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Effects and mechanisms of total glucosides of paeony on synoviocytes activities in rat collagen-induced arthritis

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ABSTRACT

The aim of the study was to investigate the effects of TGP, an active compound extracted from the roots of *Paeonia lactiflora* Pall, on the activities of synoviocytes in rats with collageninduced arthritis (CIA) and its possible mechanisms. CIA was induced in male Sprague–Dawley (SD) rats immunized with chicken type II collagen (CII) in Freund's complete adjuvant (FCA). Synoviocytes proliferation was determined by 3-(4, 5-2dimethylthiazal-2yl) 2, 5-diphenyltetrazoliumbromide (MTT) assay. Tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), prostaglandin E_2 (PGE_2) and cyclic adenosine monophosphate (cAMP) levels in synoviocytes were measured by radioimmunoassay (RIA). E-prostanoid $(EP)_2$ and EP_4 receptors were analyzed by Western blot analysis. The results showed that TGP significantly inhibited the proliferation of synoviocytes, decreased the production of IL-1, TNF-α and PGE_2 and elevated the levels of cAMP. Further study showed that TGP could up-regulate the expression of EP_2 and EP_4. These results indicated that TGP might exert its anti-inflammatory effects through inhibiting the production of pro-inflammatory mediators in synoviocytes of CIA rats, which might be associated with its ability to regulate cAMP-dependent EP_2/EP_4-mediated pathway.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease with unknown etiology. The main pathological changes of RA include hyperplasia of synovial membrane, infiltration of inflammatory cells, and neovascularization, which ultimately lead to cartilage erosion and articular destruction. Proinflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α) as well as prostaglandin E_2 (PGE_2) are expressed at very high levels in synovial membrane, which are considered to be the important participants in the pathophysiology of RA.

Prostaglandins (PGs) act as important mediators of inflammation and joint destruction in RA, especially PGE_2. Studies show that excessive production of PGE_2 in synovial tissues is associated with the edema and erosion of cartilage and juxtaarticular bone in RA. PGE_2 mediates its biological activities through at least four different E-prostanoid (EP) receptors, designated EP_1, EP_2, EP_3, and EP_4. Molecular cloning studies have indicated that these four receptors have seven transmembrane domains and belong to the G protein-coupled receptors (GPCRs) superfamily (Coleman et al., 1994). EP_1 is coupled with the G_q protein, which signals through the phospholipase C pathway, increasing intracellular Ca^{2+} concentration. EP_2 and EP_4 are coupled with the G_s protein and activate adenylate cyclase, increasing cyclic adenosine monophosphate (cAMP) levels and signaling through the protein kinase A pathway. EP_3 has different isoforms which coupled to different signaling pathways, including an increase or decrease in intracellular cAMP and an increase in intracellular Ca^{2+} concentration. The four EP receptors are expressed in diverse tissues and play essential roles in a variety of pharmacological and biochemical processes. Many studies indicated that EP_2 and EP_4 involved in the pathophysiology of RA (Kurihara et al., 2001; Yoshida et al., 2001; Inoue et al., 2002; Honda et al., 2006; Largo et al., 2004; McCoy et al., 2002; Akaogi et al., 2004).

Paeonia lactiflora Pall root is one of the most well-known herbs in China, Korea and Japan for more than 1200 years. Total glucosides of paeony (TGP) which consists of more than 90% paeoniflorin, extracted from the root of *Paeonia lactiflora* Pall (family Ranunculaceae), has been recognized as a valuable traditional herb for the treatment of RA, systemic lupus erythematosus and hepati-

Abbreviations: TGP, total glucosides of paeony; CIA, collagen-induced arthritis; CII, type II collagen; PGE_2, prostaglandin E_2; IL-1, interleukin-1; TNF-α, tumor necrosis factor alpha; cAMP, cyclic adenosine monophosphate; EP, E-prostanoid.

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tis (Zhang et al., 2001). The anti-inflammatory, anti-oxidative, analgesic, and immunoregulatory activities of TGP have been extensively confirmed in our laboratory for many years. Recently, we found that the anti-inflammatory and immunoregulatory effects of TGP were likely due to the elevation of intracellular cAMP levels via EP_2/EP_4 *in vivo* (Xu et al., 2007).

The present study was designed to further evaluate the effects of TGP on the proliferation and secretion of activated synoviocytes *in vitro*. In addition, we investigated whether TGP exert its effects on the activities of synoviocytes by regulating EP_2/EP_4-cAMP pathway.

2. 2Materials and methods

2.1. Drugs and reagents

TGP was extracted from *Paeoniae radix* through the methods of ethanol reflux, ethyl acetate extraction, and absorption resin chromatography (Xu et al., 2007). PF was one of main effective component of TGP, accounting for more than 90% by high performance liquid chromatography (HPLC) method analysis. Recombinant rat IL-1α (rIL-1α) (CYTOLAB/PEPROTECH ASIA) and recombinant rat TNF-α (rTNF-α) (CytoLab Ltd) occurs as white powder. The compound was dissolved in phosphate buffered saline. The ^{125}I-PGE_2 radioimmunoassay (RIA) kit was the product of blood institute of Suzhou College (Suzhou, China). ^{125}I-TNF-α radioimmunoassay (RIA) kit was the product of Beijing Biotinge Biomedicine Company (Beijing, China). The ^{125}I-cAMP RIA kit was the product of nuclear medicine laboratory of Shanghai College (Shanghai, China). Rabbit polyclonal anti-EP_2 and rabbit polyclonal anti-EP_4 antibody were purchased from Santa Cruz Biotechnology Inc.USA. Bacillus Calmette Guerin (BCG) from Shanghai Biochemical Factory. Chicken CII from Institute Jacques Boy (Reims, France). Type IA collagenase, trypsin, lipopolysaccharide (LPS) and 3-(4,5-2dimethylthiazal-2yl) 2,5-diphenyltetrazoliumbromide (MTT) from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) from GIBCO was supplemented with penicillin G (100 U ml^{-1}), streptomycin (100 ng ml^{-1}), and 15% fetal bovine serum (FBS, heat inactivated) and PH was adjusted to 7.2.

2.2. Animals

SD rats (male, 180 ± 20 g) were obtained from Shanghai BK Experimental Animal Center (Grade II, certificate no. D-65). All rats were housed under standard laboratory conditions. During the experimental period, rats were given standard laboratory chow and tap water ad libitum and kept on sawdust in plastic-bottomed cages at 20–25 °C. All experiments were approved by Ethics Review Committee for Animal Experimentation of Institute of Clinical Pharmacology, Anhui Medical University.

2.3. Induction of CIA

CII was dissolved in 0.1 M acetic acid at 0.4 mg ml^{-1} by stirring overnight at 4 °C and emulsified with an equal volume of FCA which had been prepared by heat-killed BCG in Freund's incomplete adjuvant (Sigma) to a final concentration of 0.1 mg ml^{-1}. Rats were injected intradermally twice with 1 ml of the emulsion (containing 200 μg of CII). The first injection was made in the left hind paw with 0.1 ml and the tail and other 3–5 sites on the back with 0.9 ml; provocation test was done 7 days later with similar methods. The day of the first immunization was defined as d0, and the second day as d1 and so on.

2.4. Culture of synoviocytes

Rats were anaesthetized and sacrificed on day 36 after immunization. Synovial tissue was collected from knee joints of rats. Tissue were minced aseptically into 1–2 mm^2, then digested in DMEM containing 5% (v/v) fetal bovine serum (FBS, Gibco) and 0.4% type IA collagenase at 37 °C, 5% CO_2. Two hours later, adherent cells were discarded and non-adherent tissue were digested in serum-starved DMEM containing 0.25% trypsin for 30 min. Then they were transferred through sterile 108-μm^2 nylon mesh into a sterile centrifuge tube and centrifuged at 300 × *g* for 10 min. Cells were washed extensively with 5% FBS-DMEM and cultured in 20 ml flat-bottomed culture bottles (Sumitomo Bakelite, Tokyo, Japan) with 15% FBS-DMEM, 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin and L-glutamine, at 37 °C, 5% CO_2. At confluence, adherent cells were trypsinized, split in a 1:3 ratio, and recultured in medium. The spindle-shaped cells were used from passages 3 through 9 in subsequent experiments, during which time they were a homogeneous population of synoviocytes.

2.5. Proliferation assay by MTT

Synoviocytes were obtained using above method. After cells adherence, the culture medium was replaced and then cells were resuspended in 200 μl 15% FBS-DMEM medium containing 5 μg ml^{-1} LPS and TGP (12.5, 62.5, 312.5 μg ml^{-1}) at a concentration of 1.0 × 10^5 cells ml^{-1} in 96-well flat-bottomed culture plates. The cultures were incubated at 37 °C, 5% CO_2 for 48 h. Two hours before the termination of culture, MTT (5 mg ml^{-1}) 10 μl was added to each well. After incubation at 37 °C for an additional 2 h, the cells were centrifuged at 760 × *g* for 10 min and all the supernatants were aspirated without disturbing the pellet. The formazan crystals were dissolved by the addition of 120 μl dimethylsulfoxide (DMSO) and oscillated for 30 s. The absorbance (A) was measured on EJ 301 enzyme-linked immunosorbent assay (ELISA) Microwell Reader at 570 nm and the results were expressed as mean of triplicate wells.

2.6. Measurement of IL-1, TNF-α, PGE_2 and cAMP

Synoviocytes of CIA rats were obtained using above method. The adherent cells were resuspended in 15% FBS-DMEM with rTNF-α or rIL-1α and TGP and added to 24-well flat-bottomed culture plates at 1.0 × 10^6 cells ml^{-1} and incubated at 37 °C, 5% CO_2 for 72 h and centrifugation at 300 × *g* for 10 min. The supernatants were collected to measure the production of IL-1, TNF-α, and PGE_2 by RIA, according to the manufacturer's instructions, respectively. The supernatants were discarded and the adherent cells were scraped and disrupted by sonication. The cell lysates were harvested for detected the level of cAMP by RIA, according to procedures offered in ^{125}I cAMP kit.

2.7. Western blot analysis

Synoviocytes were cultured in 15% FBS-DMEM with TGP (12.5, 62.5, 312.5 μg ml^{-1}) in 6-well flat-bottomed culture plates at 5 × 10^6 cells ml^{-1} and incubated at 37 °C, 5% CO_2 for 72 h. Samples were homogenized in a buffer consisting of 50 mM Tris–HCl, pH 7.4, 1% Tween 20, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin. Debris was removed by centrifugation in a Beckman GPKR at 2200 × *g* for 20 min at 4 °C. A total of 50 μg of precipitated and denatured protein was separated by 10% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS–PAGE) and transferred electrophoretically to polyvinylidene fluoride membranes (Immun-Blot PVDF membrane, 0.2 μm, Bio-