Icariin inhibits MMP-1, MMP-3 and MMP-13 expression through MAPK pathways in IL-1β-stimulated SW1353 chondrosarcoma cells

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Abstract. Osteoarthritis (OA) is the most common type of arthritis and is a leading cause of disability worldwide, resulting in pain, reduced quality of life and socioeconomic burden. Current therapies for OA focus on mitigating the symptoms of advanced disease, but novel therapeutic agents are needed to inhibit the processes leading to OA. The present study aimed to investigate the effects of Icariin on matrix metalloproteinase (MMP)-1, MMP-3 and MMP-13 expression in interleukin (IL)-1β-stimulated human SW1353 chondrosarcoma cells, and to investigate the possible mechanism underlying the chondroprotective effects of Icariin. In the present study, IL-1β was applied on SW1353 chondrosarcoma cells to mimic the microenvironment of osteoarthritis. The cells were treated with Icariin and mitogen-activated protein kinase (MAPK) signaling pathway activators or inhibitors. MMP-1, MMP-3, MMP-13, phosphorylated (P)-p38, P-c-Jun N-terminal kinase (JNK) and P-extracellular signal-regulated kinase (ERK) expression was assessed using reverse transcription-quantitative polymerase chain reaction, ELISA and western blot analysis. The results of the present study demonstrated that Icariin inhibited the expression of MMP-1, MMP-3, MMP-13, P-p38, P-ERK and P-JNK. Furthermore, it was revealed that the inhibition of p38 and ERK contributed to the inhibition of MMP-1 and MMP-3 by Icariin, whereas the inhibition of p38 and JNK contributed to the inhibition of MMP-13. The present results suggested that Icariin may have a chondroprotective effect, exerted through the inhibition of MMP-1, MMP-3 and MMP-13 via MAPK pathways. Therefore, Icariin may have potential as a novel therapeutic strategy for the treatment of osteoarthritis.

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Introduction

Osteoarthritis (OA) is the most prevalent chronic form of arthritis among older people, and is characterized by cartilage degradation, synovitis and remodeling of the subchondral bone (1,2). Existing therapeutic options include non-steroidal anti-inflammatory drugs and selective cyclooxygenase-2 inhibitors. These drugs provide symptomatic relief from the pain and inflammation associated with the later phases of OA; however, they do not target the dysregulated molecular processes responsible for the onset of OA. Furthermore, pharmacological interventions fail to prevent cartilage damage and the associated destruction of joint tissue, whereas they produce extensive adverse effects (1,2). Therefore, the development of agents that exhibit improved therapeutic and safety profiles for the treatment of OA is of critical importance.

OA is characterized by the enhanced degradation of critical extracellular matrix (ECM) components, such as aggrecan and collagen, in joint tissue (3). It has previously been suggested that an excess of matrix metalloproteinases (MMPs), such as MMP-1, MMP-2, MMP-3, MMP-8, MMP-9 and MMP-13, may serve a central role in the breakdown of ECM components, due to their ability to cleave various macromolecules, including collagen and aggrecan (4). Numerous complex pathways and mechanisms are involved in ECM degradation (5). It has previously been reported that mitogen-activated protein kinases (MAPKs) serve a critical role in the cytokine-mediated regulation of MMP expression, and consequent cartilage degradation (6). Furthermore, it has been demonstrated that the levels of phosphorylated-MAPKs, including p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) are upregulated in osteoarthritic cartilage. A number of inflammatory mediators have been identified in OA joint tissues and fluids; interleukin (IL)-1β expression was revealed to be mediated by the transcription factors nuclear factor-κB and AP-1, resulting in the increase expression of IL-1β in OA cartilage (1,7). In primary human chondrocytes and the SW1353 human chondrosarcoma cell line, MMP-1,

MMP-3 and MMP-13 were strongly induced by IL-1β. IL-1β-stimulated human SW1353 chondrosarcoma cells appear to be a valuable *in vitro* chondrocytic experimental system (8).

Icariin is a prenylated flavonol glycoside, and the main active component of Herba Epimedii. It possesses antioxidant and anti-inflammatory properties, and can also promote osteoblast differentiation (9-11). In addition, Icariin has been reported to interfere with the activation of MAPKs (12). Therefore, it may be hypothesized that Icariin holds potential as a novel treatment for OA.

The present study investigated the effects of Icariin on the expression of MMP-1, MMP-3 and MMP-13 in IL-1β-stimulated SW1353 chondrosarcoma cells. In addition, transcription factors possibly involved in the process, including phosphorylated (P)-p38, P-JNK, and P-ERK, were also assessed.

Materials and methods

Cell cultures. Human SW1353 chondrosarcoma cells (Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% v/v fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. The following treatments were applied: SB203580, a p38 inhibitor (10 μM); PD98059, an ERK inhibitor (10 μM); SP600125, a JNK inhibitor (10 μM) (Beyotime Institute of Biotechnology, Haimen, China); U-46619, a p38 and ERK activator (50 μM; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); Anisomycin, a p38 and JNK activator (10 μg/ml; Santa Cruz Biotechnology, Inc.); or Icariin (20 μM; Merck KGaA, Darmstadt, Germany). Control cells received no treatment. After 1 h at 37˚C in 5% CO_2 cell incubator, cells were then stimulated with the addition of IL-1β (10 ng/ml; PeproTech, Inc., Rocky Hill, NJ, USA) for 24 h.

Enzyme-linked immunosorbent assay (ELISA). Culture media were centrifuged at 450 x *g* for 20 min, and the resultant supernatants were transferred to a clean tube. The concentrations of MMP-1, MMP-3 and MMP-13 in the cell-free supernatants were determined using ELISA kits (cat. nos. SEA097HU, SEA101HU and SEA099HU; Uscn Life Sciences, Inc., Wuhan, China) according to the manufacturer's protocols.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol® reagent (cat. no. 15596018; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using the ReverTra Ace-α-kit (cat. no. FSK-101; Toyobo Co., Ltd., Osaka, Japan). RT-qPCR was performed using a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR-Green Realtime PCR Master Mix (cat. no. QPK-201; Toyobo Co., Ltd.). The following primers were used for RT-qPCR: MMP-1, forward 5'-CCCCAAAAGCGTGTGACAGTAAG-3', reverse 5'-AAGGGATTTGTGCGCATGTAG-3'; MMP-3, forward 5'-CTCGGTTCCGCCTGTCTCAAG-3', reverse 5'-GGAAGAGATGGCCAAAATGAAGAGA-3'; MMP-13, forward 5'-GCGTCATGCCAGCAAATTC-3', reverse 5'-GTTCCAGCCACGCATAGTCAT-3'; and β-actin, forward 5'-CTCCATCCTGGCCTCGCTGT-3' and reverse 5'-GCTGTCACCTTCACCGTTCC-3'. Cycling conditions were as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec, 59˚C for 30 sec and 72˚C for 1 min. Dissociation curves were obtained using a thermal melting profile performed after the last PCR cycle as follows: 59˚C for 30 sec followed by a constant increase in the temperature from 60 to 95˚C. β-actin was used as an endogenous control, and the $2^{-\Delta\Delta Cq}$ method was used to calculate the relative fold-changes in mRNA expression (13).

Western blot analysis. Proteins were isolated using an extraction kit (Beyotime Institute of Biotechnology). Protein concentration was determined by BCA Protein Assay kit (cat. no. 23227; Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Extracted protein samples (30 μg) were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). After blocking in 5% non-fat milk for 3 h at room temperature, the membrane was probed with the following primary antibodies (all antibodies were used at 1:1,000) overnight at 4˚C: Anti-MMP-1 (cat. no. 26585-1-AP), anti-MMP-3 (cat. no. 17873-1-AP), anti-MMP-13 (cat. no. 18165-1-AP) and anti-β-actin (cat. no. 20536-1-AP) were purchased from ProteinTech Group, Inc. (Chicago, IL, USA); and anti-P-p38 (cat. no. YP0203), anti-P-JNK (cat. no. YP0157) and anti-P-ERK (cat. no. YP0100) were purchased from ImmunoWay Biotechnology Company (Plano, TX, USA). Subsequently, membranes were washed with Tris-buffered saline containing 0.05% Tween-20, and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1000; cat. no. A0208; Beyotime Institute of Biotechnology) for 1 h at 37˚C. The bands were visualized using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology). Optical density was analyzed by Quantity One software version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). β-actin was used to normalize target protein expression.

Statistical analysis. All experiments were performed in triplicate using independent samples. Data are expressed as the mean ± standard deviation. The statistical significance of the difference between groups was assessed by one-way analysis of variance and Tukey's multiple comparisons test. $P<0.05$ was considered to indicate a statistically significant difference. The analysis was performed using SPSS software version 18.0 (SPSS, Inc., Chicago, IL, USA).

Results

Effects of Icariin on MMP-1, MMP-3 and MMP-13 expression in IL-1β-stimulated SW1353 cells. IL-1β-stimulated SW1353 cells were treated with Icariin and the mRNA expression levels of MMP-1, MMP-3 and MMP-13 were assessed using RT-qPCR and ELISA. Treatment with 20 μM Icariin was revealed to significantly inhibit the increase in MMP-1, MMP-3 and MMP-13 mRNA and protein expression levels in response to stimulation with IL-1β compared with the control group (Fig. 1).

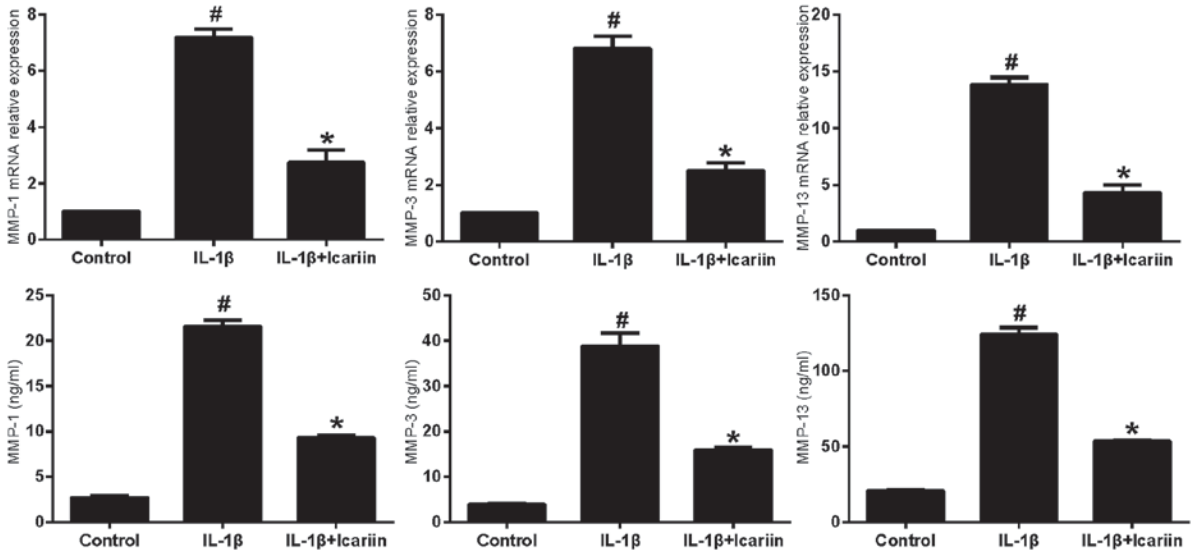

Figure 1. Effects of Icariin on MMP-1, MMP-3 and MMP-13 expression in IL-1β-stimulated SW1353 cells. Human SW1353 chondrosarcoma cells were pretreated with Icariin (20 μM) for 1 h, and then stimulated with 10 ng/ml IL-1β for 24 h. Control cells received no treatment and no stimulation. The levels of MMP-1, MMP-3 and MMP-13 were significantly increased following stimulation with IL-1β compared with in the control group. IL-1β-induced MMP-1, MMP-3 and MMP-13 expression was significantly inhibited by treatment with 20 μM Icariin compared with untreated IL-1β-stimulated cells. Data are presented as the mean ± standard deviation. *P<0.05 compared with IL-1β group; #P<0.05 compared with control group. MMP, matrix metalloproteinase; IL, interleukin.

Effects of Icariin on P-p38, P-ERK and P-JNK levels in IL-1β-stimulated SW1353 cells. In order to examine the effects of Icariin on the MAPK signaling pathway, western blot analysis was used. The levels of the phosphorylated forms of p38, ERK and JNK appeared to be markedly increased in SW1353 cells stimulated with IL-1β. Conversely, treatment with Icariin prior to stimulation appeared to prevent the increase in P-p38, P-ERK and P-JNK levels (Fig. 2).

Effects of MAPK pathway inhibitors and Icariin on MMP-1, MMP-3 and MMP-13 in IL-1β-stimulated SW1353 cells. As aforementioned, Icariin treatment decreases the IL1β-stimulated expression levels of MMP-1, MMP-3 and MMP-13 (Fig. 1). To further investigate the role of Icariin in the regulation of MMP expression, inhibitors of the MAPK signaling pathway were used. Treatment with Icariin produced the greatest decrease in MMP-1 and MMP-3 levels, followed by the p38 inhibitor and the ERK inhibitor (Fig. 3). The JNK inhibitor did not significantly affect the expression of MMP-1 and MMP-3. In addition, Icariin produced the greatest decrease in MMP-13 levels, followed by the JNK inhibitor and the p38 inhibitor. However, the ERK inhibitor did not significantly affect the expression of MMP-13 (Fig. 3). These results suggested that, compared with the single MAPK inhibitor, Icariin had a better inhibitory effect on the expression of MMP-1, MMP-3 and MMP-13. In addition, the induction of MMP-1, MMP-3 and MMP-13 in IL-1β-stimulated SW1353 cells may depend on different combinations of MAPK signaling pathways, which is consistent with previous results (14).

Effects of MAPK pathway activators on MMP-1, MMP-3 and MMP-13 in IL-1β-stimulated SW1353 cells. Based on the results of the present study and previously published reports (14,15), Icariin exhibited an inhibitory effect on the expression of P-p38, P-ERK, P-JNK, MMP-1, MMP-3 and MMP-13, and it was speculated that p38 and ERK were required for MMP-1 and MMP-3 expression, whereas p38 and JNK were required for MMP-13 expression in IL-1β-stimulated SW1353 cells.

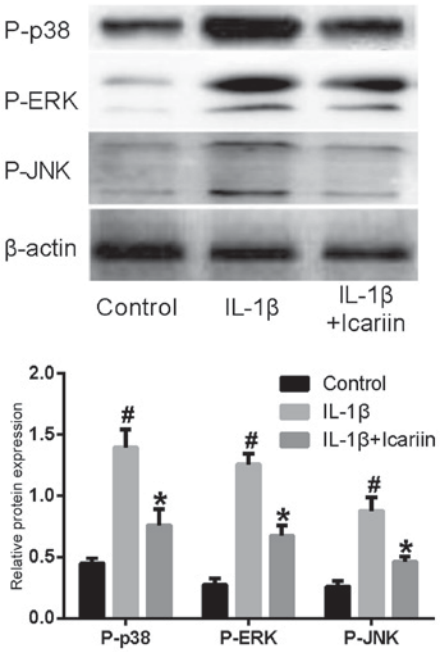

Figure 2. Effects of Icariin on P-p38, P-ERK and P-JNK levels. Human SW1353 chondrosarcoma cells were pretreated with Icariin (20 μM) for 1 h, and were stimulated with 10 ng/ml IL-1β. Control cells received no treatment and no stimulation. After 24 h, protein expression levels of P-p38, P-ERK and P-JNK were assessed using western blot analysis. The levels of P-p38, P-ERK and P-JNK were increased following stimulation with IL-1β compared with the control group. IL-1β-induced P-p38, P-ERK and P-JNK upregulation was inhibited by treatment with 20 μM Icariin compared with untreated IL-1β-stimulated cells. *P<0.05 compared with IL-1β group; #P<0.05 compared with control group. P-, phosphorylated; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IL, interleukin.

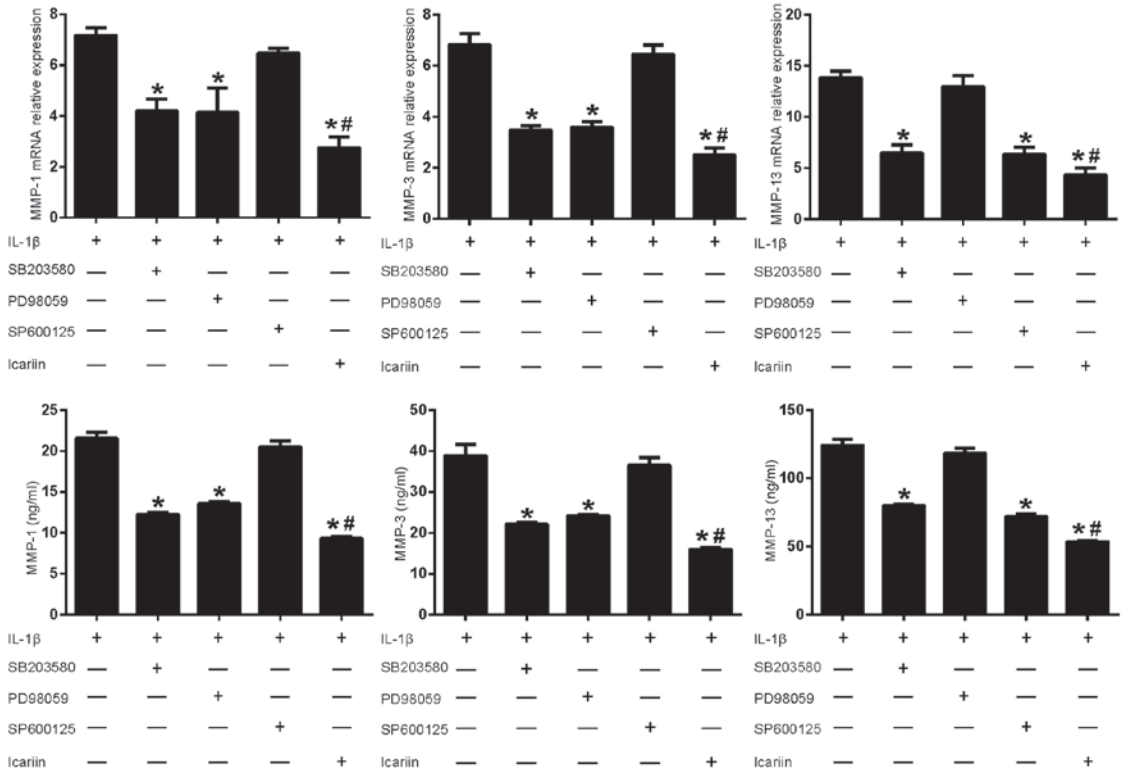

Figure 3. Effects of mitogen-activated protein kinase pathway inhibitors and Icariin on MMP-1, MMP-3 and MMP-13 expression. Human SW1353 chondrosarcoma cells were pretreated with Icariin (20 μM), the p38 inhibitor SB203580 (10 μM), the ERK inhibitor PD98059 (10 μM) or the JNK inhibitor SP600125 (10 μM) for 1 h. IL-1β (10 ng/ml) was then added to stimulate the cells. After 24 h, mRNA and protein levels of MMP-1, MMP-3 and MMP-13 were detected using reverse transcription-quantitative polymerase chain reaction and ELISA, respectively. Data are expressed as the mean ± standard deviation. For both mRNA and protein expressions *P<0.05 compared with untreated IL-1β group; $^{\#}$P<0.05 compared with inhibitor-treated groups. MMP, matrix metalloproteinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IL, interleukin; ELISA, enzyme-linked immunosorbent assay.

According to this hypothesis, an activator of p38 and ERK may be able to reverse the inhibitory effects of Icariin on MMP-1 and MMP-3, and an activator of p38 and JNK may reverse the inhibitory effect of Icariin on MMP-13. Therefore, cells were treated with Icariin with or without U-46619 (a p38 and ERK activator) co-treatment, and changes to the expression levels of MMP-1 and MMP-3 were detected. The expression of MMP-13 was detected after the cells were treated with Icariin with or without co-treatment with Anisomycin (a p38 and JNK activator). Western blot analysis demonstrated that levels of P-p38, P-ERK and P-JNK were increased by treatment with the activators U-46619 and Anisomycin (Fig. 4). Furthermore, treatment with MAPK pathway activators appeared to produce a corresponding increase in MMP-1, MMP-3 or MMP-13 expression, which was not observed when cells were treated with Icariin alone (Fig. 5).

Discussion

OA is a heterogeneous and complex joint pathology, characterized by the progressive degradation of cartilage, ultimately resulting in complete loss of articular cartilage (16). The precise mechanism of OA pathogenesis has not yet been elucidated, and no effective treatments to block the progression of the disease are currently available. Inhibition of the enzymatic degradation of ECM components and the maintenance of the cellular phenotype are two of the main therapeutic strategies that are currently under investigation (17).

MMPs serve a crucial role in the degradation of articular cartilage. Among the various MMPs, MMP-1, MMP-3 and MMP-13 can be found primarily in cartilage (18), where they target and degrade collagen, proteoglycan, osteonectin and perlecan, thus participating in OA progression (19). Previous research has investigated the potential of several MMP inhibitors as candidates for the treatment of OA and other diseases. However, the development of most of these compounds has been discontinued due to various reasons, such as toxicity, low specificity, severe off-target effects, poor bioavailability and efficacy (20). Plant-derived compounds have received considerable attention as potential therapeutic strategies for the treatment of OA, due to their beneficial properties (21,22). The proinflammatory cytokine IL-1β, which is one of the most critical catabolic factors that participate in OA pathogenesis, can enhance the production of MMPs. In the present study, IL-1β was used to develop a cellular OA model. The present results confirmed that IL-1β represents a potent inflammatory stimulus that can lead to overexpression of MMP-1, MMP-3 and MMP-13 in human SW1353 chondrocytes. The inhibitory effect of Icariin on the IL-1β-induced upregulation of MMP-1, MMP-3 and MMP-13 was also demonstrated.

It has previously been demonstrated that in OA cartilage, levels of P-MAPKs, including p38, JNK and ERK are upregulated (7). MAPK pathways can activate the downstream production of MMPs, including MMP-1, MMP-3 and MMP-13 (23,24). Mengshol *et al* demonstrated that the induction of MMPs by IL-1 depends on different signaling pathways (14).

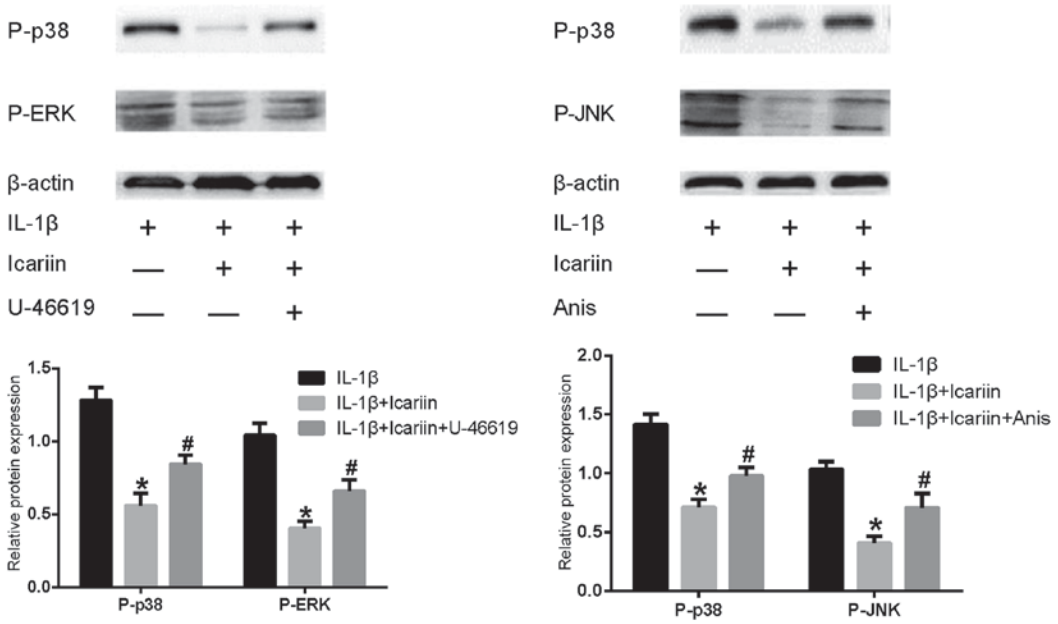

Figure 4. Effects of MAPK pathway activators and Icariin on P-p38, P-ERK and P-JNK levels. Human SW1353 chondrosarcoma cells were pretreated with Icariin (20 μM), the p38 and ERK activator U-46619 (50 μM) or the p38 and JNK activator Anisomycin (10 μg/ml) for 1 h. IL-1β (10 ng/ml) was then added to stimulate the cells. After 24 h, levels of P-p38, P-ERK, P-JNK were assessed by western blot analysis. Densitometry of the protein bands revealed that the MAPK pathway activators significantly increased P-p38, P-ERK and P-JNK levels compared with IL-1β-stimulated cells treated with Icariin alone. *P<0.05 compared with combined activator group; #P<0.05 compared with untreated IL-1β group. MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IL, interleukin; Anis, anisomycin.

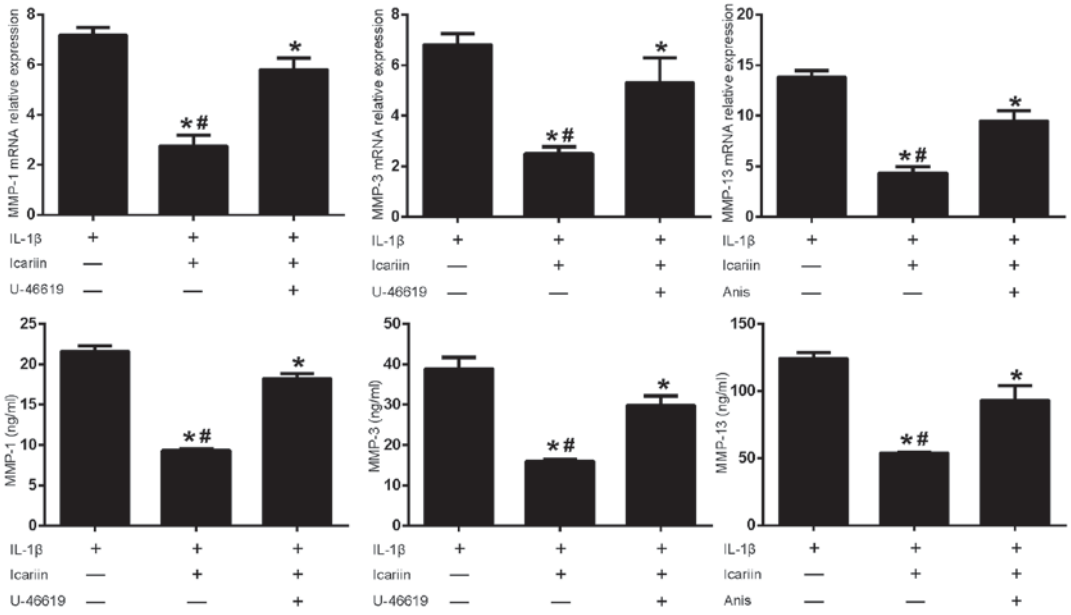

Figure 5. Effects of MAPK pathway activators and Icariin on MMP-1, MMP-3 and MMP-13 expression. Human SW1353 chondrosarcoma cells were pretreated with Icariin (20 μM), the p38 and ERK activator U-46619 (50 μM) or the p38 and JNK activator Anisomycin (10 μg/ml) for 1 h. IL-1β (10 ng/ml) was then added to stimulate the cells. After 24 h, mRNA and protein expression levels of MMP-1, MMP-3 and MMP-13 were assessed using reverse transcription-quantitative polymerase chain reaction and ELISA. Data are expressed as the mean ± standard deviation. Levels of MMP-1, MMP-3 and MMP-13 increased when MAPK pathway activators where used in conjunction with Icariin. For both mRNA and protein expressions *P<0.05 compared with untreated IL-1β group; #P<0.05 compared with activator-treated group. MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IL, interleukin; Anis, anisomycin; ELISA, enzyme-linked immunosorbent assay.

For example, IL-1 induction of MMP-13 requires p38 and JNK activity. The p38 inhibitor SB203580 has been reported to protect the cartilage against degeneration, via inhibiting the expression of MMP-3 and MMP-13 in the anterior cruciate ligament transection rat model of OA, and in IL-1-stimulated cartilage explant culture (25). Results from the present study demonstrated that the expression levels of MMP-1, MMP-3, MMP-13, P-p38, P-ERK and P-JNK were downregulated by Icariin in IL-1β-stimulated SW1353 cells. Icariin exhibited a better inhibitory effect on the expression of MMP-1, MMP-3 and MMP-13

compared with the single MAPK inhibitor. However, the JNK inhibitor SP600125 did not significantly affect the expression of MMP-1 and MMP-3, and the ERK inhibitor PD98059 did not significantly affect the expression of MMP-13. Based on the present results and previously published reports (14,15), it was speculated that p38 and ERK were required for MMP-1 and MMP-3 expression, whereas p38 and JNK were required for MMP-13 expression in IL-1β-stimulated SW1353 cells. Subsequently, treatment with U-46619 (a p38 and ERK activator) was demonstrated to reverse the inhibitory effect of Icariin on MMP-1 and MMP-3. And treatment with Anisomycin (a p38 and JNK activator) was revealed to reverse the inhibitory effect of Icariin on MMP-13. Therefore, that the suppressive effects of Icariin on MMP-1 and MMP-3 were hypothesized to be partly achieved by inhibiting the activation of p38 and ERK, whereas its effects on MMP-13 were partly achieved by inhibiting the activation of p38 and JNK. This was consistent with a previous study (14). The present study demonstrated that Icariin inhibited the IL-1β-induced expression of MMP-1, MMP-3 and MMP-13, and the phosphorylation of p38, ERK and JNK in SW1353 cells. These results reveal the ability of Icariin to block numerous pathways participating in degenerative cartilage damage, and suggest a potential for Icariin as an alternative strategy for OA treatment.

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