Inhibiting the PI3K/AKT/NF-κB signal pathway with nobiletin for attenuating the development of osteoarthritis: *in vitro* and *in vivo* studies

Linzhen Xie,† Huanguang Xie,† Chunhui Chen, Zhenyu Tao, Chuanxu Zhang and Leyi Cai*†

Osteoarthritis (OA), an age-related degenerative disease, is characterized by progressive degradation of the articular cartilage. There is increasing evidence that nobiletin (NOB) exerts special biological functions in a variety of diseases. However, whether it protects against OA remains unknown. In this study, we investigated the anti-inflammatory and chondroprotective effects of NOB on IL-1β-induced human OA chondrocytes and in the surgical DMM mice OA models. *In vitro*, NOB treatment completely suppressed the overproduction of pro-inflammatory mediators, including PGE2, NO, COX-2, iNOS, TNF-α and IL-6 in IL-1β-induced human OA chondrocytes. Moreover, NOB exerted a potent inhibitory effect on the expression of MMP-13 and ADAMTS-5 as well as the degradation of aggrecan and collagen-II, which leads to the degradation of the extracellular matrix. Furthermore, NOB dramatically suppressed the IL-1β-stimulated phosphorylation of PI3K/Akt and activation of NF-κB in human OA chondrocytes. In addition, treatment with NOB not only prevented the destruction of cartilage and the thickening of subchondral bone but also relieved synovitis in mice OA models. In conclusion, our study suggests that NOB holds novel therapeutic potential for the treatment of OA.

1. Introduction

Osteoarthritis (OA), an irreversible degenerative joint disease, is characterized by the progressive degradation of the articular cartilage, formation of osteophytes, subchondral bone rebuilding and synovial inflammation, and becomes more problematic as the population ages.† It is one of the most prevalent forms of chronic articular disease and is accompanied by joint pain and stiffness, which can affect work and normal daily activities.‡ Previous studies showed that about 12% of the Western aging population are suffering from OA and 25% people aged over 55 have an episode of persistent knee pain.§ Multiple risk factors contribute to the occurrence and development of osteoarthritis, such as age, obesity, inflammation, trauma and hereditary factors.|| However, the pathogenesis of OA is far from being fully understood. It has been known that inflammation and inflammatory cytokines play vital roles in the development of OA.⁶ Among these cytokines, interleukin-1β (IL-1β) has been implicated as the principal instigator of OA as it contributes to cartilage matrix degradation through inducing the expression of matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), which results in decreased synthesis of collagen and proteoglycan during the pathogenesis of OA.⁷ Additionally, stimulation of chondrocytes with IL-1β could induce the release of inflammatory mediators nitric oxide (NO) and prostaglandin E2 (PGE2).⁸,⁹ Overproduction of NO and PGE2 has been considered to be closely related to the clinical manifestations of OA.¹⁰ Inhibition of IL-1β production or activity by IL-1β receptor antagonists or soluble receptors has been tested as a therapeutic strategy for OA.¹¹,¹² Additionally, the attenuation of the IL-1β-stimulated inflammatory mediator could be a potential treatment strategy for OA.

Nobiletin (NOB, Fig. 1), a widely distributed citrus polymethylated flavonoid, has attracted much attention recently for its beneficial effects on human health.¹³ NOB has been reported to possess numerous potent beneficial biological effects such as, anti-cancer, anti-inflammatory and anti-insulin resistance activities.¹⁴–¹⁶ Previous studies have reported important anti-inflammatory properties of NOB. For instance, NOB dose-dependently inhibited lipopolysaccharide (LPS)-induced production of pro-inflammatory mediators NO, PGE2, IL-1β and IL-6 as well as the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) at both
protein and mRNA levels in RAW 264.7 macrophages. In addition, NOB treatment markedly suppressed LPS-induced iNOS and COX-2 expression and NO release along with TNF-α and IL-1β production in BV-2 microglia. NOB was also found to suppress the production of PGE2, the expression of COX-2 mRNAs and nuclear factor kappa B (NF-κB) activity in mouse primary osteoblasts. Furthermore, an in vivo study showed that NOB could inhibit the development of rheumatoid arthritis by inhibiting the degree of angiogenesis and inflammatory infiltration by down-regulating the protein expression level of the p38/NF-κB signaling pathway in the synovium of rats with arthritis induced by collagen. However, the anti-inflammatory effect of NOB on OA is still unclear. Therefore, in the present study, we investigated the anti-inflammatory effect and the potential mechanism of NOB on IL-1β-stimulated human OA chondrocytes in vitro as well as the protective role of NOB in a mouse model of OA in vivo.

2. Materials and methods

2.1. Chemicals and reagents

NOB (purity >98%) was purchased from Beijing Solarbio Science & Technology Co, Ltd (Beijing, China). Recombinant human IL-1β, collagenase type II, and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Cell-Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). The primary antibodies against type II collagen (#ab34712), aggrecan (#ab36861), MMP-13 (#ab39012), ADAMTS-5 (#ab41037), iNOS (#ab15323), p-Pi3K (#ab74136) and β-actin (#ab8226) were from Abcam (Cambridge, UK) and antibodies against COX-2 (#12282), IκBα (#4812), p65 (#8242), P3K (#4249), Akt (#4691) and p-Akt (#4060) were from Cell Signaling Technology (Danvers, MA, USA). Goat anti-rabbit and goat anti-mouse horseradish peroxidase conjugates were purchased from Bio-Rad Laboratories (Calif., USA). Fetal bovine serum (FBS), bovine serum albumin (BSA), Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F12 medium and 0.25% trypsin-ethylenediaminetetraacetic acid (trypsin–EDTA) were purchased from Gibco (Life Technologies Corp. Carlsbad, Calif., USA). The TRIZol reagent was purchased from Invitrogen (Carlsbad, Calif., USA). A QuantiTect Reverse Transcription kit was purchased from Qiagen (Valencia, CA). SYBR Green Master Mix was purchased from Bio-Rad Laboratories (Calif., USA). ELISA kits of PGE2 (#KGEO04B), TNF-α (#MTA00B) and IL-6 (#D6050) were obtained from R&D Systems (Minneapolis, MN, USA). The Griess reagent was purchased from Beyotime Institute of Biotechnology (Shanghai, China). All other chemicals were of reagent grade.

2.2. Primary human chondrocyte isolation and culture

Articular cartilage sample collection was performed according to the terms of the Medical Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University (ethics number: LCKY-2017-13) and following the guidelines of the Declaration of Helsinki and Tokyo. OA human cartilage tissues were obtained from ten OA patients (age: 50 ± 10 years) who underwent total knee replacement surgery at the Second Affiliated Hospital of Wenzhou Medical University. Osteoarthritis was diagnosed according to the Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. Full ethical consent was obtained from all patients. Primary chondrocytes were isolated from articular cartilage as described previously. Cartilage pieces were digested with 0.2% collagenase type II for 5 h at 37 °C, and then centrifuged at 1000 rpm for 5 min and the supernatant was discarded. The inner cell mass was obtained and suspended in DMEM/F12 with 10% FBS and a 1% antibiotic mixture (penicillin and streptomycin). Finally, cells were plated at a density of 1 × 10⁵ cells per ml in a 6-well plate and incubated under a humidified atmosphere of 5% CO₂ at 37 °C. The media were changed every 2–3 days. Cells were passaged when at 80 to 90% confluence using a 0.25% trypsin–EDTA solution. Only passages 1 to 2 were used in our study to avoid the phenotype loss.
2.4. Measurement of NO, PGE2, TNF-α and IL-6

The NO levels in the culture medium were determined by the Griess reaction as previously described. The levels of PGE2, TNF-α and IL-6 in the culture medium were investigated using commercial ELISA kits according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). All assays were performed in duplicate.

2.5. Quantitative real-time polymerase chain reaction

Total RNA was isolated from chondrocytes using the TRIzol reagent according to the manufacturer’s instructions. Its concentration was determined spectrophotometrically at 260 nm (Thermo Scientific NanoDrop 2000). The A260/A280 ratio was calculated to verify the quality and purity. First-strand cDNA was synthesized using 1 µg of total RNA and the Quantitect Reverse Transcription kit. A quantitative real-time PCR (qPCR) was performed using a CFX96 Real-Time PCR System (Bio-Rad Laboratories, California, USA), under the following conditions: 10 min, 95 °C, followed by 40 cycles of 15 s, 95 °C and 1 min, 60 °C. The reaction was performed in a total volume of 10 µL, containing 4.5 µL diluted cDNA, 0.25 µL forward primer, 0.25 µL reverse primer and 5 µL SYBR Green Master Mix. The level of target mRNA was normalized to the level of GAPDH and compared with the control. Data were analyzed using the 2-ΔΔCT method. Each gene analysis was performed in triplicate. Sequences of primers of the targeted genes are listed in Table 1.

2.6. Western blot analysis

Proteins were extracted from chondrocytes with RIPA lysis buffer. Lysates were sonicated on ice and centrifuged at 12 000 rpm for 30 min at 4 °C. The protein concentration of the supernatant was determined using a BCA protein assay kit. Equal amounts of protein (40 µg) were separated by 12% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with blocking buffer 5% non-fat milk or 3–5% BSA (for phosphorylated anti-body) in TBS containing 0.1% Tween-20 (TBST) for 2 h at room temperature and then probed with the primary antibodies against COX-2, iNOS, collagen-II, aggrecan, MMP-13, ADAMTS-5, p65, p-p65, lkBα, p-lkBα, PI3K, p-PI3K, Akt and p-Akt (dilution 1 : 1000) overnight at 4 °C. After washing three times with TBS containing 0.1% Tween-20 for 5 min, the membranes were incubated with HRP-conjugated secondary antibodies (1 : 3000) for 2 h. Finally the membranes were detected using an Enhanced Chemiluminescence (ECL) kit and quantified by the Quantity ONE (Bio-Rad, Hercules, CA, USA) software. β-Actin was used as an internal control.

2.7. Immunofluorescence

Chondrocytes were seeded on 6-well plates on glass coverslips and incubated for 24 h. Glass coverslips with chondrocyte monolayers were rinsed three times in PBS. Then the cells were fixed with 4% paraformaldehyde for 15 min at room temperature and rinsed with PBS again. Cells and nuclear membranes were permeabilized with 0.1% Triton X-100 for 5 min at room temperature. Later, the cells were overlaid with 5% protease-free BSA for 1 h at room temperature, rinsed with PBS and incubated with the primary antibody against p65 (1 : 200) for 4 °C overnight. After washing with PBS, the cells were incubated with fluorescein-conjugated goat anti-rabbit IgG antibody (1 : 500) for 1 h at room temperature. Finally, the cells were washed three times with PBS and mounted in a medium containing DAPI (Invitrogen). Slides were viewed with a confocal laser scanning microscope (Leica Microsystems, Germany).

2.8. Transient transfection and luciferase activity assay

1 µg of NF-κB promoter/luciferase DNA (Stratagene, Santa Clara, CA, USA) along with 20 ng of control pRL-TK DNA was transiently transfected into 1 × 105 chondrocytes per well in a 6-well plate using lipofectamine/plus reagents for 24 h. Cells pretreated with NOB (20, 40, 80 µM) were stimulated with IL-1β (10 ng ml⁻¹) for 2 h. Each well was then washed twice with ice-cold PBS and harvested in 100 µl of lysis buffer (0.5 mM

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<th>Table 1</th>
<th>Primer sequences used in the qRT-PCR</th>
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<tr>
<td>Gene</td>
<td>Forward primer</td>
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<tr>
<td>COX-2</td>
<td>5'-GAGAGGATGTACCTCCCATCAGTCA-3'</td>
</tr>
<tr>
<td>iNOS</td>
<td>5'-CCCTTACAGGGCGGAGAAGGACAG-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-GTCAGATCATCTTCGAAACC-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-GACAGCCGACTCACCTCTTCA-3'</td>
</tr>
<tr>
<td>Collagen-II</td>
<td>5'-CTCAAGTGCTGAGAACCA-3'</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>5'-AAATGTGCTATGCCTGCTGTGTT-3'</td>
</tr>
<tr>
<td>MMP-13</td>
<td>5'-CCGAACTTCTCACCACAT-3'</td>
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<td>ADAMTS-5</td>
<td>5'-GCAGAATCGGCACTCGA-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-TCTCCCTGACTTCAACAGCGAC-3'</td>
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MgCl2) and used for the measurement of luciferase activity using a dual-luciferase assay kit. Luminescence was measured on a top counter microplate scintillation and luminescence counter in single photon counting mode for 0.1 min per well, following a 5 min adaptation in the dark. The luciferase activity was normalized with the expression of control pRL-TK.

2.9. Mice OA models

Eight-week-old C57BL/6 male wild-type (WT) mice were purchased from the Animal Center of Chinese Academy of Sciences, Shanghai, China. The protocol for animal care and use conformed to the Guide for 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 experimental mice were subjected to surgically induced OA by destabilization of the medial meniscus (DMM) as previously described. Briefly, the mice were anesthetized with an intraperitoneal injection of 2% (w/v) pentobarbital (40 mg kg−1); then, the joint capsule of the right knee was incised just medial to the patellar tendon and the meniscal ligament was cut with microsurgical scissors. A sham operation, involving an arthrotomy without transection of the medial meniscotibial ligament, was performed in the right knee joint of the mice in the sham control group.

2.10. Experimental animal design

The mice were randomly divided into three groups of 10 mice to establish a sham control group (control), an osteoarthritis group (OA) and an osteoarthritis treated with NOB group (NOB). Mice OA models were made by DMM surgery. NOB was dissolved in 0.5% sodium carboxymethyl cellulose (0.5% CMC-Na). Mice in the sham control group were subjected to sham operation. Mice in the phloretin group received a gavage of NOB (20 mg kg−1) daily for 8 weeks after surgery while the mice in the OA group received a gavage of a vehicle (0.5% CMC-Na). The dose of NOB was chosen based on a previous study and our preliminary experiment. Food and water were available ad libitum. Mice were maintained under a constant temperature of 20 ± 2 °C, a relative humidity of 50% ± 10%, and a 12 h light/dark cycle. All animals were sacrificed by cervical dislocation at 8 weeks after surgery. Knee joint tissues were collected for further evaluation.

2.11. Histological assessment

Collected joint samples were fixed in 4% paraformaldehyde for 24 hours at 4 °C and decalcified in 10% EDTA solution at 4 °C for two weeks. After that, the samples were dehydrated through an alcohol gradient, cleared, and embedded in paraffin blocks. Frontal serial sections (5 μm thick) across entire joints were obtained and 10 slides per joint at every 50 μm were selected and stained with safranin-O/fast green and hematoxylin–eosin to assess cartilage destruction. The stained sections were photographed digitally under a microscope. To determine the extent of cartilage degeneration, we used multiple separate scoring systems for articular cartilage destruction, synovitis and subchondral bone thickness. The destruction of the articular cartilage was graded using the Osteoarthritis Research Society International (OARSI) scoring system for medial femoral condyle and medial tibial plateau. The section was assigned as follows (six OA grades): 0 = surface intact, cartilage intact; 1 = surface intact; 2 = surface discontinuity; 3 = vertical fissures; 4 = erosion; 5 = denudation; and 6 = deformation. Then we used a summed OARSI score (0–12) from medial femoral condyle and medial tibial plateau to evaluate the degree of articular cartilage destruction. The severity of synovitis was graded using a scoring system which was previously described: enlargement of the synovial lining cell layer on a scale of 0–3 (0 = 1–2 cells, 1 = 2–4 cells, 2 = 4–9 cells, and 3 = 10 or more cells) and the density of cells in the synovial stroma on a scale of 0–3 (0 = normal cellularity, 1 = slightly increased cellularity, 2 = moderately increased cellularity, and 3 = greatly increased cellularity). We applied the Axioscope software to measure the thickness of the medial subchondral bone plate according to safranin-O stained sections.

2.12. Statistical analysis

All experiments were performed independently at least three times. Data are presented as mean ± standard deviation (SD). Comparisons between groups were analyzed using the SPSS 17.0 software via ANOVA followed by Dunnett’s test. P < 0.05 was considered statistically significant.

3. Results

3.1. Effect of NOB on human OA chondrocyte viability

The chemical structure of NOB is shown in Fig. 1A. The effect of NOB on the viability of chondrocytes was determined by CCK-8 assay. The chondrocytes were cultured with increasing concentrations of NOB (0, 10, 20, 40, and 80 μM) for 24 h and 48 h, followed by the CCK-8 analysis. The results showed that NOB at the concentration range of 10–80 μM did not have any cytotoxic effects on human OA chondrocytes (Fig. 1B and C). Consequently, NOB (20, 40 and 80 μM) was used in the subsequent experiments.

3.2. Effect of NOB on the expression and production of IL-1β-induced inflammatory mediators in human OA chondrocytes

Next, we investigated the effects of NOB on IL-1β-induced iNOS and COX-2 expression in human OA chondrocytes using qRT-PCR and western blot analysis. NOB significantly inhibited the mRNA and protein levels of iNOS and COX-2 following stimulation by IL-1β (Fig. 2A, B and E–G). Moreover, the production of endogenous NO and PGE2 was up-regulated following IL-1β stimulation. Chondrocytes exhibited obvious down-regulated PEG2 and NO production after NOB treatment (Fig. 2H–I). Furthermore, the IL-1β-stimulated group showed increased production of IL-6 and TNF-α at both the mRNA and
protein levels according to the RT-PCR and ELISAs compared with the control group. However, the effects were suppressed following the treatment with NOB in a dose-dependent manner (Fig. 2C, D and J, K). Taken together, these results indicate that NOB exerts its anti-inflammatory effects by inhibiting inflammatory mediators and the production of inflammation-related enzymes in human OA chondrocytes under pathological conditions in a dose-dependent manner.

3.3. Effect of NOB on IL-1β-induced ECM degradation in human OA chondrocytes

Next, we analyzed the effect of NOB on ECM degradation in human OA chondrocytes. As shown in Fig. 3A, B, E and G, chondrocytes revealed an obvious down-regulation of mRNA and protein expression ofaggrecan and collagen-II after IL-1β stimulation. In contrast, NOB clearly dose-dependently inhibited the mRNA and protein down-regulation of aggrecan and collagen-II (Fig. 3A, B, E and G). On the other hand, chondrocytes showed obvious up-regulation of the mRNA and protein expression of MMP-13 and ADAMTS-5 after IL-1β stimulation in contrast to the control group. However, treatment of NOB markedly reduced the mRNA and protein up-regulation of MMP-13 and ADAMTS-5 in a dose-dependent manner (Fig. 3C, D, E, H and I).

3.4. Effect of NOB on IL-1β-induced NF-κB activation in human OA chondrocytes

To further elucidate the mechanism underlying the anti-inflammatory effect of NOB, western blot and NF-κB-dependent gene reporter assay were performed to study the changes
in the NF-κB signaling pathway. IL-1β stimulation significantly induced the phosphorylation of NF-κB p65 and IκBα in chondrocytes. Moreover, stimulation of chondrocytes with IL-1β resulted in conspicuous degradation of IκBα. In contrast, NOB exhibited a concentration-dependent inhibitory effect on IL-1β-induced NF-κB activation and IκBα degradation in human OA chondrocytes (Fig. 4A–C). On the other side, the results of luciferase assays showed that NOB significantly reduced NF-κB promoter luciferase activity induced by IL-1β in a dose dependent manner (Fig. 4D). The effect of NOB on NF-κB p65 nuclear translocation was examined using immunofluorescence in chondrocytes in response to NF-κB activation by IL-1β. Chondrocytes remained unstimulated or treated with IL-1β alone for 30 min or were co-treated with IL-1β and 80 μM NOB for 30 min. Control chondrocytes showed the labeling of p65 mostly restricted in the cytoplasm. Once IL-1β caused stimulation, chondrocytes showed clear and enhanced nuclear staining for p65 and indicated nuclear translocation of the NF-κB p65 subunit (Fig. 4E). Nevertheless, NOB resulted in the inhibition of the translocation of the p65 subunits into the nuclei and showed a decrease in the activation of NF-κB. These immunofluorescence findings suggested that NOB is able to reverse the nuclear translocation of NF-κB p65 in IL-1β-stimulated chondrocytes, which is consistent with the inhibitory effect of NOB on NF-κB observed by western blot.

Fig. 3 Effect of NOB on IL-1β-induced ECM degradation in human OA chondrocytes. Human OA chondrocytes were pretreated for 2 h with various concentrations of NOB (20, 40, and 80 μM) and then stimulated or not stimulated with IL-1β (10 ng ml⁻¹) for 24 h. The mRNA expression levels of collagen-II (A), aggrecan (B), MMP-13 (C) and ADAMTS-5 (D) were assayed by the qRT-PCR. The protein expression levels of collagen-II, aggrecan, MMP-13 and ADAMTS-5 were determined by western blot (E) and quantification analysis (F–I). The values are mean ± SD. *p < 0.05. **p < 0.01 compared with the control group, #p < 0.05 compared with the IL-1β group, n = 6.
3.5. Effect of NOB on IL-1β-induced PI3K/AKT phosphorylation in human OA chondrocytes

To further investigate the anti-inflammatory mechanism of NOB, the effects of NOB on IL-1β-induced PI3K/AKT phosphorylation were detected by western blot analysis. As shown in Fig. 5A–C, IL-1β significantly up-regulated the phosphorylation of PI3K and AKT compared to the control group. However, treatment of NOB dose-dependently suppressed IL-1β-induced PI3K/AKT phosphorylation (Fig. 5A–C).

3.6. Effect of NOB on destabilization of the medial meniscus (DMM)-induced OA in mice

To evaluate whether NOB has any preventive effect against the induction and progression of osteoarthritis in vivo, DMM OA models were established in mice, followed by a gavage of NOB (20 mg kg⁻¹) daily for 8 weeks after surgery. As shown in Fig. 6, the safranin-O staining and hematoxylin–eosin staining showed that the cartilage surface was smooth and intact in the sham control group. However, the OA group exhibited cartilage...
superficial destruction, massive proteoglycan loss and apparent hypocellularity compared to the sham control group. In contrast, treatment of NOB remarkably protected the structure of articular cartilage and maintained the proteoglycan in cartilage. On the basis of the results of safranin O staining, the OARSI scores of the OA group were clearly higher (9.80 ± 1.32) than those of the sham control group (0.20 ± 0.42). In contrast, the NOB group showed transparently lower OARSI scores (4.90 ± 1.45) than the OA group (Fig. 6A). Moreover, it was found that NOB obviously lowered the subchondral bone thickness (Fig. 6B) and ameliorated the inflammatory changes in synovium (Fig. 6C) compared to the OA group. Altogether, these results indicate that NOB attenuates the development of OA in the mouse DMM model.

4. Discussion

Osteoarthritis is a severe and debilitating joint disease which is characterized as resulting from the damage and degeneration of the articular cartilage of joint surfaces. However, to date, there is no curative treatment for OA. Despite the wide application of nonsteroidal anti-inflammatory drugs (NASIDs) for OA treatment in clinical practice, these agents only provide symptomatic relief and always give rise to lots of side effects. Therefore, there is an urgent need to secure a valid medication for endogenous repair and to attenuate the processes of OA. There is already growing evidence that compounds extracted from natural plants have become the potential and reasonable agents of choice for OA patients because of their potent anti-inflammatory activities and less toxicity. NOB, a polymethoxylavone, is common in the citrus genus and particularly prevalent in citrus peels, and has been reported to exhibit powerful anti-inflammatory effects. Many previous studies have reported the effect of nobiletin on different kinds of cell, such as RAW 264.7 cells, human intestinal fibroblasts, microglial cells and hepatocytes. However, in this study, it is the first time that the effect of nobiletin on chondrocytes has been evaluated. We first screened out the suitable dose of nobiletin from different kinds of cells according to previous studies. Then we chose the potential dose of nobiletin for a chondrocyte based on the characteristic of the chondrocyte. Finally, we used the potential dose of nobiletin to perform the CCK-8 assay, which could detect the viability of the chondrocyte and

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**Fig. 5** Effect of NOB on IL-1β-induced PI3K/AKT phosphorylation in human OA chondrocytes. Chondrocytes were pretreated with NOB (20, 40, and 80 μM) for 2 h, followed by stimulation with or without IL-1β (10 ng ml⁻¹) for 1 h. The protein expression levels of PI3K, p-PI3K, AKT and p-AKT were determined by western blot (A) and quantification analysis (B–C). The values are mean ± SD. #p < 0.05 compared with the control group, *p < 0.05, **p < 0.01 compared with the IL-1β group, n = 6.
figure out the most appropriate dose of nobiletin for the chondrocyte. The dose of nobiletin for the chondrocyte was chosen based on our preliminary experiment and other animal models, such as rheumatoid arthritis, \(^{19}\) colitis \(^{20}\) and osteoporosis. \(^{18}\) However, in the present study, we did not determine the potential toxicity in the \textit{in vivo} study, and this was a limit-
ation of our research. This problem will be solved in our further study. In this study, we demonstrated that the effects of NOB (0–80 μM) on human OA chondrocytes were not attributable to cytotoxic effects. We found that NOB significantly inhibited the IL-1β-induced inflammatory mediators and ECM degradation, including the expression of NO, PGE2, TNF-α, IL-6, iNOS, COX-2, MMP-13 and ADAMTS-5 along with the degradation of aggrecan and collagen-II in human OA chondrocytes. Besides, our results showed that NOB remarkably blocked the IL-1β-induced NF-κB activation and PI3K/AKT phosphorylation. Furthermore, NOB showed a protective effect against cartilage degradation in DMM-induced OA in mice.

NO and PGE2 have long been demonstrated to be strongly associated with the pathophysiology of OA because they can expedite the development of OA through facilitating the synthesis of MMPs and inhibiting the synthesis of collagen and proteoglycan in chondrocytes. NO is catalyzed by iNOS. It has been reported that NO promotes the production and activation of MMPs and suppresses collagen II and proteoglycan synthesis, leading to ECM degradation in OA. PGE2 is an inflammatory mediator produced from IL-1β-induced endogenous arachidonic acid by cyclooxygenase-2 (COX-2), and is involved in the degeneration of cartilage during the pathophysiology of OA due to its potent excitation on MMPs and ADAMTS-5 in cartilage. Studies have shown that the inhibition of the production of inflammatory mediators such as NO and PGE2 is capable of attenuating the progression of OA. In the current study, we found that NOB significantly inhibited IL-1β-induced NO and PGE2 production as well as iNOS and COX-2 expression at the mRNA and protein levels in human OA chondrocytes. Our findings agree with the previous studies which have demonstrated that NOB significantly inhibited LPS-induced expression of pro-inflammatory proteins NO, iNOS, COX-2 and PGE2 in RAW 264.7 macrophages. TNF-α and IL-6 are also considered important factors in the development of OA in that they could activate macrophages, which subsequently synthesize pro-inflammatory chemokines to maintain inflammation in the development of OA. In our study, we found that the induction of TNF-α and IL-6 production by IL-1β was abrogated by NOB. The results are consistent with Li et al.’s results. They found that NOB treatment markedly inhibited the production of TNF-α and IL-6 in LPS-stimulated A549 cells. Taken together, our results suggest the feasibility of NOB in the treatment of OA on the basis of the strong inhibition of the overproduction of NO, PGE2, TNF-α and IL-6 as well as the overexpression of iNOS and COX-2 in the progression of OA.

During the progression of OA, a variety of MMPs and aggrecanases are produced by OA chondrocytes, which regulate a wide variety of functions including degradation and destruction of the ECM in OA. Among all MMPs, MMP-13 have been demonstrated to play vital roles in the progression of OA in that they strongly and irreversibly break down collagen-II and proteoglycans which constitute the main structure of the ECM. Hence, MMP-13 appears to be one of the most important candidates as a therapeutic target. It has been demonstrated that selective inhibitors of MMP-13 provided a potential therapy that could slow the progression of OA. Growing evidence showed that ADAMTS-5 is considered as the primary aggrecanase responsible for the cleavage of aggrecan in the pathogenesis of OA, which has made it a potential therapeutic target for treating OA. Inhibition of ADAMTS-5 by small interfering RNA (siRNA) decreased aggrecan loss from human OA cartilage explants. Therefore, agents targeting MMP-13 and ADAMTS-5 will help ECM homeostasis and are an effective therapeutic intervention for OA. Interestingly, our results showed that the up-regulated MMP-13 and ADAMTS-5 expression in IL-1β-stimulated human OA chondrocytes were dramatically decreased by NOB in a dose-dependent manner. Our results are partly supported by Imada et al. who found that NOB suppressed IL-1β-mediated ADAMTS-5 mRNA expression in cultured human synovial fibroblasts. Consequently, we speculated that NOB may exert its chondroprotective effects through abolishing the expression and activation of MMPs and ADAMTS in the pathogenesis of OA. The major structural components of the cartilage tissue are collagen-II and aggrecan which can provide tensile strength and shock absorption under mechanical damage. Loss of collagen-II and aggrecan results in expedition of the progression of OA. Therefore, inhibiting the degradation of collagen-II and aggrecan may be a new therapeutic choice for OA. In our study, NOB exhibited a potent inhibition on the down-regulation of aggrecan and collagen-II protein expression in human OA chondrocytes, which suggests that NOB inhibits cartilage degradation through increasing the expression of aggrecan and collagen-II.

It is widely accepted that the NF-κB signaling pathways play key roles in the regulation of inflammatory mediators associated with the pathogenesis of OA. Normally, NF-κB is present in the cytoplasm in an inactive form combined with the inhibitory subunit IκBα. Once induced by IL-1β, IκBα is phosphorylated and degraded and NF-κB p65 translocates from the cytoplasm to the nucleus to cause the expression of inflammation-related genes, including iNOS, COX-2, NO, PGE2, TNF-α, IL-6, MMPs and ADAMTS. Therefore, targeted strategies interfering with NF-κB signaling could offer novel potential therapeutic options for OA treatment. Previous reports showed that the suppression of NF-κB activation had the ability to attenuate the progression of OA. In addition, a study also reported that NF-κB p65-specific siRNA inhibited the expression of COX-2, iNOS and MMP-9 in IL-1β-induced chondrocytes. Therefore, in our study, we also investigated the molecular mechanisms by which NOB inhibited inflammatory mediators in response to IL-1β in human OA chondrocytes. We found that NOB significantly inhibited IL-1β-induced NF-κB activation and IκBα degradation in human OA chondrocytes. Furthermore, NOB reversed the translocation of NF-κB p65 from the cytoplasm to the nucleus when detected by immunofluorescence staining. Our results are partly supported by Li et al. who found that NOB pre-treatment dramatically inhibited the phosphorylation of IκBα and NF-κB p65 in LPS-stimulated A549 cells. In addition, NOB markedly inhibited the LPS-induced pro-inflam-
matory NF-κB signaling pathway by suppressing nuclear NF-κB translocation from the cytoplasm and subsequent expression of NF-κB in the nucleus in BV-2 microglia.\textsuperscript{14}

The PI3K/Akt signaling regulates various processes including inflammatory responses, cell growth, survival and metabolism in response to growth factors, and has also been demonstrated to be involved in both cellular and ECM changes in OA pathogenesis.\textsuperscript{51} Activation of the PI3K/Akt pathway can increase MMP production by chondrocytes via its multiple downstream target proteins such as NF-κB. Upon stimuli by receptors such as cytokine receptors, the membrane protein PI3K could directly or indirectly induce the phosphorylation of AKT and then the activation of NF-κB.\textsuperscript{52} Moreover, inhibition of the PI3K/Akt pathway attenuated cartilage degradation and inflammation response in OA rats.\textsuperscript{53} Indeed, the inhibition of p-Akt and NF-κB by curcumin decreased IL-1β stimulating MMP secretion and COX-2 expression in chondrocytes.\textsuperscript{54} In our study, we found that NOB greatly suppressed IL-1β-induced PI3K/Akt phosphorylation in human OA chondrocytes, which was partly supported by a previous study showing that NOB showed potent inhibitory effects on the phosphorylation of PI3K and Akt in LPS-treated RAW 264.7 macrophages.\textsuperscript{17} Therefore, the previous studies together with our findings suggest that NOB inhibited IL-1β-induced inflammatory response through suppressing the PI3K/Akt phosphorylation and NF-κB activation in human OA chondrocytes and the underlying mechanisms are illustrated specifically in Fig. 7. However, further studies are needed to clarify the exact mechanism of the regulation of NOB on the inflammatory process in human chondrocytes.

The mouse model of DMM has been used previously to demonstrate similarities between animal models and human OA.\textsuperscript{55} Articular cartilage destruction, changes in subchondral bone and synovitis are key factors in the mechanisms leading to joint damage in OA.\textsuperscript{56,57} As a result, agents that specifically block the mechanisms associated with cartilage destruction, subchondral bone remodeling and synovitis may be beneficial for developing targeted therapy for OA. In the current study, our results showed that the mice in the OA group exhibited severe cartilage destruction and extensive proteoglycan loss, which was obviously alleviated by treatment with NOB. Moreover, NOB reduced the OARSI scores and subchondral bone plate thickness and ameliorated the severity of synovitis in mice OA, indicating that NOB had the ability to attenuate

![Fig. 7](image-url)  
**Fig. 7** Working model for the inhibitory effects of NOB on IL-1β-induced PI3K/AKT phosphorylation and NF-κB activation resulting in the destruction of cartilage in human OA chondrocytes in vitro.
the progression of OA. These results along with the *vitro* findings provide the evidence that NOB exhibits anti-inflammatory effects in OA both *in vitro* and *in vivo*.

Nobiletin has been demonstrated to be metabolized by the P450 CYP1-enzyme. CYP1 enzymes convert nobiletin into one main metabolite O-demethylnobiletin.\(^5^8\) Seven metabolites including 3’-demethylnobiletin, 4’-demethylnobiletin, 3’,4’-didehydronobiletin (DTF), 5-demethylnobiletin, 5,3’-didehydronobiletin, 5,4’-didehydronobiletin and 5,3’,4’-tridehydronobiletin have been identified as the major metabolites from the urine of mouse by employing the optimized HPLC method.\(^5^9\) DTF has showed antioxidative and anti-inflammatory activity. DTF exhibits neuroprotective functions through attenuation of NF-κB signaling, decreasing the production of reactive oxygen species and upregulation of GCL and heme oxygenase-1 (HO-1), which is independent of Nrf2. DTF also triggers the activation of ERK, Akt, and JNK signaling.\(^6^0\) 4’-Demethylnobiletin shows stronger bioactivities such as anti-inflammation and anticancer activities. In LPS-treated RAW 264.7 macrophages, 4’-demethylnobiletin downregulates the expression of proinflammatory cytokines IL-1, IL-6, PGE2, iNOS, and COX-2 and inhibits NF-κB and AP-1 nuclear translocation, while upregulating the expression of transcription factor Nrf2 and its dependent gene HO-1.\(^1^7\) This is supported by the anti-inflammatory effects of 4’-demethylnobiletin on TPA-treated mice ear inflammation through inhibition of PI3K/Akt/ERK phosphorylation.\(^6^1\) Therefore, in this study, we have a reason to believe that the main component that exerts anti-inflammatory effects in OA is 4’-demethylnobiletin.

5. Conclusion

In conclusion, our findings demonstrated that NOB inhibited IL-1β-induced inflammation and ECM degradation by suppressing the PI3K/Akt/NF-κB pathway in human OA chondrocytes. Furthermore, NOB exerted a protective effect against the degradation of cartilage and synovitis in mice OA. Taken together, these results indicate that NOB may serve as an alternative therapeutic agent for the treatment of OA in the future.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

The authors thank all the staff in the Laboratory of Orthopedic Research Institute and Scientific Research Center of Second Affiliated Hospital of Wenzhou Medical University. This study was supported by the National Natural Science Foundation of China (81771928), Zhejiang Province Medical and Health Technology Project (2017KY480) and Wenzhou Science and Technology Bureau (Y20160040).

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