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Sinomenine contributes to the inhibition of the inflammatory response and the improvement of osteoarthritis in mouse-cartilage cells by acting on the Nrf2/HO-1 and NF-κB signaling pathways

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ABSTRACT

Pathological changes, such as articular cartilage degeneration, destruction, and hyperosteogeny, are regarded as the main features of osteoarthritis (OA). Sinomenine (SIN) is a monomeric component purified from the plant *Sinomenium acutum* which has been found to have anti-inflammatory effects, however, the mechanism of action of SIN on OA is not clear. In this study, we evaluated whether SIN could regulate the inflammatory response induced by interleukin (IL)-1β and improve outcomes in the instability model of OA (medial meniscus mice (DMM)) by acting on the Nrf2/HO-1 and NF-κ B signaling pathways in chondrocytes. From our experiments, which include Griess reaction, ELISA, Western blot, and immunofluorescence, we found that SIN not only down-regulated the expression of pro-inflammatory factors induced by IL-1β, including; inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), nitricoxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), but also decreased the production of IL-1β-induced cartilage matrix catabolic enzymes including; ADAMTS-5 and MMPs, in mouse chondrocytes. In addition, the degradation of aggrecan and type II collagen protein in the extracellular matrix (ECM) stimulated by IL-1β was reversed. Most importantly, we have revealed for the first time that in OA, SIN inhibited the inflammatory response and ECM degradation by activating the Nrf2/HO-1 signaling pathways and inhibiting NF-κB activity in mouse-cartilage cells. In in vivo experiments, SIN treatment helped to improve the cartilage destruction in OA model mice. In conclusion, this study has demonstrated that SIN inhibits the IL-1β-induced inflammatory response and cartilage destruction by activating the Nrf2/HO-1 signaling pathway and inhibiting the NF-κB signaling pathway in mouse chondrocytes, suggesting a new use for SIN in the treatment of OA.

1. Introduction

Osteoarthritis (OA) is a chronic arthropathy mainly seen in the elderly, and the incidence of this disease increases with age [1–3]. The onset of OA is slow, mostly occurring in load-bearing joints (i.e. hip joint, knee joint, etc.) and joint pain and limited movement are the main characteristics of OA [7]. Although several causes of OA have been identified, including obesity, trauma, and heredity, the pathogenesis of OA is not clear [5,6]. In addition to this, current drug treatments can only temporarily improve clinical symptoms, and so finding a drug that reverses the progression of OA with minimal side effects [4,8] is of critical importance.

A growing number of studies have identified that the local inflammatory response is a key factor in initiating and promoting the progression of OA [9,10]. It has been found that an important feature of early OA patients is the production of inflammatory cytokines and that these cytokines contribute to the deterioration of the disease [11]. Among the many inflammatory cytokines, IL-1β is oversecreted by chondrocytes and synovial cells and acts as the main pro-inflammatory cytokine, which leads to the degradation of articular cartilage by promoting the secretion of cartilage matrix degrading enzymes [13,14,21]. This means that the level of catabolism of the intra-articular matrix exceeds that of anabolic metabolism [12]. As important components of the ECM, aggrecan and type II collagen have lubricating and protective effects on articular cartilage [15–17]. The secretion of IL-1β leads not only to the degradation of the ECM, but also to excess production of

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pro-inflammatory factors and catabolic substances, such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (Cox-2), matrix metalloproteinases (MMPs), and thrombin sensitive protein motifs (ADAMTS) [18–20]. There is increasing evidence that NF-κB is involved in many biological processes such as IL-1β-induced inflammation, and apoptosis by regulating the expression of multiple genes [22,23]. The primary role of IκB is to bind to the NF-κB dimer and prevent it from entering the nucleus to bind to DNA, thereby inhibiting the NF-κB pathway [30,31]. Following IL-1β stimulation, IκBα rapidly degrades, releasing multiple NF-κB dimers and causing inflammation [28,29]. Previous studies have shown that the activation of the Nrf2/HO-1 signal transduction pathway can inhibit NF-κB mediated effects [25–27]. It was found that deletion of the antioxidant gene transcription regulator, nuclear factor-erythroid 2-related factor (Nrf2), can lead to a more severe form of OA in mice, which provides further evidence for the role of the Nrf2 signaling pathway in the pathogenesis of OA [24]. Therefore, a treatment strategy focused on Nrf2 may provide a breakthrough for OA treatment.

Sinomenine (SIN), used as a traditional Chinese medicine, is a monomeric component purified from the plant *Sinomenium acutum* and studies have found that SIN has a wide range of pharmacological properties including analgesic and anti-inflammatory [33,34]. We found that TNBS-induced inflammatory bowel disease was improved following oral administration of SIN in a mouse colitis model by down-regulating the levels of miR-155 and related inflammatory cytokines [32]. SIN effectively reduces inflammation and balances oxidative stress by activating Nrf2 to inhibit acute lung injury and kidney injury [35,36]. SIN has been used clinically in the treatment of rheumatoid arthritis and regulates the secretion of various inflammatory cytokines and monocyte/macrophage subsets, leading to a reduction in the progression of rheumatoid arthritis [37]. In addition, SIN has been shown to protect chondrocytes from apoptosis by significantly reducing the expression of caspase-3 [39]. It is important to note that currently, the role of the Nrf2/HO-1 signaling pathway in the progression of OA remains unclear. The purpose of this study was to examine whether SIN can reduce inflammation levels in OA patients by regulating the Nrf2/HO-1 signaling pathway, thereby slowing down the process of OA.

2. Materials and methods

2.1. Reagents

Sinomenine (purity≧98%) was obtained from Solarbio (Beijing, China), and collagenase type II and dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich (St Louis, MO, USA). SIN was dissolved in DMSO and stored at 5 °C. Recombinant rat IL-1β was purchased from PeproTech (NJ, USA). Primary antibodies against aggrecan, collagen II, MMP-13, ADAMTS5, HO-1, Nrf2, Lamin B1, and GAPDH were purchased from Abcam (Cambridge, UK) and rabbit anti-cyclooxygenase-2 (COX-2), rabbit anti-inducible nitric oxidesynthase (INOS), mouse anti-matrix metalloproteinase-3 (MMP-3), rabbit anti-IL-6, and rabbit anti-TNF alpha were obtained from Proteintech Group (WuHan, China). Antibodies against NF-κB p65, IκBα, p-IκBα and p-NF-κB p65 were purchased from Cell Signaling Technology Sigma Aldrich (St Louis, MO, USA). Goat anti-rabbit and anti-mouse IgG-HRP were supplied by Boster (Wuhan, China). Alexa Fluor®488-labeled and Alexa Fluor®594-labeled goat anti-rabbit IgG (H + L) secondary antibody was obtained from Jackson ImmunoResearch (West Grove, PA, USA). The nuclear stain, 4′,6-diamidino-2-phenylindole (DAPI), was obtained from Beyotime (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits to detect prostaglandin E2 (PGE2) were purchased from R& D systems (Minneapolis, MN, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's Medium F12 (DMEM/F12) were purchased from HyClone (Grand Island, NY, United States).

2.2. Primary mice chondrocyte culture

Ten C57BL/6 mice within 2 weeks of age were purchased from the Animal Center of Chinese Academy of Sciences (Shanghai, China) and euthanized with 10% chloral hydrate. Articular cartilage pieces from the knee joint were carefully cut and separated by surgical aseptic surgery, the cartilage was dissected and washed with PBS for 2 to 3 times, then treated with 0.2% collagenase type II for 4 to 6 h at 37 °C. The digested cartilage tissue was centrifuged at 1000 rpm for 3 min, the supernatant was aspirated and the sediment resuspended in DMEM/F-12 (Gibco, Life Technologies, Carlsbad, Calif, USA). The cell culture medium contained 10% FBS and 1% penicillin and streptomycin antibiotics. Resuscitated cells were evenly distributed into several culture plates and then incubated in 5% CO2 at 37 °C. When the chondrocytes reached 80% ~ 90% confluency, 0.25% trypsin-EDTA (Gibco, Invitrogen) was used to harvest the cells. The cells were then seeded into a 10 cm^2 culture plate at an appropriate concentration (about 1×10^5 cells/ml). The cell culture medium was changed every 1–2 days. To reduce the error of Western blot experiment, only the second-generation chondrocytes were used.

2.3. Cell viability assay

Cell counting kit-8 (CCK-8; DojindoCo, Kumamoto, Japan) was used to detect the cytotoxicity of SIN to chondrocytes in accordance with manufacturer's operating guidelines. Briefly, 100 μl of chondrocyte suspension was seeded into wells of a 96-well plates at 5×10^4 cell/cm^2 and precultured for 24 h at 37 °C, 5% CO_2. The chondrocytes were pretreated with SIN at various concentrations (0, 6.25, 12.5, 25 and 50 μg/ml) for 24 h and 48 h after which 10 μl of the CCK-8 solution was added to each well and incubated at 37 °C. for 4-6 h. After incubation, the absorbance at 450 nm was measured using a microplate system. All experiments were performed at least five times.

2.4. NO and PGE2 measurement

To detect the levels of NO and PGE2, chondrocytes resuspended in DMEM/F-12 were exposed to different concentrations (0, 6.25, 12.5 and 25 μg/ml) of SIN and incubated at 37 °C for 24 h after which prediluted IL-6 at the same concentration were added, and the cells cultured for another 24 h. Cell culture supernatant was collected, centrifuged, and the Griess reagent [40] used as previously described to measure the concentration of NO in the supernatant. The enzyme-linked immunosorbent assay (ELISA) kit was used to detect the concentration of PGE2 in accordance with the manufacturer's guidelines. All tests were performed five times.

2.5. Western blot analysis

Chondrocytes were cultured to about 80% confluency, washed 3 times with precooled PBS then dissociated with 0.05% trypsin and collected by centrifugation. The total protein extracted from chondrocytes was isolated adding a proper amount of RIPA lysis buffer with 1 mM PMSF (phenylmethanesulfonyl fluoride). After incubating on ice for 10–20 min the sample was centrifuged at 12000 rpm and 4 °C for 15 min, and the supernatant stored at −80 °C. Protein concentration was determined using the BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). 40 μg of total protein extracted from chondrocytes was loaded into well on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, 5 μl prestained protein marker was added to both sides of the gels which were then separated at a constant voltage of 80 V. Samples were then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, USA) at constant current (300 mA) for 1 to 2 h. The membrane was immersed in 5% skim milk prepared in TBST (0.1% Tween-20) and gently shaken at room temperature for 2 h. The blocked membrane was incubated using

the primary antibodies at the specified concentrations; IL-6 (1:500), TNF-α (1:500), INOS (1:500), COX-2 (1:500), Collagen II (1:1000), Aggrecan (1:500), MMP-3 (1:500), MMP-13 (1:1000), ADAMTS-5 (1:1000), HO-1 (1:1000), NRF-2 (1:1000), NF-κB p65 (1:1000), p-NF-κB p65 (1:1000), IκB (1:1000), p-IκB (1:1000), Lamin B1 (1:1000) and GAPDH (1:1000) overnight at 4 °C. After primary antibody incubation, the membrane was washed with TBST 3 times for 5-10 min and after that incubated with the relevant secondary antibody (1:3000) in 5% skim milk in TBST for 2 h at room temperature. After washing with TBST, the membranes were developed using Enhanced Chemiluminescence (ECL) kit, the gray values of the membranes were quantified using Image Lab 3.0 software (Bio-Rad).

2.6. Immunofluorescence

Chondrocytes seeded on glass coverslips were incubated with or without SIN (25.0 g/ml) for 24 h, then co-incubated with or without IL-1β (10 ng/ml) for 24 h. The coverslips were then fixed with 4% paraformaldehyde solution for 15 min at room temperature, rinsed three times with PBS then treated with 0.1% TritonX-100 at room temperature for 5 min. Coverslips were then transferred into a wet box, blocked with 5% bovine serum albumin for 1 h at 37 °C, then incubated with a primary antibody (MMP-13 (1:200), collagen II (1: 200), NF-κB p65 (1: 200) and Nrf2 (1: 100)) diluted with PBS for 12 h at 4 °C. The coverslips were washed with PBS and incubated at room temperature with fluorescein-conjugated goat anti-rabbit IgG antibody (1:400, Bio-Rad Laboratories, Calif, USA) in darkness for 1 h, before the nuclei were stained with DAPI. After aspirating the liquid from the glass coverslips, the cells were overlaid with anti-fluorescence-quenching agent, and imaged using a fluorescence microscope (Olympus Inc., Tokyo, Japan).

2.7. siRNA transfection

Nrf2 small-interfering RNA (siRNA) was purchased from Invitrogen (Carlsbad, CA, USA) with the specific following sequences synthesized: sense, 5′-UUGGGAUUCACGCAUAGGAGCACUG-3′; antisense, 5′-CAGUGC.

UCCUAUGCGUGAAUCCCAA-3′. Chondrocytes were transfected with Lipofectamine 2000 siRNA transfection reagent (Thermo Fisher, UT, USA) and negative control siRNA in accordance to the manufacturer's guidelines.

2.8. Establishment of mice OA model and animal experiment design

Ten-week-old male C57BL/6 wild-type (WT) mice were purchased from the Animal Center of Chinese Academy of Sciences (Shanghai, China). Mice were fed in a clean environment, where water and fodder were freely consumed. 60 mice were randomly divided into 3 groups with 20 mice in each group. The first group was the blank control group, the second group the osteoarthritis (OA) group, and the third group was the OA treated with SIN group. The mice in the control group were subjected to a sham operation in which the articular capsule was incised without damaging the meniscus and articular cartilage. In the OA group and SIN group, destabilization of the medial meniscus (DMM) as previously described [41] was caused by surgical treatment. After surgery, mice without fixed limbs were allowed to move freely and given antibiotics to prevent infection.

From the first month after the operation, mice in the third group were given intraperitoneal injections of SIN(10 mg/kg/d)dissolved in 0.5% carboxymethylcellulose sodium for two months while the first and second groups were receiving intraperitoneal injections of 0.5% carboxymethylcellulose sodium in equal doses. The establishment of the animal model, and the follow-up experiments, were carried out under the guidance of the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the Animal Care and Use Committee of Wenzhou Medical University. The animals were killed at the 12th week after the operation and the knee joints of the mice were collected for analysis and evaluation.

2.9. Histological analysis

After fixing the tissue with 4% paraformaldehyde and embedding it in paraffin blocks, the knee joints were made into histological sections with a thickness of 5 μm. The two sagittal sections selected from the knee joint including the subchondral bone were considered to be the most representative samples. Modified Safranine O-Fast Green FCF Cartilage Stain (S—O) Kit was purchased from Solarbio (Beijing, China) and the stained knee sections were observed under a microscope to evaluate the extent of articular cartilage damage. In order to improve the accuracy of the experiment further independent scoring systems were used to determine the extent of articular cartilage degeneration. A group of histologic researchers were randomly selected to evaluate the histological sections using the osteoarthritis Research Society International (OARSI) scoring system as the criterion as previously described [42]. The recommended semi-quantitative scoring system can also be utilized as a separate scoring criterion for osteoarthritic damage. 20 mice in each group were used for histomorphometric scoring.

2.10. X-ray imaging and specimen photographs

Three groups of mice were examined using X-rays during the eighth week after the operation. After an intraperitoneal injection of pentobarbital sodium (35 mg/kg weight), the mice were placed on a digital X-ray machine (Kubtec Model XPERT.8; KUB Technologies Inc.) and ray imaging technology was used to evaluate the obtained images. Joint space, cartilage surface calcification and osteophyte formation were used to evaluate the degree of articular cartilage degeneration. After the mice were sacrificed, the knee joint was taken, pictures were made and observed under macroscopical conditions.

2.11. Statistical analysis

All experiments were repeated at least five times. The data from the experimental results were expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS statistical software version 16.0. The difference between the three groups (the control, OA, and treatment group) was examined by one-way analysis of variance (ANOVA) followed by Tukey's test. The OARSI score obtained by statistics were analyzed with a Kruskal-Wallis H test. A P value < 0.05 was judged to be statistically significant.

3. Results

3.1. Effects of SIN on chondrocyte viability

The molecular chemical structure of SIN and the cell morphology of mouse chondrocytes are shown in Fig. 1A and D. To detect the cytotoxicity of SIN on mouse chondrocytes, chondrocytes were cultured in different concentrations (0, 6.25,12.5, 25,50,100 μM) of SIN for 24 h and 48 h respectively, before cell viability was detected using a CCK-8 assay. From Fig. 1B and C, we find that SIN could reduce the viability of cartilage when the concentration was > 50 μM. After 48 h, the viability of chondrocytes was obviously inhibited compared with that of 24 h. This also means that SIN had no obvious toxic effect on mouse chondrocytes at concentrations $\leqq 25$ μM after 24 h or 48 h. This shows that SIN at 6.25,12.5 and 25 μM can be utilized in subsequent in vitro experiments.

3.2. SIN down-regulated the levels of inflammatory mediators in chondrocytes stimulated by IL-1β

To investigate the effect of SIN on chondrocytes stimulated by

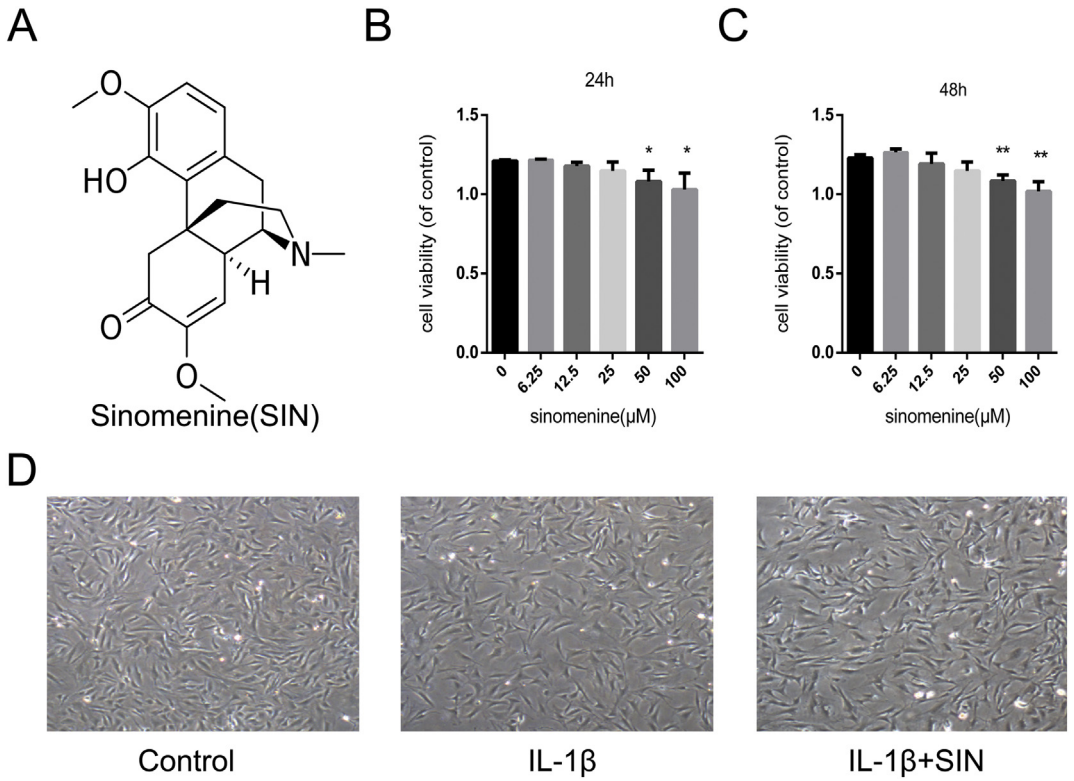

Fig. 1. Effects of SIN on the cell viability of mouse chondrocytes. (A) Chemical structure of SIN. (B, C) CCK-8 assay was used to determine the effect of increasing concentrations of SIN (6.25,12.5,25,50,100 μM) on the viability of mouse chondrocyte cells after 24 and 48 h incubations. (D) Mouse cartilage cell morphology was observed via microscopy after treatment with IL-1β (10 ng/ml) with or without SIN (25 μm) for 24 h. The values presented are the means ± S.D. and statistical difference between groups shown as follows; *P < 0.05 vs. control group; **P < 0.01 vs. control group, $n = 5$.

inflammation, mouse chondrocytes were pretreated for 24 h with different concentrations of SIN and an equal concentration of IL-1β. Western blotting and ELISA were used to determine the expression of inflammatory mediators, including; PGE2, NO, iNOS, COX-2, TNF-α, and IL-6. From Fig. 2A, we found that the production of endogenous NO and PGE2 stimulated by IL-1β was significantly up-regulated compared with the control group as shown by ELISA, however, this dose-dependent trend was reversed by SIN at 12.5 μM and 25 μM, respectively. We obtained further support for this observation using western blots to detect the expression of iNOS and COX-2. The results showed that the levels of the IL-1β group were higher than that of the control group, but the same concentration of SIN (12.5 μM and 25 μM) down-regulated the expression of iNOS and COX-2 at the protein level in a similar manner (Fig. 2C). The expression of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) was determined by western blot. SIN at concentrations of 12.5 and 25 μM inhibited the generation of TNF-α and IL-6 at the protein level compared to a lower SIN concentration (6.25 μM). These results suggest that SIN could inhibit the IL-1β induced production of inflammatory mediators and cytokines in chondrocytes in a concentration dependent manner.

3.3. Protective effect of SIN on IL-1β-induced ECM degradation in chondrocytes

To determine whether SIN can protect against IL-1β-induced ECM degradation, western blotting and immunofluorescence were used to detect the expression of ECM components in chondrocytes treated with or without SIN including; type II collagen, aggrecan, ADAMTS-5, and MMPs (MMP3, MMP13). As shown in Fig. 3 A to D, type II collagen and aggrecan synthesis, which were contributing to the survival of chondrocytes, were significantly inhibited by IL-1β. IL-1β enhanced expression of ADAMTS-5 and MMPs (MMP3, MMP13), which are the major cartilage degradation enzymes in the development of OA. The trend induced by IL-1β was reversed after SIN pretreatment in a dose-dependent manner. The results of immunofluorescence of MMP-3 were, in general, consistent with those of the western blot (Fig. 3E and F), which further supports observations of SIN's ability to block ECM degradation.

3.4. SIN inhibited IL-1β-induced NF-κB activation in chondrocytes

The immunofluorescence results showed that after treatment with IL-1β, the green fluorescence of the chondrocyte inflammation-associated protein p-NF-κB p65 was found in the nucleus of chondrocytes, and was significantly different to that of the control group. SIN treatment down-regulates the p-NF-κB p65 green fluorescence in the nucleus (Fig. 4B). Western blot results (Fig. 4A and C) showed that the p-NF-κB p65 level was significantly increased in the IL-1β group (P < 0.01), and significantly decreased with SIN (6.25, 12.5 and 25 μM). In addition, Fig. 4 A and D showed that pretreatment with SIN (6.25, 12.5 and 25 μM) inhibited expression of p-IκBα at the protein level in IL-1β-induced chondrocytes in a dose-dependent manner.

3.5. Effect of SIN on the IL-1β-induced Nrf2/HO-1 signaling pathway in chondrocytes

Western blots and immunofluorescence were used to determine whether the Nrf2/HO-1 signaling pathway acted on chondrocytes. According to the western blots, we found that there was no significant difference in the expression of Nrf2 and HO-1 between the IL-1β treatment group and control group (Fig. 5B and C), however, SIN significantly increased the expression of Nrf2 and HO-1 in a concentration-dependent manner (Fig. 5B and C). In addition to this, the results of immunofluorescence staining showed that SIN could promote the

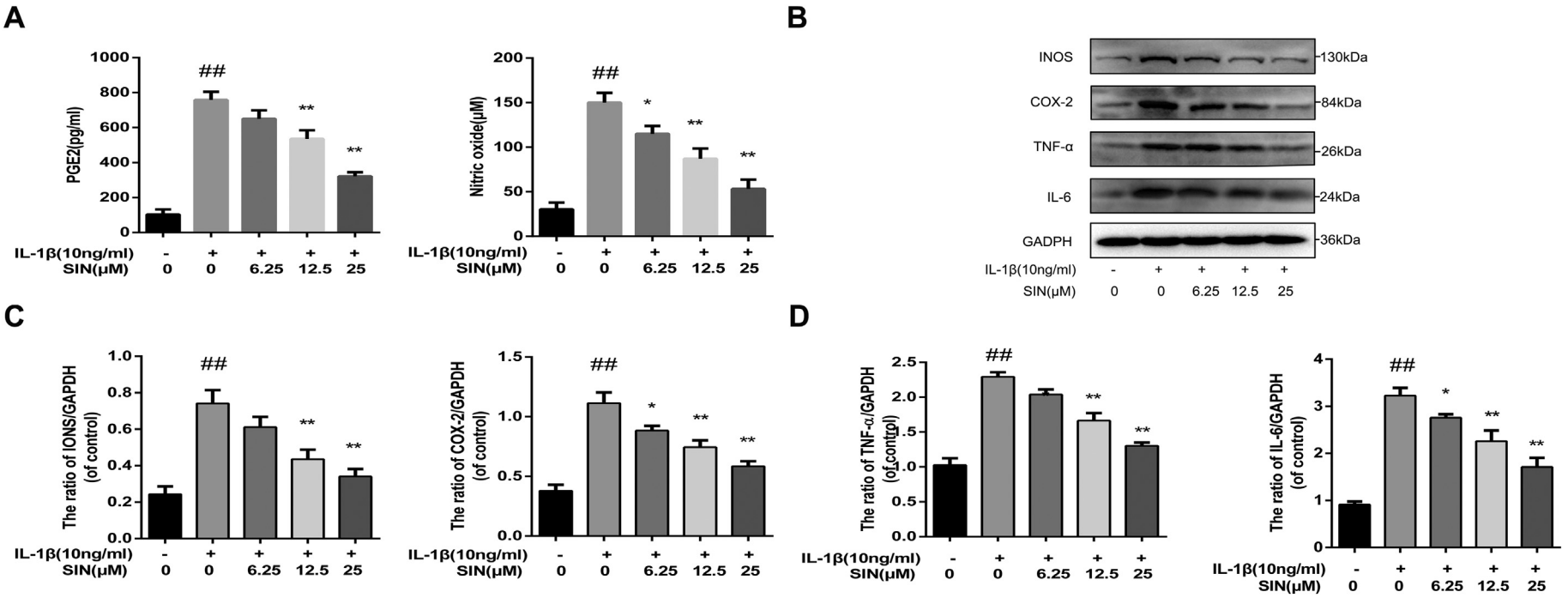

Fig. 2. Effects of SIN on IL-1β-induced expression of NO, PGE2, iNOS, COX2, TNF-α, and IL-6 in mouse chondrocytes. (A) The production of NO and PGE2 were measured by ELISA. (B) The expression of iNOS, COX-2, TNF-α, and IL-6 was visualized by western blot and (C, D) quantified using ImageJ. The data in the figures indicate the averages ± S.D. Statistically significant differences between groups were shown as follows: $^{\#\#}P < 0.01$, vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. IL-1β alone treatment group, n = 5.

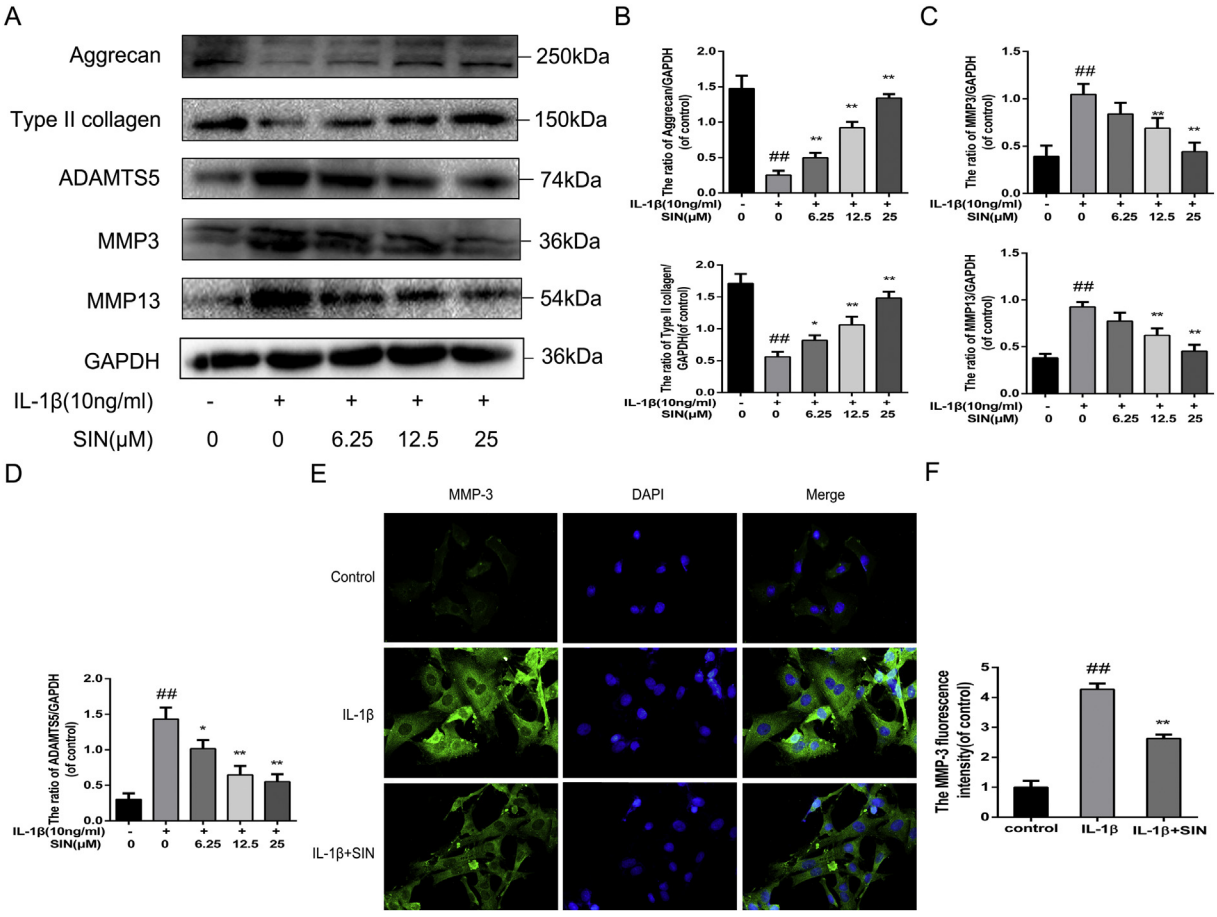

Fig. 3. Effects of SIN on IL-1β induced ECM degradation. The expression levels of type II collagen, aggrecan, ADAMTS-5, and MMPs (MMP3 and MMP13) were visualized by western blot and (B, C, D) quantified using ImageJ. (E) After IL-1β treatment (10 ng/ml) with or without SIN (25 μM) for 24 h, the expression of MMP-3 was observed by immunofluorescence (scale bar: 10 μm) and the fluorescence intensity analyzed using Image J (F). The data in the figures indicate the averages ± S.D. Statistically significant differences between different groups were shown as follows: $^{\#\#}P < 0.01$, vs. control group; $^{**}P < 0.01$, vs. IL-1β alone treatment group, n = 5.

nuclear translocation of Nrf2 compared to the IL-1β treatment and control groups, which was consistent with the results of western blot (Fig. 5A). To further verify that SIN could activate the Nrf2/HO-1 pathway and that the activated Nrf2/HO-1 pathway in mouse chondrocytes could inhibit the inflammatory response, Nrf2-siRNA was transfected into chondrocytes and were co-treated with IL-1β and SIN. The sinomenine-induced Nrf2/HO-1 signaling activation was inhibited by Nrf2 siRNA transfection, as shown in Fig. 5D and E. However, the results of western blot after Nrf2 siRNA transfection showed that the expression of p-NF-κB p65, iNOS, and COX-2 in was up-regulated at the protein level, indicating that the Nrf2/HO-1 pathway inhibited the inflammatory response (Fig. 5D and E).

3.6. SIN played a protective role in the development of a DMM OA model

To verify the protective effect of SIN against the development of OA, a surgically induced mouse DMM model was established and treated with SIN for eight weeks after operation by intraperitoneal injection. Among the sham, OA, and SIN groups, we used X-ray analysis, safranin O staining, and OARSI scores to analyze the morphological structure of cartilage. As shown in Fig. 6 A, X-ray analysis revealed that knee-joints in the sham-operated group had a normal joint space and bone mineral density. The OA group exhibited narrowing of the joint space and hyperosteogeny while in the SIN group these were reversed. As shown in Fig. 6 C, according to the ratio of red-dyed area to total area, the OA group showed an evident reduction in the cartilage matrix and articular cartilage thickness compared to the sham control group. A significant increase in articular cartilage thickness and amelioration of matrix degradation was observed in the sinomenine-treated group compared to the OA group. As shown in Fig. 6 B, the OARSI scores of the OA group were significantly higher than the sham control group ($P < 0.05$). In contrast, the OARSI scores of SIN group were significantly lower than those of the OA group ($P < 0.05$). Thus, these results showed that SIN played a protective role in the development of a DMM OA model.

4. Discussion

At present, it is increasingly obvious that the inflammatory mechanism plays an important role in regulating biomechanical disorders of joint tissue which lead to the development of OA [44]. New studies have shown that cartilage, bone, and synovium play pivotal roles in OA. As mechanical dampers, cartilage, bone, and synovium are involved in the pain process of OA and are the source of inflammatory mediators [43]. Many studies have shown that down-regulation of inflammatory mediators may have a protective effect against the progression of OA [44]. Although some breakthroughs have been made in elucidating the pathogenesis of the disease, treatment remains a challenge. As opposed to inflammatory joint disease, there is no effective drug treatment for OA [45]. Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used clinically, but will only temporarily relieve OA symptoms and can increase the risk of myocardial infarction [46]. SIN, used as a traditional Chinese medicine, has been reported to have a positive therapeutic effect on rheumatoid arthropathy (RA) [47,48]. It has been shown that SIN has a protective effect against cartilage degradation and

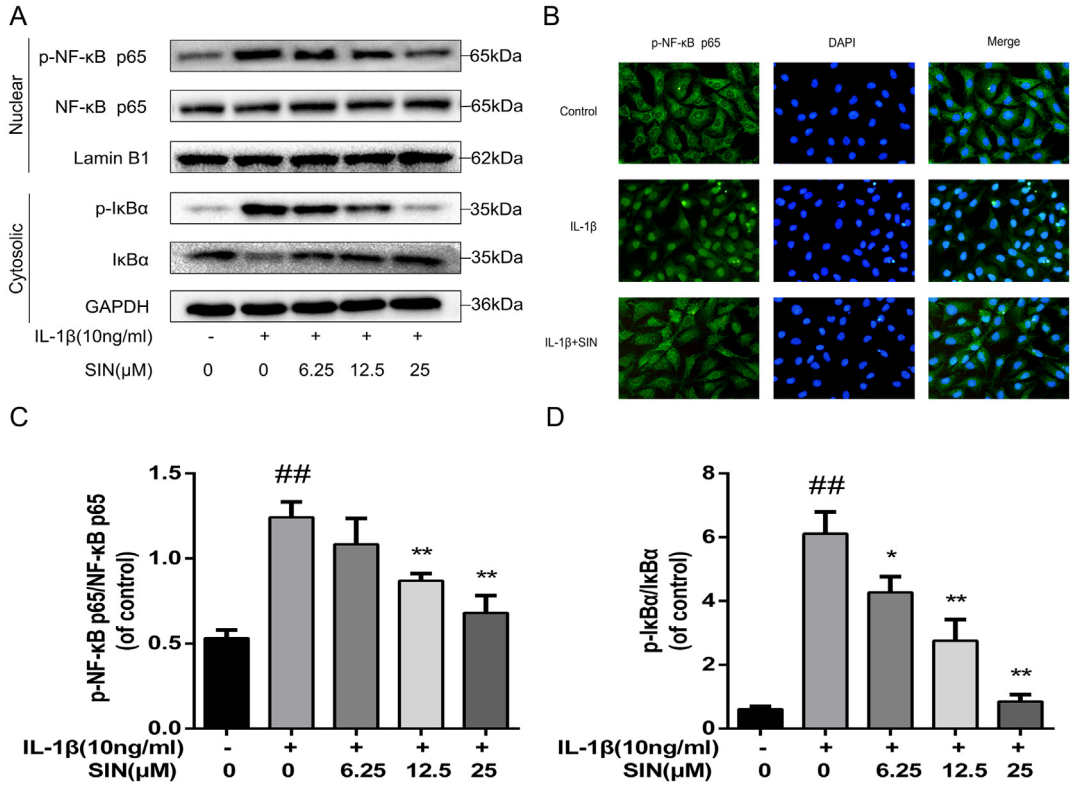

Fig. 4. Effects of SIN on IL-1β-induced NF-κB activation. The expression levels of p-NF-κB p65 in the nuclei and p-IκBα in the cytoplasm of chondrocytes were determined by western blot and (C,D) quantified using ImageJ (B) After IL-1β treatment (10 ng/ml) with or without SIN (25 μM) for 24 h, the expression of p-NF-κB p65 was observed by immunofluorescence (scale bar: 10 μm). The data in the figures indicate the averages ± S.D. Statistically significant differences between groups were shown as follows: $^{\#\#}P < 0.01$, vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. IL-1β alone treatment group, n = 5.

chondrocyte apoptosis during OA, but its mechanism of action needs to be further studied [8]. In this study, the anti-inflammatory effects of SIN on IL-1β-induced inflammatory response were confirmed both in vitro and in vivo.

According to our study, we concluded that SIN could reverse the imbalance of IL-1 β-induced inflammation and ECM metabolism by activating the Nrf2/HO-1 pathway, and blocking the NF-κB pathway as shown in (Fig. 7). The regulation of NF-κB by SIN was achieved by activating Nrf2. Our study also showed that SIN alleviated the progress of OA in a mouse DMM model.

Nitric oxide (NO) has long been thought to be a breakdown factor that promotes inflammation and osteoarthritis pathology [49]. The over-expression of inducible nitric oxide synthase (INOS) is due to the stimuli of proinflammatory cytokines, including tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) [50]. It has been shown that NO can not only increase the expression of active matrix metalloproteinase (MMPs), but can also inhibit the synthesis of matrix protein, which can induce chondrocyte apoptosis under certain conditions [51]. COX-2 participates in the inflammatory response by promoting the synthesis of prostaglandin E2 (PGE2) and COX-2 expression not only affects normal cell function, but also promotes the imbalance of proteoglycan metabolism induced by inflammatory cytokines and thus, promotes the occurrence of arthritis [52]. These inflammatory cytokines play an important role in the development of OA. In this study, we found that SIN significantly inhibited the oversecretion of NO, PGE-2, IL-6, and TNF-α stimulated by IL-1β and down-regulated the expression of iNOS and COX-2. These results showed that SIN can effectively down-regulate inflammatory cytokines and alleviate the development of OA.

It has been reported that NF-κB signaling is involved in the pathophysiological process of OA and is activated by the inflammatory process of OA [53]. NF-κB is the upstream regulatory factor of cytokines such as TNF-α and IL-1β. As a target of OA, NF-κB binds to IκBα. The main function of IκB protein is to mask the nuclear localization signal of NF-κB, block the nuclear transport of NF-κB p65, and make NF- κB exist in the cytoplasm of cells in an inactive form. When cells are stimulated by various intracellular and extracellular stimuli, IκB kinase was activated, resulting in the degradation of IκBα and the transport of NF-κB p65 to the nucleus [54]. The NF-κB pathway induces a variety of inflammatory-related factors, including MMP proteins, inducible nitric oxide synthase (INOS), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α). IL-1β and TNF-α play an important role in cartilage degradation, they cause a series of events and further activate signal cascades [55]. NO is a core inflammatory medium, which not only activates MMPs and ADAMTS 5 which promote the degradation of ECM, but also reduces the production of IL-1 receptor antagonist (IL-RA), and mediates chondrocyte apoptosis [56]. In addition to inducing a variety of inflammatory-related factors, NF-κB is also involved in the degradation of the extracellular matrix (ECM). NF-κB mediates the chondrocyte activation triggered by fibronectin fragments, which contributes to the increased expression of MMPs and ADAMTS-5, resulting in ECM damage and cartilage erosion [57]. Type II collagen and aggrecan, the two most abundant components in the ECM, are degraded by MMP-13 cleaving the helical protein [58]. ADAMTS-5 is a zinc-metalloproteinase with thrombin motif and it has been shown that it degrades aggrecan which reduces the integrity of cartilage [59]. In this study, we found that SIN inhibited the excessive secretion of inflammatory factors such as NO, PGE-2, IL-6 and TNF-α using Western blot. Nuclear translocation of NF-κB p65 protein was detected by immunofluorescence and the results showed that SIN could inhibit the nuclear translocation of p-NF-κB p65. These results suggest that SIN may play an anti-inflammatory role by inhibiting the activation of NF-κB signaling pathway, which was consistent with studies of the anti-inflammatory mechanism of SIN in chondrocytes by Yu-Ren Zhou [61] and Zhou Lan [60].

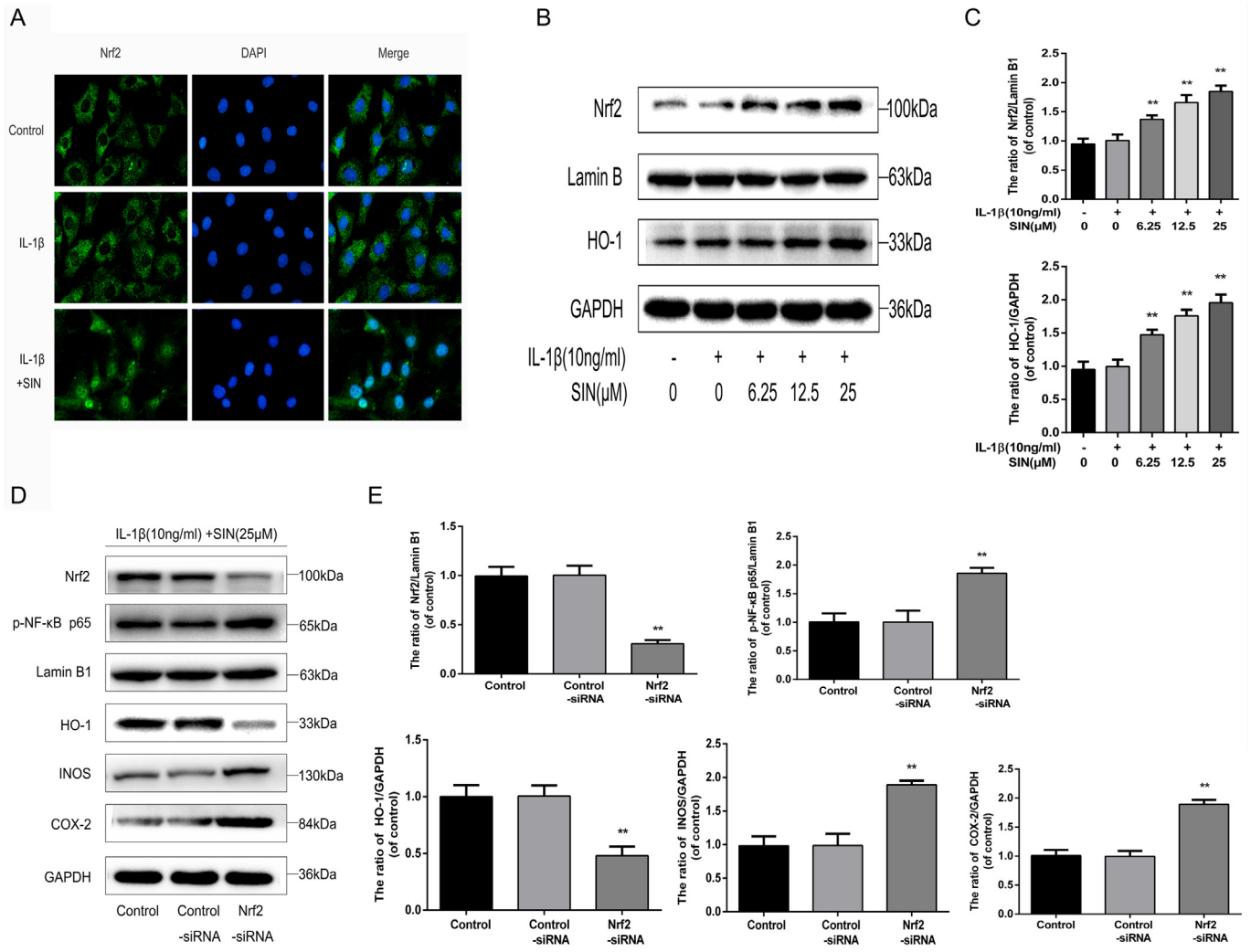

Fig. 5. Nrf2 knockdown reverses the protective effect of SIN. (A) Nrf2 nuclear translocation was detected by immunofluorescence (original magnification ×400, scale bar: 20 μm). The expression levels of Nrf2 in the nuclei and HO-1 in the cytoplasm of the chondrocytes were determined by western blot (B) and quantified (C) using ImageJ. After Nrf2 knock down, western blot was used to detect (D) and quantify (E) expression levels of NRF2 and p-NF-κB p65 in the nuclei and of HO-1, iNOS, and COX-2 in the cytoplasm. The data in the figures indicate the averages ± S.D. Statistically significant differences between groups were shown as follows: $^{\#\#}P < 0.01$, vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. IL-1β alone treatment group, n = 5.

The Nrf2-ARE signaling pathway is the main bearer of antioxidant stress and protects the organism from various pathological changes caused by oxidative stress. It was reported that activation of Nrf2 not only inhibited the secretion of inflammatory cytokines (TNF-α, IL-1β, COX-2, and iNOS), but also decreased the expression of MMPs [62]. Studies on Sulforaphane have shown that its anti-inflammatory effect is mainly achieved through activating Nrf2 to inhibit the expression of TNF-α, IL-1β, cox-2, and iNOS [63]. In addition, the Nrf2/HO-1 signaling pathway can significantly inhibit the expression and activity of MMPs in OA, which is beneficial to the treatment of OA [64,65].

In this study, to investigate whether sinomenine-mediated cartilage protection depends on Nrf2, we transfected chondrocytes with Nrf2-siRNA to reduce Nrf2 levels. The results showed that Nrf2-siRNA significantly upregulated the expression of COX-2 and iNOS, similar to the findings of Khor et al. [66]. Antioxidant response element (ARE) is an DNA-promoter binding sequence located in the upstream regulatory region of some protective genes, and Nrf2 is an activator of this sequence. When Nrf2 was activated in vivo and transferred into the nucleus, it could bind to ARE to form a Nrf2-ARE signaling pathway and thus, regulate the expression of downstream genes [68]. Nrf2 could induce the expression of HO-1 gene, which has been widely used in vivo and in vitro. For instance, many studies have shown that HO-1 and its metabolites have anti-inflammatory effects which are induced by Nrf2 and that the final metabolites of HO-1 can inhibit the nuclear translocation of NF-κB [67]. Previous studies have shown that SIN plays a protective role in kidney damage by activating Nrf2 upstream to inhibit NF-κB signaling [69]. These in vivo studies showed that Nrf2 plays a negative role in the regulation of the NF-κB signaling pathway. To further explore the anti-inflammatory mechanism of SIN in OA we investigated the Nrf2/HO-1 pathway. Our data showed that SIN significantly upregulated the expression of HO-1 and Nrf2 at the protein level, but this was reversed when Nrf2-siRNA was transfected into chondrocytes and it was also shown that Nrf2-siRNA promoted p-NF-κB p65 expression. In addition to this, immunofluorescence results showed that SIN enhanced the nuclear translocation of Nrf2. Taken together, these observations show that SIN plays a protective role against OA in mice by activating the Nrf2/HO-1 signaling pathway and indirectly inhibiting the NF-κB pathway. Previous studies have reported that SIN protects against cartilage degradation and chondrocyte apoptosis by increasing TIMP-1 activity and decreasing MMP-13 activity [70] and some scholars believe that SIN further prevents the development of OA by inhibiting miR-223-3p/NLRP3 inflammasome signaling [38]. One of

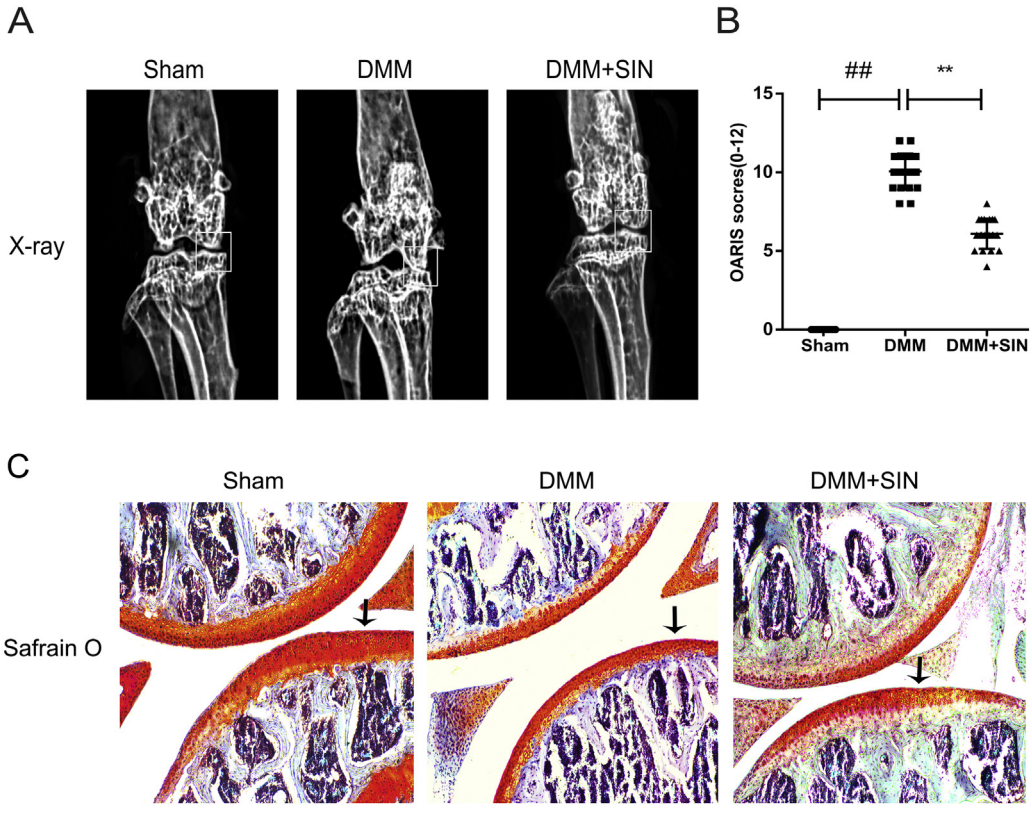

Fig. 6. SIN relieves OA development in a surgically induced mouse DMM model. (A) Digital X-ray images of the degree of degeneration of mouse knee joint samples of the three experimental groups. (B) The OARSI scores of the sham, DMM and DMM + SIN groups. The data in the figures indicate the averages ± S.D. Statistically significant differences between groups were shown as follows: $^{\#\#}P < 0.01$, vs. sham group; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. DMM group, $n = 20$. (C) Representative S–O staining of cartilage from three groups (scale bar: 200 μm).

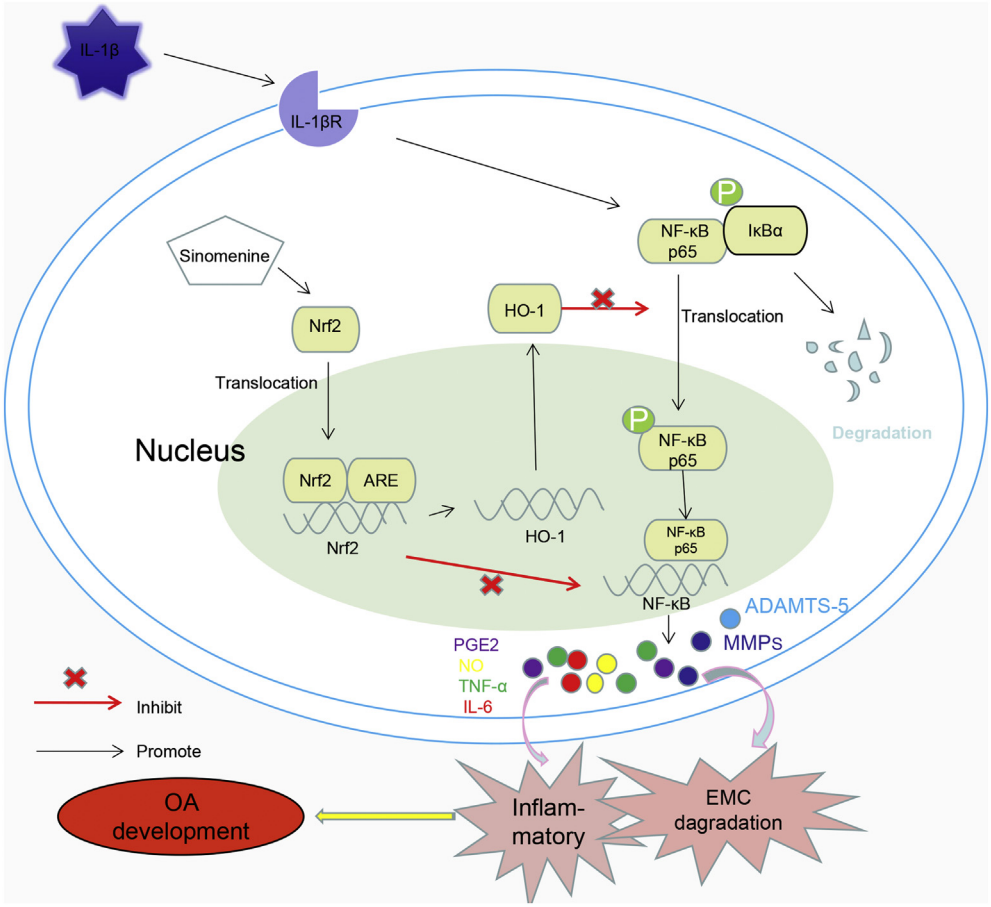

Fig. 7. Molecular mechanism of sinomenine on IL-1β-induced Mouse chondrocytes.

the disadvantages of our study is the failure to determine which pathway plays a more important role in protecting against cartilage degradation and inhibiting the inflammatory reaction. Therefore, future research will be directed towards revealing the potential mechanisms of action of these signaling pathways on the progression of OA.

The surgery induced mouse model of DMM is widely used in in vivo studies of OA, and mainly presented pathological changes such as joint space stenosis, cartilage surface calcification, hyperosteogeny, and cartilage erosion. The treatment of SIN could reverse the development of OA and protect against the degradation of cartilage and ECM, as shown by X-ray and histological section staining analysis, and reduce the OARSI score of DMM mice. The in vitro and in vivo results were consistent, suggesting that SIN has a protective effect against the progression of OA.

5. Conclusion

In conclusion, our study explored the role of SIN in alleviating the development of OA by activating the Nrf2/HO-1 signaling pathway and blocking the activity of NF-κB in mouse cartilage cells. Our findings contribute to a new appreciation of SIN and its use in the treatment of OA and provide a new research direction for the development of future OA therapies.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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