Drug Design, Development and Therapy

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ORIGINAL RESEARCH

Effect of Eriodictyol on Collagen-Induced Arthritis in Rats by Akt/HIF-1α Pathway

This article was published in the following Dove Press journal:
Drug Design, Development and Therapy

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Purpose: The aim of the experiment was to explore the effect of eriodictyol (ERI) on arthritis.
Methods: We established a rat model of collagen-induced rheumatoid arthritis (CIA) using type II collagen plus Freund's complete adjuvant. We evaluated the degree of paw swelling, joint pathology, inflammatory cytokine levels, and the Akt/hypoxia-inducible factor (HIF)-1α signaling pathway in the CIA rats.
Results: ERI significantly ameliorated joint swelling; improved joint pathology; and suppressed the release of interleukin-6, interleukin-1 beta, and tumor necrosis factor-alpha. Moreover, ERI inhibited the Akt/HIF-1α pathway in the joints of rats and in lipopolysaccharide-treated RAW264.7 cells.
Conclusion: ERI ameliorated arthritis in a manner involving the Akt/HIF-1α signaling pathway.
Keywords: Eriodictyol, arthritis, inflammation, Akt/HIF-1α pathway

Introduction

Rheumatoid arthritis (RA) is a chronic, systemic, autoimmune disease. Its main pathological features are synovial cell hyperplasia, thickening of the lining layer, infiltration of various inflammatory cells, formation of vasospasm, and subsequent destruction of cartilage and bone tissue. These effects lead to joint deformity and loss of function.[1] The clinical manifestations of RA patients include joint swelling and pain and stiffness in the morning.[2] In China, the incidence of RA is 0.26–0.5%. RA can occur at any age; the incidence increases with age.[3] Because its etiology and pathogenesis are unclear, and specific diagnostic methods are lacking, the treatment of RA is problematic. The drugs used to treat RA fall into the following three categories: steroidal anti-inflammatory drugs, non-steroidal anti-inflammatory drugs, and slow-acting anti-rheumatic drugs.[4,5] However, patients with RA frequently suffer adverse reactions such as nausea, vomiting, and osteoporosis.[6] The pathogenesis of RA is closely related to a variety of types of immune cells.[7]

RA usually erodes bones and destroys cartilage, which greatly reduces patients' quality of life (QoL) and can lead to disability if RA patients are not treated properly. Among various treatment methods, conventional anti-disease anti-rheumatic drugs (cDMARD) such as methotrexate (MTX) and sulfasalazine are considered the first choice for RA treatment, and many patients with RA are unresponsive or intolerant to these drugs and accompanied by other side effects such as gastrointestinal reactions. So we are looking for more effective drugs with less side effects. Chinese medicine has shown advantages in treating chronic diseases such as RA. Eriodictyol (ERI,

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http://doi.org/10.2147/DDDT.S239662

Supplementary material), a natural flavonoid, is found in many fruits and vegetables.[8] ERI has a variety of beneficial biological effects, such as antioxidant,[9] anti-inflammatory,[10] anti-diabetic[11] and anti-tumor[12] activity. Therefore, we explored the anti-inflammatory effects, and the underlying mechanisms, of ERI in collagen-induced rheumatoid arthritis (CIA) rats. Our findings will facilitate the development of novel anti-rheumatic agents.

Materials and Methods

Reagents and Kits

ERI (purity ≥ 98%) was obtained from Tauto Biotech Co., Ltd (Shanghai, China). Freund's complete adjuvant and bovine type II collagen were purchased from Sigma-Aldrich (St. Louis, MO, USA). ELISA kits of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β were purchased from Nanjing KeyGEN Biotech. CO., LTD (Nanjing, China). Primary antibodies of Akt, p-Akt, HIF-1α and GAPDH were purchased from Cell Signaling Technology (Danvers, USA).

Animal and Experimental Protocol

Fifty specific pathogen-free (SPF) grade SD rats (180–220 g) were purchased from the Charles River (Beijing, China). Rats were maintained under controlled temperature (22–24°C) and a 12 h light/12 h dark cycle circumstance. All animal experimental procedures were reviewed and approved by the Medical Ethics Committee of Southern Medical University. All animal experimentations in the present research were followed through in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Health's National Organizations.

The rats were randomly divided into five groups (10 rats each group): control group (Control), collagen-induced rheumatoid arthritis (CIA) group, CIA+ Diclofenac Sodium (DS, 5 mg/kg) group, CIA + ERI 20 or 40 mg/kg group. After Freund's complete adjuvant and bovine type II collagen in a 1:1 ratio with the concentration of 1g/L, 0.1 mL of emulsifier was subcutaneously injected with the sub-plantar region of right hind paw of each rat and then immunized with 0.1 mL of emulsifier again after 14 days. On day 15, rats in treatment groups were orally administrated with DS (5 mg/kg) or ERI (20 mg/kg or 40mg/kg) once a day for 4 weeks.

Evaluation of Arthritis

After the rat model of joint swelling was completed, the hind limbs of rats in each group were assessed by water volume method at regular intervals every week.

Detections of Cytokines

At the end of the animal experiment, blood was collected and centrifuged at 3500 rpm for 15 min to get supernatant for the following detection of cytokine. The levels of cytokines inducing IL-6, IL-1β and TNF-α were detected by ELISA kits in accordance with the manufacturer's directions.

RAW 264.7 cultured in the Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL. Co. Ltd., Grand Island, New York, USA) containing 10% fetal bovine serum (FBS, Beyotime Biotech, Shanghai, China) and supplementing with 1% penicillin-streptomycin (Beyotime Biotech, Shanghai, China) at 37°C and 5% CO_2. ERI at concentrations of 5–100 μM was treated with the cultured cells (about 10^4/96-well). In this part of experiment, the level of IL-1β, IL-6 and TNF-α were detected after cells were treated with LPS (1 μg/mL) with or without drugs (ERI, 10 μM, 20 μM, and 40 μM), exclude the control group (the cells without drug and LPS), for 24 h using ELISA assay kits according to the manufacturer's instructions.

Histological Study

Joint tissue was fixed in neutral-buffered formalin for 48 h at room temperature, decalcification, embedded in paraffin. And then sections were routinely deparaffinized by normal method and then cut into 4μm –slice and stained with hematoxylin and eosin (HE). The pathological injury scoring criteria are as follows: According to the severity of lesions in each part, the quantitative score is 0~4 points in turn. 1 point (mild), 2 points (moderate), 3 points (severe), 4 points (extremely severe), 0 points (basically normal), and the total score of lesions is 24 points. Accumulate all scores and calculate the average score (±SD) of each animal in each group. The lower the score, the lighter the lesion degree.

Western Blot Analysis

The CIA-synovium tissue or RAW264.7 cells were lysed with the radioimmunoprecipitation assay (RIPA) (Beyotime Biotech, Shanghai, China). The obtained lysing products were separated with a 15% sodium dodecyl sulphate-polyAcrylamide gel electrophoresis (SDS-PAGE) (Beyotime Biotech, Shanghai, China). The proteins were then electro-transferred onto the polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Little Chalfont, UK). PVDF membranes were subsequently incubated with the primary antibodies Akt, p-Akt, HIF-1α and GAPDH at 4°C overnight. The PVDF membranes were then treated with the horseradish peroxidase-conjugated secondary antibody at room

temperature for 2 h. Finally, PVDF membranes were incubated using a commercial ECL Kit (Cat. No. 32106, Thermo-Pierce, Rockford, IL, USA) in dark for 2 min at room temperature. The grey density of the bands was calculated with the image analysis software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical Analysis

In the present research, data were defined as mean ± standard deviation (SD) and analyzed using a professional SPSS software 20.0 (SPSS Inc., Chicago, IL, USA). The Tukey's post-hoc test validated analysis of variance (ANOVA) was applied to analyze the differences among multiple groups. And the student's *t* test was applied to analyze the differences between two groups. The p value less than 0.05 was defined as a significant difference.

Results

Effect of ERI on Paw Swelling

As shown in Figure 1, the degree of paw swelling was significantly increased in the CIA group compared to the control group ($P < 0.01$). The degree of paw swelling was significantly decreased after treating with DS and ERI (40 mg/kg).

Effect of ERI on Histological Parameters

Rats in the control group did not exhibit joint damage. However, marked changes in pannus formation, synovial hyperplasia, cartilage degeneration, and bone erosion were found in the CIA group. In contrast, ERI (20 and 40 mg/kg) and DS (5 mg/kg) ameliorated those effects, suggesting that ERI exerts an anti-inflammatory effect on arthritis (Figure 2).

Effect of ERI on RAW264.7 Cells

As shown in Figure 3, the results showed ERI did not exhibit cytotoxicity and then we selected the concentration range for use in subsequent experiments.

Effect of ERI on the Levels of Cytokines in vivo and in vitro

The levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β in serum and culture supernatant were increased in the CIA group compared to the control group. The levels of TNF-α, IL-6, and IL-1β in serum and culture supernatant were significantly reduced in the ERI groups, particularly the high-dose group (Figure 4).

Effect of ERI on Akt/HIF-1α Signaling in vivo and in vitro

To investigate the anti-inflammatory mechanism of ERI, we evaluated the levels of factors of the Akt/HIF-1α pathway in the joints of CIA rats and lipopolysaccharide (LPS)-treated RAW264.7 cells. The levels of p-Akt and HIF-1α were upregulated in CIA rats and LPS-treated

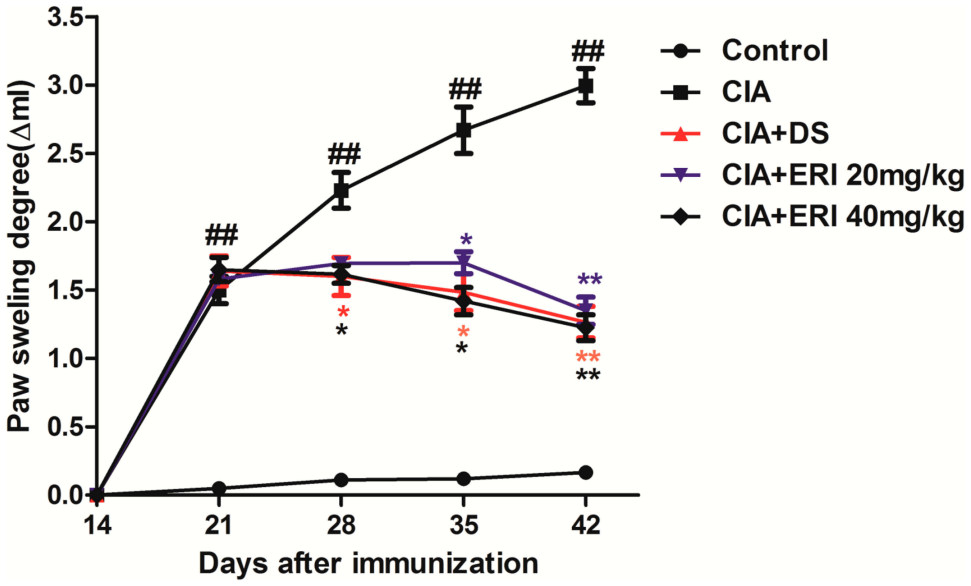

Figure 1 Effect of ERI on the paw swelling degree. Rats were given an intragastric injection of ERI (20 and 40 mg/kg) once a day for 4 weeks after the initial immunization. The paw swelling degree was assessed every 7-day intervals from day 1 to 42. Values represent the mean ± SD and are representative of two independent experiments. ##P < 0.01 versus control group; *P<0.05, **p < 0.01, versus CIA group.

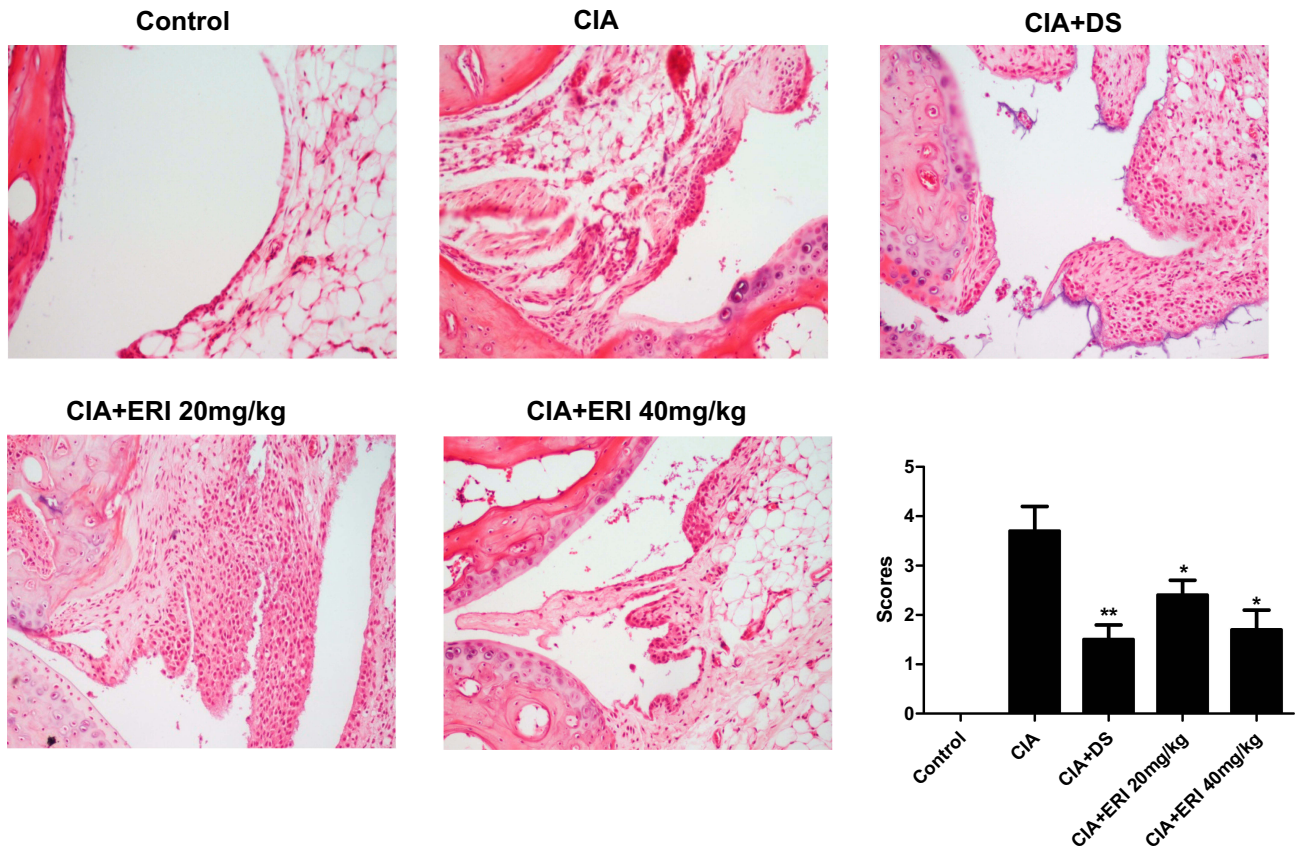

Figure 2 Histological assessment (×200). Rats were given an intragastric injection of ERI (20 and 40 mg/kg) once a day for 4 weeks after immunized again. Representative histological changes of joint tissues obtained from mice of different groups. Values represent the mean ± SD and are representative of two independent experiments. *P<0.05, **p < 0.01, versus CIA group.

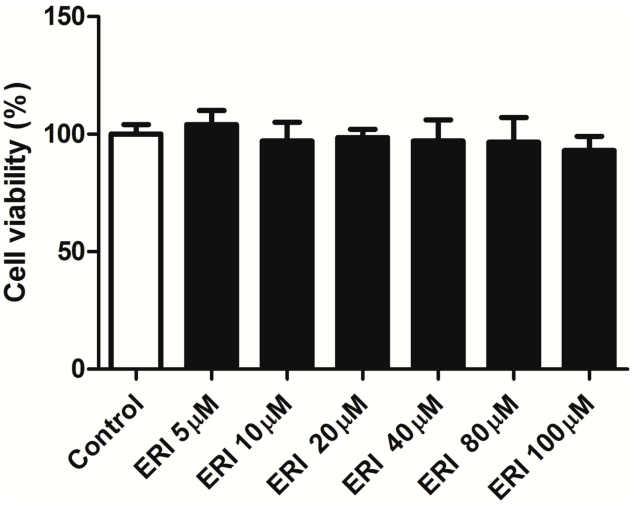

Figure 3 Effects of ERI on RAW264.7 cells by MTT assay. RAW264.7 cells were treated with ERI at concentrations of 5–100 μM and then the cytotoxicity was detected by MTT assay. Values represent the mean ± SD and are representative of two independent experiments.

RAW264.7 cells, while ERI or DS downregulated the levels of these proteins, suggesting that ERI affects Akt/HIF-1α signaling in arthritis (Figure 5).

Discussion

RA is a refractory disease in orthopaedic diseases, which troubles many orthopaedic doctors, and arthritis is a chronic disease with a long course of disease.[13,14] Its main pathological feature is chronic synovial inflammation. RA not only markedly reduces the patient's quality of life but also imposes a considerable economic and medical burden on their family and on society.[15] The pathogenesis of RA is unclear. In the west, RA is typically treated using hormone drugs. However, the adverse reactions caused by their long-term administration reduce compliance, resulting in treatment failure.[16] However, traditional Chinese medicine started from the root cause of its pathogenesis "malady" and treated it dialectically, based on the principle of enhancing body resistance and eliminating pathogenic factors, grasping its incidence trend as a whole, and achieving a gratifying curative effect.[17,18] In this study, ERI exerted a protective effect on RA.

Western medicines for the treatment of RA have a variety of side effects. In this study, rats in the diclofenac

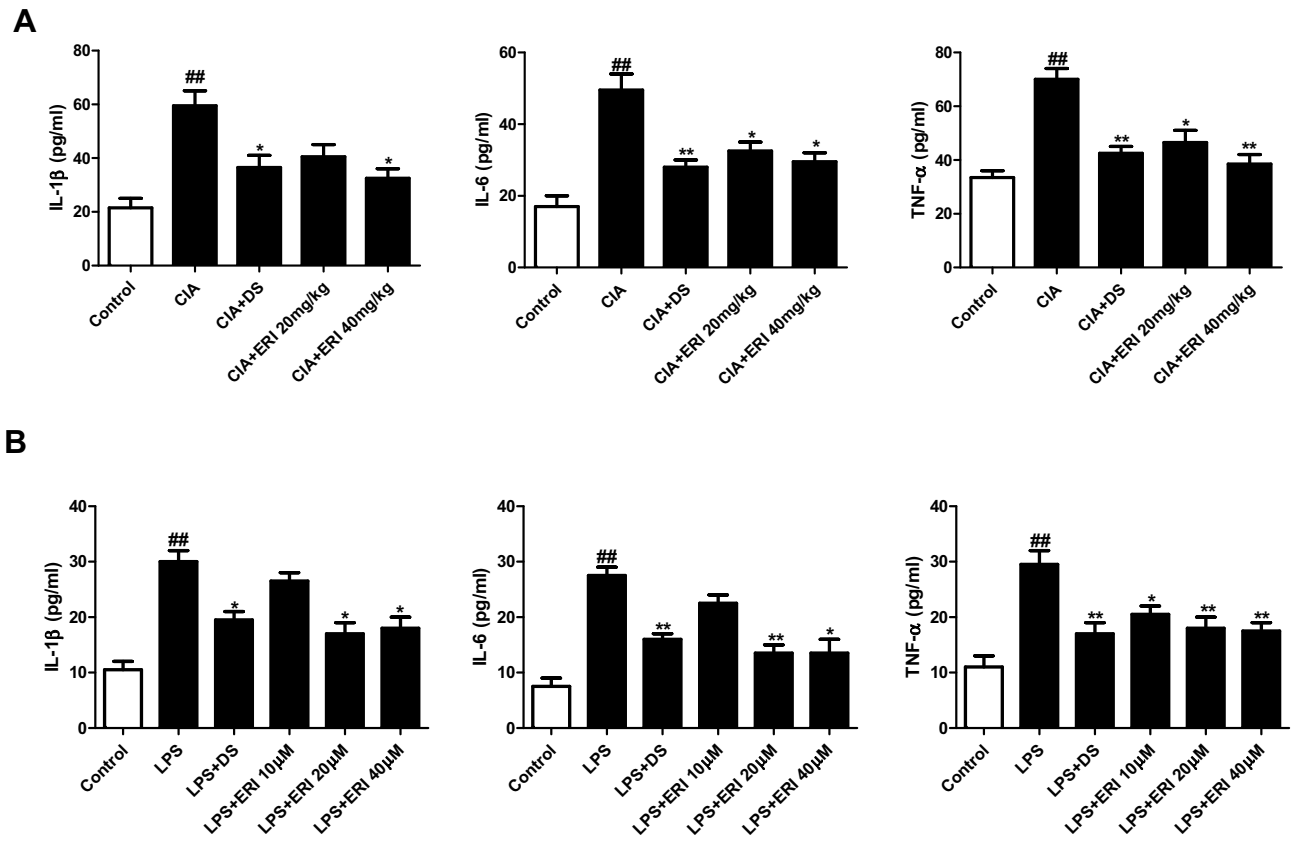

Figure 4 Effect of ERI on inflammatory cytokine levels in vivo and in vitro. (**A**) Rats were given an intragastric injection of ERI (20 and 40 mg/kg) once a day for 4 weeks after the immunized again. The concentrations of IL-1β, IL-6 and TNF-α were determined in serum. (**B**) RAW264.7 cells were treated with LPS. The concentrations of IL-1β, IL-6 and TNF-α were determined in culture supernatant. Values represent the mean ± SD and are representative of two independent experiments. $^{\#\#}P < 0.01$ versus control group; *P<0.05, **p < 0.01, versus CIA group.

sodium group exhibited decreased appetite, weight loss, hair loss, and listlessness, while those in the ERI group did not. Therefore, ERI showed both the anti-inflammatory effect of diclofenac sodium and few side effects, suggesting its therapeutic potential for RA.

Multiple signaling pathways play important roles in the production and secretion of inflammatory cytokines, which are implicated in the pathogenesis of RA. The PI3K-Akt intracellular signaling pathway is closely related to the development of RA.[19] The activation state of this signaling pathway is regulated negatively by phosphatase and tensin homolog and Src-homology-2-domain-containing inositol polyphosphate 5-phosphatase, and positively by TNF-α, transforming growth factor-β, and TNF-related apoptosis-inducing ligand.[20] The PI3K-Akt pathway is abnormally activated in synovial cells of patients with RA, leading to elevated expression of downstream anti-apoptotic genes and affecting multiple downstream effector molecules. These effects play a key role in the imbalanced proliferation and apoptosis of synovial cells in patients with RA.[21] Hyperproliferative synovial cells infiltrate articular cartilage and bone tissue, resulting in joint deformity and dysfunction in patients with RA.[22] Moreover, synovial angiogenesis is important in the initiation of RA and the subsequent pathological changes.[23] The resulting new microvessels not only recruit inflammatory cells but also provide nutrients for proliferating synovial cells and cover the erosion of articular cartilage, generating the RA joint cavity. The result is an anoxic microenvironment that leads to joint destruction and deformity typical of RA.[24] In this state, HIF-1α regulates the expression of other growth factors and participates in angiogenesis.[25] On this basis, we performed Western blotting to determine the protein levels of phosphorylated-Akt and HIF-1α. ERI significantly reduced the protein levels of p-Akt and HIF-1α in CIA rats. Therefore, ERI may regulate the Akt/HIF-1α signaling pathway, and so exert a protective effect on RA.

These inflammatory cells aggregate into specific cavities, producing mediators and inflammatory cytokines. These released substances build a complex network through narrow

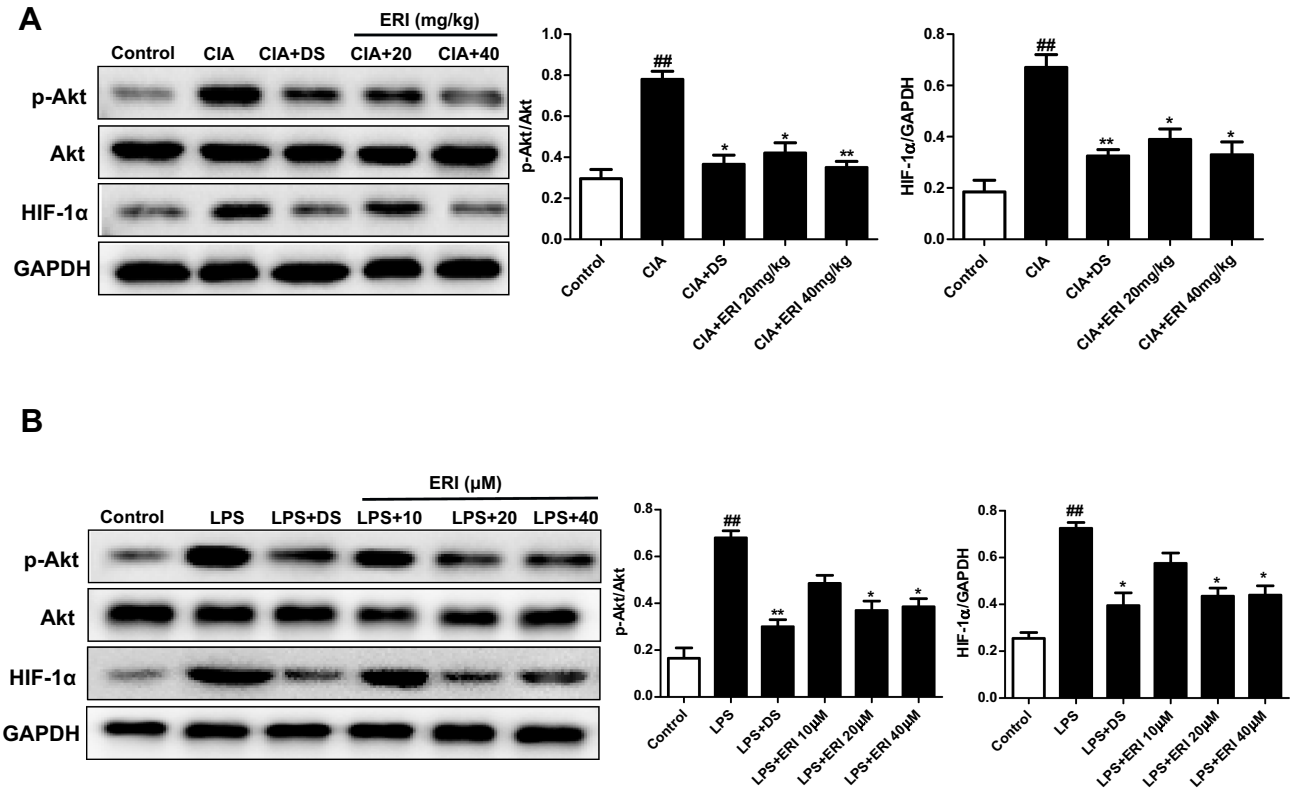

Figure 5 Effects of ERI on the expression of Akt/HIF-1α signaling in synovial tissue and RAW264.7 cells. (**A**) Rats were given an intragastric injection of ERI (20 and 40 mg/kg) once a day for 4 weeks after immunized again. Protein samples were analyzed by Western blot with specific antibodies. (**B**) Cells were treated with LPS (1 μg/mL) with or without drugs (ERI, 10 μM, 20 μM, and 40 μM) for 24 h. Protein samples were analyzed by Western blot with specific antibodies. Values represent the mean ± SD and are representative of two independent experiments. $^{\#\#}P < 0.01$ versus control group; *P<0.05, **p < 0.01, versus CIA group or LPS group.

joint space, vasculature formation, synovial hyperplasia, and bone destruction to accelerate joint damage. Tumor necrosis factor-α stimulates the innate immune cascade and mimics the secretion of other inflammatory cytokines.[26] There is evidence that anti-tumor necrosis factor-alpha antibodies and soluble tumor necrosis factor-alpha receptors are effective in the pathogenesis of rheumatoid arthritis.[27] Interleukin-1 is required for cartilage destruction and synovial cell self-proliferation in arthritic rats. Interleukin-6 is a cytokine overexpressed in rheumatoid synovium, causing joint damage. Our data implied that the inhibition of EIR could reduce the contents of inflammatory cytokines in RA arthritis.

In summary, ERI exerted a protective effect on RA, as evidenced by decreased hind-paw swelling and amelioration of the histological changes in joint tissues. The anti-inflammatory effect of ERI may be mediated by this modulation of the Akt/HIF-1α pathway.

Acknowledgments

The study was supported by the Nature Science Foundation of Hubei Province (2018CFB423).

Disclosure

The authors declare that they have no conflicts of interest to disclose.

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