International Immunopharmacology 50 (2017) 161–167

Contents lists available at ScienceDirect

International Immunopharmacology

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

Cryptotanshinone protects against IL-1β-induced inflammation in human osteoarthritis chondrocytes and ameliorates the progression of osteoarthritis in mice

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ARTICLE INFO

Keywords:
Cryptotanshinone
Osteoarthritis
Chondrocytes
NF-κB
IL-1β
Inflammation

ABSTRACT

Osteoarthritis (OA) is a common degenerative disease characterized by progressive erosion of articular cartilage, subchondral bone sclerosis and synovitis. Cryptotanshinone (CTS), an active component extracted from the root of Salvia miltiorrhiza Bunge, has been shown to have potent anti-inflammatory effects. However, its effects on OA have not been clearly elucidated. This study aimed to assess the effect of CTS on human OA chondrocytes and mice OA models. Human OA chondrocytes were pretreated with CTS (5, 10 and 20 μM) for 2 h and subsequently stimulated with IL-1β for 24 h. Production of NO, PGE2, IL-6, TNF-α was evaluated by the Griess reaction and ELISA. The protein expression of COX-2, iNOs, MMP-3, MMP13, COX-2, ADAMTS-5, JNK, p-JNK, ERK, p-ERK, p38, p-p38, p-IKKα/β, p65, p-p65, IκB-α, and p-IκB-α was tested by Western blot. In vivo, the severity of OA was determined by histological analysis. We found that CTS significantly inhibited the IL-1β-induced production of NO and PGE2; expression of COX-2, iNOS, MMP-3, MMP-13, and ADAMTS-5. Furthermore, CTS in dramatically suppressed IL-1β-stimulated NF-κB and MAPK activation. Immunofluorescence staining demonstrated that CTS could suppress IL-1β-induced phosphorylation of p65 nuclear translocation. In vivo, treatment of CTS prevented the destruction of cartilage and the thickening of subchondral bone in mice OA models. These results indicate that the therapeutic effect of CTS on OA is accomplished through the inhibition of both NF-κB and MAPK signaling pathways. Our findings provide the evidence to develop CTS as a potential therapeutic agent f or patients with OA.

1. Introduction

Osteoarthritis (OA) is a common degenerative disease caused by joint instability and is wide spread in the world [1]. The main characteristic of OA includes progressive erosion of articular cartilage, subchondral bone sclerosis and synovitis, leading to severe joint pain and loss of function [2,3]. The exact etiology of OA remains unknown, but studies showed that inflammation are closely integrated processes in OA [4]. Inflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) play critical roles in the progression of OA [5]. Elevated levels of IL-1β has been found in synovial fluid, synovial membrane, subchondral bone, and cartilage of patients suffered from OA [6,7]. IL-1β could induce the production of matrix metalloproteinases (MMPs) and inflammatory mediator PGE_2 and NO production in chondrocytes [8,9]. Accumulated evidences proved that MMPs play vital roles in extracellular matrix (ECM) degradation during the progression of OA because they are responsible for the degradation of collagen-II and proteoglycans in articular cartilage [10]. Therefore, there is reason to believe that inhibition of IL-1β and IL-1β induced inflammatory mediators may attenuate the progression of OA.

Cryptotanshinone (CTS) is a quinonoid constituent that is found in the root of a Chinese herbal medicine, Salvia miltiorrhiza Bunge [11]. A growing body of studies indicated that CTS has diverse multiple pharmacological activities such as anti-inflammatory [12], antitumor [13,14], and antioxidative [15]. Previous studies showed that CTS inhibited LPS-induced inflammation through the inactivation of the nuclear factor-kappa B (NF-κB) in RAW 264.7 cells and mitogen-activated protein kinase (MAPK) [16]. Moreover, CTS ameliorated TNF-α-induced LOX-1 expression through ROS/NF-κB signaling pathway [17]. Additionally, CTS has been reported to attenuate the severity of collagen-induced arthritis (CIA) through the inhibition of NF-κB signaling

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http://dx.doi.org/10.1016/j.intimp.2017.06.017
Received 28 April 2017; Received in revised form 30 May 2017; Accepted 17 June 2017
Available online 27 June 2017

[18]. Meanwhile, a study also showed that CTS reduced the expression levels of IL-6 and TNF-α in primary cultured neonatal rat myocardial cells [19]. However, the anti-inflammatory effect of CTS in OA remains unknown. Therefore, we investigated the anti-inflammatory effects and the underlying mechanism of CTS on IL-1β-stimulated human OA chondrocytes in vitro and the role of CTS in mice OA models in vivo.

2. Materials and methods

2.1. Reagents

CTS, recombinant human IL-1β, collagenase type II, Safranin O/Fast Green, and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Cell-Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamo, Japan). Primary antibodies against COX-2, iNOS, MMP-3, MMP-13, ADAMTS-5, and p-IKKα/β were purchased from Abcam (Cambridge, MA, USA). Primary antibodies against p65, p-p65, IκB, p-IκB, JNK, p-JNK, ERK, p-ERK, p38, and p-p38 were purchased from CST (MA, USA). Dulbecco's modified Eagle's medium (DMEM)/F12, fetal bovine serum (FBS), and bovine serum albumin (BSA) were purchased from Healthcare life Sciences (Hyclone; Logan, UT, USA). Griess reagent was purchased from Beyotime Institute of Biotechnology (Shanghai, China). ELISA kits of PGE2, IL-6, and TNF-α were purchased from R & D systems (Minneapolis, MN, USA).

2.2. Isolation and culture of chondrocytes

Tissue collection was according to the terms of the Medical Ethical Committee of the Second Affiliated Hospital, Wenzhou Medical University and following the guidelines of the Declaration of Helsinki. Human cartilage samples were obtained from OA patients (aged 50–62 years, three men and three women) who underwent total knee replacement surgery at the Second Affiliated Hospital of Wenzhou Medical University. Full ethical consent was obtained from all patients. Cartilage was separated from underlying bone and connective tissues were cut into small pieces. The pieces were digested with a 0.25% trypsin-EDTA solution for 30 min and then incubated with 0.1% collagenase II in DMEM/F12 at 37 °C for 4 h. Then centrifuged at 1000 rpm for 5 min and the supernatant was discarded. The inner cell mass was obtained and suspended in DMEM/F12 with 10% FBS and 1% antibiotic mixture and incubated in an atmosphere of 95% air and 5% CO_2 at 37 °C. The medium was changed every 2–3 days. Cells were passaged when at 80 to 90% confluence using 0.25% trypsin-EDTA solution. Only passage 1 to 2 was used in our study to avoid the phenotype loss.

2.3. CCK-8 assay

The effects of CTS to chondrocytes were determined using a CCK-8 kit according to the manufacturer's instructions. Human OA chondrocytes were seeded onto 96-well plates (6000/well) for 12 h and then treated with various concentrations (5, 10 and 20 μM) Of CTS for 24 h. Afterwards, 10 μl CCK-8 was added to each well and incubated at 37 °C for 4 h. Absorbance at 450 nm was then measured using a microplate reader (Leica Microsystems, Gremany).

2.4. NO, PGE2, IL-6, and TNF-α measurement

The NO levels in the culture medium were determined by Griess reaction as previously described [20]. The level of PGE2, IL-6, and TNF-α in the culture medium was evaluated using commercial ELISA kit according to the manufacturer's instruction. All assays were performed in duplicate.

2.5. Western blotting

Western blotting was used to detect the protein level of iNOS, COX-2, MMP-13, ADAMTS-5, p65, JNK, ERK, p38, p-IKKα/β, and IκB. Total proteins were extracted from chondrocytes using RIPA and PMSF. Lysates were sonicated on ice and centrifuged at 12,000 rpm for 30 min at 4 °C. Protein concentration was determined using the BCA protein assay kit (Beyotime). 40 μg of the protein were loaded onto an SDS-PAGE gel and transferred to a PVDF membrane (Bio-Rad, USA). The membranes were blocked with 5% non-fat dry milk for 2 h at room temperature and subsequently washed three times for 5 min in TBS with Tween-20 (TBST). The membranes were incubated sequentially with primary antibodies against p65, p-p65, p-IκB, IκB, iNOs, MMP-3, MMP-13, COX-2, ADAMT-5, JNK, p-JNK, ERK, p-ERK, p38, p-p38, p-IKKα/β, and β-actin overnight at 4 °C, followed by incubation in specific antibodies (1:3000) for 2 h. All primary antibodies were used at 1:1000 dilution. After washing three times with TBST for 5 min, the membranes were incubated with HRP-conjugated secondary antibodies (1:3000) for 2 h. Finally, the intensity of these membranes was quantified with Image Lab 3.0 software (Bio-Rad).

2.6. Immunofluorescence microscopy

Chondrocytes were seeded on slices in a 6-well plate at a density of 3×10^5 cells/ml and incubated for 24 h. Glass coverslips with chondrocyte monolayers were rinsed three times in PBS. Then, cells were fixed with the 4% paraformaldehyde for 15 min and permeabilized in PBS containing 0.5% Triton X-100 for 15 min at room temperature. Later, cells were overlaid with 5% goat serum for 1 h at room temperature, rinsed with PBS, and incubated with primary antibody against p65 (1:200) at 4 °C overnight. On the next day, cells were incubated with fluorescein-conjugated goat anti-rabbit IgG antibody (1:400) for 1 h after washing with PBS and then labeled with DAPI (Invitrogen) for 1 min. Finally, images were captured using a fluorescence microscope (Olympus Inc., Tokyo, Japan).

2.7. Animal experiments

Ten-week-old C57BL/6 male wild-type (WT) mice were obtained from the Animal Center of Chinese Academy of Sciences, Shanghai, China. Care and use of all animals conformed to the Guidelines set forth by the Chinese National Institutes of Health, with relevant study protocols also approved by the Animal care and Use Committee of Wenzhou Medical University. OA induction was performed as previously described [21]. In this study, mice were randomly divided into three groups of 10 mice to establish a sham control group (sham), an osteoarthritis group (OA), an osteoarthritis treated with CTS group (CTS). CTS was dissolved in 0.5% carboxymethylcellulose sodium to form oral suspension. Mice in sham group were made by sham operation. Mice in CTS group received a gavage of CTS (10 mg/kg/d) for 16 days after surgery while mice in OA group received a gavage of 0.5% carboxymethylcellulose sodium.

2.8. Histological analysis

Knee joints were fixed in 4% paraformaldehyde for 24 h at 4 °C and decalcified in 10% EDTA solution at 4 °C for 4 weeks. The specimens then were embedded in paraffin and cut into 5 μm thick sections. The sections were stained with Hematoxylin and Eosin (H & E) and Safranin-O-Fast green staining to assess cartilage condition. The histological assessment was performed according to the grading of Osteoarthritis Research Society International (OARSI) scoring system in a blinded manner [20]. We applied AxioVision software to measure the thickness of the medial subchondral bone plate according to Safiranin O stained sections.