Blocking of the $P2X_7$ receptor inhibits the activation of the MMP-13 and NF-κB pathways in the cartilage tissue of rats with osteoarthritis

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Abstract. P2X purinoceptor 7 ($P2X_7$) receptor ($P2X_7R$) is known to play a significant role in inflammation and pain-causing diseases, including osteoarthritis (OA). However, the mechanisms of action of $P2X_7R$ and its role in OA remain unclear. The articular cartilage is the crucial region in which pathological changes occur in OA, involving the dysregulation of degradation and maintenance mechanisms. In this study, we aimed to reveal the molecular mechanisms of action of $P2X_7R$ in articular cartilage in OA-induced pain and inflammation by using AZD9056, an antagonist of $P2X_7R$. We created an animal model of OA by using Wistar rats administered (by intra-articular injection) monosodium iodoacetate (MIA), and the rats with OA were then treated with the $P2X_7R$ antagonist, AZD9056. We found that treatment with AZD9056 exerted pain-relieving and anti-inflammatory effects. Importantly, we found that the upregulated expression of interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), matrix metalloproteinase-13 (MMP-13), substance P (SP) and prostaglandin E_2 (PGE_2) which was induced by MIA in cartilage tissues was reversed by AZD9056. Western blot analysis was used to examine the expression of inhibitor of nuclear factor-κB (NF-κB) kinase (IKK)α, IKKβ, inhibitor of NF-κB (IκB)α, NF-κB p65 and their phosphorylation forms; they were found to be significantly increased in the knee cartilage tissues from rats with OA; however, opposite effects were observed by the injection of AZD9056. These results implied that $P2X_7R$ was associated with the activation of the NF-κB pathway in the development of OA. Our results also revealed that helenalin, an NF-κB pathway inhibitor, decreased the expression of $P2X_7R$, IL-1β, IL-6, TNF-α, SP, PGE_2 and MMP-13, which was induced by MIA, in the knee cartilage tissues of rats with OA. On the whole, our findings suggest that $P2X_7R$ regulates the MMP-13 and NF-κB pathways in cartilage tissue and mediate OA-induced pain and inflammation.

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Introduction

A number of factors have been implicated in the development of osteoarthritis (OA), such as age, obesity, trauma and genetics. However, the pathogenesis of OA remains poorly understood. Mild OA frequently manifests with symptoms of inflammation, severe or chronic pain, edema and stiffness. In severe cases, patients are unable to move due to joint deformity. Idiopathic and secondary OA causes great suffering and severely affects the quality of life of patients (1). The study of OA has led to the realization that the pathological changes occurring in OA mainly include the degeneration of articular cartilage, subchondral bone destruction and synovial tissue hyperplasia.

Arthritis is usually characterized by dysfunctions in cytokines, immune factors and neurotransmitters (2). Cytokines, such as interleukin (IL)-1β are key inducers of OA, which involves the excessive production of catabolic enzymes and leads to the metabolic imbalance of chondrocytes. These catabolic enzymes abnormally destroy the matrix and physiological function of articular cartilage (3-5). It has been shown that that chondrocytes obtained from patients with OA actively produce prostaglandins (PGs), tumor necrosis factor-α (TNF-α), IL-1β and IL-6 (6). The synovial membrane also produces inflammatory cytokines, which diffuse into the cartilage and manifest structural alterations associated with the development and progression of OA (7). In joint inflammation, prostaglandin E_2 (PGE_2), one of the PG subtypes, degenerates cartilage tissue via matrix metalloproteinases (MMPs) (8); MMP-13 is specifically expressed in cartilage tissues from patients with OA (9). Additionally, substance P (SP), an important neuropeptide, plays an important role in the regulation of arthritis-induced pain and inflammation (10).

At present, the treatment of OA includes pain control, treatment with anti-inflammtory agents and the re-establishment of joint mobility. Non-steroidal anti-inflammatory

drugs (NSAIDs) are widely used in the treatment of OA. However, researchers have found that NSAIDs often lead to a greater risk of complications. Other medications, such as glucosamine hydrochloride, chondroitin sulfate and hyaluronic acid, are able to increase joint lubrication and joint activity, although their effects are not convincing. The exploration of potential analgesic and anti-inflammatory drugs has gradually emerged as a new focus of research in OA.

As is well known, the purinergic membrane receptor superfamily may be divided into the P1 and P2 receptor families. The P2 family, which can be further divided into the P2X and P2Y subtypes, is closely related to OA-induced pain (11,12). Importantly, a previous study reported that P2 receptor signaling is altered in OA, and that it is associated with an elevated ATP level (12). P2X ($P2X_{1-8}$) are ligand-gated ion channel receptors, whereas P2Y are G-protein coupled receptors. The expression of several P2X members has been identified in cartilage tissues, including $P2X_2$, $P2X_3$, $P2X_4$ and $P2X_7$ (13,14). P2X receptors have also been identified in peripheral glial cells and are known to play a role in neuropathic pain. It has been demonstrated that $P2X_7$ receptor ($P2X_7R$) is involved in regulating inflammation and pain (15). Moreover, $P2X_7R$ upregulation in macrophages and $P2X_7R$ upregulation in spinal cord following peripheral nerve damage has been reported (16,17). Pain sensitivity has also been linked to $P2X_7R$ gene polymorphisms in women with post-mastectomy pain syndrome (PMPS) and OA (11,18,19). Evidence suggests that $P2X_7R$ is an important therapeutic target in the treatment of rheumatoid arthritis (RA) (20,21). Although accumulating evidence has indicated that $P2X_7R$ may be closely associated with pain in OA, the effects of $P2X_7R$ and the underlying mechanisms involved in the development and progression of OA and the damage to cartilage tissue, however, remain unclear.

The administration of monosodium iodoacetate (MIA) leads to some OA-related pathological alterations, including the degeneration and necrosis of chondrocytes and the damage to cartilage tissue (22). Beyreuther *et al* demonstrated that OA was induced in the knee joints of rats upon MIA injection for 5 days (23). In our study, an animal model of OA was established by administering MIA to Wistar rats. In addition, we examined the effects of AZD9056, a $P2X_7R$ antagonist, on the cartilage tissue of rats with OA.

Our study demonstrates the role of $P2X_7R$ in MIA-induced OA. Our results demonstrate that the inhibition of $P2X_7R$ exerts protective effects against OA, and our finidngs may provide the basis of a promising targeted therapeutic approach for the treatment of articular cartilage degradation-related diseases, such as OA.

Materials and methods

Antibodies and reagents. All materials were purchased from Gibco (Rockville, MD, USA), unless otherwise stated. MIA, AZD9056 (a $P2X_7R$ selective antagonist) and helenalin [a nuclear factor-κB (NF-κB) signaling pathway inhibitor] were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following antibodies were used: rabbit anti-P2X7R (ab48871), anti-MMP-13 (ab39012), anti-SP (ab67006), anti-PGE_2 (ab2318), anti-IL-1β (ab9787), anti-IL-6 (ab6672), anti-TNF-α (ab9635), anti-inhibitor of NF-κB kinase (IKK)α (ab4111), anti-phosphorylated (p)-IKKα (ab38515), anti-IKKβ (ab124957), anti-p-IKKβ (ab59195), anti-NF-κB p65 (ab16502) and anti-p-NF-κB p65 (ab86299) antibodies were purchased from Abcam (Cambridge, UK); rabbit anti-inhibitor of NF-κB (IκB)α (sc-371) and anti-p-IκBα (sc-101713) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, G9545) antibodies were obtained from Sigma. HRP-conjugated goat anti-rabbit antibodies (ab97051) were purchased from Abcam.

Animals. Male Wistar rats (9-14 weeks old, weighing 293-411 g; Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China, n=93) were used. Each rat was housed alone in a plastic box under a 12-hour light dark cycle (light from 06:00 a.m to 6:00 p.m). Humidity (55±10%) and room temperature (19-23˚C) were governed to be constant. Food and water were available *ad libitum*. All rats were handled for several 10-min sessions daily for 2 weeks so that they could adjust to the testing environment. All animal experiments were conducted according to the Committee of the Chinese Academy of Sciences, and the methods complied with the code of conduct for conscious animal pain published by the International Association for the Study of Pain (IASP). The protocol of the experiments was approved by the local ethics committee of Weinan Central Hospital (approval number no. WNZXHLAC019).

Induction of OA and general grouping of animals. The rats were anesthetized with isoflurane (2% in O_2) and received a single intra-articular injection of MIA (5 mg/kg), as previously described (24) in sterile 0.9% saline (MIA, n=30; 18 rats for behavioral tests, 3 rats for western blot analysis on day 7, 3 rats for western blot analysis on day 14, 3 rats for ELISA, 3 rats for RT-qRCR on day 7; after the behavioral tests on day 21, the 18 rats were also sacrificed and used for western blot analysis and ELISA). MIA solution was administered through the infrapatellar ligament of the left hind knee using a 26G needle. Non-osteoarthritic rats, including a blank control group (control, n=15; 9 rats for behavioral tests, 3 rats for ELISA, 3 rats for RT-qPCR; after the behavioral tests, the 9 rats were also sacrificed and used for western blot analysis and ELISA) and a solvent group (vehicle, n=18; 9 rats for behavioral tests, 3 rats for western blot analysis on at day 7, 3 rats for western blot analysis on day 14; 3 rats for ELISA; after the behavioral tests on day 21, the 9 rats were sacrificed and used for western blot analysis and ELISA), were also employed. In the present study, we sought to examine the role of P2X7R in mediating the development and progression of OA. The other treatment groups were as follows: the AZD9056 (12.5 mg/kg) [as previously described (20,25)] treatment group (MIA + AZD; AZD9056 was injected every 2 days for 7 days at 2 weeks after MIA injection, n=24, 18 rats for behavioral tests, 3 rats for western blot analysis on day 14, 3 rats for ELISA; after the behavioral tests on day 21, the 18 rats were sacrificed and used for western blot analysis and ELISA), and helenalin (0.1 mg/kg) [as previously described (26)] treatment group (MIA + HEL; helenalin was injected every 2 days for 7 days at 2 weeks after MIA injection, n=6, 3 rats for western blot analysis on day 14 and 3 rats for western blot analysis on day 21). Testing was done by a researcher blinded to the randomized treatments.

Table I. Sequences of primers used for RT-qPCR.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
$P2X_1R$	AGG CTC AGG CTG TCA TTG TC	GTG GCA CAA GAA CAC ACG AC
$P2X_2R$	TTC GAC AAG GTG CGT ACT CC	GTC CCA TAT GCT GGC CAA GT
$P2X_3R$	GAA GGG TAC TGC GTC AAC CA	AGC GCC TAA CCA TGG CTT TC
$P2X_4R$	GTG GCG GAC TAT GTG ATT CCA	TGA CAG ACG CAG TAG CCA TC
$P2X_5R$	CCG TGA CCT GAT GAA AGC CT	CAT CTC GTT GGC CTC AAC CT
$P2X_6R$	ATG TGG CTG ACT TCG TGA GG	GAG CAG TCA GAG CCT TTC GT
$P2X_7R$	AAC AGC CAA TGA GTC CGA GG	TAG GGA CGG CTC AGT GGT TA
$P2X_8R$	CAT GCC ATG GGG CAG GTG TCC TGG AAG	GGC TTG GAT CTT TTC TCC AC
GAPDH	AGG AGC AAT GAT CTT GAT CTT	TGC CAA CAC AGT GCT GTC T

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Reverse transcription-quantitative PCR (RT-qPCR). The rats used for RT-qPCR were sacrificed by spinal dislocation and the skin of the limbs was disinfected. Under aseptic conditions, the femur was intercepted 5 cm above the femoral condyle, the tibia was cut out under 3 mm of the tibial plateau, and the knee joint was carefully removed. The surrounding soft tissue was removed and the aponeuroses on both sides of the patella were cut open, and the knee joint was openened. A no. 15 conventional scalpel blade was used to cut off the cartilage on the articular surface.

Total RNA from the rat left knee joint cartilage was extracted using Unizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Approximately 5 μg of RNA from each sample was used as a template for cDNA synthesis with a reverse transcription kit (Fermentas, St. Leon-Rot, Germany). Subsequently, SYBR-Green Master mix (Life Technologies, Carlsbad, CA, USA) was used for the quantitative analysis of gene expression. Amplification involved a denaturation step (95˚C for 5 min, 1 cycle), and amplification and quantification were repeated for 40 cycles (95˚C for 5 sec and 60˚C for 1 min, respectively). The primers used for amplification are listed in Table I. The data of the relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and are presented as the fold change of transcripts for genes. The sample mean value was calculated and expressed as the cycle threshold (Ct). mRNA expression was calculated as the difference (ΔCt) between the Ct value of the target gene and the Ct value of the inner control. $2^{-\Delta\Delta Ct}$ means the fold change in the target gene expression, as previously described (27,28). GAPDH was used as internal control for normalization in RT-qPCR.

Behavioral assessments. On the one hand, pain-related behaviors induced by MIA were tested at different time points; the test for the hindlimb weight-bearing asymmetry and paw withdrawal thresholds were evaluated at 3, 7, 10, 14, 15, 17, 19 and 21 days after the injection of MIA or the vehicle, as previously described (24). On the other hand, the intra-articular injection of AZD9056 was administered at 14, 16, 18 and 20 days after the injection of MIA. Subsequently, in the presence of AZD9056, the test for the hindlimb weight-bearing asymmetry was evaluated at 15, 17, 19 and 21 days and paw withdrawal thresholds were evaluated at 14, 15, 17, 19 and 21 days after the injection of MIA. Weight-bearing asymmetry was presented as the percentage in the weight distribution of the left and right hindlimb, as previously described (29). Baseline levels were measured immediately prior to the intra-articular injection.

Specifically, for the test of paw withdrawal threshold, 30 min before the test, the rats were placed in a transparent organic glass box (20x20x30 cm^3) on steel wire mesh (1x1 cm^2). Paw withdrawal threshold was tested by using Von Frey monofilaments (58011, Stoelting Co., USA) (from 0.6 to 26 g). When the bilateral hind limbs came into contact with the steel wire mesh and the rat was settled, the paw withdrawal threshold test was performed. Von Frey monofilaments were applied, in ascending order of bending force (the maximum value is 26 g). Von Frey monofilaments contact and push the plantar surface of the left hind paw; the monofilament is bent at an appropriate degree and is held for 2-3 sec. The lowest weight (g) of monofilament that elicited a withdrawal reflex was recorded as the paw withdrawal threshold.

For the test of hind paw weight-bearing asymmetry, LE7900 - Incapacitance Tester (NatureGene Corp., Medford, NJ, USA) was used. The rats were kept in an upright position while the hind paws rested on the separate small electronic balance of the Incapacitance Tester so that the weight distributed on the right and left hind paws could be measured. Once the rat was settled, three consecutive readings (each measured over 3 seconds) were recorded. The average of a total of 3 readings was determined for each hind limb for each rat and used for subsequent analyses. Weight of test hindlimb (%) = [readings of right weight-bearing (no-injection side) - readings of left weight-bearing (injection side)]/[the readings of total weight-bearing of both hind limbs]x100.

Evaluation of knee edema size. The knee edema sizes at 3, 7, 10, 14, 15, 17, 19 and 21 days after the injection of MIA were assessed in randomly selected rats. The AZD9056-treated rats were evaluated at 14, 15, 17, 19 and 21 days after the injection of MIA. Knee diameter was measured using calibrated digital calipers, and differences in the diameter between the right and left knees were determined by the value of the left (MIA-injected) side minus the value of the right side, as previously described (30).

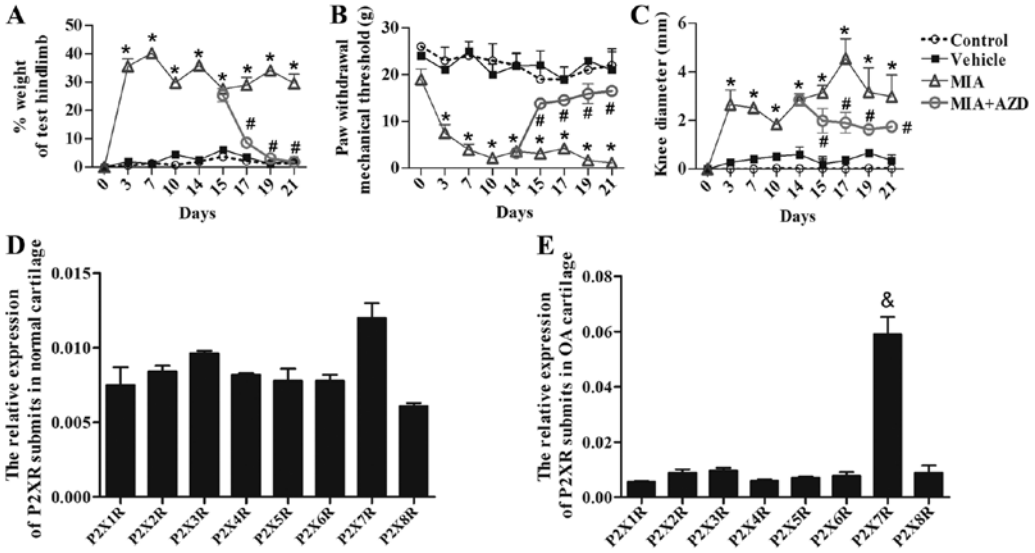

Figure 1. Evaluation of the rat model of monosodium iodoacetate (MIA)-induced osteoarthritis (OA) and the expression of P2XR subunits in knee articular tissues. The MIA injection led to a continuous increase in pain behavior. (A) The weight-bearing asymmetry was increased by thye MIA injection, but decreased by the AZD9056 injection. (B) The paw withdrawal thresholds were downregulated by MIA, but these effects were reversed by AZD9056. (C) The knee edema size was reduced by AZD9056 injection compared with the MIA group. n=6 in each group of animal experiments. $^*p<0.05$, MIA vs. control; $^\#p<0.05$, MIA + AZD vs. MIA. (D and E) Results of RT-qPCR for the expression of P2X$_1$R-P2X$_8$R in (D) normal cartilage and (E) in OA-affected cartilaged on the 7th day after the MIA injection. The expression of P2X$_7$R was markedly increased in OA-affected cartilage ($^\&p<0.05$). n=3 in each group.

Measurement of cytokine levels. The rats were anaesthetized with 1% mebumal sodium (Sigma-Aldrich). Blood samples were obtained from the carotid artery and centrifuged at 3,500 x g for 15 min. The supernatant was then collected and stored at -80˚C for the analysis of serum cytokine levels. The levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) were analyzed using enzyme-linked immunosorbent assay (ELISA) kits (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) according to the manufacturer's instructions.

Western blot analysis. The protein expression levels of P2X$_7$R, MMP-13, SP, PGE$_2$, IL-1β, IL-6 and TNF-α were detected in the knee joint cartilage tissues of rats with OA by western blot analysis. The expression levels of IKKα, IKKβ, IκBα, NF-κB p65 and their corresponding phosphorylated forms was also detected. Briefly, the minced, homogenized and frozen left side cartilage tissues in each group were triturated in lysis buffer. The debris were removed by centrifugation at 12,000 x g for 5 min at 4˚C. The total protein concentration in the supernatant was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology, Nanjing, China). A total of 20-30 μg of protein was loaded on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then electroblotted onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked by 3% non-fat milk for 1.5 h at 37˚C, and then incubated with primary antibodies against P2X$_7$R (1:800), MMP-13 (1:1,000), SP (1:1,200), PGE$_2$ (1:1,000), IL-1β (1:500), IL-6 (1:500), TNF-α (1:500), IKKα (1:600), p-IKKα (1:500), IKKβ (1:1,000), p-IKKβ (1:400), IκBα (1:800), p-IκBα (1:500), NF-κBp65 (1:600), p-NF-κBp65 (1:500) and GAPDH (1:2,000) at 4˚C overnight. Following incubation with HRP-conjugated secondary antibody for 1.5 h at room temperature, protein bands were visualized using an enhanced chemiluminescence detection system. Densitometry values were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA), which were then normalized to GAPDH.

Statistical analysis. The data are presented as the means ± standard deviation (SD). Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Bonferroni tests for multiple groups, or Student's t-tests for differences between 2 groups using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Differences with a P-value <0.05 were regarded as statistically significant.

Results

Induction of OA by an intra-articular injection of MIA. We first investigated whether the model of OA model was successfully established. The weight-bearing asymmetry and paw withdrawal thresholds were evaluated. As shown in Fig. 1A and B, the two indexes remained unaltered in the vehicle group; however, in the rats administered MIA, the weight-bearing asymmetry remained >27.6 % of the high level from days 3 to 21 (Fig. 1A; $p<0.05$), and the paw withdrawal thresholds were markedly reduced compared with those of the control and vehicle group rats from days 3 to 21 (Fig. 1B; $p<0.05$). In addition, notable increases in knee edema size were observed from days 3 to 21 after the injection of MIA (Fig. 1C; $p<0.05$).

P2X$_7$R expression is elevated in rats with OA. To determine whether P2X$_7$R is involved in regulating OA development, we analyzed the expression of P2X$_{1\text{-}8}$R in rat cartilage tissues from rats with OA by RT-qPCR. The results revealed that the mRNA expression of the mRNA expression of P2X$_7$R was slightly higher than that of the other subtypes in normal rats (Fig. 1D). However, in rats with OA, the expression of P2X$_7$R was significantly increased following the injection of

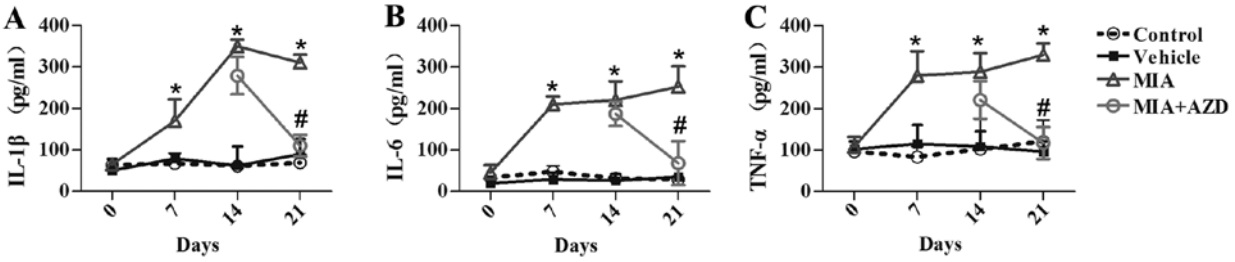

Figure 2. The investigation of cytokines (TNF-α, IL-6 and IL-1β) in serum via enzyme-linked immunosorbent assay (ELISA). The levels of (A) Interleukin (IL)-1β, (B) IL-6 and (C) tumor necrosis factor-α (TNF-α) were increased in the monosodium iodoacetate (MIA) group in comparison with those in the control group; however, AZD9056 reversed these effects. n=3. *p<0.05, MIA vs. control; #p<0.05, MIA + AZD vs. MIA.

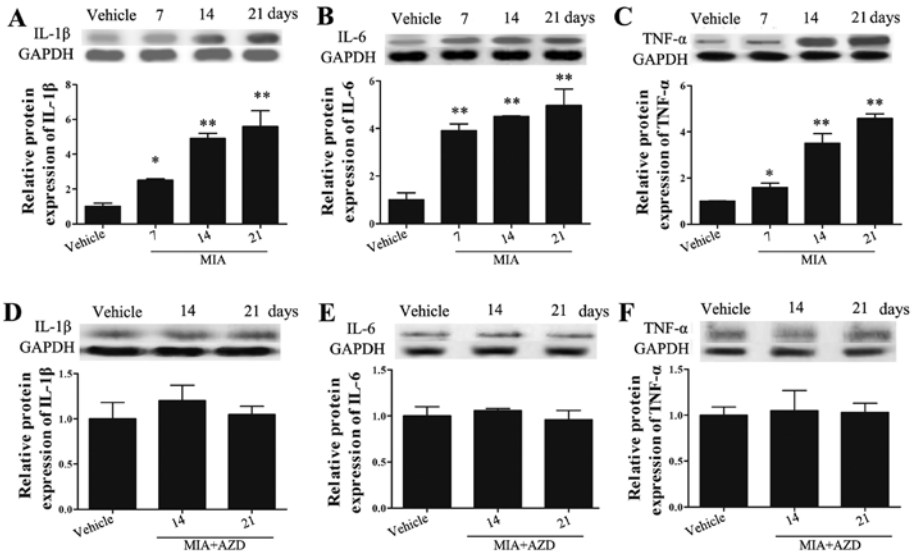

Figure 3. Expression of inflammatory factors in the presence of monosodium iodoacetate (MIA) and AZD9056 injections. The expression of (A) interleukin (IL)-1β, (B) IL-6 and (C) tumor necrosis factor-α (TNF-α) in knee cartilage tissue was notably upregulated in rats with osteoarthritis (OA) in comparison with those in the vehicle group. However, the expression of (D) IL-1β, (E) IL-6 and (F) TNF-α was reversed by the administration of AZD9056 every 2 days for 7 days at 2 weeks after the MIA injection. n=3, *p<0.05 and **p<0.01 vs. vehicle.

MIA (Fig. 1E; p<0.05); however, the expression of other subtypes of P2X receptors was not significantly altered (Fig. 1E). These results suggest that $P2X_7R$ plays an important role in the development of OA.

Effects of P2X7R antagonist on OA-induced pain and inflammation. The $P2X_7R$ antagonist, AZD9056, was then used to further examine the role of $P2X_7R$ in OA. As shown in Fig. 1A, weight-bearing asymmetry was markedly reduced after day 15 by continuous treatment with AZD9056 compared with MIA administration alone (p<0.05). The paw withdrawal thresholds were also almost restored in a time-dependent manner by continuous treatment with AZD9056 (Fig. 1B; p<0.05). Edema is the clinical symptom of tissue inflammation. Treatment with AZD9056 resulted in a statistically significant decrease in knee edema size compared with MIA administration alone (Fig. 1C; p<0.05). These results demonstrated that the $P2X_7R$ antagonist, AZD9056, exerted pain-alleviating and anti-inflammatory effects in our rat model of MIA-induced OA.

Effects of AZD9056 on cytokine levels in serum. The cytokine levels in serum were then evaluated in order to further investigate the anti-inflammatory effects of AZD9056. As shown in Fig. 2, TNF-α, IL-6 and IL-1β were highly expressed in the serum of rats with OA compared with those in the control group (p<0.05). However, the levels of IL-1β, IL-6 and TNF-α were notably decreased following the intra-articular injection of AZD9056 (Fig. 2A-C; p<0.05).

Effects of AZD9056 on cytokine levels in knee joint cartilage tissues from rats with OA. We then examined the expression levels of cytokines in the knee joint cartilage tissues of rats with OA by western blot analysis. As shown in Fig. 3A-C, the levels of IL-1β, IL-6 and TNF-α in the knee joint cartilage tissues of rats with OA were continuously increased compared with those in the rats in the vehicle group (p<0.05). We then wished to examine the effects of AZD9056 on the levels of inflammatory factors in the knee joint cartilage tissues of rats with OA. As shown in Fig. 3D-F, the protein expression levels of IL-1β, IL-6 and TNF-α in the knee joint cartilage tissues following treatment with AZD9056 were reversed and down-regulated (compare values in Fig. 3A-C for MIA with those in Fig. 3D-F MIA + AZD on days 14 and 21). These results suggested that treatment with AZD9056 exerted an inhibitory effect on inflammation.

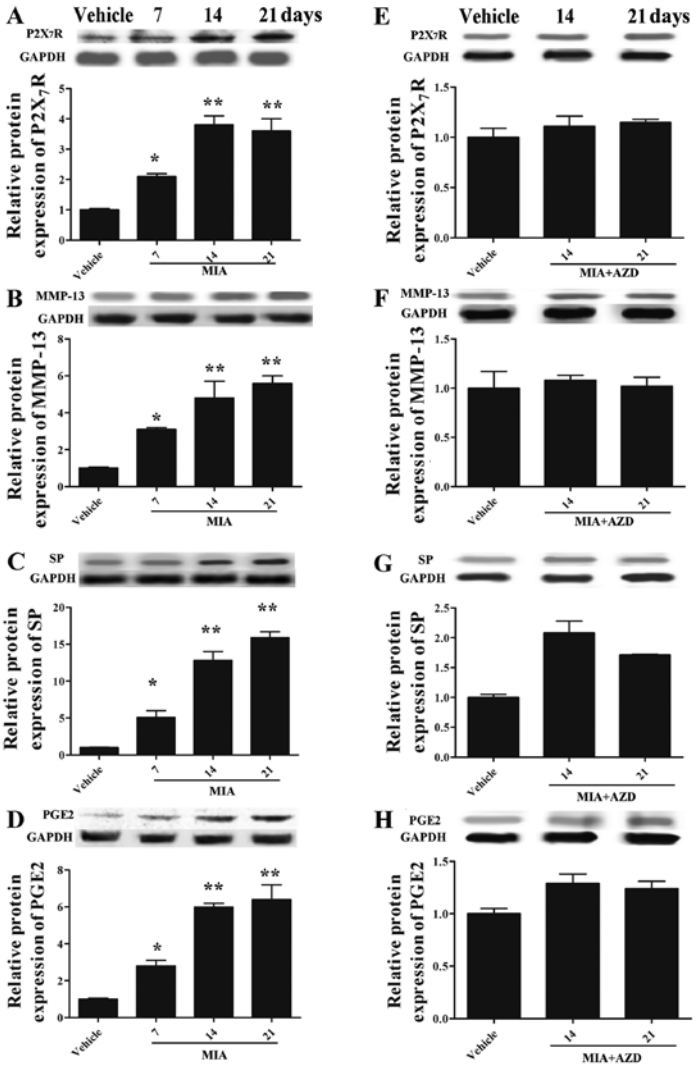

Figure 4. Expression of $P2X_7$ receptor ($P2X_7R$), matrix metalloproteinase-13 (MMP-13), substance P (SP) and prostaglandin E_2 (PGE_2) in the knee articular tissues. (A) The levels of $P2X_7R$, (B) MMP-13, (C) SP and (D) PGE_2 were significantly increased in rats with osteoarthritis (OA); however, the injection of AZD9056 returned the expression of (E) $P2X_7R$, (F) MMP-13, (G) SP and (H) PGE_2 back to a normal levels with no statistically significant compared to the vehicle group. n=3, $^*p<0.05$ and $^{**}p<0.01$ vs. vehicle.

AZD9056 reverses the MIA-induced increase in the expression of P2X$_7$R, MMP-13, SP and PGE$_2$. We examined the expression of $P2X_7R$, MMP-13, SP and PGE_2 in order to evaluate the effects of AZD9056 on OA development. As shown in Fig. 4A-D, MIA markedly increased the expression levels of $P2X_7R$, MMP-13, SP and PGE_2 in the cartilage tissues ($p<0.05$), while this trend was reversed by treatment with AZD9056 (Fig. 4E-H) (compare values in Fig. 4A-D for MIA with those in Fig. 4E-H MIA + AZD on days 14 and 21). These results collectively demonstrated that AZD9056 played an important role in inhibiting OA development, suggesting that $P2X_7R$ is a key regulator of OA.

Administration of MIA activates the NF-κB signaling pathway. To explore whether the NF-κB signaling pathway is involved in regulating the development of OA, we detected the expression of IKKα, IKKβ, IκBα, NF-κB p65, which are the effectors of the NF-κB signaling pathway, and their corresponding phosphorylated forms (Fig. 5). The results of western blot analysis revealed that the levels of these signaling molecules were upregulated in the joint cartilage tissues of rats with OA (Fig. 5A-H; $p<0.05$). Specifically, the expression of IKKα (Fig. 5A and E), IκBα (Fig. 5C and G) and NF-κBp 65 (Fig. 5D and H) was significantly increased on days 7, 14 and 21 after the MIA injection, and that of IKKβ (Fig. 5B and F) was notably upregulated on dayd 14 and 21 ($p<0.05$). Moreover, the phosphorylated forms of IKKα (Fig. 5A and I), IKKβ (Fig. 5B and J), IκBα (Fig. 5C and K) and NF-κB p65 (Fig. 5D and L) were also markedly upregulated in the rats with OA ($p<0.05$). These results suggested that the NF-κB signaling pathway was activated upon MIA stimulation in the model of OA.

AZD9056 inhibits the activation of the NF-κB signaling pathway. To further delineate the role of $P2X_7R$ in mediating the NF-κB pathway, the $P2X_7R$ antagonist, AZD9056, was used. The results of western blot analysis (Fig. 6A-D) revealed that treatment with AZD9056 inhibited the expression of IKKα, IKKβ, IκBα and NF-κB p65 (Fig. 6A-H), as well as that of their corresponding phosphorylated forms (Fig. 6A-D

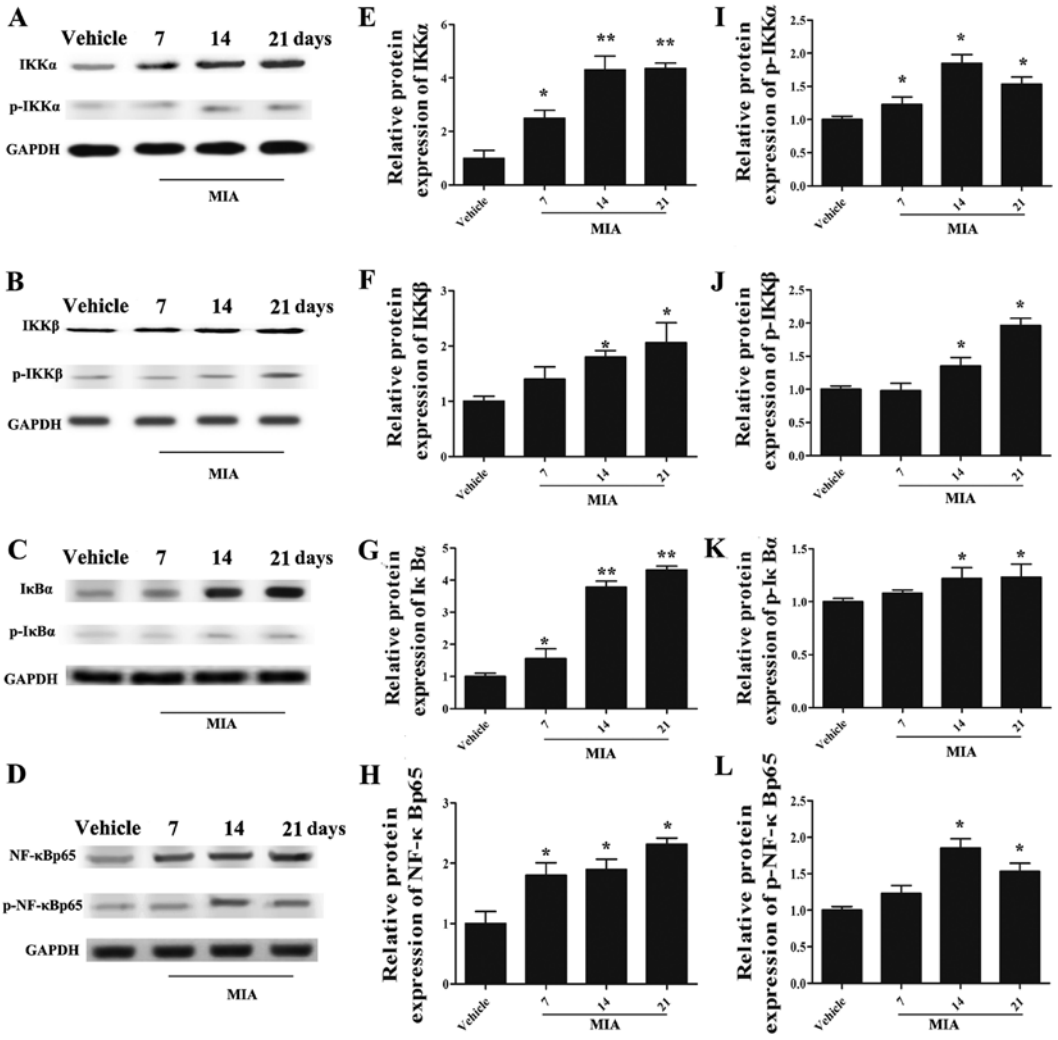

Figure 5. Expression of [inhibitor of nuclear factor-κB (NF-κB) kinase (IKK)α], IKKβ, inhibitor of NF-κB (IκB)α, NF-κBp65, p-IKKα, p-IKKβ, p-IκBα and p-NF-κB p65 in rats with monosodium iodoacetate (MIA)-induced osteoarthritis (OA). The protein levels of IKKα, IKKβ, IκBα, NF-κB p65 and those of their phosphorylation forms were markedly incrased by MIA injection compared with the vehicle group. (A) The protein bands of IKKα and p-IKKα and (E and I) corresponding densitometric analysis, (B) IKKβ and p-IKKβ and (F and J) corresponding densitometric analysis, (C) IκBα and p-IκBα and (G and K) corresponding densitometric analysis, (D) NF-κB p65 and p-NF-κB p65 and (H and L) corresponding densitometric analysis are shown. n=3, *p<0.05 and **p<0.01 vs. vehicle.

and I-L) in the presence of MIA, indicating that AZD9056 suppressed NF-κB signaling (compare values in Fig. 5 for MIA with those in Fig. 6 MIA + AZD on days 14 and 21). Thus, the above results confirm the role of $P2X_7R$ in regulating NF-κB signaling.

Helenalin reverses the MIA-induced expression of $P2X_7R$, MMP-13, SP and PGE_2. To further investigate the role of NF-κB signaling in the development of OA, the NF-κB signaling pathway inhibitor, helenalin, was used to evaluate the expression of $P2X_7R$, MMP-13, SP and PGE_2. As shown in Fig. 7, in the presence of helenalin, the protein expression levels of $P2X_7R$ (Fig. 7A), MMP-13 (Fig. 7B), SP (Fig. 7C) and PGE_2 (Fig. 7D), which had been increased following the MIA injection (Fig. 4A-D) were returned to levels similar to those of the vehicle group on the 21st day (compare values in Fig. 4A-D for MIA with those in Fig. 7 MIA + HEL on day 21). Due to the delayed drug effects of helenalin, the levels of $P2X_7R$, MMP-13, SP and PGE_2 remained high on the 14th day compared to the vehicle ($p<0.05$). These results provide further evidence that the activation of NF-κB signaling participates in OA development, indicating that $P2X_7R$ is a regulator of OA by targeting the NF-κB signaling pathway.

Discussion

In the present study, we discovered the following results: i) the mRNA expression of $P2X_7R$ was slightly higher than that of the other subtypes in normal rats. Correspondingly, the mRNA expression of $P2X_7R$ was markedly increased in the rats with MIA-induced OA; ii) the $P2X_7R$ antagonist, AZD9056, relieved hindlimb weight-bearing asymmetry and increased paw withdrawal thresholds, and also reduced the swelling of the knee joint in the rats with OA; iii) AZD9056 reversed the upregulated expression of IL-β, IL-6 and TNF-α in both serum and the knee articular cartilage tissues of rats

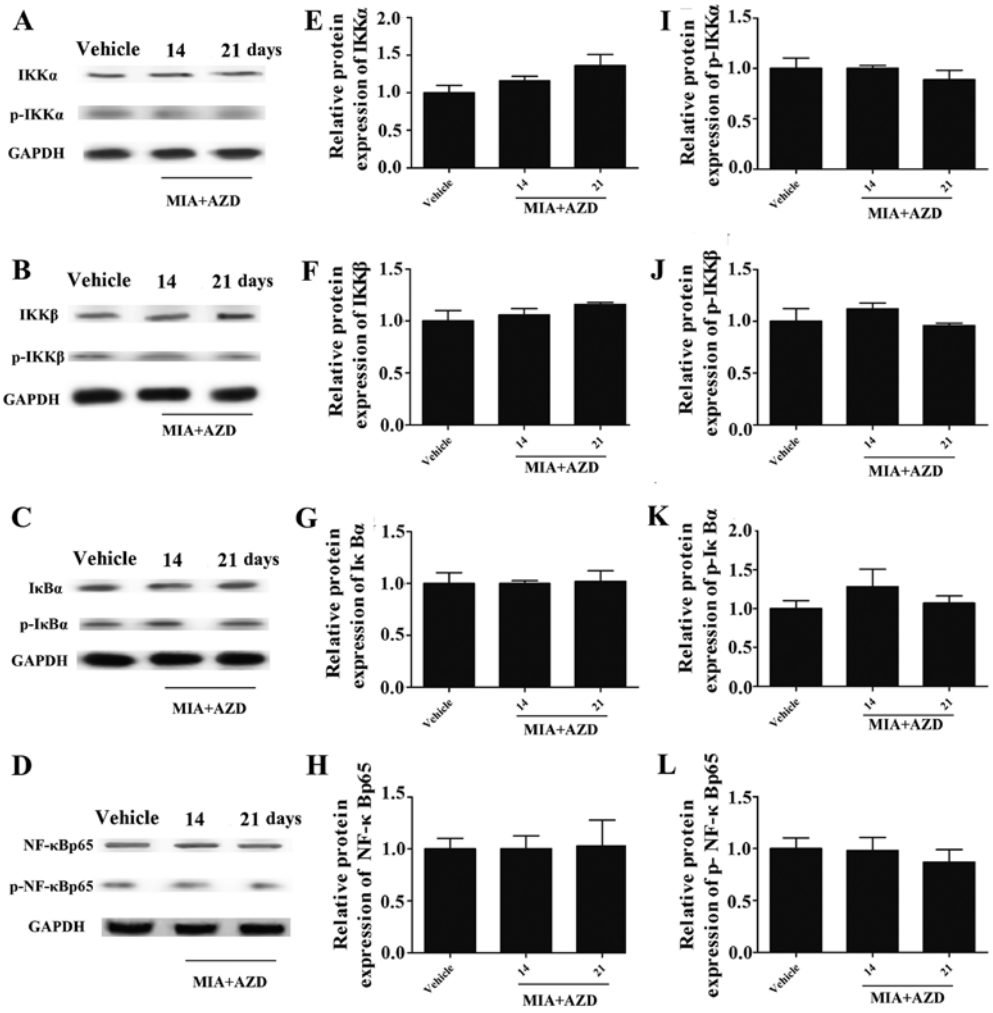

Figure 6. Expression of [inhibitor of nuclear factor-κB (NF-κB) kinase (IKK)α], IKKβ, inhibitor of NF-κB (IκB)α, NF-κBp65, p-IKKα, p-IKKβ, p-IκBα and p-NF-κB p65 in rats with monosodium-iodoacetate (MIA)-induced osteoarthritis (OA) under AZD9056 treatment conditions. (A-D) The protein bands analyzed by western blot analysis. (E) After the AZD9056 injection, the expression of IKKα, (F) IKKβ, (G) IκBα, (H) NF-κBp65 and (I-L) their phosphorylation forms returned to levels similar to those of the vehicle group. n=3.

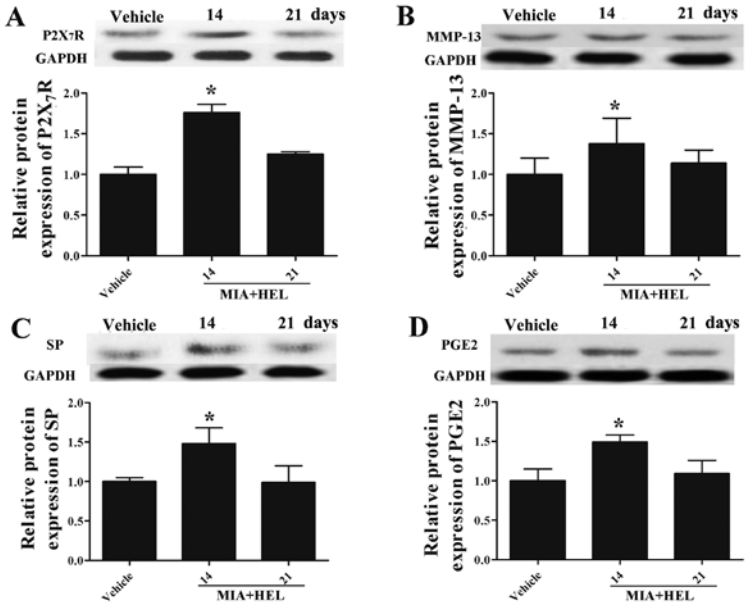

Figure 7. Expression of P2X$_7$ receptor (P2X$_7$R), matrix metalloproteinase-13 (MMP-13), substance P (SP) and prostaglandin E$_2$ (PGE$_2$) in the presence of helenalin. Helenalin was injected every 2 days for 7 days since 2 weeks after the MIA injection. (A) The protein expression of P2X$_7$R, (B) MMP-13, (C) SP and (D) PGE$_2$ were returned levels similar to those of the vehicle group on the 21st day. n=3, *p<0.05 vs. vehicle.

with OA; iv) further experiments indicated that the expression of $P2X_7R$, MMP-13, SP and PGE_2 was increased in the knee joint cartilage tissues of rats with OA, but this trend was significantly reversed by AZD9056; v) the expression of IKKα, IKKβ, IκBα and NF-κBp65 and that of their corresponding phosphorylation forms was significantly increased in the knee cartilage tissues of rats with OA, and AZD9056 reversed these effects; and vi) the NF-κB pathway inhibitor, helenalin, reversed the increased expression of $P2X_7R$, SP, PGE_2 and MMP-13 induced by MIA in rats with OA. Thus, we discovered an important role of $P2X_7R$ in rats with OA, and also found that a $P2X_7R$ antagonist can be used to regulate OA by targeting the NF-κB pathway.

AZD9056, an adamantane amide, is regarded as a selective $P2X_7R$ antagonist. In the present study, AZD9056 was shown to effectively relieve OA-induced pain, as shown by the results of behavioral tests. Moreover, the sustained use of AZD9056 played a role in eliminating edema induced by OA, indicating that AZD9056 had a prominent suppressive effect on inflammation. Similarly, recent evidence from another study indicated that AZD9056 possessed anti-inflammatory and pain-relieving properties (25). It was also indicated that AZD9056 inhibited inflammatory factors, protected the synovial tissue and prevented the degradation of the cartilage matrix in mammals with RA (25). However, OA differs from RA, as RA is an autoimmune disease, but OA is usually caused by the 'wear and tear' of joints. However, there is evidence to suggest that OA is associated with arthritis (11). OA frequently leads to inflammation and the degeneration of articular cartilage, the destruction of subchondral bone and hyperplasia of synovial tissue (31). In addition, it has been reported that chondrocytes have a direct effect in inflammatory pain through the activation of neurons (4,31). It has also been shown that chondrocytes obtained from patients with OA actively produce inflammatory cytokines, such as nitric oxide (NO), PG, IL-1β, TNF-α, IL-6, and IL-8 (6). Moreover, the activation of $P2X_7R$ in mouse mast cells has been shown to increase the expression of IL-4, IL-6, IL-13 and TNF-α (32). Another study demonstrated that the activation of $P2X_7R$ in rat immune cells promoted the release of IL-6, IL-1β and TNF-α (33). However, the blockade of TNF-α (using adalimumab) and the silencing of TNF-α has been shown to lead to a prolonged inhibitory effect on inflammation in animal models (34-36). Our data demonstrated that the increasing trend in the expression of IL-1β, IL-6, and TNF-α in the knee joint cartilage tissue of rats with OA was reversed by the $P2X_7R$ antagonist, AZD9056. These results suggest that $P2X_7R$ plays an important role in the modulation of OA-induced inflammation in cartilage.

Inflammatory cytokines affect a range of ion channels; however, the role of ion channels in OA development remains unclear. However, $P2X_7R$ has been reported to be associated with joint inflammation and OA (11). SP is a neuropeptide which has been reported to exert anti-inflammatory and analgesic effects via its antagonists in OA or RA (10,37,38). Furthermore, PGE_2 has been reported to promote cartilage degeneration by increasing the production and secretion of proteinases, such as MMPs and aggrecanases (8). MMP-13, which is the main expressed MMP in OA-affected cartilage, is specifically expressed in OA-affected cartilage but is not present in normal adult cartilage; during the process of OA, MMP-13 is one of the most effective collagenase II and is considered to be the marker of cartilage degeneration (9,39-41). The activation of MMPs increases the degradation of cartilage matrix and cartilage cell apoptosis, eventually leading to cartilage damage (40,42). In this study, we confirmed the increased expression of MMP-13 in knee joint tissues from rats with OA and this was reversed by AZD9056. The results suggest that AZD9056 may play protective role in cartilage in OA. Our findings strongly suggest that antagonists of $P2X_7R$ have potential for clinical and therapeutic use in the alternative management of OA. However, further studies are required to confirm our results.

In terms of arthritis and relative inflammatory diseases, the NF-κB pathway is among the most attractive targets for such therapeutic intervention. In previous studies, the NF-κB pathway has been shown to be activated when chondrocytes are stimulated with IL-1β (43-45). It has been found that $P2X_7R$ triggers the activation of the NF-κB signaling pathway, and the activation of $P2X_7R$ is believed to have a close association with inflammatory diseases, such as arthritis (46). These findings raise the possibility that the modulation of the NF-κB pathway is a viable path for improving the treatment efficacy of OA. In the present study, we found that $P2X_7R$ regulated OA by targeting the NF-κB signaling pathway. Firstly, our results demonstrated that the levels of IKKα, IKKβ, IκBα, NF-κB p65 and their phosphorylated forms were upregulated in rats with OA. However, it was shown that the protein expression of these molecules was reversed by AZD9056. According to previously published results, inactive NF-κB is present in the cytoplasm as a heterotrimer complex consisting of two subunits, p65 and p50, bound to an additional inhibitory subunit IκBα, which prevents NF-κB from entering the nuclei. In particular, purinergic signals activate NF-κB through P2X7R by selectively targeting NF-κB p65 (47). Consistent with these results, we found that the blockade of $P2X_7R$ downregulated NF-κB p65 and the corresponding phosphorylation level. Another study demonstrated that in complete Freund's adjuvant (CFA)-induced arthritis, IKKα and IKKβ were activated by $P2X_7R$, causing the activation of NF-κB and the phosphorylation of IκBα (48). These reports and our results demonstrate that $P2X_7R$ is involved in the regulation of the NF-κB signaling pathway in cartilage tissues in OA.

Secondly, we aimed to inhibit NF-κB signaling using the NF-κB signaling inhibitor, helenalin. Our results indicated that helenalin suppressed the expression of $P2X_7R$, MMP-13, SP and PGE_2 in the left side knee joint cartilage tissues of rats with OA. Existing evidence indicates that the $P2X_7R$-mediated signal is necessary for the activation of the NF-κB pathway (49-52). The above-mentioned results suggest that the effects of helenalin are closely related to $P2X_7R$. In general, our data suggested that $P2X_7R$ regulates OA by targeting the NF-κB pathway, which is considered one of the most attractive targets for the prevention, treatment and prognosis of arthritis and cartilage degenerative diseases.

In conclusion, in this study, $P2X_7R$ was identified not only as a regulator of OA-induced pain and inflammation, but that it can also influence the expression of MMP-13 and NF-κB signaling in OA-affected cartilage tissues, providing evidence that $P2X_7R$ may serve as a potential therapeutic target for the management of OA-related cartilage degenerative diseases.

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