Echinocystic Acid Inhibits IL-1β-Induced COX-2 and iNOS Expression in Human Osteoarthritis Chondrocytes

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Abstract—Echinocystic acid (EA), a pentacyclic triterpene isolated from the fruits of Gleditsia sinensis Lam, displays a range of pharmacological activities including anti-inflammatory and antioxidant effects. However, the effect of EA on IL-1β-stimulated osteoarthritis chondrocyte has not been reported. The purpose of this study was to assess the effects of EA on IL-1β-stimulated human osteoarthritis chondrocyte. Chondrocytes were stimulated with IL-1β in the absence or presence of EA. NO and PGE2 production were measured by Griess reagent and ELISA. The expression of COX-2, iNOS, nuclear factor-κB (NF-κB), inhibitory kappa B (IκBα), c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) were detected by Western blot analysis. The results showed that EA suppressed IL-1β-induced collagenase-3 (MMP-13), NO, and PGE2 production in a dose-dependent manner. IL-1β up-regulated the expression of COX-2 and iNOS, and the increase was inhibited by EA. Furthermore, IL-1β-induced NF-κB and mitogen-activated protein kinase (MAPK) activation were inhibited by EA. In conclusion, EA effectively attenuated IL-1β-induced inflammatory response in osteoarthritis chondrocyte which suggesting that EA may be a potential agent in the treatment of osteoarthritis.

KEY WORDS: echinocystic acid; osteoarthritis chondrocyte; IL-1β; NF-κB.

INTRODUCTION

Osteoarthritis (OA), the most prevalent form of arthritis, is a leading cause of disability among elderly patients [1]. The prevalence of OA increases with age [2]. Previous studies showed that OA affects at least 25% of men and 40% of women older than the age 75 years [3]. Accumulating evidence suggests that pro-inflammatory cytokines play critical roles in the development of OA [4]. Elevated IL-1β and TNF-α were observed in synovial fluid of patients suffered from OA [5, 6]. Stimulating of human osteoarthritis chondrocytes by IL-1β could induce nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) activation [7]. The activation of NF-κB and MAPKs could induce the production of inflammatory mediators such as NO and COX-2 [8]. These inflammatory mediators lead to clinical manifestations. Studies showed that inhibition of IL-1β-induced inflammatory responses has the ability to attenuate the development of OA [9].

Echinocystic acid (EA), a pentacyclic triterpene isolated from the fruits of Gleditsia sinensis Lam, has been reported to have antivirus and antioxidant effects [10, 11]. Recently, several studies confirmed that EA had anti-inflammatory effects [12]. It has been reported that EA suppressed inflammatory cytokines TNF-α and IL-6 production in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Studies also showed that EA inhibited TNF-α and IL-1β production in alveolar macrophages [13]. Previous reports showed that EA had a protective
effect on LPS-induced acute lung injury. In addition, EA was found to inhibit TNBS-induced colitis in mice [12, 13]. However, the protective effect of EA on osteoarthritis remains unclear. Thus, in this study, we used human osteoarthritis chondrocyte to assess the anti-inflammatory effect of EA and to investigate the underlying mechanisms.

**MATERIALS AND METHODS**

**Chemicals and Reagents**

Echinocystic acid was purchased from Shanghai PureOne Biotechnology Co., Ltd (Shanghai, China). Recombinant human IL-1β, ELISA kit of PGE2 was purchased from R&D systems (Minneapolis, MN, USA). Antibodies against p38, p-p38, extracellular signal-regulated kinase (ERK), phosphorylated ERK (p-ERK), c-Jun N-terminal kinase (JNK), phospho-JNK (p-JNK),

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**Fig. 1.** Effects of EA on the cell viability of chondrocytes. Cells were cultured with different concentrations of EA (0–10 μM) for 24 h. The cell viability was determined by MTT assay. The values presented are the means±SEM (n=8) of three independent experiments.

**Fig. 2.** EA inhibits MMP-13, NO, and PGE2 production, as well as iNOS and COX-2 expression up-regulated by IL-1β. The data presented are the means±SEM of three independent experiments, and differences between mean values were assessed by Students’ t test. #p<0.05 vs. control group; **p<0.01 vs. IL-1β group.
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p65, p-p65, inhibitory kappa B (IκBα), and phospho-IκBα (p-IκBα) were purchased from Cell Signaling Technology Inc. (Beverly, MA). Antibodies against iNOS and COX-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against aggrecan and type II collagen were obtained from Abcam (Cambridge, MA, USA). Griess reagent was purchased from Beyotime Institute of Biotechnology (Shanghai, China). All other chemicals were of reagent grade.

**Cell Culture**

Articular cartilage samples were obtained from 25 patients (age 57±13) undergoing total knee replacement surgery. The patients gave their informed consents, and the study was approved by the local ethic committee. The experiment was in accordance with the Declaration of Helsinki and Tokyo. Primary chondrocytes were isolated from articular cartilage as described previously [14]. Briefly, the cartilage was harvested from non-lesional areas and further minced. The tissues were digested with 0.25 % trypsin for 30 min. After that, the tissues were further digested by 2 mg/ml collagenase II in Dulbecco’s Modified Eagle’s Medium (DMEM) with antibiotics for 6 h at 37 °C. The cells were suspended in DMEM containing 10 % fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 mg/ml of streptomycin and cultured at 37 °C with 5 % CO2. The cells between passages 1 and 3 were used in this study [15, 16].

**MTT Assay**

The effect of EA on the viability of cells was assessed using 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Chondrocytes (6×10^3/well) seeded in a 96-well plate were treated with various concentrations of EA for 24 h. The medium was removed, and 20 μl MTT (5 mg/ml) was added to each well and cultured for an additional 4 h. After that, 150 μl of DMSO was added to each well. Absorbance at 570 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA).

**ELISA Assay**

The chondrocytes were treated with EA 1 h prior to IL-1β stimulation for 6 h. The concentration of PGE2 and MMP-13 in the culture medium was measured using an ELISA kit (R & D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**NO Measurement**

The chondrocytes were pretreated with EA 1 h and then stimulated with IL-1β for 6 h. The concentration of NO in supernatant was determined using the Griess reagent according to the manufacturer’s instructions.
Western Blot Analysis

The chondrocytes were pretreated with EA 1 h and then stimulated with IL-1β for 30 min. The total proteins from chondrocytes were extracted by Total Protein Extraction Kit (BestBio). Protein concentration was determined using the BCA protein assay kit (Thermo). Equal amounts of protein (40 μg) were separated on 12 % SDS-PAGE and transferred to PVDF membranes (Roche). After blocked with 5 % nonfat dry milk, the membranes were probed with primary antibodies (diluted 1:1000) against p38, p-p38, ERK, p-ERK, JNK, p-JNK, p65, p-p65, p-IκBα, IκBα, and β-actin overnight at 4 °C and probed with the HRP-conjugated secondary antibodies at room temperature for 2 h. The signals on the membrane were visualized using enhanced chemiluminescence reagents (ECL).

Statistical Analysis

Data are presented as means±SEM. Comparison between groups was made with ANOVA followed by Dunnett’s test, and *p<0.05 were considered to indicate statistical significance.

RESULTS

Effects of EA on Cell Viability

The potential cytotoxicity of EA on chondrocytes was evaluated by MTT assay. As shown in Fig. 1, EA at the doses of 0–10 μM contributed no cytotoxicity on chondrocytes. Thus, we chose EA (0–10 μM) in the subsequent experiments.

Effects of EA on IL-1β-induced NO and iNOS Expression

The effects of EA on IL-1β-induced NO production was measured using the Griess reagent. The results demonstrated that NO levels in the medium of chondrocytes increased significantly after IL-1β treatment. The treatment of EA suppressed IL-1β-induced NO production in a dose-dependent manner. iNOS has been reported to play a critical role in NO production during inflammation. Thus, in this study, we detected the effects of EA on iNOS expression by Western blot analysis. Our results showed that EA dose-dependently inhibited IL-1β-induced iNOS expression (Fig. 2).

Effects of EA on IL-1β-induced MMP-13, PGE2, and COX-2 Expression

MMP-13 plays critical roles in degrading cartilage. In this study, the results showed that EA dose-dependently suppressed IL-1β-induced MMP-13 expression. PGE2 is an important inflammatory mediator that plays a vital role in the pathogenesis of OA. To investigate the anti-inflammatory effects of EA, the production of PGE2 was detected by ELISA. As shown in Fig. 2, the production of PGE2 increased after IL-1β treatment. EA significantly inhibited IL-
1β-induced PGE₂ production. The production of PGE₂ is mainly through the COX-2 pathway. Thus, the expression of COX-2 was also detected. The results showed that EA dose-dependently suppressed IL-1β-induced COX-2 expression (Fig. 2).

**Effects of EA on Aggrecan and Type II Collagen Expression**

The effects of EA on aggrecan and type II collagen expression were detected by Western blot analysis. As shown in Fig. 3, IL-1β significantly decreased the expression of aggrecan and type II collagen. However, treatment of EA markedly up-regulated the expression of aggrecan and type II collagen (Fig. 3).

**Effects of EA on IL-1β-Induced MAPK Activation**

MAPKs also play an important roles in the regulation of inflammatory mediators NO and PGE₂. In this study, the effect of EA on IL-1β-induced MAPK activation was detected. The results showed that EA
dose-dependently inhibited IL-1β-induced MAPK activation (Fig. 5).

DISCUSSION

Osteoarthritis (OA) is a widespread musculoskeletal disease which is characterized by erosion of articular cartilage and subchondral bone sclerosis [17]. Pharmaceutical and dietary strategies have targeted these disorders to control OA [18, 19]. Recently, studies showed that many natural products are good candidates for the control or treatment of OA [20-22]. Echinocystic acid (EA), a pentacyclic triterpene isolated from the fruits of G. sinensis Lam, has been reported to have anti-inflammatory effects. In this study, we found that EA inhibited IL-1β-induced inflammation in chondrocytes. These results suggested that EA may be a potential agent in the treatment of osteoarthritis.

Inflammatory cytokines such as IL-1β have been reported to play important roles in OA [23]. Stimulating of chondrocytes by IL-1β could up-regulate the expression of iNOS and COX-2, which promotes the production of NO and PGE₂ [24]. NO has the ability to induce the production of PGE₂ and inflammatory cytokines [25]. PGE₂ is implicated in the degeneration of articular cartilage [26]. Elevated NO and PGE₂ were observed in patients with OA [27]. Previous studies showed that attenuation of the production of inflammatory mediators such as NO and PGE₂ had the ability to attenuate the development of OA [28]. In this study, we found that EA dose-dependently inhibited the production of NO and PGE₂, as well as iNOS and COX-2 expression. Previous studies showed that MMP-13 played an important role in cartilage degradation during OA. IL-1β has the ability to up-regulate the expression of MMP-13. In this study, our results showed that EA significantly inhibited IL-1β-induced MMP-13 production.

NF-κB, one of the most ubiquitous and important transcription factors, has been shown to be involved in the regulation of inflammatory mediators such as NO and PGE₂ production [29, 30]. Under normal conditions, NF-κB is sequestered in the cytoplasm and bound to its inhibitor IκB. Upon certain stimuli, such as IL-1β or LPS, NF-κB p65 translocates from the cytoplasm to the nucleus to regulate the production of inflammatory mediators [31]. MAPKs, including ERK, P38, and JNK, are a group of signaling molecules that also play important roles in the regulation of inflammatory mediators [32]. To investigate the mechanisms of EA on the inhibition of inflammatory mediator production in chondrocytes, the effects of EA on LPS-induced NF-κB and MAPK activation were detected. The results showed that EA inhibited IL-1β-induced inflammatory response in chondrocytes via inhibition of NF-κB and MAPK activation.

In conclusion, the present study demonstrated that EA inhibited IL-1β-induced MMP-13, NO, and PGE₂ production, as well as iNOS and COX-2 expression in chondrocytes via inhibition of NF-κB and MAPK activation. These results suggested that EA may be a potential agent in the treatment of OA. Previous studies showed that EA protected against LPS-induced acute lung injury and TNBS-induced colitis through intraperitoneal or oral administration [12, 13]. These results indicated that EA has a short-time effect and may have systemic anti-inflammatory effects. In the following studies, we will consider local application by intra-articular injections as cartilage does not have a blood supply and it might not reach the chondrocytes. EA will be given for multiple intra-articular injections due to its short-time effect.

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REFERENCES

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