Original Paper

Baicalein Inhibits MMPs Expression via a MAPK-Dependent Mechanism in Chondrocytes

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Key Words

Baicalein • Osteoarthritis • Matrix metalloproteinase • Chondrocytes • Interleukin-1 β

Abstract

Background: Baicalein is a flavonoid isolated from *Scutellaria baicalensis* Georgi. Here, we investigated the anti-osteoarthritic effect of baicalein *in vitro* and *in vivo*. **Methods:** Interleukin-1 beta (IL-1 β)-induced chondrocytes were treated with different concentrations of baicalein, real-time PCR and ELISA were performed to detect the matrix metalloproteinases (MMPs) expression. Western blot was used to evaluate the mitogen-activated protein kinase (MAPK) expression. In experimental osteoarthritis (OA), rabbits were treated with baicalein, gross morphological and histological assessment was performed to evaluate the cartilage damage. **Results:** Baicalein significantly reduced the expression of MMPs *in vitro* and *in vivo*. Moreover, baicalein significantly reduced the phosphorylation of p38 and extracellular signal regulated kinase (ERK), but not of c-Jun N-terminal kinase (JNK). In addition, intra-articular injection of baicalein ameliorated the cartilage damage in a rabbit model of OA induced by anterior cruciate ligament transection (ACLT). **Conclusions:** The results indicate that baicalein may be considered as a potential agent for OA treatment.

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Introduction

Osteoarthritis (OA) is the most prevalent joint disorder in the elderly and often results in joint pain, stiffness and dysfunction. The disease is characterized by cartilage degradation, synovial inflammation and subchondral sclerosis, among which, cartilage

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degradation is considered the central feature of OA. It is believed that cartilage degradation results from the homeostatic imbalance between matrix anabolism and catabolism. Matrix metalloproteinases (MMPs) and interleukin-beta (IL-1 β) are known to play pivotal roles in cartilage degradation and OA pathogenesis [1, 2]. MMPs is a family of enzymes that contribute to cartilage degradation through degrading extracellular matrix (EMC) while IL-1 β is a cytokine that contributes to cartilage degradation by inducing the expression of MMPs and other proteases [3, 4]. Previous studies demonstrated that the inhibition of IL-1 β or MMP expression using pharmacologic inhibitors exerted beneficial effects on OA [5, 6].

A number of drugs, including nonsteroidal anti-inflammatory drugs (NSAIDs), cyclooxygenase-2 inhibitors, glucosamine, steroids and hyaluronan, have been used in OA treatment; however, current drugs are unable to reverse cartilage damage. Therefore, it is considerable interest in discovering a novel potential agent for OA treatment, with natural drugs receiving increasing interest due to their tolerance and safety.

Baicalein is a flavonoid isolated from *Scutellaria baicalensis Georgi* (Huangqin) which possesses anti-inflammatory, anti-oxidative and anti-carcinogenic activities [7-10]. In addition, baicalein has been shown to attenuate MMP-1 expression in human keratinocytes [11]. Furthermore, baicalein has been reported to inhibit the proliferation of human rheumatoid arthritis fibroblast-like synoviocytes [12]. Whether baicalein possesses anti-osteoarthritic properties remains unknown. In the present study, we investigated the anti-osteoarthritic properties of baicalein and the related mechanisms. We demonstrated that baicalein inhibited IL-1 β -induced MMP expression via regulation of the extracellular signal regulated kinase (ERK) and p38 pathways. The results of the *in vivo* study showed that baicalein reduced cartilage degradation in OA.

Materials and Methods

Reagents

Baicalein, dimethyl sulfoxide (DMSO), recombinant human IL-1 β and 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, fetal bovine serum, 0.25% trypsin and collagenase II were obtained from Gibco BRL (Grand Island, NY, USA). Baicalein was dissolved in DMSO for stock preparation.

Chondrocytes culture

This study was approved by the local Ethics Committee. Following patient consent, cartilage was obtained from OA patients undergoing total knee arthroplasty (n = 5, mean age, 67.2 years; range, 64–75 years). Chondrocytes were isolated from the cartilage as described previously [13]. Confluent cells were passaged at a ratio of 1:3, and third-passage cells were used.

Cells viability assay

Chondrocytes were cultured in 96-well plates (5 $\times$ 10³/well) and incubated with different concentrations of baicalein for 24 h. Cell viability was determined using the MTT assay as described previously [14].

Cell stimulation by IL-1 β and baicalein treatment

Cells at 80% confluency were serum-starved overnight and pretreated with baicalein for 1 h prior to stimulation with IL-1 β (10 ng/ml) for 24 h. The cells were harvested for MMPs gene expression analysis. The culture media were collected for enzyme-linked immunosorbent assay (ELISA). In a further study, cells were pretreated with baicalein (50 μ M) for 24 h, stimulated with IL-1 β for 30 min and harvested for western blotting analysis.

Quantitative real-time polymerase chain reaction (PCR)

PCR was performed as reported previously [13]. In brief, total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using the Moloney murine leukemia virus Reverse Transcriptase cDNA synthesis kit (Promega, Madison, WI, USA). The expression of messenger RNAs (mRNAs)

was quantified using real-time PCR, with actin as an internal control. Each gene analysis was performed in triplicate. Primer sequences of the targeted genes are listed in Table 1.

Measuring MMPs by ELISA

The levels of MMP-1, MMP-3 and MMP-13 in culture media were determined by ELISA using human MMP-1, MMP-3 and MMP-13 ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA).

Western blotting analysis

Western blotting analysis was performed as described previously [13]. Briefly, cells were washed using ice-cold phosphate-buffered saline (PBS) and the protein was extracted. The protein was transferred to polyvinylidene fluoride (PVDF) membranes and the membranes were incubated with blocking buffer followed by antibodies (ERK1/2, p-ERK1/2, P38, p-P38, c-Jun N-terminal kinase (JNK) and p-JNK (Cell Signaling Technology, Beverly, MA, USA). Finally, the filter was incubated with the electrochemiluminescence substrate and exposed to X-ray film (Kodak).

Animal experiments

New Zealand rabbits weighing 2.0–2.5 kg were used. The rabbits were purchased from the animal center of Zhejiang University and the study was approval by the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China). Thirty-two rabbits underwent anterior cruciate ligament transection (ACLT) in the right knee joint as described previously, and the animals were randomly assigned to four groups of eight rabbits each [15]. The rabbits received intra-articular injection of DMSO (as vehicle) or different concentration of baicalein (5, 25, or 50 μ M) in the right knee. Treatment was initiated on the day of the operation, and an injection was performed once weekly for 6 consecutive weeks. Another eight rabbits were used as a normal group, and received no injection. The rabbits were sacrificed and the right femoral condyles were harvested for assessment.

Gross morphological changes

Gross morphological changes were observed and graded by two independent blinded researchers according to following criteria: Grade 1: intact surface; Grade 2: minimal fibrillation; Grade 3: overt fibrillation; Grade 4: erosion [16].

Histological assessment

The samples were fixed in 10% neutral-buffered formalin, decalcified and embedded. Sections of 5 μ m were cut and stained using Safranin O for histological assessment according to the Mankin score system in a blinded manner [17](Table 2).

Table 1. Primers of targeted genes. * S = Sense; A = Antisense

Targeted genes	Sequence(5'-3')*	Amplicon length(bp)	Accession number
MMP-1	S: GGGATAAGTACTGGGCTGTTTCAG A: CCTCAGAAAGAGCAGCATCGATATG	125	NM_002421
MMP-3	S: CCTCTATGGACCTCCACAGAATC A: GGTGCTGACTGCATCGAAGGACAAA	121	NM_133523
MMP-13	S: CTGGCCTGCTGGCTCATGCTT A: CCTCAGAAAGAGCAGCATCGATATG	162	NM_002427
Actin	S: CACCTTCTACAATGAGCTGCGTGT A: CACAGCCTGGATAGCAACGTACA	100	NM_002046

Table 2. Mankin Score System

Items	Score
1. Structure integrity	
Intact	0
Surface irregularity	1
Irregular surface plus pannus	2
Fissures to middle zone	3
Fissures to deep zone	4
Fissures to subchondral zone	5
Totally disrupted	6
2. Chondrocytes	
Normal	0
Slight loss of chondrocytes clusters	1
25% of the clusters	2
No clusters	3
3. Safranin-O staining	
Normal	0
Slight loss	1
Moderate	2
Slight	3
None	4
4. Tidemark	
Intact	0
Vascular penetration	1

Table 3. Primers of targeted genes. S = Sense; A = Antisense

Targeted genes	Sequence(5'-3')*	Amplicon length(bp)	Accession number
MMP-1	S: ATGGCCTAGCAGTTGAGAAGCTGA A: TCCACACCTGGGTTGCTTCATCAT	110	AH005676
MMP-3	S: ACACCGGATCTGCCAAGAGA A: CTGGAGAACGTGAGTGGAGTCA	89	NM_001082280
MMP-13	S: CAGATGGGCATATCCCTCTAAGAA A: CCATGACCAATCTACAGTCTCTAC	88	NM_001082037
18SrRNA	S: GACGGACACAGCGGAAAGC A: CGCCAGTCGGCATCGTTTATG	119	EU236696

Detection gene expression in cartilage

Cartilage was collected and stored in liquid nitrogen for the detection of MMP-1, MMP-3 and MMP-13 gene expression using quantitative real-time PCR as indicated above. Primer sequences of the targeted genes are listed in Table 3.

Statistical analysis

Data are expressed as means $\pm$ standard deviation. Data were analyzed using a one way-ANOVA test. Dunnett-t method was used as the post-test in the ANOVA. A value of $P < 0.05$ was taken to indicate statistical significance

Results*Effect of baicalein on IL-1 β -induced MMPs expression in chondrocytes*

The MTT assay demonstrated that 50 μ M baicalein had no adverse effects on cell viability (data not shown). Therefore, we investigated the effects of baicalein on IL-1 β -induced MMP expression in human chondrocytes. Baicalein treatment resulted in a dose-dependent reduction in MMP-1, MMP-3 and MMP-13 gene expression (Fig. 1). In addition, baicalein reduced the levels of MMP-1, MMP-3 and MMP-13 in the culture media (Fig. 2).

Effects of baicalein on IL-1 β -induced mitogen-activated protein kinase (MAPK) activation in chondrocytes

To evaluate the underlying mechanisms responsible for the baicalein-mediated reduction of MMPs, we investigated the effects of baicalein on MAPK in IL-1 β -stimulated chondrocytes. IL-1 β induced JNK, p38 and ERK phosphorylation, which were reduced by baicalein for p38 and ERK, whereas JNK was unaffected (Fig. 3).

Fig. 1. Baicalein treatment inhibited interleukin (IL)-1 β -induced matrix metalloproteinases (MMPs) gene expression. Chondrocytes were pre-treated with various concentrations of baicalein for 1 h, followed by stimulation with IL-1 β (10 ng/ml) for 24 h. The chondrocytes were collected for gene expression analysis by PCR. Baicalein significantly inhibited MMP-1, MMP-3 and MMP-13 expression. Data are expressed as means $\pm$ standard deviation (SD). * $P < 0.05$ compared to cells stimulated with IL-1 β alone.

Fig. 2. Baicalein treatment inhibited interleukin (IL)-1 β -induced matrix metalloproteinases (MMPs) production. Chondrocytes were pre-treated with various concentrations of baicalein for 1 h, followed by stimulation with IL-1 β (10 ng/ml) for 24 h. The supernatants were collected and the concentrations of MMP-1, MMP-3 and MMP-13 were determined using an enzyme-linked immunosorbent assay. Baicalein significantly reduced the synthesis of MMP-1, MMP-3 and MMP-13. Data are expressed as means $\pm$ standard deviation (SD). * $P < 0.05$ compared to cells stimulated with IL-1 β alone.

Fig. 3. Baicalein inhibited interleukin (IL)-1 β -induced extracellular signal regulated kinase (ERK) and p38 but not c-Jun N-terminal kinase (JNK) activity. Chondrocytes were pretreated with baicalein for 24 h, followed by stimulation with IL-1 β (10 ng/ml) for 30 min. Western blotting was performed to evaluate phosphorylation of JNK, p38 and ERK. Baicalein inhibited IL-1 β -induced ERK and p38, but not JNK phosphorylation.

Fig. 4. Gross morphological grading. Rabbits received intra-articular injections of baicalein or vehicle once weekly for 6 weeks, initiated 1 day after the operation. There was no significant difference between OA group and baicalein-treated group in grading ($P > 0.05$).

Gross morphological cartilage changes in a rabbit OA model

In the normal group, the articular cartilage was smooth and no lesions were observed on the surface. In contrast, in the OA group, the articular cartilage exhibited varying degrees of damage, the cartilage surface was rough and ulcers. In the baicalein-treated groups, the

Fig. 5. Histological evaluation. Safranin-O staining was performed on cartilage sections. Typical changes in cartilage lesions are shown in. A: osteoarthritis (OA) group; B: baicalein (5 μ M); C: normal; D: baicalein (25 μ M); E: baicalein (50 μ M). (Original magnification $\times 50$). The Mankin scores were also assessed (F). The Mankin scores of medium and high-baicalein-concentration groups were lower than that of the osteoarthritis (OA) group. * $P < 0.05$ when compared to cartilage from the OA group.

Fig. 6. Baicalein inhibited matrix metalloproteinases (MMPs) gene expression in cartilage. The expression of MMP-1, MMP-3 and MMP-13 in cartilage was analyzed using quantitative real-time polymerase chain reaction and normalized to that of 18S rRNA. Baicalein inhibited the expression of MMP-1, MMP-3 and MMP-13. Data are presented as means $\pm$ standard deviation (SD). * $P < 0.05$ when compared to cartilage from the osteoarthritis (OA) group.

articular cartilage also showed lesions. And there was no significant difference between OA group and baicalein-treated group (Fig. 4).

Histological evaluation

The cartilage of the normal group had a normal histological appearance, whereas that of the OA group displayed clear hypocellularity and fissures to the deep zone. Cartilage from the low-concentration baicalein-treated group was similar to the OA group. Intra-articular injection of medium and high baicalein concentrations significantly ameliorated the cartilage

damage compared to the OA group (Fig. 5). The Mankin scores for the cartilage are presented in Fig. 5.

Cartilage gene expression

MMP-1, MMP-3 and MMP-13 expression in the OA group was significantly increased compared to that in normal cartilage. Baicalein treatment reduced MMP-1, MMP-3 and MMP-13 expression in a dose-dependent manner (Fig. 6).

Discussion

NSAIDs were used extensively in the treatment of OA to ameliorate pain. However, long-term use of NSAID can lead to serious side effects, particularly in the gastrointestinal and cardiovascular systems. Therefore, a new, safer agent which can ameliorate pain is required for OA. Phytochemicals are potential candidates because of their safety for long-term use.

Scutellaria baicalensis Georgi (Huangqin) is a herb with anti-inflammatory properties which was used widely for many centuries in China. Baicalein, a flavonoid isolated from *Scutellaria baicalensis* Georgi (Huangqin), is reported to possess multiple biological activities. In the present study, we evaluated its anti-osteoarthritic properties *in vitro* and *in vivo*. Baicalein inhibited the expression of key molecules, including MMP-1, MMP-3 and MMP-13 in IL-1 β -stimulated articular chondrocytes. This anti-osteoarthritic effect of baicalein was due in part to its inhibition of the MAPK pathway. *In vivo*, we found that baicalein significantly reduced cartilage damage as well as MMP-1, MMP-3 and MMP-13 expression in cartilage. Therefore, the findings were consistent in both models: *in vitro* in IL-1 β -induced chondrocytes and in an ACLT-induced OA rabbit model.

MMPs is a family of enzymes responsible for ECM degradation. Previous studies demonstrated that MMP-1, MMP-3 and MMP-13 play important roles in OA. In particular, MMP-13 is considered a potent protease for cartilage damage because of its ability to breakdown type II collagen, the main ECM component. Therefore, inhibiting MMP activities would be expected to ameliorate OA progression. In the present study, we demonstrated that baicalein effectively suppressed the overexpression of MMP-1, MMP-3 and MMP-13 in IL-1 β -stimulated chondrocytes. Previous studies showed that baicalein reduced MMP-2 and MMP-9 expression in glioma and hepatocellular carcinoma cells [18, 19]. In addition, MMP-1 was inhibited by baicalein in human keratinocytes [11]. Moreover, Zhang et al. reported recently that baicalein inhibited MMP-3 and MMP-13 expression in chondrocytes stimulated using a mixture of IL-1 β and tumor necrosis factor- α (TNF- α) [20]. Therefore, we hypothesized that baicalein may exert beneficial effects in OA via reducing MMPs activities and expression.

In the present study, we also investigated the mechanisms underlying the inhibition by baicalein of IL-1 β -induced MMP expression in chondrocytes. Many signaling pathways are known to be involved in cartilage degradation in OA; Of these, we focused on the MAPK signaling pathway because of its importance in OA progression. The MAPK signaling pathway is involved in MMPs regulation [21]. For example, the p38 signaling pathway was reported to induce MMPs expression, leading to ECM degradation [22]. In the present study, we demonstrated that IL-1 β stimulation induced the phosphorylation of p38, ERK and JNK, while baicalein reduced the phosphorylation of p38 and ERK, whereas there was no significant inhibition of phospho-JNK formation. Our findings are at least in part consistent with previous reports using other cell types. Zhang et al. demonstrated that baicalein inhibited p38 activation in glioma cells, whereas ERK and JNK were unaffected [23]. Liu et al. found that baicalein inhibited p38 activation in human melanocytes, whereas ERK activation was unaffected [24]. In a further study, Chen et al. reported that baicalein exerted its effect via down-regulation of the ERK pathway in hepatocellular carcinoma cells [25]. These studies indicate that baicalein can affect the activation of MAPK. The reasons for the discrepancies among the studies may be use of different cell types and/or experimental conditions. Therefore, we hypothesized that baicalein inhibits MMPs expression in chondrocytes at least in part through the inhibition of p38 and ERK activation. However, the exact

mechanism associated with baicalein and MAPK is still unclear, it is still unknown whether ERK and p38 are direct targets of baicalein, thus, further studies are needed to explore the underlying mechanism by which baicalein regulates MAPK signaling pathway. In addition, the mechanism underlying the regulation of MMPs in chondrocytes by baicalein remains unclear. Further studies are required to elucidate the mechanism of the anti-osteoarthritic effects of baicalein.

We investigated the *in vivo* effects of baicalein on cartilage in experimental OA over a period of 6 weeks, which is similar to the durations used in previous studies [26, 27]. We found that the cartilage displayed OA-like changes; Therefore, 6 weeks was sufficient to induce OA using ACLT. However, a longer study may provide more information regarding the effects of baicalein on cartilage damage in experimental OA. Indeed, some studies investigated three stages of ACLT-induced OA for up to 12 weeks [28, 29]. Therefore, further studies are required to obtain a better understanding of the *in vivo* effects of baicalein on cartilage damage in OA.

In conclusion, we demonstrated that baicalein inhibits MMPs expression in chondrocytes, which is mediated at least in part by the suppression of the activation of the p38 and ERK signaling pathways. Moreover, intra-articular injections of baicalein in experimental OA reduced cartilage damage. These findings indicate a potential therapeutic role for baicalein in OA.

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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