Isoliquiritigenin Inhibits IL-1β-Induced Production of Matrix Metalloproteinase in Articular Chondrocytes

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Osteoarthritis (OA) is a major joint disease in which inflammatory cytokine interleukin-1β (IL-1β) and matrix metalloproteinases (MMPs) play a pivotal role. Isoliquiritigenin has been reported to have anti-inflammation activity. In this study, the effect of isoliquiritigenin on IL-1β-induced production of matrix metalloproteinase and nuclear factor κB (NF-κB) activation was analyzed. We treated primary cultured articular chondrocytes with isoliquiritigenin and the expressions of MMPs were analyzed on mRNA and protein level. The phosphorylation of IκBα and p65 was analyzed to detect NF-κB activation. We also used in vivo model by treating mice with isoliquiritigenin and detecting the level of MMPs. IL-1β induced NF-κB activation and MMP-1, MMP-3, MMP-9, MMP-13, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and ADAMTS-5 production on chondrocytes. A 10-µM isoliquiritigenin treatment significantly inhibited IL-1β-induced NF-κB activation and these MMPs production on chondrocytes. Injecting isoliquiritigenin into rat knee joint also inhibited IL-1β-induced NF-κB activation and MMPs production in articular cartilage. Isoliquiritigenin treatment inhibited IL-1β-induced MMPs production and NF-κB activation both in vitro and in vivo, suggesting a potential therapeutic role of isoliquiritigenin to treat osteoarthritis.

INTRODUCTION

Osteoarthritis (OA) is the most common degenerative joint disease, affecting more than 25% of the population over 18 years old.1 OA is a progressive and dynamic process in joint tissues, including cartilage, underlying bone, the entire synovial joint, synovium, and muscle. One of the main symptoms in OA is progressive degeneration of articular cartilage including chondrocyte loss and degradation of the extracellular matrix (ECM). This phenomenon represents deregulation of chondrocyte metabolism due to the actions of inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α), which is responsible for the downregulation of collagen as well as proteoglycan biosynthesis and stimulation of their degradation.

OA affects predominantly articular cartilage, which degrades by gradual loss of its ECM composed mainly of aggrecan and type II collagen. Loss of large proteoglycan aggrecan decreases cartilage compressive stiffness and precedes the damage to collagen fibrillar network, which is responsible for tensile properties of the tissue.2 The activation of degradative enzymes leads to the loss and degradation of proteoglycan and collagen in articular cartilage and the MMPs play a pivotal role in the destruction of articular cartilage in OA.3 MMPs can be classified into collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), and stromelysins (MMP-3, -7, -10, and -11).4 Among these MMPs, the role of MMP-1, -13, -2, -3, and -9 in OA has been reported.5 In addition, the anti-OA effects of inhibitors targeting these MMP have also been reported,6–11 indicating inhibiting MMP activities should be an effective therapy to treat OA.

Isoliquiritigenin (ISL) is a flavonoid derived from Glycyrrhiza uralensis. It has diverse biological activities including anti-allergic,12 anti-angiogenesis,13 anti-tumor growth,14 and anti-inflammation.15–18 For the anti-inflammatory activity, ISL has been reported to inhibit LPS-induced nuclear factor κB (NF-κB) activation16 and MAPK activation.18,19 IL-1β can activate NF-κB and induce MMPs production,20,21 playing an essential role in OA pathophysiology. Thus, in this study, we tested the potential role of ISL on IL-1β-induced MMPs production and NF-κB activation.

RESULTS

ISL Showed No Effects on Primary Rat Chondrocytes Proliferation (Cytotoxicity Assay)

The primary rat chondrocytes were treated with 5, 10, and 20 µM ISL for 72 hr and then SRB assay was performed to determine the cytotoxicity of ISL. As shown in Figure 1, there was no significant difference of cell viability in cell samples treated with 5, 10, and 20 µM ISL when compared to control. These data indicated that the selected concentration of ISL did not affect the cell viability of primary rat chondrocytes in long-term cultures and we used these concentrations for following experiments.

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ISL Inhibited IL-1β-Induced NF-κB Activation

IL-1β can initiate several signal transduction pathways, leading to an increase in intracellular Ca2+, activation of PKC, p38, ERK1/2, and JNK, and nuclear translocation of NF-κB, activating transcription factor (ATF), and activator protein 1 (API). As ISL has been shown to be able to inhibit the NF-κB activation in several different models, we continued to test whether ISL also inhibited the IL-1β-induced NF-κB activation in primary cultured articular chondrocytes. As shown in Figure 4A, IL-1β treatment activated NF-κB activation as increased phosphorylated-p65 and phosphorylated-IκBα detected in IL-1β alone samples. In the presence of ISL, the phosphorylation of both p65 and IκBα decreased. After quantitation, we detected significantly decreased protein level of p-p65 and p-IκBα in the presence of 5-μM ISL (Figure 4B), indicating ISL inhibited IL-1β-induced NF-κB activation, which was in a dose-dependent manner.

DISCUSSION

OA, a common form of arthritis, is an age-related degenerative disease characterized by the chronic joint pain, inflammation and the damage of joint cartilage. The degeneration is mainly characterized by a progressive degradation of extracellular matrix (ECM) components, followed by chondrocyte death, tissue fibrillation, and erosion. OA affects predominantly articular cartilage, which degrades by gradual loss of its ECM composed mainly of aggrecan and type II collagen. The progressive loss of aggrecan and collagen leads to cartilage degradation and development of OA. In our study, we detected the inhibition of MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4, and ADAMTS-5 as well as NF-κB production in articular cartilage tissues. The treatment of ISL inhibited NF-κB activation and decreased protein levels of MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4, and ADAMTS-5 production in articular cartilage tissues. To investigate whether ISL also shows the potential effect in vivo, we examined the effect of intra-articular injection of ISL into the knee joint of rats on IL-1β-stimulated production of MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4, and ADAMTS-5 as well as NF-κB activation from articular cartilage tissues. As shown in Figures 5A and 5C, treatment with IL-1β (20 ng/20 μL) activated NF-κB and increased MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4, and ADAMTS-5 production in articular cartilage tissues. The treatment of ISL inhibited NF-κB activation and decreased protein levels of MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4, and ADAMTS-5 production in articular cartilage tissues. After quantitation, treatment of 20 mg/kg ISL significantly inhibited IL-1β-induced NF-κB activation and suppressed MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4, and ADAMTS-5 production (Figures 5B and 5D). The inhibition effects were in a dose-dependent manner; treatment of 100 mg/kg ISL showed the best inhibition effects. Thus, our data indicated that ISL also functioned in vivo.
collagen. Loss of large proteoglycan aggrecan decreases cartilage compressive stiffness and precedes the damage to collagen fibrillar network, which is responsible for tensile properties of the tissue. Aggrecan degradation is associated with upregulation of aggreganases ADAMTS-4 and -5 as well as MMPs. The excessive cleavage of type II collagen in OA is assumed to be caused by the upregulation of the synthesis and activities of collagenases, in particular MMP-13. Presently, it is believed that articular cartilage destruction in OA results from excessive loading, age-related changes, and metabolic imbalance in the tissue.

OA also exhibits features of a systemic disease as it has been shown to involve vascular pathology as well as T cell immune response associated with upregulation of cytokines such as IL-1β and TNF-α, which aggravate cartilage resorption. IL-1β has been reported to induce MMP-1, MMP-3, MMP-13 and ADAMTS-4 in human tendon cells and promote MMP-1 and MMP-2 expression in human aortic valve myofibroblasts. In this study, we also detected that IL-1β activated NF-κB and induced production of MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4, and ADAMTS-5 in articular chondrocytes both in vivo and in vitro (Figures 2, 3, and 5), suggesting the essential role of IL-1β and MMPs in OA.

As the mechanism of OA development is not completely understood, the disease manifestations, which are associated with cartilage resorption and inflammation, suggest a treatment involving inhibition of proinflammatory cytokines or MMP activity to prevent matrix destruction. IL-1β activity is mediated solely by binding to its specific receptor, IL-1RI and induces phosphorylation of dependent signaling pathways with p38 MAP kinase and NF-κB as principal pathways that regulate various gene expression, including the synthesis of several inflammatory cytokines and MMPs. Specific inhibitions of IL-1β through the application of IL-1 receptor antagonist protein, soluble IL-1 receptors, monoclonal antibodies against IL-1β, blocking the formation of active IL-1β, blocking the IL-1β cellular signaling pathways have been used as treatment to OA. Using MMP-13 inhibitor CL82198, Wang and colleagues found CL82198 inhibited MMP-13 activity in media from primary murine chondrocytes and intraperitoneal injection of CL82198 decelerated OA progression.

Figure 2. Effect of Isoliquiritigenin on the Gene Expression of MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4, and ADAMTS-5 in Chondrocytes

Primary cultured articular chondrocytes were pretreated with varying concentrations of isoliquiritigenin for 2 hr and then stimulated with IL-1β (10 ng/mL) for 24 hr. The gene expression levels of MMP-1 (A), MMP-3 (B), MMP-9 (C), MMP-13 (D), ADAMTS-4 (E), and ADAMTS-5 (F) were measured by real-time PCR. Three independent experiments were performed and the representative data were shown. Each bar represents a mean ± SEM of three independent experiments in comparison with that of the control set at 100%. *Significantly different from control (p < 0.05). Significantly different from IL-1β alone (#p < 0.05).
increased type II collagen and proteoglycan levels. Baragi et al. found ALS 1-0635, a MMP-13 inhibitor, inhibited bovine articular cartilage degradation in a dose-dependent manner and modulated cartilage damage in rat model. Thus, the inhibitors exert chondroprotective effects and can potentially modulate joint pain, and are, therefore, uniquely suited as potential disease-modifying OA drugs. However, these treatments were not entirely satisfactory, and searching for new drugs is required to achieve the desired goals of therapy.

ISL is a flavonoid derived from licorice compounds and showed various biological activities including antioxidant and anti-inflammatory properties. A previous study has revealed ISL could significantly inhibit cytokine-induced endothelial cell adhesion molecule expression through NF-κB. ISL could also dampen MMP-1 and MMP-2 production via inhibition of MAPK-responsive signaling pathway. These previous studies strongly suggested ISL, which can inhibit MMPs production and NF-κB activation, could have the potential to treat OA. Thus, we tested the possible inhibitory effect of ISL on MMPs production and NF-κB activation in our in vitro primary cultured articular chondrocytes model. We found that ISL is quite safe as the highest concentration of 20 μM did not cause toxicity on chondrocytes (Figure 1) while 5 μM ISL treatment significantly prevented IL-1β-induced MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4, and ADAMTS-5 production (Figures 2 and 3) and NF-κB activation (Figure 4). The inhibition effect of ISL on MMPs production was reflected on both mRNA and protein level, suggesting the ISL inhibited the transcriptions of MMPs. As we detected the inhibition of NF-κB at the same time, it is clear that the inhibition of MMPs production by ISL was through NF-κB pathway. Furthermore, we found the inhibitory effect of ISL on IL-1β-induced MMPs production and NF-κB activation also existed in vivo. We detected decreased protein levels of MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4, and ADAMTS-5 from rat articular cartilage lysate, together with decreased NF-κB activation (Figure 5). Taking together, our data showed ISL exerts chondroprotective effects both in vivo and in vitro, suggesting that ISL as a novel agent for the control of cartilage damage in OA.

In summary, our experiments showed that the chondroprotective effects of ISL are produced by inhibiting IL-1β-induced production of MMPs through NF-κB.

**MATERIALS AND METHODS**

**Primary Cultures of Chondrocytes from Rat Articular Cartilage**

A modified method for harvesting chondrocytes was performed as previously described. Briefly, rat chondrocytes were isolated from articular cartilage in 3-week-old male Sprague Dawley rats. Cartilage was removed from animals that were subsequently pretreated with varying concentrations of isoliquiritigenin for 2 hr and then stimulated with IL-1β (10 ng/mL) for 24 hr. The protein levels of MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4, and ADAMTS-5 were measured by western blotting (A). Relative protein expressions to control were shown as histogram in (B). Six independent experiments were performed and the representative data were shown. Each bar represents a mean ± SEM of three independent experiments in comparison with that of the control set at 100%. Significantly different from control (*p < 0.05). Significantly different from IL-1β alone (#p < 0.05).
euthanized by an overdose of anesthesia. The cartilage was cut into thin slices and then washed with sterilized PBS and soaked in 5% penicillin-streptomycin-neomycin (Sigma, St. Louis, MO, USA) for 15 min. The cartilage slices were washed with PBS to remove residual antibiotic solution and digested with 0.02% collagenase type II (Sigma, USA) in DMEM (HyClone, Logan, UT, USA) for 3 hr in a 37°C water bath. Digested cartilage was collected and centrifuged. The pellet was resuspended in DMEM and filtered through 70 μm nylon mesh. The resultant rat chondrocytes were cultured in DMEM supplement with 10% fetal bovine serum and 1% penicillin-streptomycin-neomycin in a 5% CO2 incubator at 37°C.

Treatment of Cells with ISL
Chondrocytes were seeded on 6-well culture plates with a density of 2 × 10^5 cells/mL. After 2 days in monolayer culture, the cells were incubated for 2 hr in growth medium with 5, 10, or 20 μM ISL, followed by incubation in the presence or absence of 10 ng/mL IL-1β for 24 hr. ISL was purchased from Shaanxi Green Bio-Engineering (Xi'an, China) and dissolved in 1% sodium carboxymethyl cellulose (CMC-Na).

Cytotoxicity Assay
Chondrocytes were seeded at a density of 2 × 10^5/mL (0.1 mL/well) in a 96-well microtiter plate and allowed to attach for 24 hr to keep the log phase growth at the time of drug treatment. After incubation with the indicated drug concentrations for 72 hr, cell proliferation was determined using the sulforhodamine B (SRB) assay (Sigma, St. Louis, MO, USA).

Quantitative Real-Time PCR
RNA isolation was performed by using the RNeasy mini kit (QIAGEN, Valencia, CA, USA). The Quatitect reverse transcription kit (QIAGEN, USA) was used to synthesize the subsequent cDNA. Real-time PCR was performed using SYBR Green Master Mix (QIAGEN, USA). Samples were normalized to internal control GAPDH. Primer sequences used for real-time PCR are listed in Table 1.

In Vivo Experiments
Male Sprague-Dawley rats weighing 200–210 g were used to investigate the effect of ISL in articular cartilage in vivo. Animals were housed five per cage, provided with distilled water and food ad libitum, and kept under a 12 hr light/dark cycle at constant temperature (22.5°C) and humidity (55%). Animals were cared for by the Care and Use of Laboratory Animals of the National Institutes of Health of Cangzhou Central Hospital. The rats were randomly divided into six groups: control, IL-1β only, 20 mg/kg ISL plus IL-1β, 50 mg/kg ISL plus IL-1β, 100 mg/kg ISL plus IL-1β or 100 mg/kg ISL alone. Rats were anesthetized with vaporized diethylether, and those from the ISL plus IL-1β or ISL alone groups received a 30 μL injection of different amounts of ISL, respectively, into the right knee joint. After 3 hr, rats received a 30 μL injection of 20 ng IL-1β in sterile PBS into the right knee joint. Rats from the control group were injected with 30 μL of sterile PBS. Rats were euthanized via CO2 asphyxiation 72 hr after injections. Articular cartilage (tibial plateau and femoral condyle) was isolated from each animal, homogenized, and prepared for measurement of protein by western blot analysis.

Western Blot
A total of 50 μg of proteins from either cell lysate or articular cartilage homogenate were loaded onto a 12% SDS-PAGE gel. After transfer, membranes were blocked by 5% non-fat milk and incubated with
different primary antibodies for overnight at 4°C. Next day, corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies were incubated. After washing, the membranes were finally washed with distilled water.

**Table 1. Primer Sequences Used for Real-Time PCR**

| Gene    | Primer          | Sequences (5′-3′) |
|---------|----------------|------------------|
| MMP-1   | forward         | CCGGCAAGAATGGAAACAG |
|         | reverse         | GCCGATTTTGGCCCTGGTTT |
| MMP-3   | forward         | TTTGCGCCTCTTCATCCATC |
|         | reverse         | GGAGGCCAGAGTGGGATG |
| MMP-9   | forward         | AGGGGCCCTTCTTTATGCCC |
|         | reverse         | CGAGTAAAGCTCCTGGGATC |
| MMP-13  | forward         | GAAGATCGCTTGGTTGCGT |
|         | reverse         | GGATCCGGAAGAGTACCA |
| ADAMTS-4| forward         | CATCCTAGCGCGAAGATGC |
|         | reverse         | AAGCGGAGGCTTGTTCG |
| ADAMTS-5| forward         | CCCAAATACGGCTGGTCT |
|         | reverse         | ACCAGCGAGGTGGCTAGG |
| Gapdh   | forward         | TGGGACGGAATTTGGCCTG |
|         | reverse         | TGAACCCTGCGCTGGTAGAG |

Statistical Analysis

One-way ANOVA analysis, followed by a Tukey’s post hoc test was used to determine the related protein levels. Statistical difference was considered as significant only if p < 0.05.

AUTHOR CONTRIBUTIONS

Designed the study, did the experiments, and wrote the manuscript: L.Z.; did the experiments and analyzed the data: S.M., H.S., and J.C.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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