Anti-arthritic activity of luteolin in Freund’s complete adjuvant-induced arthritis in rats by suppressing P2X4 pathway

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ABSTRACT

To investigate anti-arthritic activity of luteolin (Lut) in Freund’s complete adjuvant (FCA)-induced arthritis (AA) in rats. AA was induced by injecting with Freund’s complete adjuvant (FCA). Male rats were randomly divided into five groups with 10 mice in each group: (1) control group (saline), (2) AA group, (3) AA + Diclofenac Sodium (AA + DS, 5 mg/kg), (4) AA + Lut (20 mg/kg), (5) AA + Lut (40 mg/kg). Male SD rats were subjected to treatment with Lut at 10 and 20 mg/kg from days 18 to 24 after immunization. Arthritic scores, tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-17 (IL-17), paw histopathology and the proteins of P2X4 pathway were assessed at the end of the experiment. Lut reduced the severity of arthritic scores during the experimental period as compared with positive control (RA). Lut significantly suppressed TNF-α, IL-6, IL-1β and IL-17 as compared with RA group. Histopathological examination indicated that Lut alleviated infiltration of inflammatory cells and synovial hyperplasia as well as protected joint destruction. Lut significantly suppressed P2X4, NLRP1, ASC, and Caspase-1p10. Lut may be a potential preventive or therapeutic candidate for the treatment of inflammation and arthritis.

1. Introduction

Arthritis and related disorders, including rheumatoid arthritis (RA), are common diseases affecting millions of people [1]. RA is characterized by articular injuries having an inflammatory propagation of synovial cells, attaining a nearly complete functional defect. It affects about 1% of the general population [2]. RA is a chronic inflammatory autoimmune disease.

P2X4 receptor is a membrane cation-selective receptor channels and interacts with extracellular ATP [3]. It has been reported that the P2X4 expression promotes inflammatory response during experimental autoimmune encephalomyelitis [4]. P2X4 receptor is markedly upregulated by LPS due to activation of Toll-like receptors [5]. Of interest, recent work has demonstrated that P2X4-induced Ca2+ influx is required for effective production of IL-1β and IL-6 via activation of P2X7 receptor [6]. And thus P2X4 receptor has become a focus of attention in its role in inflammatory responses. However, the functional role of P2X4 in RA remains unknown.

Freund’s complete adjuvant (FCA)-induced arthritis in rats has been employed widely as a model for chronic systemic inflammation and possesses many features in common with human rheumatoid arthritis.

Recently, the multi-protein complex called the inflammasome has attracted researchers’ attention [7]. NLR family, pyrin domain containing 3 (NLRP3) inflammasome is the best studied inflammasome and has been involved in rheumatic and autoimmune diseases including RA [8–10]. However, little attention is paid to another member NLRP1. NLRP1 inflammasome is made up of NLRP1, PYD and CARD domain containing (ASC), and Caspase-1 [7]. NLRP1 is associated multiple autoimmune diseases [11]. Sui et al. reported that gene polymorphism of NLRP1 is a risk factor for RA [12].

Luteolin (3,4,5,7-tetra-hydroxylflavone), a flavonoid isolated from Lonicera japonica, exhibits a strongly anti-inflammatory activity, can effectively inhibit the lipopolysaccharide (LPS)-induced tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and inducible nitric oxide (NO) production in vitro [13]. As the literature survey shows that Lut may show antiinflammatory activity, therefore the objective of the present study was to determine the efficacy of Lut in Freund’s complete adjuvant (FCA)-induced arthritis in rats.
2. Materials and methods

2.1. Animals

Male SD rats were purchased from the Animal Department of China Pharmaceutical University (Nanjing, Jiangsu Province, China). All animals were housed under specific pathogen-free conditions with a 12-h light/dark cycle in a temperature-controlled room at 23 °C (±1) and allowed food and water ad libitum in each group of no more than ten.

All experiments using rats were performed in accordance with protocols approved by the Ethics Review Committee for Animal Experimentation of China Pharmaceutical University.

2.2. Reagents

Lut (pure: 99%) were purchased from National Institutes for Food and Drug Control (Beijing, China). Diclofenac Sodium was purchased from Xiansheng drug Store (Nanjing, China). The enzyme-linked immunosorbent assay (ELISA) kits for determination of IL-6, IL-17, IL-1β, and TNF-α were produced by Nanjing KeyGEN Biotech. CO., Ltd. (Nanjing, China).

2.3. Induction of AA

AA was induced as previously described [14]. Briefly, rats were immunized on day 0 by a single intradermal injection into the left hind paw with 100 μL of FCA for each rat.

2.4. Treatment of AA

Before the onset of arthritis, rats were divided into five groups randomly, in which the AA rats were given intragastrically Lut (10, 20 mg/kg) and DS (5 mg/kg) from days 14 to 24 after immunizations. While in groups of normal and AA model, rats were given an equal volume of water at the same time.

2.5. Arthritis assessment

Rats were assessed daily for signs of arthritis by two independent observers who were blinded to the experimental design. Non-injected hind paw volume was determined with YLS-7A volume meter (Shandong Academy of Medical Sciences Equipment Station, China).

The severity of arthritis in each paw was graded on a scale of 0–4: 0, no swelling; 1, swelling of finger joints; 2, swelling of phalanx joint and digits; 3, severe swelling of the entire paws; 4, deformity or ankylosis. The maximum arthritis score was 12 including three secondary arthritis paws for each rat [15,16].

2.6. Detection of cytokines

Rats were sacrificed using a dose of 30 mg/kg of pentobarbital after the end of the experiment, blood samples were centrifuged at 3000 rpm for 10 min at 4 °C, the supernatants were stored in −80 °C for analysis of cytokine levels of IL-6, IL-17, IL-1β and TNF-α were evaluated using ELISA kits in accordance with the manufacturer’s instructions.

2.7. Histological analysis

At the end of the experiment hind paws were removed above the knee joint and were fixed in 10% formalin saline solution. The paws were then decalcified in 10% EDTA for 14 days at 4 °C, embedded in paraffin, and sectioned in a mid-sagittal plane. The sections of articulation of the tarsal joints were stained with hematoxylin and eosin. The histological damage evaluated microscopically was defined according to system evaluated cartilage and bone destruction by pannus formation, mononuclear cell infiltration, and vascularity in synovial tissues. The histological damage evaluated microscopically was defined according to Omoto et al. [17].

2.8. Western blot analysis

The frozen joints were pulverized and proteins were isolated from the homogenized joints using a protein extraction kit (Invitrogen). The bicinchoninic acid (BCA) assay was used for protein quantification. Equal amount of proteins was loaded onto a 10% SDS–PAGE gel and subjected to electrophoresis. The proteins were transferred onto a PVDF membrane and blocked with 5% skim milk. Primary antibodies against anti-P2X4, anti-NLRP1, anti-ASC, anticaspase-1p10, and anti-GAPDH (all from Abcam) were used. The membrane was incubated overnight at 4 °C and then washed by PBST, followed by incubation with peroxidase-labeled secondary antibody. Protein visualization was achieved using enhanced chemiluminescence (ECL) Western blotting detection reagents (Pierce). Immunoreactive bands were visualized via a photoperoxidase Western blot detection system (Cell Signaling Technologies, Beverly, MA) and quantified via densitometry using Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA).

2.9. Statistical analysis

Data was expressed as mean ± SD and statistical analysis was carried out by using GraphPad 5.0 software (GraphPad, San Diego, USA) by applying one-way ANOVA with Dunnett’s test. P < 0.05 was considered to be significant.

3. Results

3.1. Effect of Lut on arthritis and body weight in AA rats

There was a remarkable inflammatory response in the model, which accompanied with paw swelling and polyarthritis. The administration of Lut (10, 20 mg/kg) significantly suppressed the hind paw swelling (Fig. 1A). While a significant decrease in polyarthritis index was observed when the AA rats were treated with Lut at 10 and 20 mg/kg and Lut at 20 mg/kg was better than Lut at 10 mg/kg. The efficacy was similar to that of DS (5 mg/kg) (Fig. 1B). The body weight was considered as apparent indicators of arthritis to evaluate the arthritic progression of adjuvant-induced arthritis. As shown in (Fig. 1C), we found that while the body weight of control rats increased steadily during the experimental period, the AA rats showed significantly lowered body weight from day 7 after adjuvant injection. From the 14th day after adjuvant injection, the body weight of Lut groups was significantly higher than that of AA; while, DS failed to increase the body weight of rat.

3.2. Effect of Lut on production of IL-6, IL-17, IL-1β and TNF-α in AA rats

The cytokines of AA rats were determined on day 24 after immunization. The production of IL-6, IL-17, IL-1β and TNF-α were increased in AA rats, the administration of Lut (10, 20 mg/kg) significantly suppressed IL-6, IL-17, IL-1β and TNF-α; but Lut at 20 mg/kg had no different between Lut at 10 mg/kg (Fig. 2).
3.3. Effect of Lut on histopathology in AA rats

Rats were sacrificed on day 24 after immunization and subjected to histopathological examination. The increased numbers of inflammatory cells were observed in AA rats; the administration of Lut (10, 20 mg/kg) significantly reduced inflammatory cells (Fig. 3).

3.4. Effect of Lut on P2X4, NLRP1, ASC, Caspase-1p10

The production of P2X4, NLRP1, ASC, Caspase-1p10 were increased in AA rats, the administration of Lut (10, 20 mg/kg) significantly suppressed P2X4, NLRP1, ASC, Caspase-1p10 (Fig. 4).
4. Discussion

RA is a common autoimmune disease, which is characterized by joint swelling and pain, joint stiffness, deformity and serious functional damage. AA could act as an experimental model in our study to demonstrate the effects of Lut on human RA because of its similarity to human RA in both clinical and histopathologic features.

Lut, which is the most abundant flavonoid in dandelions, has been reported to possess strong anti-oxidative and anti-inflammatory activities [18,19], for instance Lut inhibited LPS-induced inflammation in RAW 264.7 cells [20].

Proinflammatory cytokines play important parts in RA. IL-1β, as a crucial mediator in the promotion of synovial inflammation and pannus, promotes joint inflammation and bone destruction in RA patients [21,22]. IL-1β is also a pivotal factor that can induce expression of other proinflammatory cytokines [23]. IL-1β has previously been shown to be increased in synovial tissue [24] and correlated with histological features of arthritis [25]. IL-17 promotes inflammation via enhancing the production of IL-1β, TNF-α, and IL-6 [26]. Accumulated data suggest that IL-17 contributes to the pathogenesis of RA. Our study showed Lut significantly suppressed IL-17, IL-1β, TNF-α, and IL-6.

P2X4 receptor is expressed on the human osteoblast-like cells. However, the role of P2X4 in RA remains unknown; our study showed Lut significantly suppressed P2X4.

Recently, the role of NLRP1 inflammasome has attracted great interest in immunity and inflammation. NLRP1 polymorphisms confer risk for multiple autoimmune diseases including RA [27]. NLRP1 inflammasome-mediated IL-1β production is associated with autoimmune disorder [28]. Our study showed that Lut significantly suppressed NLRP1 inflammasome.

Fig. 3. Effect of Lut on histopathology in AA rats (Light microscopy, 200×). (A) (control); (B) (model); (C) (DS, 5 mg/kg); (D) [Lut (10 mg/kg)]; (E) [Lut (20 mg/kg)]. Values are expressed as means ± SDs. Compared with control: *P < 0.05, **P < 0.01; Compared with model: *P < 0.05, **P < 0.01.
Taken together, our findings suggest that Lut inhibited P2X4 and ameliorated the joint inflammation and destruction in RA, and these effects were due to inhibition of NLRP1 signaling pathways. Lut be regarded as a potential drug in the treatment of RA.

Conflict of Interest

The authors declare that there are no conflicts of interest.

References


