Original Article

Osthole attenuates adjuvant-induced arthritis in rats by suppressing inflammation and oxidative stress via the SIRT1/TLR4/NF-κB pathway

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Abstract: Osthole (Ost) is a natural coumarin, mainly isolated from Cnidium monnieri. It has effects in many diseases, including tumours, osteoporosis, and inflammation. However, its molecular mechanism in arthritis remains unknown. This study investigated the effects and mechanism of Ost in preventing arthritis in Freund’s adjuvant-induced arthritis (AA) rats. The rats were injected with diclofenac sodium (DS; 5 mg/kg, i.p.) or Ost (20 or 40 mg/kg, i.p.) daily. The hind paw volumes (HPV) were measured. Inflammatory cytokines (IL-10, IL-1β, IL-6, and TNF-α), oxidant stress makers [malondialdehyde (MDA) and nitric oxide (NO)], and eicosanoids (LTB4 and PGE2) were determined at the end of the experiment. The expression of SIRT1, TLR4, and NF-κB in synovial tissue was analysed using Western blotting. Ost at doses of 20 and 40 mg/kg significantly reduced the HPV in AA rats. It also reduced the production of TNF-α, IL-6, IL-10, and IL-1β and altered the histopathological changes. Ost significantly increased the anti-inflammatory production of LTB4 and PGE2. Moreover, it inhibited oxidative stress by increasing the serum MDA and SOD levels in the rats. Moreover, Ost inhibited the expression of TLR4, and NF-κB, increased the SIRT1 expression in this model. These findings suggest that Ost suppresses the inflammatory response of adjuvant-induced arthritis rats via the SIRT1/TLR4/NF-κB signalling pathway.

Keywords: Osthole, arthritis, inflammation, oxidative stress, SIRT1/TLR4/NF-κB signalling pathway

Introduction

Rheumatoid arthritis (RA) is a polyarticular autoimmune disease that affects 0.5-1% of the population worldwide [1]. Many factors contribute to the disease progress, including immunological deregulation [2], genetic [3] and environmental factors [4, 5]. RA is characterised by joint swelling, synovial membrane inflammation, and cartilage destruction [6]. It is a clinical syndrome that includes several disease subsets [7], which involve several inflammatory cascades [8]. These all lead towards a final pathway in which persistent synovial inflammation and associated damage to articular cartilage and underlying bone are present.

Osthole (Ost) is a coumarin compound that is mainly isolated from Cnidium monnieri (L.) Cusson. It is used in traditional Chinese medicine. The fruit has anti-inflammatory and antioxidant effects and Ost is one of its important components. Recent studies have demonstrated the anti-inflammatory [9-12], anti-apoptotic [13], and anti-fibrotic [11, 14] properties of Ost.

The SIRT1/TLR4/NF-κB pathway is involved in inflammation and affects the pathogenesis of RA [15, 16]. It controls the production of pro-inflammatory cytokines in macrophages. The overexpression of Tissue necrosis factor-α (TNF-α) is important in inflammatory RA [17]. Sirtuin1 (SIRT1), an NAD⁺ dependent histone deacetylase, is a member of the conserved Sir2 (silent information regulator 2) family [18]. The expression of SIRT1 is increased in the synovium of RA patients [19]. In this study, we examined whether Ost suppresses inflammation and oxidative stress via regulation of the SIRT1/TLR4/NF-κB pathway in rats with arthritis.
Materials and methods

Experimental animals

Male 7-week-old Sprague-Dawley (SD) rats (n=50) weighing 200-250 g were raised under specific-pathogen-free conditions at 24 ± 2°C, 50 ± 10% relative humidity, and a 12 h light-dark cycle, with free access to water. The university ethics committee approved this animal study. The SD rats were assigned to five groups randomly: normal, Freund’s adjuvant-induced arthritis (AA), AA + diclofenac sodium (DS; 5 mg/kg), AA + Ost (20 mg/kg), and AA + Ost (40 mg/kg) groups with 10 rats per group. Ost (purity > 98%) was purchased from the National Institute for Food and Drug Control (Beijing, China). The RA rat model was made using Freund’s adjuvant-arthritis, as described previously [20]. The palmar surface of the left hind paw was injected with 0.1 mL of Freund’s adjuvant (BD Biosciences, Oxford, UK) on day 0, except for the control group. The Ost (20 or 40 mg/kg) and DS (5 mg/kg) was administered intraperitoneally (i.p) from day 21 to 28; the control and AA groups were treated with an equal volume of normal saline.

Hind paw volume measurement

We measured the hind paw volume (HPV) using an electronic water plethysmometer in all groups 0, 7, 14, 21, and 28 days after the start of the experiment. The HPV was calculated using the method of Coelho et al. [21].

Histological analysis

All animals were anesthetised with 3% chloral hydrate and sacrificed on day 29, and serum and ankle samples were collected. The ankle tissues were fixed with 4% paraformaldehyde, decalcified in hydrochloric acid for 1 week, and embedded in Paraffin. Tissue sections (5 μm) were stained with haematoxylin and eosin to examine the influx of inflammatory cells. An inflammation score was calculated based on the thickness of the lining cell layer, cellular density of the synovial stroma, and leukocytic infiltrate: 0, no inflammation; 1, slight thickening of the lining layer; 2, slight thickening of the lining layer plus some infiltrating cells in the sublining layer; and 3, thickening of the lining layer, influx of cells in the sublining layer, cells in the synovial space, and many inflammatory cells in the synovium [22].

Cytokine assay

The serum levels of the cytokines interleukin (IL)-6, IL-10, and IL-1β, and TNF-α were determined on day 28 using ELISA kits (Angene Biotech, Nanjing, China), according to the manufacturer’s instructions, as described in Ma et al. [23]. Briefly, 0.1 mL of serum was incubated in 96-well ELISA plates at 37°C. After 2 hours, the plates were washed, biotinylated detection antibodies (0.1 mL) were added, and the plate was incubated for 1 h at 37°C, and then washed extensively. Streptavidin-conjugated alkaline phosphatase was added for 1 h at 37°C. After washing, the alkaline phosphatase substrate was added to each well and incubated at 37°C for 10 min in the dark. The reaction was stopped with stop solution. Finally, the optical density of the samples was measured at a wavelength of 450 nm in a microplate spectrophotometer (KHBST-360, Shanghai, China) at room temperature. The cytokine levels are expressed as picograms per millilitre based on calibration curves plotted using standard concentrations of each cytokine.

Measurement of MDA and SOD activity

Superoxide dismutase (SOD) activity was measured using commercial assay kits (Jiancheng Biotech, Nanjing, China) and the malondialdehyde (MDA) levels were measured using a thio-barbituric acid reactive substances assay kit (Jiancheng Biotech, Nanjing, China). Both assays were performed according to the manufacturers’ instructions.

Measurement of LTB4 and PGE2

The serum eicosanoids were analysed using ELISA kits. The leukotriene B4 (LTB4) and prostaglandin E2 (PGE2) levels were determined from serum samples obtained on day 28 using ELISA kits (R & D Systems, Germany), according to the manufacturer’s instructions.

Western blotting

At the end of the experiment, rat synovial tissue was lysed on ice using chilled lysis buffer at 4°C. Then, the mixture was homogenised and centrifuged (13,000 rpm) at 4°C. The extract was extracted from the homogenised rat syno-
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Figure 1. Effect of Ost on rat HPV. Values are the mean ± SD and are representative of two independent experiments. *P<0.05, **P<0.01, significantly different from the values in the control group; †P<0.05, ††P<0.01, significantly different from the values in the AA group, n=6, Student’s t-test.

Result

Effects of osthole on joint damage in CFA-induced arthritic rats

To examine the effects of Ost in RA, we established a complete Freund’s adjuvant (CFA)-induced arthritis rat model by injecting CFA into the rat paw, which induced a mono-articular arthritis characterized by joint inflammation. Ost was administered once a day to the AA rats at doses of 20 or 40 mg/kg/day i.g. for 28 days. There were no significant differences among the groups for the first few days after injecting the CFA. On days 7, 14, 21, and 28, there was a significant increase in inflammation in the AA group compared with the healthy controls (P<0.01) (Figure 1). In the other groups, the HPV increased more slowly than in the AA group. A reduction in the swelling in the hind paw was seen in the Ost-treated AA rats. On the last day, there was a significant difference in HPV in the 40 mg/kg Ost group compared with the AA group (P<0.01) (Figure 1).

Histologically, the AA rats showed a massive inflammatory cell infiltration in the synovial lining layer and cartilage destruction, pannus formation, and narrowed joint space (Figure 2). Treatment with Ost significantly (P<0.05) inhibited the inflammatory cell infiltration in the AA rats.

Statistical analysis

The statistical analyses were performed using GraphPad Prism 5.0. Values are expressed as the mean ± SD and analyzed by using one-way analysis of variance (ANOVA) and Student’s t-test. P<0.05 was deemed statistically significant.

Discussion

To summarize, the current study demonstrates the potential of Ost as a novel therapeutic agent for the treatment of RA. Further studies are needed to elucidate the molecular mechanisms underlying the anti-inflammatory effects of Ost, and to evaluate its efficacy in clinical settings.
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Cytokine assays

We used ELISA kits to determine the serum levels of IL-6, IL-10, IL-1β, and TNF-α. TNF-α is the earliest and most important mediator of the inflammatory reaction. Compared with the controls, the AA rats had significantly \( P<0.01 \) elevated levels of all of the inflammatory cytokines (Figure 3). However, the treatment with 20 or 40 mg/kg Ost significantly \( P<0.05 \) and \( P<0.01 \), respectively) reduced the inflammatory cytokine levels compared with the AA rats. The cytokine levels were related to the dose of Ost.

Effect of Osthole on oxidative stress

Rheumatoid arthritis is characterised by excessive oxidative stress. SOD is an antioxidant enzyme that is inactivat-
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Rheumatoid arthritis can lead to increased production of inflammatory mediators such as the eicosanoids LTB4 and PGE2 [24], which cause vasodilation/contraction, plasma exudation, mucus secretion, bronchial contraction, and the accumulation of inflammatory cells. The serum LTB4 and PGE2 levels were significantly ($P<0.05$) reduced in the group treated with 40 mg/kg Ost (Figure 5).

**Effect of osthole on LTB4 and PGE2**

Rheumatoid arthritis can lead to increased production of inflammatory mediators such as the eicosanoids LTB4 and PGE2 [24], which cause vasodilation/contraction, plasma exudation, mucus secretion, bronchial contraction, and the accumulation of inflammatory cells. The serum LTB4 and PGE2 levels were significantly ($P<0.05$) reduced in the group treated with 40 mg/kg Ost (Figure 5).

Effects of osthole on the SIRT1/TLR4/NF-κB signalling pathway

To clarify the inflammatory signalling pathway involved, we quantified the protein levels of SIRT1, p-NF-κB, p-IκBα, and TLR4 in synovial tissue. Compared with the controls, the expression of p-NF-κB, p-IκBα, and TLR4 was significantly increased in AA rats, while the SIRT1 expression was significantly lower (Figure 6A). Moreover, in the Ost-treated group, the TLR4, p-NF-κB, and p-IκBα protein expression was higher than in the AA rats, the SIRT1 level was significantly reduced. Moreover, in further to determine the mechanism of Ost in the arthritis rats, the mRNA expression of SIRT1, TLR4 and NF-κB were detected. As shown in the Figure 6B, the mRNA expression of TLR4 and NF-κB was significantly increased in AA rats compared with the controls and SIRT1 mRNA expression was significantly lower. Ost could reverse the effects. Therefore, these results suggest that Ost can suppress inflammation and oxidative stress via regulation of the SIRT1/TLR4/NF-κB pathway in rats with arthritis.

Discussion

Osthole [7-methoxy-8-(3-methylpent-2-enyl) coumarin] is a derivative of coumarin that is extracted from the herb C. monnieri (L.) Cusson, a well-known traditional Chinese medicine used to treat skin rashes, eczema, and ringworm. Ost has anti-inflammatory effects and it improves lipid metabolism and fatty liver [25-27]. In this study, we tested the hypothesis that Ost suppressed inflammation and oxidative stress via the SIRT1/TLR4/NF-κB pathway in adjuvant-induced arthritis (AA) rats. We treated AA rats with 20 or 40 mg/kg Ost and found that the effect of Ost was dose dependent.

Rheumatoid arthritis is a chronic inflammatory joint disease that usually leads to cartilage and
bone damage, as well as disability. Many RA patients do not respond to existing therapies [28]. The etiology of RA remains unclear. The inflammatory and matrix regulatory responses affect the balance of chondrocyte catabolism and synovial osteoclastogenesis, which promotes articular destruction [29, 30]. Joint swelling, one of the most important characteristics of RA, is caused by synovial membrane inflammation. The leukocytes, including innate and adaptive immune cells, infiltrate the normally sparsely populated synovial compartment triggering the disease process. The HPV reflects the severity of joint swelling. As Figure 2 shows, Ost alleviated the joint swelling.

A complex network of cytokines and chemokines regulates the inflammation in RA. The pro-inflammatory cytokine IL-1β is closely related to inflammatory pain. The IL-1β level is very low in normal conditions, while it increases dramatically in many diseases, like RA [31]. In our study, the IL-1β levels were significant lower in the rats treated with diclofenac sodium and Ost, while they were high in the AA rats (Figure 4). IL-10 is a cytokine with multiple, pleiotropic effects in immunoregulation and inflammation. IL-6 is involved in inflammatory and fever reactions. TNF-α and IL-6 may play roles in stimulating granulocyte-monocyte colony. The IL-10, IL-6, and TNF-α levels were reduced in the groups treated with diclofenac sodium and Ost (Figure 4). These results showed that Ost can regulate inflammatory cytokine expression and reduce the inflammatory reaction.

SIRT1 is a mammalian homologue of yeast chromatin silencing factor that is an NAD⁺-dependent class III histone deacetylase with a highly conserved catalytic domain structure.
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that catalyses the acetylation of many substrates in a wide variety of physiological processes. It also regulates the activity of transcription factors like FOXO3 and NF-κB via deacetylation and regulates inflammatory metabolites [32]. NF-κB is an important signalling pathway in innate and adaptive immunity that is regulated by SIRT1; the NF-κB signalling pathway is a hub in the stimulation of inflammatory cytokines and lymphocyte activation [33]. SIRT1 deacylates the lysine 310 residue (K310) of P65, which decreases NF-κB transcription.

Toll-like receptor-4 (TLR4) is a transmembrane receptor in innate immune cells that binds non-specifically to pathogen-associated molecules and initiates signal transduction [34, 35]. TLR4/NF-κB induces the synthesis and release of inflammatory cytokines via both MyD88-dependent and MyD88-independent signalling pathways [36]. SIRT1/TLR4/NF-κB may be involved in inflammation and oxidative stress and affect the pathogenesis of RA [37]. As shown in Figure 6, the expression of SIRT1 in the Osthole-treated groups was significantly lower than that in the AA rats, and TLR4 and NF-κB were upregulated significantly. Therefore, Osthole plays a role in the SIRT1/TLR4/NF-κB signalling pathway and inhibits inflammation and oxidation in AA rats.

In this study, we examined whether Osthole suppresses the inflammatory and oxidation response of AA rats via the SIRT1/TLR4/NF-κB signalling pathway and showed that Osthole improves inflammation and oxidative stress via this pathway.

Disclosure of conflict of interest

None.

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