

Original Article

Shikonin protects chondrocytes from interleukin-1beta-induced apoptosis by regulating PI3K/Akt signaling pathway

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Abstract: Chondrocyte apoptosis is mostly responsible for the development and progression of osteoarthritis. IL-1 β is generally served as an agent that induces chondrocyte apoptosis. Shikonin exerts its anti-inflammatory effect on cartilage protection in vivo. We aimed to explore the protective effect of shikonin on interleukin-1beta (IL-1 β)-induced chondrocyte apoptosis and the potential molecular mechanisms. Chondrocytes were isolated from the joints of newborn Sprague-Dawley rats. The MTT assay and LDH cell death assay were used to determine the cell viability and chondrocyte apoptosis was detected by Annexin-V/PI staining and nucleosomal degradation. The contents of phosphorylated-PI3K (p-PI3k), phosphorylated-Akt (p-Akt), Bcl-2, Bax, and cytochrome c were detected by Western blotting. A quantitative colorimetric assay was used to detect the caspase-3 activity. Our results showed that pretreatment with shikonin (4 μ M) inhibited cytotoxicity and apoptosis induced by IL-1 β (10 ng/ml) in chondrocytes. Shikonin pretreatment also decreased the activity of IL-1 β that decreased Bcl-2 expression and levels of p-PI3K and p-Akt, and increased Bax expression, cytochrome c release, and caspase-3 activation. It also reversed the activity of IL-1 β that promoted the synthesis of matrix metalloproteinase-13 and inhibited the expression of tissue inhibitor of metalloproteinase-1 expression, with the net effect of suppressing extracellular matrix degradation. These data suggested that shikonin may protect chondrocytes from apoptosis induced by IL-1 β through the PI3K/Akt signaling pathway, by deactivating caspase-3.

Keywords: Shikonin, osteoarthritis, chondrocytes, IL-1 β , apoptosis, PI3K/Akt signaling

Introduction

Osteoarthritis (OA) is a degenerative disease with age-related joint disorder characterized by the progressive loss of articular cartilage and degradation of the cartilage matrix. Proinflammatory cytokines such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) play a critical role in the development of OA pathological changes by inducing chondrocytes to secrete matrix metalloproteinases (MMPs), which degrade the extracellular matrix [1] and promote chondrocyte apoptosis [2]. For instance, the expression of type I IL-1 receptor (IL-1R) is evidently augmented in chondrocytes of OA, making these cells a higher sensitivity to stimulation by IL-1 β [3, 4]. IL-1 has specially

been proved to induce chondrocyte apoptosis [2]. Therefore, IL-1 β is generally served as an agent that induces chondrocyte apoptosis [5-7]. Moreover, previous reports have indicated that the major causes of OA are excessive chondrocyte apoptosis-induced a loss of chondrocytes and degeneration of cartilage tissue [8].

Apoptosis is an autonomous programmed cell death process regulated by multiple signaling pathways. Chondrocyte apoptosis plays an important role in the development of cartilage degeneration in OA and is increasingly considered as one of the potential targets for the treatment of OA [9-12]. Thus, it is essential to elucidate the molecular mechanisms of chondrocyte apoptosis in OA. One of the consider-

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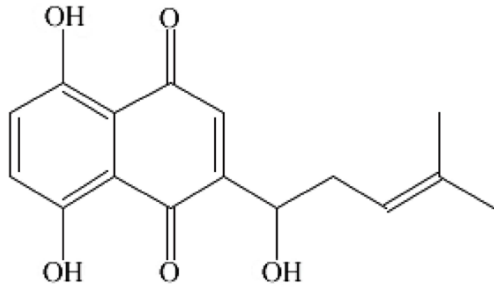


Figure 1. Chemical structure of shikonin.

able pathways during the apoptotic process involves in the activation of caspase-3, and then induces hydrolysis of nucleic acids and cytoskeletal proteins [13]. Previous reports have demonstrated that the activity of caspase-3 is dramatically enhanced in chondrocyte apoptosis induced by IL-1 β [14]. Then, caspase-3 is largely activated by cytochrome c (Cyt c) [15, 16]. These signaling events proceed by the pro-apoptotic protein Bax that is a member of the Bcl-2 family, which moves at the mitochondrial membrane to cause Cyt c release from mitochondria to cytosol. Thus, in order to inhibit cell apoptosis, the most important is to inhibit the mitochondrial pathway that can activate caspase-3, which can be realized by Bcl-2 via binding to and inhibiting Bax [17].

Protein kinase B (Akt), a serine/threonine protein kinase, can be phosphorylated and activated by extracellular factors in a phosphatidylinositol 3-kinase (PI3K)-dependent manner. As a potential signaling pathway for inhibiting apoptosis, PI3K/Akt signaling pathway performs a critical role in inhibiting chondrocyte apoptosis [18]. 17 β -Estradiol increases cell proliferation in rat OA model chondrocytes through the PI3K/Akt signaling pathway [12]. Phosphorylated PI3K/Akt increases Bcl-2 expression in chondrocytes, resulting in reducing the activity of Bax and inhibiting cell apoptosis [19, 20]. Thus, PI3K/Akt signaling pathway can available block cell apoptosis induced by the mitochondrial pathway.

Shikonin (**Figure 1**) is an active naphthoquinone derived from *Lithospermum erythrorhizon*, a Chinese medicinal herbal. It has been widely used as a traditional Chinese medicine for thousands of years in treating inflammations, burns and wounds [21]. A recent study reports that administration of shikonin inhibits rheumatoid arthritis (RA) in vivo. Shikonin

down-regulates the level of both proinflammatory cytokines IL-1 β and TNF- α , and also has significant protective effects against cartilage destruction possibly by suppressing MMP-1 synthesis and enhancing tissue inhibitors of metalloproteinase (TIMP)-1 expression, which are considered shikonin as an outstanding candidate as a cartilage protective medicine for RA [22]. However, the effect and potential molecular mechanism of shikonin on IL-1 β -induced chondrocytes in OA remain unclear.

In our research, we explored whether shikonin could suppress apoptosis and reduce the secretion of matrix metalloproteinases in IL-1 β -stimulated rat chondrocytes. We also studied the molecular mechanism of the protective effect of shikonin on IL-1 β -induced chondrocytes by researching changes in PI3K/Akt and mitochondrial signaling pathways.

Materials and methods

Materials

Purified shikonin (> 98%) was purchased from the National Institute for the Control Pharmaceutical and Biological Products (Beijing, China). Dimethylsulfoxide (DMSO) and 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (USA). Dulbecco's modified Eagle's medium (DMEM)/F12, Fetal bovine serum (FBS), trypsin and collagenase II were purchased from Gibco Co. (New York, USA). Was purchased from Gibco Co. (New York, USA). BCA Protein Assay Kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). LY294002 was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Recombinant rat IL-1 β was purchased from PeproTech (Rocky Hill, NJ, USA). Penicillin/streptomycin was purchased from Hangzhou Sijiqing Biological Engineering Materials Co. Ltd (Hangzhou, China). LDH Cytotoxicity Assay Kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Annexin V-FITC Apoptosis Detection Kit was obtained from Nanjing KeyGen Biotech Co. (Nanjing, China).

Isolation and culture of rat articular chondrocytes

The articular cartilages of new born male Sprague-Dawley rats were collected and shredded under sterile conditions before being

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digested with 0.25% trypsin. The trypsin was then removed, and the cartilage was washed with phosphate-buffered saline (PBS) three times, after which 0.2% collagenase II was added for digestion at 37°C for 4 h. A 200- μ m mesh strainer was used to filter the above solution, and the cells were harvested by centrifugation. The cells were then cultured in DMEM-F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin and incubated with 5% CO₂ at 37°C.

Cell treatment

The cells were divided into five groups and treated with shikonin at various concentrations: 0, 1, 2, 4 or 8 μ M. After incubation for 24 h, 48 h and 72 h, the MTT assays were performed. To study the protective effect of shikonin on cytotoxicity induced by IL-1 β , cells were treated with shikonin for 2 h prior to addition of 10 ng/ml IL-1 β . In the control group, cells were treated with no treatment. After 24 h treatment, the cell viability was also determined by the MTT assay and cell death was detected by estimating LDH activity.

Apoptosis, Western blotting and caspase-3 activity experiments, the cells were divided into five groups: control, cells were treated with no treatment; IL-1 β , cells were stimulated with 10 ng/ml IL-1 β for 24 h; shikonin alone, cells treated for 24 h with 4 μ M shikonin without IL-1 β ; shikonin + IL-1 β , cells were pretreated with shikonin for 2 h, and then stimulated with 10 ng/ml IL-1 β for 24 h; and inhibition group, in which the cells were pretreated with 25 μ M LY294002 and 4 μ M shikonin for 2 h followed by 24 h with 10 ng/ml IL-1 β .

Cell viability assay

Chondrocytes were plated in 96 well culture plates. After incubation with test medium for 24 h, the number of viable cells was determined using MTT reagent according to the manufacturer's instructions. In brief, MTT reagent (10 μ l) was added to the 100 μ l medium, and incubated at 37°C for 4 h. The supernatant was removed and dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. Absorbance (570 nm) of the medium was measured with Biotek Elix-800 plate reader. Cell death was detected by estimating LDH activity. According to the manufacturer's instructions, the amount of LDH released from cells in the supernatant was detected by LDH assay kit

after incubation. The absorbance was measured on a microplate reader at 490 nm. The data in each treatment group is expressed as a percentage of control.

Flow cytometric evaluation of apoptosis

After treatments, Cells were double-stained by using an Annexin V-FITC apoptosis detection kit according to the manufacturer's instructions. Samples stained with Annexin V and PI was quantitatively analyzed at 488 nm emission and 570 nm excitation by Flow Cytometry (BD FACScalibu; BD Biosciences, San Jose, CA, USA).

Measurement of apoptosis by ELISA

Apoptosis was determined by using Cell Death ELISA Detection Kit (Switzerland; Roche, Inc.) that measures cytoplasmic DNA-histone complexes generated during apoptotic DNA fragmentation. Cell apoptosis detection was performed under the manufacturer's instructions and monitored spectrometrically at 405 nm.

Western blot analysis

Cells were lysed using protein lysis buffer and protease inhibitor cocktail. The protein concentration of cell lysates was quantified by BCA Kit, and equal amounts of protein were separated by SDS-PAGE and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membranes were blocked in 5% non-fat dry milk diluted with Tri Buffered Saline Tween-20 (TBST) (in mmol/L: Tris-HCl 20, NaCl 150, PH 7.5, 0.1% Tween 20) at room temperature for 1 h and probed overnight at 4°C with anti-rabbit phospho-PI3K antibody, anti-rabbit total-PI3K antibody, anti-rabbit phospho-Akt antibody, anti-rabbit total-Akt antibody, anti-rabbit MMP-13 antibody, anti-rabbit TIMP-1 antibody (1:1000; Santa Cruz), anti-rabbit Bcl-2 antibody, anti-rabbit Bax antibody (1:1000; Cell Signaling Technology), and then incubated for 1 h with a goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1000; Santa Cruz). Incubation with monoclonal mouse β -actin antibody (1:4000; BOSTER) was performed as the loading sample control. The proteins were visualized using ECL™ western blotting detection reagents (Amersham Biosciences Corp., USA). The densitometry of the bands was quantified using the Image J 1.38X software (USA).

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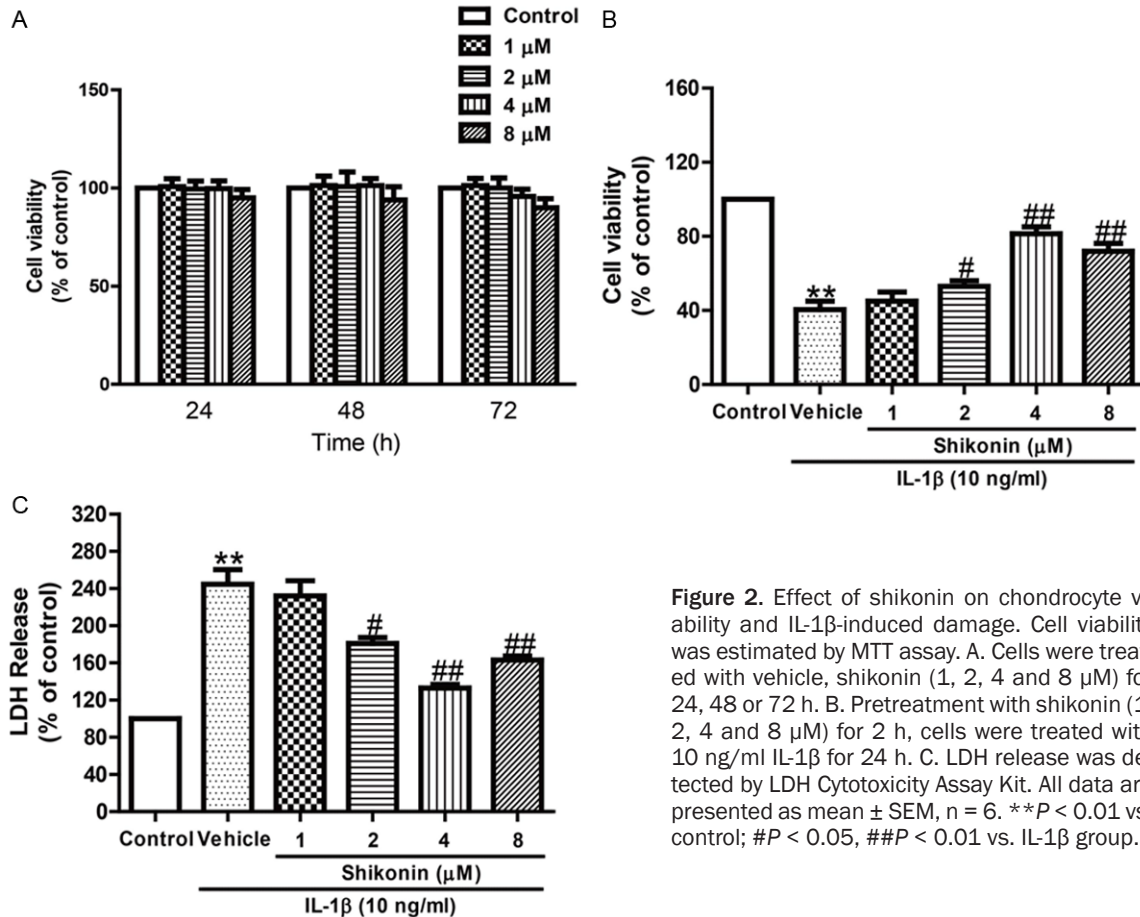


Figure 2. Effect of shikonin on chondrocyte viability and IL-1 β -induced damage. Cell viability was estimated by MTT assay. A. Cells were treated with vehicle, shikonin (1, 2, 4 and 8 μ M) for 24, 48 or 72 h. B. Pretreatment with shikonin (1, 2, 4 and 8 μ M) for 2 h, cells were treated with 10 ng/ml IL-1 β for 24 h. C. LDH release was detected by LDH Cytotoxicity Assay Kit. All data are presented as mean \pm SEM, n = 6. ** P < 0.01 vs. control; # P < 0.05, ## P < 0.01 vs. IL-1 β group.

Cytochrome c release assay

Cells were collected by centrifugation at 1000 \times g for 3 min at room temperature. Cytosol and mitochondrial extractions were isolated by using the Mitochondrial Isolation Kit (Pierce Chemical, USA) according to manufacturer's instructions. The level of cytochrome c expression was detected from cytosol and mitochondrial extractions by western blotting analysis using anti-rabbit cytochrome c antibody (1:1000, Cell Signaling).

Caspase-3 activity assay

Caspase-3 fluorescent assay kit (Nanjing KeyGen Biotech, China) was used to detect caspase activity. In brief, cells were cultured in 60-mm dishes and treated with test medium. Cells were lysed in the lysis buffer and centrifuged at 10,000 \times g for 1 min, and then the supernatants were collected. With bovine serum albumin as the control, equal amounts of protein samples were reacted with the synthetic fluorescent substrates at 37°C for 1.5 h and the reactions were read at 405 nm in a

microplate reader (Biorad, USA). Fold-increases in caspase-3 activity were determined with values obtained from the treatment samples divided by those from the controls.

Reverse transcription polymerase chain reaction

Total RNA of chondrocytes was extracted by using Trizol reagent (Life Technologies, Carlsbad, CA). Two microgram RNA was used for gene-specific reverse transcription polymerase chain reaction (RT-PCR) using one-step RT-PCR kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. The following primers were used: MMP-13 (forward, TGAGGATACAGGCAAGACTCT; reverse, CAATACGGTTACTCCAGATGC), TIMP-1 (forward, CTTCTGGCATCCTGTTGTTG; reverse, AGAAGGC-CGTCTGTGGGT) and GAPDH (forward, GAAGG-TGAAGTCCGAGTC; reverse, GAAGATGGT-GATGGGATTTC). The levels for each gene were counted by standardizing the quantified mRNA amount to the GAPDH mRNA. Each sample was assessed in triplicate.

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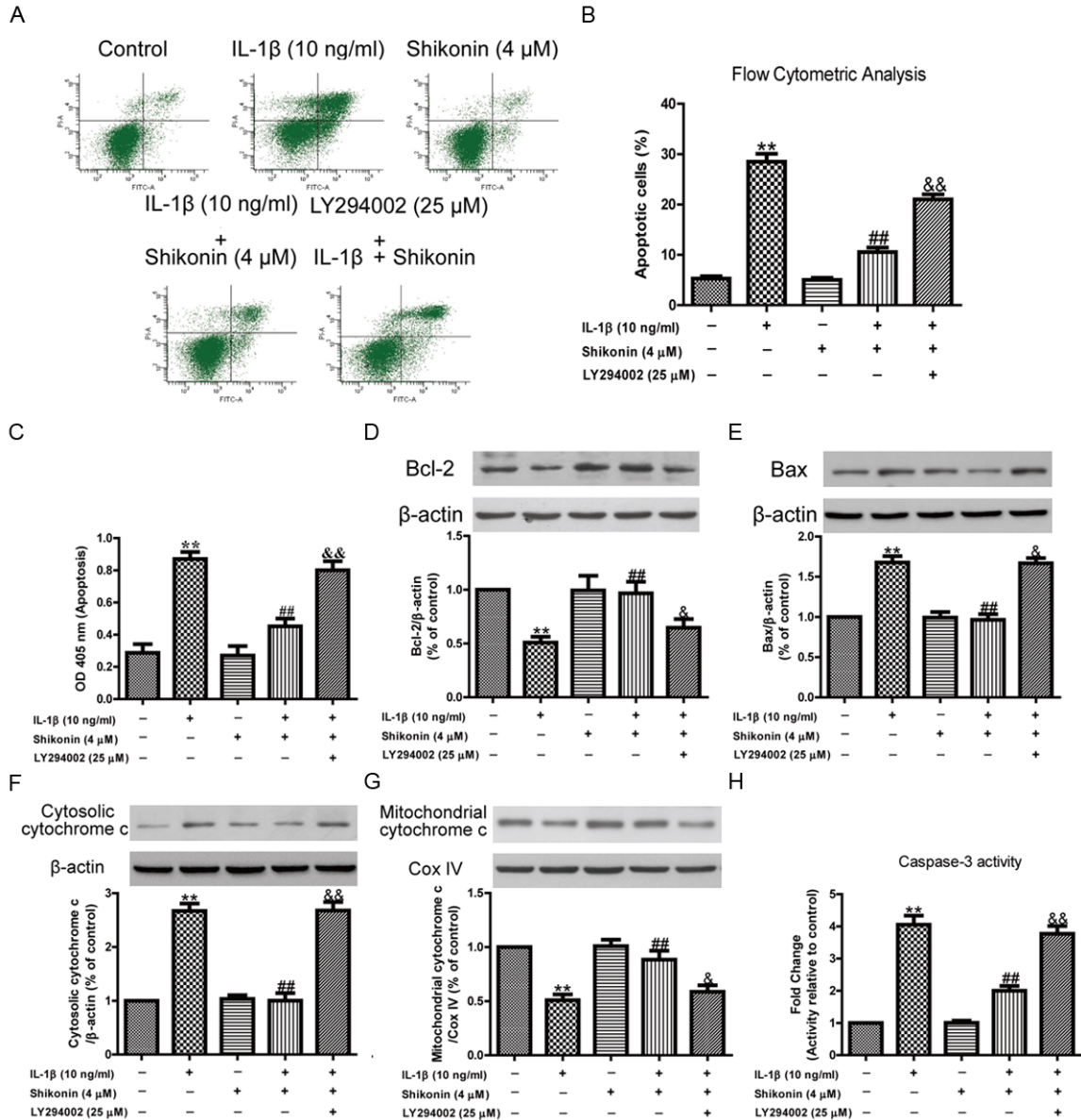


Figure 3. The effect and mechanism of shikonin on IL-1 β -induced chondrocyte apoptosis. Chondrocytes were treated with shikonin (4 μ M) with or without LY294002 (25 μ M) for 2 h prior to 24 h treatment with 10 ng/mL IL-1 β . Apoptosis of chondrocytes was measured by flow cytometric analysis of cells labeled with Annexin-V/PI double staining (A, B) and nucleosomal degradation using Roche's cell death ELISA detection kit (C). Bcl-2 (D), Bax (E) and Cyt c (F, G) were detected by Western Blot using β -actin as internal reference. (H) The activity of caspase-3 was determined by spectrophotometry. All data are presented as mean \pm SEM, n = 6. ** P < 0.01 vs. control; ## P < 0.01 vs. IL-1 β group; & P < 0.05, && P < 0.01 vs. IL-1 β + shikonin group.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., USA). Data for each study parameter from each group were presented as mean \pm standard error of the mean (S.E.M.). Data from each group were statistically analyzed by a two-tailed Student's t test or one-way analysis of variance (ANOVA). Differences were considered statistically significant at P < 0.05.

Results

Effects of shikonin on chondrocyte viability and damage induced by IL-1 β

To investigate whether shikonin has any possible cytotoxic effect on chondrocytes, the cell viability treated with shikonin was examined by MTT assay. Cells were treated with shikonin at various concentrations (0, 1, 2, 4 and 8 μ M). The cytotoxic effect of shikonin was determined

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at 24 h, 48 h and 72 h after treatment. From our results (**Figure 2A**), we found that there were no remarkable differences in absorbance among the treated groups, suggesting that there were no detectable cytotoxic effects for shikonin at the above concentrations and time on chondrocytes. Next, we determined the protective effect of shikonin on cytotoxicity induced by IL-1 β . Pretreated with shikonin (0, 1, 2, 4 and 8 μ M) for 2 h, cells were incubated with IL-1 β (10 ng/ml). After 24 h, MTT assays were performed (**Figure 2B**). Our findings showed that IL-1 β significantly reduced cell viability. However, the addition of shikonin at its various concentrations (2, 4, or 8 μ M) visibly decreased cell damage induced by IL-1 β . Similarly, results by LDH cell death assay were in accordance with those of MTT assay (**Figure 2C**). 4 μ M shikonin showed the most protective effect in the three concentrations of shikonin. Thus, 4 μ M of shikonin was used in the following experiments. These results showed the ability of shikonin to reduce the cytotoxicity induced by IL-1 β in chondrocytes.

Shikonin protected chondrocyte from apoptosis induced by IL- β 1 by inhibiting the mitochondrial apoptosis pathway and deactivating caspase-3

To confirm whether the cytotoxicity induced by IL-1 β inhibited growth arrest by inducing apoptosis, we assess apoptosis of chondrocytes detected by flow cytometric analysis of cells labeled with Annexin-V/PI double staining and nucleosomal degradation. For a negative control group, the cells were treated with IL-1 β alone and cultured for 24 h. The results shown in **Figure 3A** and **3B** showed that IL-1 β could significantly increase the percentage of apoptotic chondrocytes (28.5%) compared with the control group (5.3%). When chondrocytes were pretreated with shikonin (4 μ M) for 2 h prior to adding IL-1 β (10 ng/mL) to treat the cells for 24 h, the results exhibited that an evident decrease in the percentage of apoptotic chondrocytes (10.6%) was observed as compared with the IL-1 β alone group. However, LY294002, a PI3K inhibitor, obviously blocked the protective effect of shikonin on IL-1 β -induced chondrocytes apoptosis (21.0%). Similarly, results by DNA-histone ELISA were consistent with those of flow cytometric analysis (**Figure 3C**).

To investigate the role of the mitochondrial apoptosis pathway on shikonin-mediated pro-

tection against chondrocyte apoptosis induced by IL-1 β , expression levels of the Bcl-2 family proteins Bcl-2 and Bax were determined by Western blotting. As shown in **Figure 3**, compared to control group, the anti-apoptotic protein Bcl-2 expression was evidently decreased (**Figure 3D**) while the pro-apoptotic protein Bax expression was significantly increased in the IL-1 β group (**Figure 3E**). Similar results were acquired in cells pretreated with LY294002 and shikonin prior to IL-1 β . When 4 μ M shikonin was added before IL-1 β , Bcl-2 expression was markedly higher than that in the IL-1 β group and Bax expression was dramatically lower. Besides, we explored Cyt c release from mitochondria into the cytosol (**Figure 3F, 3G**). Our findings exhibited that after IL- β stimulation, significantly increased Cyt c was released compared to the control group, and this release was obviously reduced by the addition of shikonin. To some extent, LY294002 prohibited the inhibitory action of shikonin on Cyt c release stimulated by IL-1 β . Cells treated with shikonin alone did not show any change in this signaling pathway compared to control.

The activity of caspase-3 was determined by the caspase-3 fluorescent assay kit. Shikonin treatment alone had no effect on the activity of caspase-3. However, as shown in **Figure 3H**, addition of shikonin evidently suppressed activation of caspase-3 induced by IL-1 β , indicating that chondrocyte apoptosis was inhibited by shikonin. This effect was partially weakened by LY294002, suggesting that shikonin performed its functions by enhancing PI3K/Akt signaling.

Shikonin inhibited IL-1 β -induced apoptosis of chondrocyte through PI3K/Akt signaling pathway

To further study the molecular mechanism through which shikonin protected chondrocytes from apoptosis induced by IL-1 β , we study the changes in PI3K/Akt signaling by using. As shown in **Figure 4**, p-PI3K and p-Akt expressions were evidently reduced after 24 h with IL-1 β induction compared to the control group. However, pretreatment with 4 μ M shikonin for 2 h notably increased p-PI3K and p-Akt expressions in chondrocytes induced by IL-1 β . Total PI3K and Akt levels were not altered. To further study the effect of PI3K/Akt signaling in shikonin-mediated protection of chondrocyte apoptosis induced by IL-1 β , the LY294002 was added together with shikonin. 25 μ M LY294002

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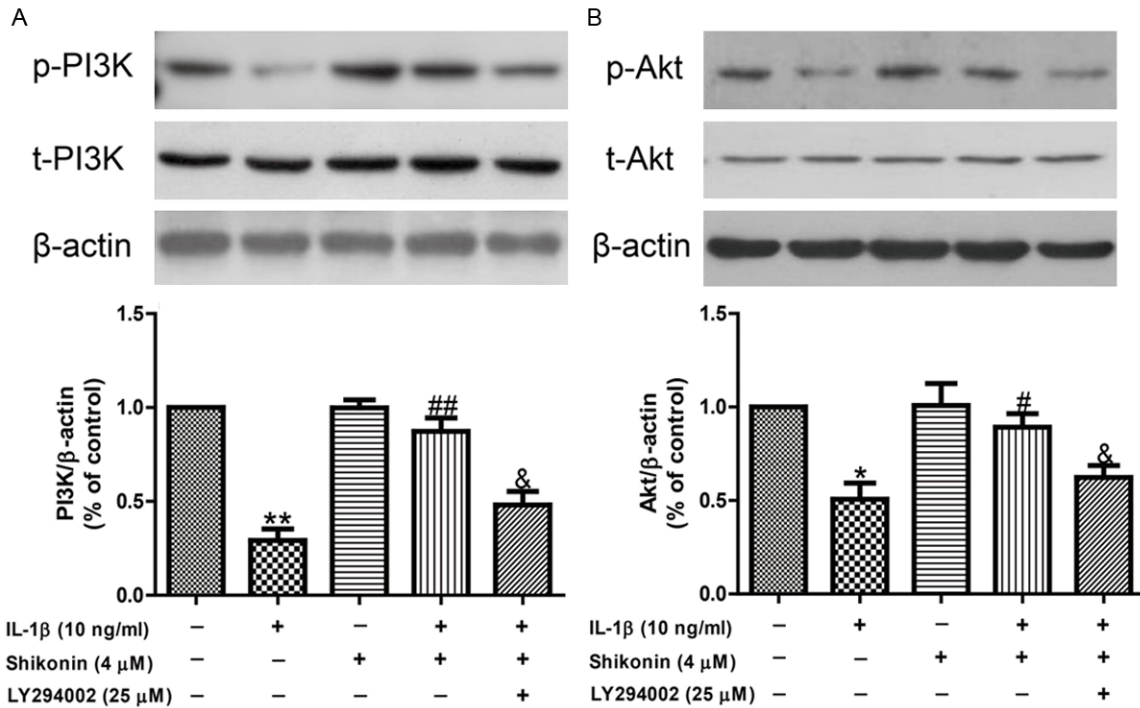


Figure 4. Shikonin suppressed IL-1 β -induced chondrocyte apoptosis via PI3K/Akt signaling. Chondrocytes were treated with shikonin (4 μ M) or shikonin (4 μ M) + LY294002 (25 μ M) for 2 h before 24 h IL-1 β (10 ng/mL) treatment. A. Total PI3K and p-PI3K were detected by Western Blot. P-PI3K levels were normalized to t-PI3K levels. B. Total Akt and p-Akt were detected by Western Blot. P-Akt levels were normalized to t-Akt levels. All data are presented as mean \pm SEM, n = 6. * P < 0.05, ** P < 0.01 vs. control; # P < 0.05, ## P < 0.01 vs. IL-1 β group; & P < 0.05 vs. IL-1 β + shikonin group.

blocked the shikonin-mediated increase of p-PI3K and p-Akt, and also decreased the inhibition of shikonin on IL-1 β -induced chondrocyte apoptosis. All above results indicated that shikonin may enhance PI3K/Akt signaling to perform its protective effects.

Shikonin decreased the synthesis of MMP-13 and increased the expression of TIMP-1

The balance between MMP-13 and TIMP-1 is directly responsible for adequate synthesis and degradation of the extracellular matrix of chondrocyte. To detect whether the protective effect of pretreatment with shikonin was due to the regulation of the balance of two proteins, we tested their expression levels in IL-1 β -induced chondrocytes. As shown in **Figure 5A** and **5B**, Western blotting assay showed that, pretreatment with shikonin significantly restrained IL-1 β -induced MMP-13 secretion and increased TIMP-1 expression compared to the IL-1 β group. However, LY294002 partially blocked the effects of shikonin. Furthermore, the results of MMP-13 and TIMP-1 at mRNA levels were similar to the results of both at protein levels (**Figure 5C, 5D**). All of above results suggested that shi-

konin should regulate expressions of MMP-13 and TIMP-1 through the PI3K/Akt signaling pathway.

Discussion

Shikonin has numerous pharmacological properties, including anti-cancer and anti-inflammatory properties and the ability to promote wound healing [23]. However, the effects of shikonin in OA have not yet been reported. Our results showed that pretreatment with shikonin increased cell viability and inhibited cell apoptosis in IL-1 β -stimulated rat chondrocytes that is one type of model OA chondrocytes, increased Bcl-2 expression, decreased Bax expression, and reduced Cyt c release and deactivated caspase-3. It also suppressed MMP-13 synthesis and augmented TIMP-1 expression, with the net effect of restraining extracellular matrix degradation. These effects by shikonin are partly mediated by promoting PI3K/Akt signaling.

IL-1 β , a pro-inflammatory cytokine, can induce chondrocyte apoptosis in rats and humans, which is associated with the happen and devel-

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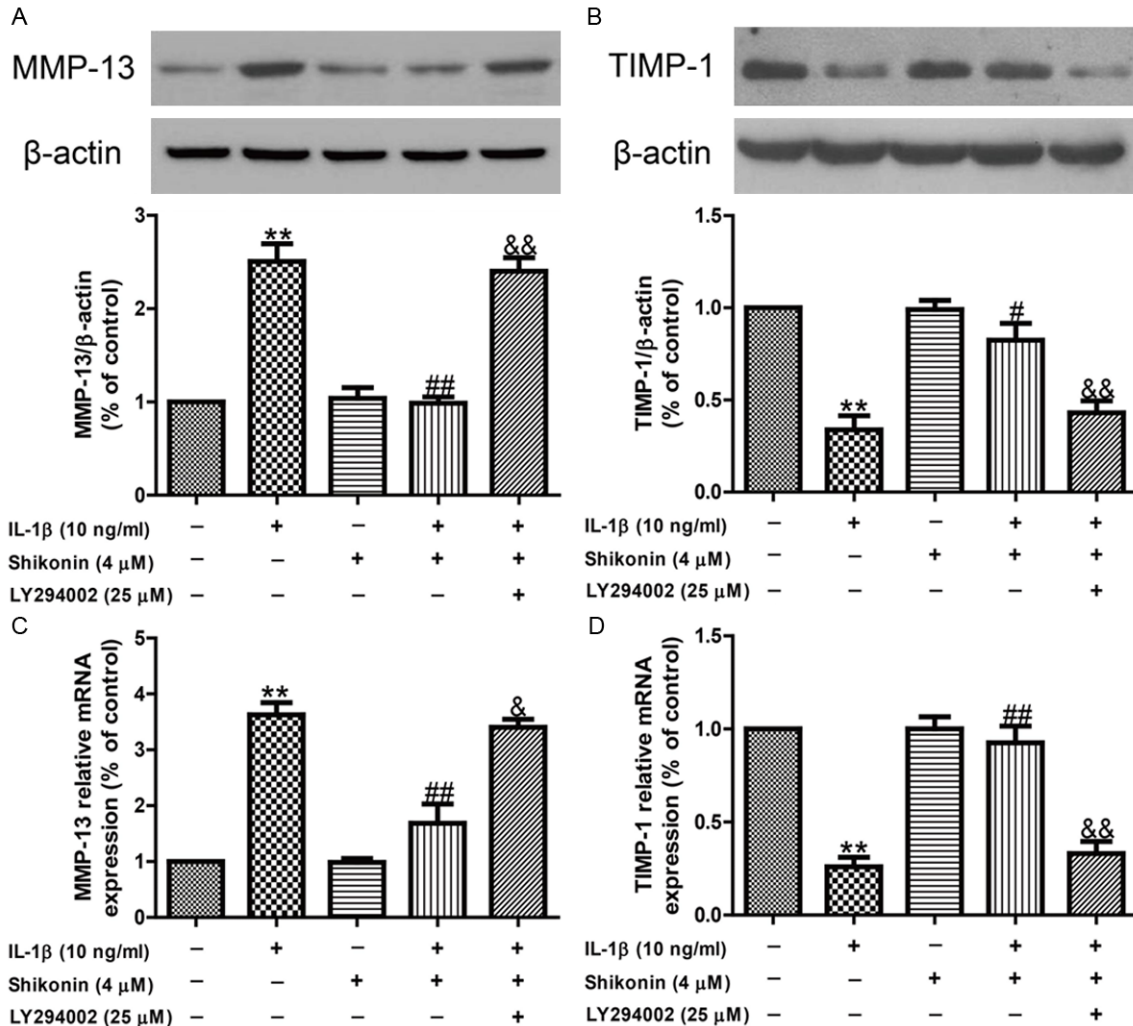


Figure 5. Shikonin inhibited MMP-13 synthesis and promoted TIMP-1 expression. Chondrocytes were treated with shikonin (4 μ M) or shikonin (4 μ M) + LY294002 (25 μ M) for 2 h prior to 24 h treatment with 10 ng/mL IL-1 β . A & B. The expressions of MMP-13 and TIMP-1 were detected by Western Blot, and β -actin was used as the internal reference. C & D. MMP-13 mRNA and TIMP-1 mRNA were determined by RT-PCR. All data are presented as mean \pm SEM, n = 6. ** P < 0.01 vs. control; # P < 0.05, ## P < 0.01 vs. IL-1 β group; & P < 0.05, && P < 0.01 vs. IL-1 β + shikonin group.

opment of OA [24]. The current report provided for the first time that shikonin (4 μ M) evidently inhibited chondrocyte apoptosis induced by IL-1 β and thus shikonin may play a protective role against IL-1 β relevant happen and development of OA.

Previous studies have showed that the PI3K/Akt signaling pathway is responsible for sustaining chondrocyte survival and promoting extracellular matrix synthesis [25-29]. For instance, phosphorylation of Akt significantly enhances proteoglycan synthesis and promotes cell survival in human articular chondrocytes [25]. Conversely, inhibition of PI3K/Akt

signaling decreases proteoglycan synthesis in chondrocytes and increases chondrocytes survival [26-29]. Further reports also confirmed that inhibition of Akt phosphorylation was a significant mechanism of chondrocyte apoptosis induced by 4-hydroxynonenal and that LY294002 blocked the effect of insulin-like growth factor-1 and induced an evident augment in chondrocyte apoptosis [20, 27]. In current research, we found that pretreatment with shikonin reversed the effects of IL-1 β on inhibition of Akt phosphorylation and chondrocyte apoptosis. Moreover, the PI3K-specific inhibitor LY294002 could significantly inhibit the anti-apoptotic effect of shikonin. Thus, above results

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suggested that PI3K/Akt signaling is a critical molecular target of OA and that shikonin may have a potential therapeutic action by activating this signaling pathway.

Increased chondrocyte apoptosis is considered an important pathological feature of OA [9-11]. Recent reports indicated that chondrocytes in OA showed the lost mitochondrial function, which preceded the classical marks of apoptosis [30, 31]. An appropriate balance between Bcl-2 and Bax is necessary for maintaining the mitochondrial function [32]. If mitochondrial function was decreased, the Cyt c release can cause a chain of signaling cascades, resulting in chondrocyte apoptosis in OA. P-Akt can activate Bad, a downstream substrate of Akt, which depolymerizes with Bcl-2, leading to Bcl-2 release and subsequent apoptosis inhibition [33]. Lee et al. [24] reported that *Clematis mandshurica* increased phosphorylation of Akt and decreased Bad activity, which upregulated the Bcl-2/Bax ratio and reduced chondrocyte apoptosis. Our current data proved that shikonin pretreatment on chondrocyte apoptosis induced by IL-1 β upregulated the Bcl-2/Bax ratio and restrained Cyt c release from mitochondria to cytosol, suggesting that shikonin may decrease mitochondrial permeability in chondrocytes to suppress Cyt c release and chondrocyte apoptosis induced by IL-1 β .

The most important executor of cell apoptosis is caspase-3 that is a member of the downstream Cyt c signaling pathway. A previous report indicated that 17 β -estradiol activated the PI3K/Akt signaling pathway and restrained the mitochondrial pathway, eventually deactivating caspase-3, and thereby inhibiting chondrocyte apoptosis [34, 35]. Thus, the crux to suppress chondrocyte apoptosis may be inhibiting the caspase-3 activation. Here, our research pointed out that shikonin could sustain mitochondrial function and restrained IL-1 β -induced caspase-3 activation.

Besides, drugs used to treat OA not only inhibited chondrocyte apoptosis, but also promoted chondrocyte matrix synthesis. Previous report had confirmed that TIMP-1 blocked MMP-13 synthesis and suppressed extracellular matrix degradation [36]. Recent reports pointed out that PI3K/Akt signaling regulated chondrocyte expression of MMP-13 and TIMP-1 [37]. The present study showed that pretreatment with

shikonin in cytotoxicity induced by IL-1 β decreased MMP-13 synthesis but increased TIMP-1 expression. Addition of LY294002 further demonstrated that the effects of shikonin on regulation of MMP-13 and TIMP-1 expressions induced by IL-1 β were blocked, indicating that PI3K/Akt signaling played a critical role during this process. Therefore, we deduced that PI3K/Akt signaling that is a target of shikonin could both restrained chondrocyte apoptosis and reduce extracellular matrix degradation.

In conclusion, our results confirmed that shikonin restrained chondrocyte apoptosis induced by IL-1 β and suppressed MMP-13 synthesis and enhanced TIMP-1 expression by its effects on PI3K/Akt/mitochondrial signaling pathways. Collectively, these findings suggested that shikonin had a potential therapeutic function for the treatment of OA.

Disclosure of conflict of interest

None.

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