Biomedicine & Pharmacotherapy 106 (2018) 255–259

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Paeoniflorin inhibits Rho kinase activation in joint synovial tissues of rats with collagen-induced rheumatoid arthritis

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ARTICLE INFO

Keywords:
Paeoniflorin
Rheumatoid arthritis
Rho kinase
Synovial tissue

ABSTRACT

Paeoniflorin (PF) has many effects, such as anti-inflammation, immune-regulation, abirritation, and so on. However, the protective mechanisms of PF on rheumatoid arthritis (RA) was not completely known. Thus, we explored deeply the protective mechanisms in a collagen-induced RA (CIA) rat model. CIA was induced in rats by intradermal injection of bovine type II collagen in complete Freund's adjuvant. Later, the CIA rats received oral administration of PF (50 and 100 mg/kg) once a day from the day 21, with the treatment lasting for 14 days. A variety of indicators were measured for evaluation of anti-rheumatism effect, including paw swelling, arthritis scores, and histopathological changes. And the contents of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) in the serum, as well as p-NF-κB p65 and p-MYPT1 in the joint synovial tissues were detected to explore the possible mechanisms. The results demonstrated that PF treatment significantly ameliorated the symptoms in CIA rats, reduced the levels of pro-inflammatory cytokines and paw swelling, down-regulated the expressions of p-NF-κB p65 and p-MYPT1. The present results revealed that PF could effectively improve collagen-induced RA in rats by inhibiting Rho kinase activation in the joint synovial tissues, in turn down-regulating expression of p-NF-κB p65 and reducing contents of pro-inflammatory cytokines. Moreover, PF may be an effective agent for RA.

1. Introduction

Rheumatoid arthritis (RA), a type of inflammatory disease with significant morbidity, is characterized by heterogeneous, chronic, immune-mediated symptoms, and is commonly associated with inflammation of the joints and destruction of the structures of the hands, feet, and large joints [1]. Patients with RA usually show symptoms of physical fatigue, pain, lower quality of life [2] and at a greater risk of dying younger [3]. Although the pathogenesis of RA is not completely clear, previous researches suggest that the pathogenesis of RA is related to the genes, infection, immune disorders, and the endocrine system [4]. It is verified that a large number of inflammatory factors play important roles in RA pathology [5].

NF-κB, a vital transcription factor, has been extensively reported to be the key to the pathogenesis of RA through regulating various genes involved in immune and acute phase inflammatory reaction [6,7]. In resting cells, NF-κB is sequestered in the cytoplasm by association with inhibitors of κB (IκBs). Upon inflammation stimulation, the inhibitor of κB kinase (IKK) complex is activated, leading to the phosphorylation, ubiquitination, and degradation of IκBs. The liberated NF-κB complex then translocates to the nucleus and binds to the cis-acting NF-κB enhancer element of genes, promoting the expression of inflammatory mediators such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) [8]. These pro-inflammatory cytokines play a significant role in the progression of RA [9].

Rho-kinase (ROCK) is a serine/threonine kinase and the crucial downstream effector protein of the small G protein RhoA. RhoA/ROCK play many roles in the regulation of cellular functions [10] including the production of pro-inflammatory molecules [11]. Researches over the past decades had shown that ROCK could mediate the production of pro-inflammatory cytokines and the proliferation of synovial fibroblasts [12–15], and the inhibitor of ROCK prevented NF-κB activation by suppressing IκB phosphorylation and degradation [16]. ROCK plays an important role in adjusting and activating NF-κB in RA [17].

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https://doi.org/10.1016/j.biopha.2018.06.130
Received 19 November 2017; Received in revised form 15 June 2018; Accepted 25 June 2018
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Paeoniflorin (PF) is the most abundant component of peony, which has been used in traditional Chinese medicine for many years. Many researches had reported that PF had multi-pharmacological effects, such as, immune-regulation [18], anti-inflammation [19], and hepatic-protection [20]. Recent studies found that PF might be a potential therapeutic agent for RA through anti-oxidative effects and the suppression of inflammatory process [21]. Besides, PF could inhibit the proliferation of lymphocytes and fibroblast-like synoviocytes, the formation of new blood vessels, and the production of matrix metalloproteinases [22]. However, it is not reported whether or not the effect of PF on CIA was related to ROCK/NF-κB pathway. In view of these findings, our study decided to explore whether PF could improve collagen-induced RA in rats by inhibiting Rho kinase activation in joint synovial tissues.

2. Materials and methods

2.1. Main reagents and kits

PF was purchased from Nanjing Zelang Biological Technology Co, Ltd. (Nanjing, China). Bovine collagen type II (CII) was purchased from Chondrex, Inc. (Redmond, WA, USA). Complete Freund's adjuvant was provided by Sigma-Aldrich (St. Louis, MO, USA). The radioimmunoassay (RIA) kits for TNF-α, IL-1β, and IL-6 were acquired from the Northern Institute of Biotechnology (Beijing, China). Antibodies of p-MYPT1 and p-NF-κB p65 were produced from Beijing Bioss Biotechnology Co. Ltd. (Beijing, China).

2.2. Animals and grouping

Male Wistar rats (6 weeks old; 160–180 g) were purchased from Hebei Experimental Animal Centre (Shijiazhuang, China), and remained in their cages under standard laboratory conditions (12-h light/dark cycle, 40–70% humidity, and a constant temperature of 22–24 °C), with unrestricted food and water. All the rats were fed according to the Animal Ethics Committee of Hebei University of Chinese Medicine (permit no. 1,703,094). All experiments were conducted in accordance with the international guidelines of the Animal Care and Use Committee. The rats were randomly assigned to four groups (n = 6): normal, CIA, PF (50 mg/kg), and PF (100 mg/kg).

2.3. Induction of CIA and PF treatment

Bovine type II collagen solution (2 g/ml) was emulsified with an equal volume of complete Freund's adjuvant to form a stable emulsion of 1 g/ml in an ice-cold water bath. Except the normal group, rats of the other groups were immunized with 200 μg of the emulsion. On day 21, those rats received a boost of an additional 100 μg to establish CIA models.

Previous study has confirmed that PF has neither adverse effects nor mortality in normal animals [20]. The CIA rats were given orally administered PF (50 and 100 mg/kg) once a day from the day 21 [23], concomitantly, the normal group and CIA group rats were orally administered the same volume of normal saline using the same method. These rats were sacrificed after 14 days of treatment.

The clinical symptoms were scored once per week from the first immunization. A scoring system was used to assess the severity of CIA [24]. Scoring was as follows: 0 = no swelling or redness; 1 = slight swelling and redness confined to the tarsals or ankle joints; 2 = mid range of swelling and redness extending from the ankle to the tarsals; 3 = serious swelling and redness extending from the ankle to metatarsal joints; and 4 = the most severe degree of swelling and redness encompassing the ankle, foot, and digits or ankylosis of the limb. The degree of severity of the disease in each paw was quantified individually; 16 was the maximum score for a single rat. Two experienced operators completed the evaluation process. The swelling degree of the paws was estimated using a plethysmometer to measure paw volume.

2.4. Histopathological evaluation of the ankle joints

The ankle joints subsequently harvested from the rats were fixed in 4% paraformaldehyde, paraffin-embedded, and cut into 4 mm slices using a microtome. Pathological improvement of the ankle joint was assessed through the tissue sections and hematoxylin and eosin (H&E) staining.

2.5. Measurement of pro-inflammatory cytokines in serum

Blood was drawn from the rats' femoral arteries and centrifuged at 5000 rpm for 15 min. The serum was obtained and stored at −80 °C for cytokines analysis. The concentration of IL-1β, IL-6, and TNF-α in the serum were analyzed using RIA kits following the manufacturer's instructions.

2.6. Immunohistochemical analyses of p-NF-κB p65 and p-MYPT1

NF-κB p65 is a representative member of NF-κB, p-NF-κB p65 is required a marker of NF-κB activation, MYPT1 is a major downstream target of ROCK, p-MYPT1 is a commonly used index of ROCK activation [25]. so we chose them as indexes to evaluate the activation of NF-κB and ROCK in the synovial tissues by Immunohistochemical analyses.

Paraffin-embedded sections (4 μm thick) of the synovial tissues separated from the rats' knee joints were incubated for 30 min with 5% bovine serum albumin (BSA). The sections were incubated with primary anti-p-NF-κB p65 and anti-p-MYPT1 in phosphate buffered saline (PBS) containing 1% serum overnight at 4 °C, secondary antibody for 30 min. The sections were then enhanced using DAB and counter stained with hematoxylin at room temperature. Images were captured using a light microscope. The values were obtained as the positive staining area and the integral optical density of each index using Image-Pro Plus. The images were analyzed by the following formula: mean optical density = integral optical density/positive staining area.

2.7. Statistical analysis

All statistical analyses were completed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). The data were presented as mean ± standard deviation (SD). Differences among the four groups were assessed using one-way analysis of variance (ANOVA) followed by Tukey's test, and were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effects of PF on macroscopic features of CIA rats

The hind paw volume was obviously increased after CIA immunization. However, A significant reduction in paw edema and redness was noticed after oral administration of PF at 50 and 100 mg/kg/day compared to the CIA group (Fig. 1A). The clinical arthritic score (Fig. 1B) and paw swelling (Fig. 1C) decreased significantly in a dose-dependent manner compared to the CIA group (both $P < 0.05$).

3.2. Effects of PF on histopathological changes

This study conducted H&E staining to evaluate the effects of PF on histological changes in the ankle joints. As shown in Fig. 2, the normal group presented clear and smooth tissues, without inflammatory cell infiltration. The CIA group demonstrated obvious hyperplasia of the synovial tissues, destruction of the articular cartilage, a narrowed articular cavity, and the infiltration of many inflammatory cells. PF 50 mg/kg and 100 mg/kg remarkably inhibited the hyperplasia of the synovial membrane and significantly reduced the infiltration of

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