Life Sciences 158 (2016) 70–77

Contents lists available at ScienceDirect

Life Sciences

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

Cynaropicrin is dual regulator for both degradation factors and synthesis factors in the cartilage metabolism

Teruaki Masutani [a,b], Yuka Tsuda Tanaka [b], Hiroyuki Kojima [b], Makoto Tsuboi [b], Akira Hara [c], Masayuki Niwa [a,d,*]

[a] Medical Science Division, United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, 1-1 Yanagido, Gifu City, Gifu 501-1194, Japan
[b] Research & Development Dept., Ichimaru Pharcos Co., Ltd, 318-1 Asagi, Motosu City, Gifu 501-0475, Japan
[c] Department of Tumor Pathology, Gifu University Graduate School of Medicine, Gifu City, Gifu 501-1194, Japan
[d] Medical Development Center, Gifu University School of Medicine, 1-1 Yanagido, Gifu City, Gifu 501-1194, Japan

ARTICLE INFO

Article history:
Received 18 February 2016
Received in revised form 23 June 2016
Accepted 28 June 2016
Available online 29 June 2016

Chemical compounds studied in this article:
Cynaropicrin (PubChem CID: 119093)

Keywords:
Osteoarthritis
NF-κB
Cynaropicrin
HIF-2α
Sox9

ABSTRACT

Aims: The molecular mechanism of osteoarthritis (OA) has never been understood clearly, but it has been suggested that imbalance of degradation and synthesis in cartilage contribute to the underlying mechanisms of OA. In this study, we investigated the effectiveness in the cartilage metabolism of the artichoke extract that includes the compound cynaropicrin.
Main methods: We evaluated the efficacy of the artichoke extract or cynaropicrin in the cartilage metabolism factors and NF-κB signaling activity stimulated by inflammatory cytokine in chondrogenic cell lines, OUMS-27 and SW1353, using qRT-PCR, immunofluorescence and immunoblotting.
Key findings: We initially found that an artichoke extract and cynaropicrin both inhibited the increase of cartilage degradation factor MMP13 and further decreased the synthesis factor aggrecan induced by TNF-α in OUMS-27. In addition, cynaropicrin suppressed the enhancement of master regulator HIF-2α on cartilage degradation and further reduced the master regulator Sox9 on cartilage synthesis induced by TNF-α. We observed that cynaropicrin suppresses NF-κB signaling, which controls HIF-2α and Sox9. Since, HIF-2α is induced by p65 (RelA), we evaluated the effect of cynaropicrin and observed that it suppressed the nuclear transport of p65 (RelA) by inhibiting phosphorylation of IκBα. Moreover, cynaropicrin not only suppressed TNF-α stimulation, it had a similar effect on IL-1β stimulation. No significant cytotoxicity with cynaropicrin was observed.
Significance: These finding suggest that cynaropicrin is an effective substance that can improve the balance of cartilage metabolism, by altering the equilibrium of cartilage degradation and synthesis induced by multiple mediators know to contribute to OA.

1. Introduction

The negative impact of osteoarthritis (OA) on quality of life (QOL) is a growing global issue as the percentage of elderly individuals in most societies continues to increase. Though OA is a chronic degenerative disorder characterized by cartilage loss, the mechanisms are not completely understood. Currently, it is thought that cartilage loss is caused by an imbalance between anabolic and catabolic factors resulting from aging, mechanical stress and proinflammatory cytokines [1–3].

Sox9 is a key transcription factor of the cartilage synthetic pathway, and directly regulates expression of type II collagen and aggrecan, which are extracellular matrix (ECM) molecules in cartilage [4–6]. Also, cartilage from OA patients exhibit decreased expression of Sox9 and the expression of key cartilage matrix genes [7]. The synovium is frequently inflamed in OA resulting in increased levels of cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α), as well as matrix metalloproteinase (MMP, from chondrocytes), which degrade collagen and proteoglycan, and also impair ECM production [8–10]. Furthermore, IL-1β and TNF-α decrease the level of Sox9 mRNA and protein in chondrocytes [5]. MMPs are involved in the degradation of the type II collagen, with MMP13 playing a key role in collagen degradation in OA [11,12]. A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), also called aggrecanases, is involved with the degradation of proteoglycan, with an enhancement of ADAMTS4 activity occurring in OA [11,13,14]. The transcription factor hypoxia-inducible factor-2α (HIF-2α, encoded by *EPAS1*) is highly enhanced in OA cartilage [2,12,15–17]. Increased HIF-2α expression occurs in human OA cartilage, and increased HIF-2α expression is correlated with the progression of OA in human knee joint samples, reaching a maximum at the initial and progressive stages [16]. HIF-2α also induces a transcription of MMP13, ADAMTS4 and type X collagen involved in hypertrophic differentiation of chondrocytes. In other words, it is thought that HIF-2α is a master regulator of cartilage

* Corresponding author at: Medical Science Division, United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, 1-1 Yanagido, Gifu City, Gifu 501-1194, Japan.
E-mail address: mniwa@gifu-u.ac.jp (M. Niwa).

http://dx.doi.org/10.1016/j.lfs.2016.06.028
0024-3205/

degradation. Because RelA, Nuclear factor κB (NF-κB) family member, induced transcription of HIF-2α, it is thought that NF-κB signal activity is directly involved in the regulation of HIF-2α [15]. Furthermore, NF-κB signal activity regulates transcription of Sox9, thus signal activation of NF-κB may be an appropriate target in the treatment of OA [5].

We previously found a strong suppressing effect on NF-κB in an extract from *Cynara scolymus* L. (edible artichoke) [18]. It has been reported that cynaropicrin, sesquiterpene lactone contained in artichoke, has *in vitro* anti-inflammatory effects *via* its inhibition of the production of inflammatory mediators [19]. Since mechanisms of inhibition of NF-κB signaling activity by sesquiterpene lactones is different depending on each species, inhibition mechanisms by cynaropicrin have not been reported yet [20,21]. Moreover, it has been reported that sesquiterpene lactones apprehensive about cytotoxicity despite being superior efficacy [22–24].

In this study, we examined whether cynaropicrin may be an effective treatment for OA, by assessing its effect on cytokine stimulation of two chondrogenic cell lines OUMS-27 and SW1353. We also studied the potential for cynaropicrin to improve the balance between anabolic and catabolic factors by evaluating its effect on suppression of HIF-2α and activation of Sox9 through investigating suppression mechanism of NF-κB signaling activity.

2. Materials and methods

2.1. Cell cultures

Human chondrogenic cell line OUMS-27 and SW1353 were purchased from the Japanese Collection of Research Bioresources Cell Bank and American Type Culture Collection (ATCC). OUMS-27 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. SW1353 cells were cultured in DMEM/Ham's F12 containing 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cells were subcultured at split ratios of 1:2 to 1:4 using trypsin/EDTA every 7 to 10 days. The medium was changed every 2 days. Cells from 7 to 12 passages were used for experiments. The viability assay was evaluated by Cell Counting kit-8 (Dojindo, Japan).

2.2. Reagents and treatment of cells

An artichoke extract was made by the leaves of *Cynara scolymus* L. The leaves were soaked in 45% ethanol (15 times the leaf weight) for 2 h at 60 °C and then filtered and concentrated by use of evaporator. The concentrate was then freeze-dried (FD), cynaropicrin content including the FD powder is 3.3%, and adjusted with dextrin to have 1%(w/w) cynaropicrin content. The HPLC analysis of cynaropicrin was carried out under the following conditions: column, Mightysil RP-18GP (5 μm, 250 mm × 4.6 mm, Kanto chemical Co., Inc., Japan); column temperature, 40 °C; flow rate, 1 mL/min; solvent, CH_3CN: 0.1%H_3PO_4 = 18:72; detection, UV absorption at 210 nm. Cynaropicrin was purchased from Extrasynthese, France. TNF-α and IL-1β were purchased from HumanZyme, USA. For most experiments, 5×10^5 cells were plated in 60-mm dishes and transferred to serum-free DMEM for 24 h. The cells were then treated with artichoke extract or cynaropicrin at the indicated concentrations for 1 h and then exposed to TNF-α (1 ng/mL) or IL-1β (1 ng/mL) for the indicated times.

2.3. RNA isolation and reverse transcription (RT)-PCR

Cultured OUMS-27 cells were rinsed and total RNA was extracted using RNeasy kit (QIAGEN). The extracted RNA was quantified using a NanoDrop (Thermo Fisher Scientific, USA). cDNA was prepared from 50 ng of total RNA using Prime Script RT reagent Kit(Takara Bio, Japan) for RT-PCR. The RT-PCR conditions were reverse transcription reaction at 37 °C for 15 min, enzyme inactivation at 85 °C for 5 s.

2.4. Real-time quantitative PCR

Real-time quantitative PCR was performed using each specific primers and the SYBR Premix EX Taq II (Takara Bio, Japan). MMP13 (using the forward primer sequences 5′-ATGCATCCAGGGGTCCTGGC-3, the reverse primer sequences 5′-TGCTGCATTCTCCTTCAGGA-3), aggrecan (using the forward primer sequences 5′-GCCTTGAGCAGTTCACCTTC-3′, the reverse primer sequences 5′-CTCTTCTACGGGGACAGCAG-3′), HIF-2α (using the forward primer sequences 5′-ACCCAGACGGATTTCAATGAGC-3′, the reverse primer sequences 5′-TTGCTTCCGGCATCAAAGAAG-3′), Sox9 (using the forward primer sequences 5′-TGGGCAAGCTCTGGAGAC-3′, the reverse primer sequences 5′-CCCTCTCGCTTCAGGTCA-3′) and β-actin (using the forward primer sequences 5′-TTCCTGGGCATGGAGTTCCT-3′, the reverse primer sequences 5′-AGGAGGAGCAATGATCTTGATC-3′), as the internal control, were purchased from Hokkaido System Science, Japan. ADAMTS4 specific primers (QT00032949) were prepared by QuantiTect Primer Assays (QIAGEN). Real-time fluorescence detection was performed using the Thermal Cycler Dice Real Time System Single (Takara Bio, Japan). PCR cycling conditions were as follows, 94 °C for 15 min followed by 40 cycles at 94 °C for 30 s and 54–60 °C for 30 s.

2.5. Immunofluorescence and image quantification

The cells cultured on glass bottom dish were fixed with aldehyde for 10 min and incubated with blocking buffer (PBS with 10% goat serum and 1% BSA) for 1 h at room temperature. p65 (RelA) antibody (Santa Cruz Biotechnology, USA) at 1:50 dilution in Can Get Signal Solution B(Toyobo, Japan) was added and incubated for 1 h at room temperature. After washing three times for 10 min with TBS-T solution, goat-rabbit IgG antibody coupled to Alexa fluor 568 (Life Technologies, USA) at 1:200 dilution in Can Get Signal Solution B was incubated for 30 min at room temperature under shading condition. After washing three times for 10 min with TBS-T solution, nuclear DNA was stained with Hoechst 33342 (Dojindo, Japan). After washing three times for 10 min, the cover slips were mounted on glass bottom dishes (AGC Techno Glass, Japan). The images were acquired using fluorescence microscope (Olympus, Japan). The obtained images were transformed using IN Cell Transfer and quantitative analysis of the transformed images were performed using IN Cell Analyzer Workstation (GE Healthcare, UK).

2.6. Immunoblotting

The cells were lysed with radioimmunoprecipitation assay (RIPA) buffer after washing with cold phosphate-buffered saline (PBS) and total protein was extracted. The cellular debris was removed from the extracted protein by centrifugation for 5 min after freezing and thawing. The BCA method (Thermo Fisher Scientific, USA) was used to test protein concentration. The obtained protein were eluted by boiling in 2xSDS sample buffer included 2-mercaptoethanol for 5 min. The samples were separated by SDS-PAGE, followed by protein blotting onto a polyvinylidene fluoride (PVDF) membrane using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, USA). The protein-blotted membranes were blocked with 5% (w/v) fat-free dry milk in TBS with 0.05% Tween20 (TBS-T) overnight at 4 °C. They were then incubated with anti-phospho IκBα antibody (Cell Signaling Technology, USA) at 1:1000 dilution, anti-Sox9 antibody (Abcam, UK) at 1:1000 dilution and anti-β-actin antibody (Sigma-Aldrich, USA) at 1: 10,000 dilution in Can Get Signal Solution 1(Toyobo, Japan) for 2 h at room temperature. After washing three times for 5 min with TBS-T solution, the membrane were further incubated for 1 h at room temperature with goat-rabbit IgG antibody coupled to horseradish peroxidase (GE Healthcare, UK) at 1:10,000 dilution in Can Get Signal Solution 2 (Toyobo, Japan) and