme assay. RDN dose-dependently suppressed the production of nitric oxide (NO), prostaglandin E_2 (PGE_2), interleukin-6 (IL-6) and interleukin-1β (IL-1β), and reduced the protein expression of inducible NO synthetase (iNOS) and cyclooxygenase-2 (COX-2), which could be related to its suppression on the phosphorylations of mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinase (ERK), c-jun NH2-terminal kinase(JNK) and p38, as well as the activation and translocation of nuclear factor-κB (NF-κB). In addition, the activity of RDN on PGE_2 was also partly attributed to the inhibition of COX-2 enzyme. Therefore, it can be concluded that RDN inhibited the production of inflammatory mediators and the macrophage activation to treat URTIs via down-regulating the activation of MAPK and NF-κB signaling pathways, which might pave a way to illustrate the molecular mechanism of herbs.

1. Introduction

As an important complementary and alternative medical system, Chinese medical herbs have been widely used in clinic for thousands of years in China (Zhang et al., 2013a). However, along with the development of modern medicine, the scientific basis and action mechanism of herbs have been questioned by medical practitioners recently (Yang et al., 2013). Fortunately, network pharmacology integrating systems biology and polypharmacology is expected to open a way to identify active ingredients and to reveal the action mechanisms of herbs at system-level, which is helpful to illustrating the scientific basis of herbs (Li et al., 2014a; Lv et al., 2014; Zhang et al., 2014).

Reduning Injection (RDN) is a traditional Chinese medicine preparation, which was developed from an herbal formula that consisted of *Lonicera Japonica* Thunb. (Jinyinhua), *Gardenia Jasminoides* Ellis (Zhizi) and *Artemisia Annua* L. (Qinghao). It has been widely used to treat various diseases in clinic such as upper respiratory tract infections (URTIs) (Liu, 2014), fever and inflammation caused by viral infection (influenza viruses, respiratory syncytial virus, EV71, Dengue) (Li et al., 2014b, 2013; Zhang et al., 2013b).

In previous work (Zhang et al., 2014), network pharmacology was employed to identify potential compounds and to uncover the mechanism of RDN on URTIs. The results revealed that the candidate active compounds of RDN could not only regulate mitogen

Abbreviations:: RDN, reduning injection; LPS, lipopolysaccharide; URTIs, upper respiratory tract infections; PG, prostaglandin; NO, nitric oxide; COX, cyclooxygenase; iNOS, inducible NO synthase; IL, interleukin; TNF-α, tumor necrosis factor-α; TLR, Toll-like receptor; MAPK, mitogen activated protein kinase; ERK, extracellular signal-related kinase; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; ELISA, enzyme linked immunosorbent assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BSA, bovine serum albumin; EMSA, electrophoretic mobility shift assay; RSV, respiratory syncytial virus RSV

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activated protein kinase (MAPK) pathways via interacting MEK1, c-jun NH2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), p38 proteins, but also inhibit some key enzymes such as cyclooxygenase (COX), inducible NO synthetase (iNOS). Moreover, the process played an important role in the production of chemokines, inflammatory cytokines (interleukin-1β (IL-1β), interleukin-6 (IL-6), prostaglandin E_2 (PGE_2), nitric oxide (NO)) when the host was attacked by respiratory virus. Previous animal research indicated that RDN had antipyretic effect on fever in rabbit and inhibited inflammation by reducing the levels of IL-6, IL-1 and PGE_2 in rat (Wang et al., 2013). According to these hints on the molecular mechanism of RDN, the molecular mechanism of RDN was clarified by ELISA, Western Blot, EMSA, immunofluorescence assay and Enzyme assay. The results would provide further reasonable practice of RDN in clinical use, and it might be a strategy to investigate the mechanism of action of herbs.

2. Materials and methods

2.1. Chemicals and reagents

RDN was supplied by Kanion Pharmaceutical Co. Ltd. (Lianyungang, China). Geniposidic Acid, Neochlorogenic Acid, Chlorogenic Acid, Cryptochlorogenic Acid, Caffeic Acid, Geniposide, Secoxyloganin, Isochlorogenic Acid B, Isochlorogenic Acid A, Isochlorogenic Acid C ($\geq$98%) were purchased from National Institute for Food and Drug Control (Nanjing, China). HPLC grade methanol was purchased from Merck (Darmstadt, Germany), and deionized water was purified using the Milli-Q system (Millipore, Bedford, MA, USA). The murine macrophage RAW264.7 cell lines were purchased from Chinese Academy of Medical Sciences (Beijing, China). Mouse tumor necrosis factor-α (TNF-α), IL-1β and IL-6 enzyme linked immunosorbent assay (ELISA) kits were from eBioscience (Vienna, Austria). PGE_2 ELISA kits were from Enzo Life Science (Farmingdale, NY, USA). Antibodies against iNOS, COX-2, p65, ERK1/2, JNK, p38, p-p65, p-ERK1/2, p-p38 and p-JNK were obtained from Cell Signaling Technology (Beverly, MA, USA). Horse radish peroxidase-conjugated secondary antibodies, DyLight 488-conjugated secondary antibody and primary antibody against β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dulbecco's modified Eagle's medium (DMEM) was produced by Kaiji Biotechnology (Nanjing, China) and fetal bovine serum (FBS) was produced by Sijiqing (Hangzhou, China). Lipopolysaccharide (LPS, Escherichia coli O55:B5), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), cell culture grade DMSO and 4′,6-diamidino-2-phenylindole (DAPI) were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. LC-MS analysis of the compounds of RDN

The compounds of RDN were identified by using an Agilent Series 1290/6538 ultra-high performance liquid chromatograph/QTOFMS (Agilent Technologies, Palo Alto, CA). The chromatographic separation of RDN was achieved by an Agilent Eclipse Plus C18 RRHD (1.8 μm, 2.1 × 100 mm^2). The mobile phase was deionized water (0.1% formic acid, v/v) and methanol with a flow rate of 0.5 mL/min. The elution program was listed as follows: 0–10 min, 12–30% methanol, 10–14 min, 30% methanol, 14–20 min, 30–49% methanol, 20–25 min, 49–100% methanol and 25–30 min, 100% methanol. Agilent 6538 Q-TOF mass spectrometer with electrospray ionization interface was used in LC-MS method. MS conditions were as follows: negative ion mode, drying gas N_2, 8 L/min, temperature 350 °C, pressure of nebulizer 40 psig and capillary voltage 3500 V, scan range 100–3000 u.

2.3. Cell culture and sample preparation

The murine macrophage RAW264.7 cell lines were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in humidified atmosphere containing 5% CO_2, and 95% air. The stock solution (200 mg/mL) of RDN was dissolved in DMSO. In order to quantify and administer accurately, RDN powder was prepared by lyophilize action for all the cell assays and was dissolved in DMSO before administration.

2.4. Cell viability assay

RAW264.7 cells were seeded in a 96-well plate at a density of 1×10^4 cells/well overnight. After treated with various concentrations of RDN (25, 50, 100, and 200 μg/mL) for 24 h, the cells were added with 20 μL MTT (5 mg/mL) each well and incubated for another 4 h. The supernatant was then removed and 150 μl DMSO was added to each well. The optical absorbance at 490 nm was measured by a microplate reader (Molecular Devices, Menlo Park, USA).

2.5. Measurement of NO

RAW264.7 cells (2×10^5 cells/well) were plated into 96-well plates overnight. The cells were then treated with various concentrations of RDN (50, 100, and 200 μg/mL) for 24 h at the presence or absence of 1 μg/mL LPS. In the supernatants, NO level was detected by Griess reaction (Luo et al., 2010). In brief, 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 2.5% phosphoric acid) was mixed with an equal volume of the supernatant. The optical absorbance was measured at 540 nm after incubation in dark for 10 min.

2.6. Measurement of cytokine levels

RAW264.7 cells were seeded in a 24-well plate at a density of 5×10^4 cells /well for PGE_2 and TNF-α, as well as 1×10^5 cells/well and 2.5×10^5 cells/well for IL-6 and IL-1β, respectively. After incubated for 24 h, RAW264.7 cells were pretreated by RDN (50, 100, and 200 μg/mL) for 1 h, and then stimulated with LPS (1 μg/mL) for 24 h. The levels of PGE_2, TNF-α, IL-6 and IL-1β in the supernatants were quantified using ELISA kits according to the manufacturer's instruction.

2.7. Western blot analysis

RAW264.7 cells were seeded in 6-well plates. The cells were pretreated with RDN at various concentrations of 50, 100 and 200 μg/mL for 3 h and stimulated with LPS (1 μg/mL) for 20 min. Then cells were homogenized in lysis buffer for 10 min. After determined by bicinchoninic acid (BCA) assay (Sigma Aldrich, St. Louis, MO, USA), the prepared protein extracts were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The PVDF membranes were blocked at room temperature for 1 h with 5% non-fat dry milk in PBS buffer, and then washed five times in PBS containing 0.1% Tween 20 (PBST) for 5 min. The blots were sequentially incubated with specific primary antibodies (iNOS, COX-2, p65, ERK1/2, JNK, p38, p-p65, p-ERK1/2, p-P38, p-JNK and β-actin) in PBST containing 3% bovine serum albumin (BSA) overnight at 4 °C. The blots were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The bands were visualized using film exposure with enhanced chemiluminescence detection reagents (Pierce, Rockford, IL, USA).