

Curcumin ameliorates H₂O₂-induced inflammatory response in chondrocytes by inducing autophagy activation

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Abstract. Relapsing polychondritis (RP) is a clinical disease characterized by inflammation of cartilage tissue and chondrocytes. The principal curcuminoid curcumin is the most active component in turmeric and has been reported to have a chondroprotective effect, including anti-inflammatory activity, which is vitally important for mitigating RP symptoms and prognosis. However, the mechanisms underlying these actions have remained to be fully elucidated. In the present study, the chondroprotective mechanisms of curcumin on hydrogen peroxide (H₂O₂)-treated primary chondrocytes were examined *in vitro*. The viability of chondrocytes treated with H₂O₂ was significantly reduced in a dose- and time-dependent manner. Cotreatment of curcumin with H₂O₂ significantly decreased growth inhibition. It was observed that curcumin inhibited the expression levels of the inflammatory mediators interleukin (IL)-1 β , IL-6 and inducible nitric oxide synthase and induced autophagy activation. Curcumin increased the protein levels of the autophagy marker beclin-1 and light chain 3-II and decreased the expression levels of P62 in H₂O₂-treated chondrocytes. The curcumin-induced anti-inflammatory effects were markedly abrogated by the autophagy inhibitor 3-methyladenine. In conclusion, the present study suggested that curcumin regulates inflammatory factors by activating autophagy in chondrocytes. The protective role of curcumin in chondrocytes was demonstrated, suggesting that it should be explored for the prophylactic treatment of RP in the clinic in the future.

Introduction

Relapsing polychondritis (RP) is a clinical disease, which is characterized by the inflammation of systemic cartilage tissues such as the external auricle, nose, respiratory tract and joints, and a pattern of repeated remission and recurrence (1). However, identifying effective treatment strategies for RP remains a challenge. It is well known that inflammatory response in chondrocytes is one of the most important risk factors in the pathogenesis of RP, which is influenced by oxidative stress (2). H₂O₂ is a common agent and may lead to DNA damage by inducing oxidative stress. It is necessary to deeply explore the molecular mechanisms underlying the suppression of inflammation in chondrocytes under oxidative stress, which is responsible for cartilage destruction in the progression of RP. Therefore, the suppression of inflammatory responses in chondrocytes may be an effective strategy to delay RP progression.

Curcumin is a promising pharmacologically active natural product, which is extracted from turmeric (*Curcuma longa*) and has significant anti-inflammatory, anti-oxidant and anti-cancer properties (3). Previous studies have reported on the anti-inflammatory effects of curcumin in various common diseases, including chronic inflammation, cancer, cardiovascular disease and osteoarthritis (4-7). Furthermore, it was suggested that curcumin may exert anti-inflammatory effects in several chronic diseases by activating the nuclear factor E2-related factor 2 (Nrf2) signaling pathway (8). However, the roles of curcumin in chondrocytes and the underlying mechanisms remain elusive.

Previous studies have indicated that the reactivation of autophagy is a promising therapeutic strategy for the suppression of inflammation (9,10). Autophagy, a cellular conservation and self-digestion system, is accurately regulated by a family of autophagy regulators and autophagy-related proteins and homologues. It is controlled by a series of different signaling pathways such as MAPK, phosphoinositide-3 kinase (PI3K) and mTOR (11-13), which coordinate autophagy by regulating autophagosome formation and autophagosome-lysosome fusion. Autophagy mediates the degradation of dysfunctional proteins and damaged organelles for energy recycling to maintain the metabolic regulation and nutrition maintenance of the cell under oxidative stress (14). Recently, pharmacological

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suppression studies to reduce cell inflammation have repeatedly demonstrated the protective effect of autophagy on cells under abnormal physiological conditions, including external pressure, hypoalimentation, hypoxia and endoplasmic reticulum stress (15,16). In rats with osteoarthritis, β -ecdysterone promoted the autophagic flux of chondrocytes by regulating the PI3K/AKT/mTOR signaling pathway and attenuated the inflammatory response (17). In addition, curcumin was reported to exert a neuroprotective effect by inducing autophagic activities via the PI3K/Akt/mTOR pathway and suppresses inflammatory reactions through the Toll-like receptor 4/p38/MAPK pathway (18). However, whether curcumin mediates the suppression of the inflammatory response by inducing autophagic activities in chondrocytes has remained elusive.

Therefore, the present study aimed to explore the role of curcumin on the inflammatory response of chondrocytes and its correlation with autophagy in a hydrogen peroxide (H_2O_2)-induced inflammation model *in vitro*.

Materials and methods

Materials. Dulbecco's Modified Eagle's Medium F-12 (DMEM-F12) and FBS were purchased from Corning Life Sciences. Curcumin (cat. no. HY-N0005, >96.0%) purchased from MedChemExpress was dissolved in DMSO and then diluted with culture medium for cell experiments. H_2O_2 solution (cat. no. 106097; 34.5-36.5%) was purchased from Merck KGaA. 3-Methyladenine (3-MA; cat. no. HY-19312) was obtained from MedChemExpress and prepared as a 100 mM stock solution in PBS. Protease inhibitors were purchased from MilliporeSigma. An ECL chemiluminescence detection kit (SuperSignal HRP; cat. no. 46640) was from Pierce (Thermo Fisher Scientific, Inc.).

Cell isolation and culture. A total of 20 male Sprague-Dawley rats (weight, 200-220 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All Sprague Dawley rats were reared under specific pathogen-free conditions. Rats were housed under laminar flow in an isolated room with controlled temperature and at a 12-h light/dark cycle. Food and water were available *ad libitum*. The rats were sacrificed by injecting 100-200 mg/kg pentobarbital sodium at the end of the experiments. Death of rats was confirmed by observation of respiration and heartbeat. Primary chondrocytes were isolated from the bilateral hip joints of 4-week-old male rats. The cartilage of the rat hip joint was cut into 1 mm³ pieces in a sterile manner and then treated with 0.25% (V/V) trypsin/EDTA (cat. no. C0201; Beyotime Institute of Biotechnology) for 1 h and digested with 0.2% (V/V) collagenase II (cat. no. C2-28; Sigma-Aldrich; Merck KGaA) in DMEM-F12 at 37°C in an atmosphere with 5% CO₂ for 6 h. Next, the suspensions were centrifuged at 1,609 x g at room temperature for 5 min and cultured at 37°C under 5% CO₂ in DMEM-F12 with 10% (V/V) FBS, 1% (V/V) penicillin and streptomycin. Primary chondrocytes from the first passage were used for *in vitro* experiments.

Cell proliferation assay. The effect of curcumin and H_2O_2 on chondrocytes was assessed with a CCK-8 (cat. no. CK04;

Dojindo Laboratories, Inc.) according to the manufacturer's protocol. In brief, after treatment, cells were cultured in 96-well plates with a density of 5x10³ cells/well for 24 h. Subsequently, CCK-8 solution was added to each well and the cells were further incubated at 37°C in the dark for 1 h. The optical density values were detected at wavelengths of 450 nm.

Monodansylcadaverine (MDC) assay. MDC was used to fluorescently label autophagic lysosomes in the cytoplasm. Cells were seeded on sterile glass slides in cell culture media. In the curcumin+ H_2O_2 +3-MA group, chondrocytes were pretreated with 20 μ M curcumin for ~2 h, followed by incubation with 20 μ M H_2O_2 and 10 mM 3-MA at 37°C for 24 h. In the rapamycin group, chondrocytes were pretreated with 7.5 μ M rapamycin. In the other groups, chondrocytes were pretreated with or without 20 μ M curcumin for ~2 h, followed by treatment with or without 20 μ M H_2O_2 at 37°C for 24 h. Subsequently, chondrocytes were treated with MDC (0.05 mM) at 37°C for 30 min and were then washed with PBS three times. The samples were immediately analyzed by confocal microscopy (Olympus Corporation). Excitation wavelengths were 360-380 nm and images were captured under a microscope at x200 magnification from 20 separate randomly selected microscopic fields. MDC-specific activity was calculated as the number of cells with morphological features of autophagy as determined by scoring 100 cells from 20 different microscopic fields.

Western blot analysis. To examine the function of curcumin on apoptosis induced by H_2O_2 on chondrocytes, the total cellular protein was extracted by using a radioimmunoprecipitation assay lysis buffer (main components, pH 7.4; 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA and leupeptin; cat. no. P0013B; Beyotime Institute of Biotechnology) supplemented with protease inhibitor cocktail (cat. no. 1081; Beyotime Institute of Biotechnology). The concentration of the protein in different groups was measured by a BCA protein assay kit (cat. no. P0009; Beyotime Institute of Biotechnology). Subsequently, 30 μ g of protein in each group was separated by 15% SDS-PAGE and electrotransferred to PVDF membranes (cat. no. 162-0177; Bio-Rad Laboratories, Inc.). Following blocking with 5% skimmed milk powder (cat. no. P0216; Beyotime Institute of Biotechnology) in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 90 min at room temperature, membranes were incubated overnight at 4°C with the following primary antibodies: IL-1 β (dilution, 1:1,000; cat. no. ab283818; Abcam), IL-6 (dilution, 1:1,000; cat. no. ab259341; Abcam), inducible nitric oxide synthase (iNOS; dilution, 1:1,000; cat. no. ab178945; Abcam), beclin-1 (dilution, 1:1,000; cat. no. ab207612; Abcam), P62 (dilution, 1:1,000; cat. no. ab109012; Abcam), light chain (LC)3 (dilution, 1:1,000; cat. no. ab192890; Abcam) and β -actin (dilution, 1:1,000; cat. no. ab6276; Abcam). The next day, the blots were washed with TBST five times for 30 min and incubated with secondary antibodies (dilution, 1:20,000; cat. no. ab288151; Abcam) at room temperature for 90 min. Then membranes were washed with TBST five times. An imaging system (Li-Cor Biosciences, Inc.) was used to detect and analyze the density of each band.

Reverse transcription-quantitative (RT-q)PCR analysis. Following the manufacturer's protocol, the total RNA was isolated by an RNA extraction kit (cat. no. 28306; Qiagen GmbH). The quality [criterion, optical density at 260 nm (OD_{260})/ OD_{280} =1.8-2.0] and concentration of RNA in each group were assessed using a NanoDrop 2000 (Thermo Fisher Scientific, Inc.), while any RNA contamination and degradation were detected on 1% agarose gels. Eventually, 1,000 ng of total RNA was reverse transcribed to synthesize cDNA with the PrimeScript™ RT Master Mix (cat. no. RR036A; Takara Biotechnology, Co., Ltd.). Real-time PCR was performed in triplicate by using SYBR green PCR Master Mix (cat. no. 640210; Takara Biotechnology, Co., Ltd.). The amplification was conducted using the following cycling conditions: 5 sec at 95°C, 20 sec at 63.5°C and 10 sec at 72°C for 40 cycles. The amplification efficiency of the qPCR was 95.6% and the relative mRNA expression level of the target gene was determined using the $2^{-\Delta\Delta C_q}$ method (19). The sequences of the forward and reverse primers of target genes are presented in Table I.

ELISA. The concentrations of IL-1 β and IL-6 in the culture supernatants from chondrocytes treated with the different stimuli were determined using commercial ELISA kits (cat. nos. KE1002 and KE1003; Proteintech Group, Inc.) following the manufacturer's instructions. The absorbance at 450 nm was detected with a Multiskan Ascent (SPECTRAFluor Plus; Tecan Group, Ltd.).

Statistical analysis. The experiments were performed as at least three independent experiments. The results are presented as the mean \pm standard deviation. SPSS 13.0 software (SPSS Inc.) was used to analyze the data. Comparisons among multiple groups were performed using a one-way or two-way ANOVA followed by a Tukey's post-hoc test. $P < 0.05$ was considered to indicate statistical significance.

Results

Effect of various concentrations of curcumin on chondrocyte viability in the presence or absence of H_2O_2 . The effect of curcumin on chondrocyte viability with or without H_2O_2 was studied at different concentrations for 24, 48 and 72 h by the CCK-8 assay. Curcumin exerted no significant cytotoxic effect at concentrations of up to 20 μ M at different time-points (Fig. 1A). The results also indicated that 20 μ M H_2O_2 significantly inhibited the viability of chondrocytes ($P < 0.05$; Fig. 1B), whereas curcumin ($< 20 \mu$ M) markedly increased the viability of chondrocytes in a dose-dependent manner ($P < 0.01$; Fig. 1C). To mimic the oxidative stress of RP *in vitro*, 20 μ M H_2O_2 was used to treat rat chondrocytes for 24 h. Thus, 20 μ M of curcumin and 20 μ M of H_2O_2 were used for the next experiments.

Curcumin inhibits H_2O_2 -induced chondrocyte inflammation. To explore the effects of curcumin on H_2O_2 -induced chondrocyte inflammation, chondrocytes were pretreated with curcumin for 2 h at 10 and 20 μ M and were exposed to H_2O_2 for another 24 h. Subsequently, it was examined whether curcumin affects H_2O_2 -induced IL-1 β , IL-6 and iNOS mRNA levels. RT-qPCR analyses indicated that curcumin treatment

Table I. Primer sequences used for real-time PCR.

Primer/gene name	Sequences (5' to 3')
IL-1 β F	CAACCAACAAGTGATATTCTCCATG
IL-1 β R	GATCCACACTCTCCAGCTGCA
IL-6 F	ACTTCCATCCAGTTGCCTTCTTGG
IL-6 R	TTAAGCCTCCGACTTGTGAAGTGG
iNOS F	AGTCAACTACAAGCCCCACG
iNOS R	GCAGCTTGTCCAGGGATTCT
GAPDH F	GCATCTTCTTGTGCAGTGCC
GAPDH R	GATGGTGATGGGTTTCCCGT

F, forward; R, reverse; iNOS, inducible nitric oxide synthase.

inhibited IL-1 β , IL-6 and iNOS mRNA expression levels as compared to the levels found in cells treated only with H_2O_2 ($P < 0.05$; Fig. 2A-C). In addition, western blot analysis also indicated that curcumin treatment inhibited the H_2O_2 -induced increases in the protein levels of inflammatory indicators, including IL-1 β , IL-6 and iNOS ($P < 0.05$; Fig. 2D and E). Furthermore, the production of IL-1 β and IL-6 in the culture supernatant was detected by an ELISA kit and the results suggested that curcumin treatment markedly inhibited the H_2O_2 -induced secretion of IL-1 β and IL-6 ($P < 0.05$; Fig. 2F). In conclusion, curcumin inhibited H_2O_2 -induced chondrocyte inflammation at the RNA and protein levels.

Curcumin treatment promotes autophagy of chondrocytes. MDC staining and western blot analysis were used to examine whether curcumin is able to induce autophagy in chondrocytes. In early endosome compartments, no MDC accumulates may be observed, but certain accumulates are present in mature autophagic vacuoles (AVs), such as autophagolysosomes (20). AVs stained by MDC appear as distinct dot-like structures distributed within the cytoplasm or localized in the perinuclear regions and were detected under a fluorescence microscope by scanning the cells. As presented in Fig. 3A, there was an increase in the number of MDC-labeled positive vesicles at 24 h after curcumin treatment. The effects of curcumin were inhibited by H_2O_2 . This observation was further confirmed by examining the expression levels of the autophagy-related markers LC3-I/II using western blot analysis. The expression of LC3II was significantly increased, while the expression of P62 was significantly reduced after curcumin treatment compared to the group without treatment, but there was no significant effect in inducing LC3II expression in the H_2O_2 group ($P < 0.01$; Fig. 3C-F). Therefore, curcumin promoted autophagy of chondrocytes.

Autophagy inhibition abrogates the anti-inflammatory and protective effects of curcumin in inflammatory chondrocytes. To study the role of autophagy in the anti-inflammatory and chondroprotective effects of curcumin, chondrocytes were treated with the inhibitor of autophagy 3-MA. Under stimulation with H_2O_2 , inhibition of autophagy abolished the curcumin-mediated downregulation of the mRNA and protein levels of IL-1 β , IL-6 and iNOS ($P < 0.05$; Fig. 4). These findings indicated

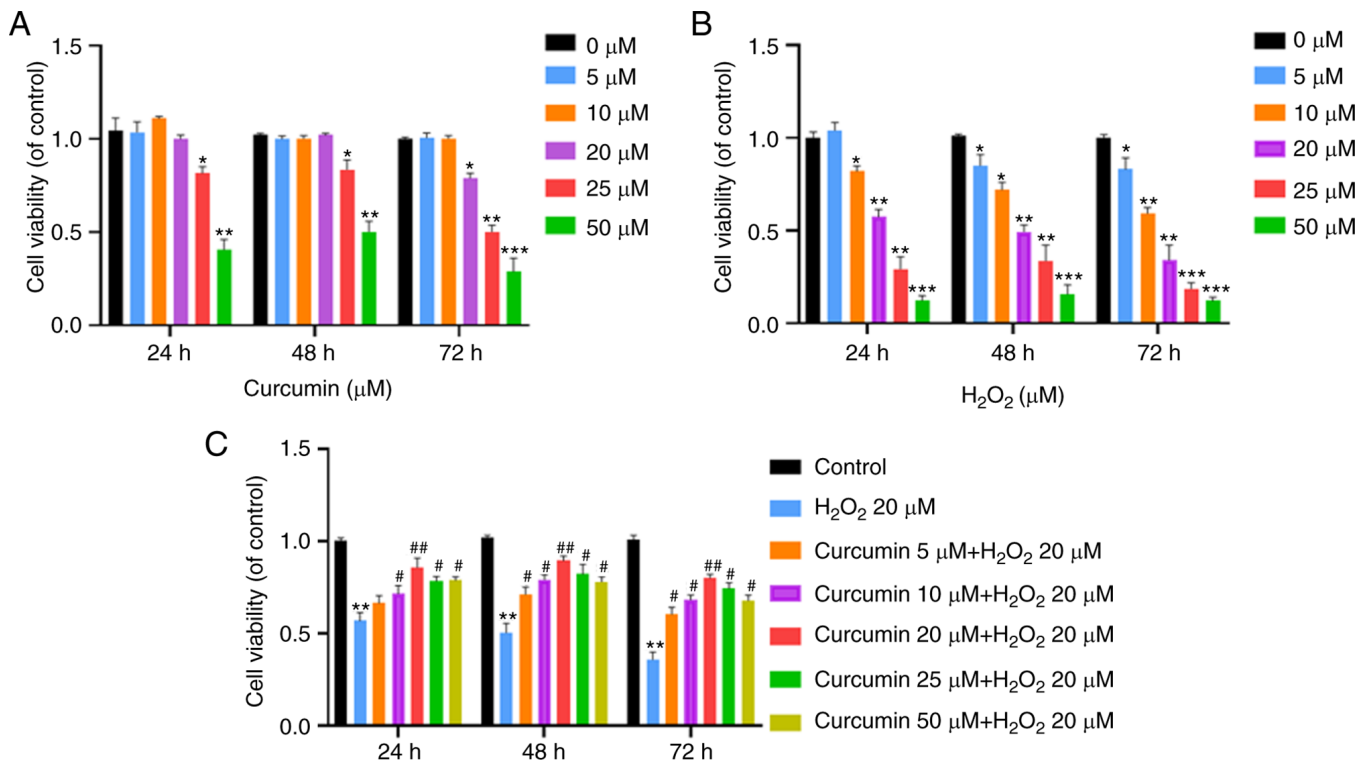


Figure 1. Effects of curcumin on chondrocyte viability with or without H₂O₂. (A) The cytotoxic effects of curcumin on chondrocytes were examined at various concentrations for 24, 48 and 72 h with a CCK-8 kit. (B) The cytotoxic effects of H₂O₂ on chondrocytes were examined at various concentrations for 24, 48 and 72 h with a CCK-8 kit. (C) Viability of H₂O₂-treated (20 μM) chondrocytes after curcumin treatment. *P<0.05, **P<0.01, ***P<0.001 vs. the control group; #P<0.05, ##P<0.01 vs. the H₂O₂ treatment group. All values are expressed as the mean ± standard deviation (n=3). CCK-8, Cell Counting Kit-8.

that autophagy is a pivotal factor in the curcumin-mediated suppression of inflammation in chondrocytes.

Discussion

The present study reported the following: i) Treatment of chondrocytes with 20 μM H₂O₂ results in viability inhibition and inflammation; ii) H₂O₂-induced chondrocyte inflammation was decreased by pretreatment with curcumin in a time-dependent manner; iii) curcumin's anti-inflammatory effects were mediated by the induction of chondrocyte autophagy; and iv) autophagy inhibitor 3-MA abolished the curcumin-mediated downregulation of inflammation factors.

The present results implied that autophagy is necessary to suppress chondrocyte inflammation. Curcumin is known for its underlying anti-inflammatory and antioxidant activity. Curcumin suppresses IL-1β secretion and prevents inflammation through inhibition of the NLR family pyrin domain containing 3 inflammasome (21). More importantly, curcumin is able to inhibit oxidative stress by regulating the Nrf2/heme oxygenase-1 signaling pathway to prevent aflatoxin B1-induced hepatotoxicity (4). In Saos-2 cells, curcumin ameliorated apoptosis by inhibiting oxidative stress and it attenuated palmitic acid-induced cell apoptosis by inhibiting endoplasmic reticulum stress (22-24). Further, related mechanistic studies suggested that curcumin is able to modulate autophagy (25). These data are consistent with the present results.

Various previous studies have reported that autophagy is constitutively active in chondrocytes (26,27). Appropriate

autophagy has a housekeeping role in preventing diseases such as cancer, cardiomyopathy, diabetes, liver diseases and autoimmune diseases, as well as neurodegeneration and infections (28-34). It is a well-conserved mechanism and has been confirmed to be important in various physical events. Beclin-1 and LC3 are major regulators and markers of the autophagy pathway among the human autophagy genes (35). The nucleation of autophagic vesicles relies on beclin-1, which may consequently lead to the formation of a complex with type III phosphatidylinositol. LC3-I is converted to LC3-II, which is then attached to the membrane of the autophagosome during autophagy activation. The BH3 domain of beclin-1 interacts with Bcl-2 and lead to inhibition of beclin-1-induced autophagy activation. Sequestosome 1 (SQSTM1/p62) is an important autophagy receptor protein. It is able to bind and deliver polyubiquitinated proteins to the autophagy pathway for degradation. It is important that SQSTM1/p62 is able to induce NF-κB signaling pathway activation by recruiting TNF receptor-associated factors (TRAFs) to TRAF binding sites in CD40 (36), but its effect may be inhibited after the silencing of p62 (37). Of note, p62 and certain other proteins that activate the NF-κB signaling pathway may also be degraded in the selective autophagy pathway (38). There is a complex regulatory relationship between p62-mediated autophagy and NF-κB signaling pathway activation (39). A study reported that p62 is able to improve the expression of antioxidant genes by binding to kelch-like ECH-associated protein 1, leading to activation of the Nrf2 signaling pathway (40). In short, p62-mediated induction of Nrf2 reduces inflammatory responses by inhibiting the activation of the NF-κB signaling pathway during

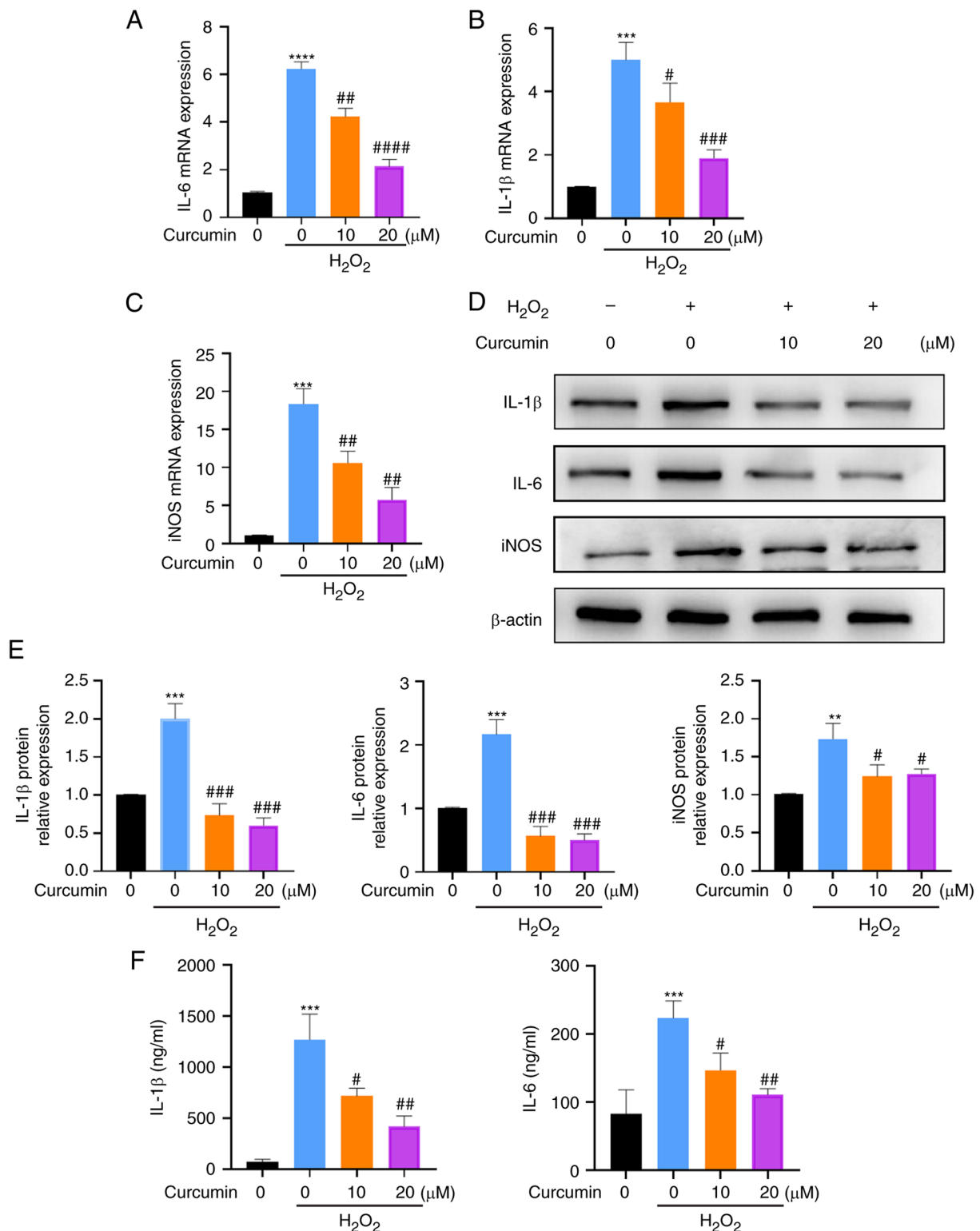


Figure 2. Curcumin inhibits the expression of H₂O₂-induced inflammatory mediators in chondrocytes. Chondrocytes were pretreated with curcumin (10 or 20 μM) for 2 h followed by H₂O₂ (20 μM) for 24 h. Protein and RNA samples were harvested. (A) IL-6, (B) IL-1β and (C) iNOS mRNA expression was measured by reverse transcription-quantitative PCR. GAPDH was used as an internal control. (D and E) IL-1β, IL-6 and iNOS protein expression was determined using western blot analysis. (D) Representative western blot and (E) quantified protein levels. (F) The production of IL-1β and IL-6 was also detected by ELISA. Values are expressed as the mean ± standard deviation (n=3). One-way ANOVA followed by Tukey's post-hoc test was used for statistical analysis. **P<0.01, ***P<0.001, ****P<0.0001 vs. H₂O₂-untreated group; #P<0.05, ##P<0.01, ###P<0.001, ####P<0.0001 vs. H₂O₂-treated group. iNOS, inducible nitric oxide synthase.

autophagy (41). In the present study, it was observed that curcumin raised the LC3-II/LC3-I ratio and decreased p62 expression in chondrocytes. This effect was reconciled with accelerated autophagy and mitigated inflammatory responses.

RP is a clinical disease characterized by a pattern of repeated remission and recurrence of systemic inflammation, in some cases followed by destruction, affecting the cartilage of the ears, nose, larynx, joints

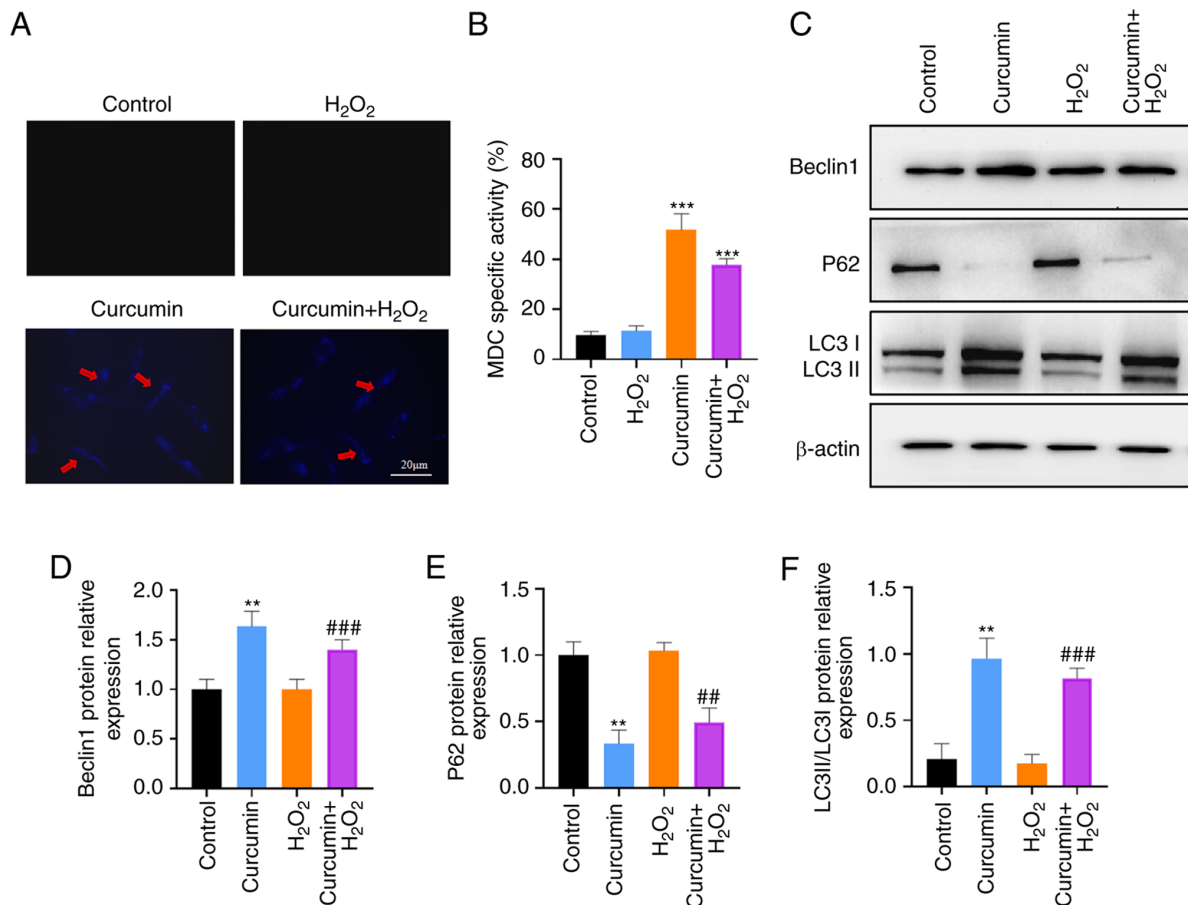


Figure 3. Curcumin activates autophagy within chondrocytes. (A and B) Curcumin-induced autophagy was detected by MDC staining. (A) Representative image of staining (arrows indicate autophagic vacuoles; scale bar, 20 μ m) and (B) quantified MDC activity at 24 h. (C-F) The levels of beclin-1, P62 and LC3-I/II were assessed using western blot analysis. (C) Representative western blot and quantified expression of (D) beclin-1, (E) P62 and (F) LC3-I/II. Values are expressed as the mean \pm standard deviation (n=3). **P<0.01, ***P<0.001 vs. control group; ##P<0.01, ###P<0.001 vs. H₂O₂ treatment group. LC3, light chain 3; MDC, monodansylcadaverine.

and tracheobronchial tree (42,43). Previous studies have revealed that inflammation, oxidative stress and matrix degradation are important factors associated with RP (2). It has been indicated that cell inflammation may be effectively inhibited by moderate autophagy activity (44). In the present study, an increase in autophagic activity induced by curcumin treatment was observed. In chondrocytes, autophagy is likely to be a self-protective process induced by curcumin in response to H₂O₂ stimulation. To confirm this, it was demonstrated that when chondrocytes were pretreated with curcumin and then cotreated with H₂O₂ and 3-MA, chondrocyte inflammation clearly increased and autophagy was decreased.

In conclusion, the present study suggested that autophagy is vital for chondrocyte inflammation, whereas the self-activation of autophagy is a protective mechanism against inflammation under curcumin treatment. This suggested that the anti-inflammatory effects of curcumin are mediated, at least in part, by the autophagy signaling pathway. The present study provided a theoretical basis for the treatment of RP in the clinic.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZL was responsible for the conception and design of the study. HLQ and ZYC were involved in data acquisition. YHQ was involved in the development of the study methodology, analysis and interpretation of the data. HLQ, ZYC, YHQ and YZL were involved in the writing, reviewing and revision of the article, and analyzed the relevant literature. HLQ and YZL confirmed the authenticity of the raw data. All authors have read and approved the final manuscript.

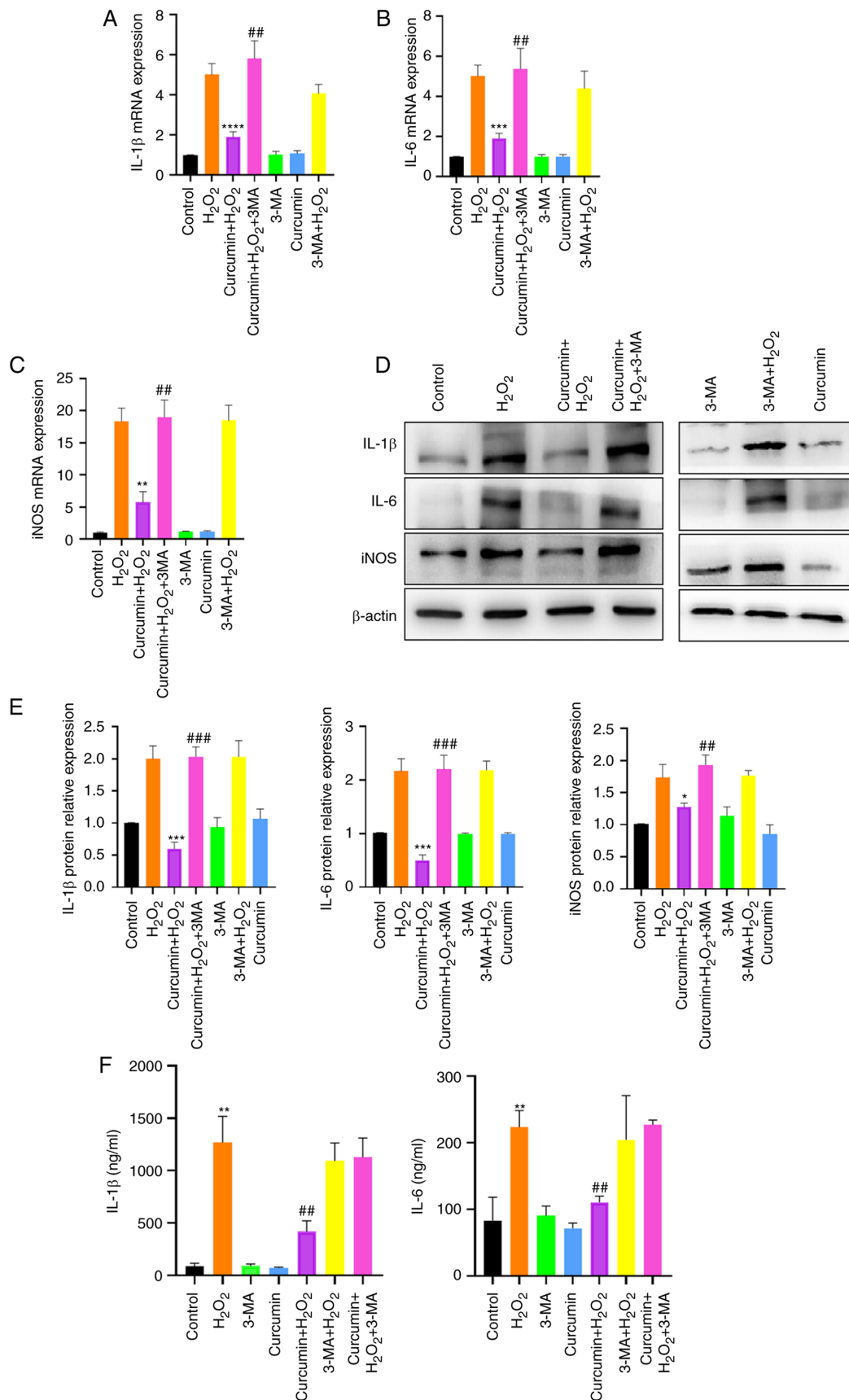


Figure 4. Autophagy inhibition abrogates the anti-inflammatory and chondroprotective effects of curcumin in inflammatory chondrocytes. Chondrocytes were pretreated with or without curcumin (20 μ M) for 2 h, followed by treatment with or without H₂O₂ (20 μ M) for 24 h. Chondrocytes were then treated with 3-MA (50 nM), curcumin (20 μ M) or 3-MA+curcumin. (A) IL-1 β , (B) IL-6 and (C) iNOS mRNA expression was measured by reverse transcription-quantitative PCR. GAPDH was used as an internal control. (D and E) IL-1 β , IL-6 and iNOS protein expression was determined using western blot analysis. (D) Representative western blot and (E) quantified protein levels. (F) The production of IL-1 β and IL-6 was detected by ELISA. Values are expressed as the mean \pm standard deviation (n=3). One-way ANOVA followed by Tukey's post-hoc test was used for statistical analysis. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. H₂O₂ treatment group; ##P<0.01, ###P<0.001 vs. curcumin + H₂O₂ treatment group. iNOS, inducible nitric oxide synthase; 3-MA, 3-methyladenine.

Ethics approval and consent to participate

All experiments were approved by the Laboratory Animal Care and Use Committee of Hebei Medical University (approval ID: HebMU 20200026; Shijiazhuang, China) and were conducted in accordance with National Institutes of Health guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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