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Phillygenin inhibits inflammation in chondrocytes via the Nrf2/NF-κB axis and ameliorates osteoarthritis in mice

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ABSTRACT

Objective: Osteoarthritis (OA), widely seen in the elderly, is featured by cartilage degradation, subchondral bone remolding, and synovium inflammation. Currently, there is no cure for OA development. Phillygenin (PHI), an active ingredient from the Forsythiae Fructus, possesses many biological properties, such as anti-inflammation and anti-oxidative stress in several diseases. However, the potential effects and underlying mechanisms of PHI on OA remain unclear.

Methods: Western blotting, RT-PCR, ELISA and tissue staining were employed to explore the mechanisms by which PHI exerted a protective effect on IL-1β-induced production of pro-inflammation cytokines and extracellular matrix (ECM) degradation in primary murine chondrocytes and destabilization of the medial meniscus (DMM) mouse models.

Results: In this study, we found that PHI inhibited the production of pro-inflammation cytokines and ECM degradation induced by IL-1β in primary murine chondrocytes. Mechanically, PHI inhibited the NF-κB pathway via activating nuclear factor (erythrluteolind-derived 2)-like 2 (Nrf2). *In vivo* experiments also confirmed the chondroprotection of PHI in DMM mouse models.

Conclusion: PHI alleviated IL-1β-induced inflammation cytokines and ECM degradation via activating Nrf2 and inhibiting NF-κB pathway.

The translational potential of this article: This study provides a biological rationale for the use of PHI as a potential candidate for OA treatment.

1. Introduction

Osteoarthritis (OA), a common degenerative joint disorder in the elderly, often leads to joint pain and dysfunction, affecting people's quality of life [1,2]. OA is usually featured by cartilage degradation, subchondral bone remolding, and synovium inflammation. Currently, the underlying etiology of OA is still far from fully elucidated and there is no cure for OA progression [3]. However, emerging research evidence has indicated that interleukin (IL)-1β plays a robust role in OA progression, due to its promotion of many inflammatory cytokines, including nitric oxide (NO), thrombospondin motifs (ADAMTS), prostaglandin E2 (PGE2) and matrix metalloproteinases (MMPs) [4,5]. Therefore, inhibiting inflammation induced by IL-1β might be an effective treatment for OA.

NF-κB is a critical transcription mediator in IL-1β-induced OA progression [4,5]. After stimulated by IL-1β, IκBα is phosphorylated and marked for degradation by proteases, causing p65 nuclear translocation, and then p65 activates some specific genes to initiate inflammatory activities. Nuclear factor (erythrluteolind-derived 2)-like 2 (Nrf2) plays an

Abbreviations: ADAMTS, thrombospondin motifs; CCK-8, cell counting kit-8; ECM, extracellular matrix; HO-1, heme oxygenase 1; IL-1β, interleukin-1β; MMPs, matrix metalloproteinases; NO, nitric oxide; NSAIDs, non-steroidal anti-inflammatory drugs; Nrf2, nuclear factor (erythrluteolind-derived 2)-like 2; NQO1, NAD(P) H quinone oxidoreductase-1; OA, osteoarthritis; OARSI, Osteoarthritis Research Society International; PGE2, prostaglandin E2; PHI, Phillygenin; ROS, reactive oxygen species; SPF, specific pathogen-free.

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essential part in maintenance of a stable internal environment [6,7]. In the inactivated state, Nrf2 localizes in the cytoplasm by binding to Keap1. Once activated by various cytokines, inflammatory molecules or stress signals, Nrf2 separates from Keap1 and translocates to the nucleus, where it associates with antioxidant response elements (ARE), followed by activation of the transcription and expressions of various antioxidants, such as heme oxygenase 1 (HO-1) and NAD(P) H quinone oxidoreductase-1 (NQO1) [8–10]. Previous studies have revealed that Nrf2 and p65 will compete for p300, a transcription co-activator, in the nucleus, and upregulation of HO-1 expression by Nrf2 activation help to inhibit p65 nuclear translocation [11–13]. Hence, activation of Nrf2/HO-1 helps to suppress the NF-κB pathway and reduce inflammation, as evidenced by a growing number of studies [14,15]. Furthermore, Nrf2-knockout mice displayed more severe cartilage damage [16]. Therefore, targeting Nrf2 may mitigate OA progression.

Phillygenin (PHI) is an active ingredient from the traditional Chinese medicine, Forsythiae Fructus, with many biological properties [17–20]. For example, previous researches have revealed the hepatoprotective effects of PHI [17,19,20]. Another study suggested that PHI exhibited anti-inflammatory activity through modulating multiple cellular behaviors, leading to the suppression of the adaptive immune response [18]. Besides, PHI inhibited the inflammation and apoptosis of pulmonary epithelial cells [21]. However, the effects of PHI on OA remains unclear. Accordingly, the current study investigated the therapeutic effects of PHI on OA.

2. Materials and methods

2.1. Chemicals, reagents, and antibodies

PHI (purity >98%, Fig. 1A) was provided from Shanghai Yuan Ye Biotechnology Co. Ltd (Shanghai, China). The antibodies of Aggrecan (13880-1-ap) and Nrf2 (80593-1-RR) were obtained from ProteinTech (Wuhan, China). The remaining antibodies such as MMP13 (ab39012), Collagen II (ab34712), HO-1 (ab13243), GAPDH (ab9485), p65 (ab32536), IκBα (ab76429), ADAMTS5 (ab41037), COX-2 (ab179800), iNOS (ab178945) and Lamin B (ab229025) were purchased from Abcam (Cambridge, UK). Cell Counting Kit-8 (CCK-8) was provided by Dojindo Laboratories (Kumamoto, Japan). Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) fluorescent dye, JC-1 dye, TRIzol reagent and Lipofectamine 2000 were obtained from Invitrogen (CA, USA). Puromycin, polybrene, the Annexin V- fluorescent-activated cell sorting (FACS) assay kit, and cell culture reagents were provided by Sigma–Aldrich (St. Louis, MO, USA). All reagents for cell culture were provided by Gibco (NY, USA).

2.2. Animals and treatment

Male C57BL/6 mice aged 8 weeks were provided from Shanghai Animal Center of the Chinese Academy of Sciences and housed in a specific pathogen-free (SPF) facility. All animal procedures were conducted in accordance with the National Institutes of Health guide for the care and use of Laboratory animals and approved by the Institutional Animal Care and Use Committee of Soochow University. The mouse OA model was established by surgical destabilization of the medial meniscus (DMM) of the right knee. In this study, mice were randomly assigned to three groups: sham group (n = 10) with sham operation, DMM group (OA group, n = 10), and DMM + PHI group (n = 10). PHI was dissolved in 0.5% carboxymethylcellulose sodium. Mice in DMM + PHI group were intragastrically administered PHI (20 mg/kg/d) for 8 weeks after surgery, while mice in the sham group and the DMM group received the same amount of 0.5% carboxymethylcellulose sodium at the same time. Afterwards, the mice were sacrificed and the knee joints were harvested.

2.3. Primary murine chondrocyte culture

Primary murine chondrocytes were isolated as previously described [8]. Briefly, immature C57BL/6 mice were sacrificed with pentobarbital

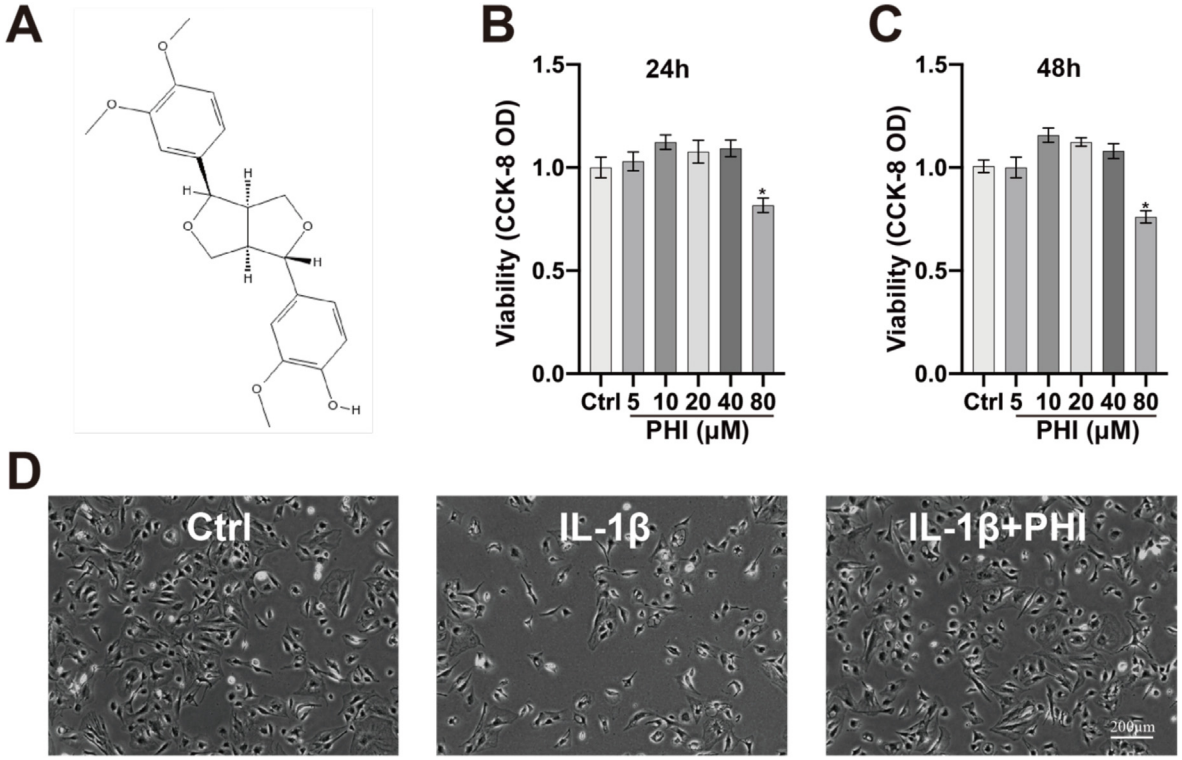

Figure 1. Effects of PHI on primary murine chondrocyte viability. The chemical structure of PHI (A). The cytotoxicity of PHI on chondrocytes determined at diverse concentrations (0, 5, 10, 20, 40, 80 μM) for 24 h and 48 h using the CCK-8 assay (B and C). Primary murine chondrocyte morphology was observed via microscopy with or without PHI (20 μM) for 24 h (D). The values are presented as mean ± S.D. *p < 0.05 compared with control group.

sodium, and the articular cartilage tissues of the femoral heads were dissected by digestion with 0.2% collagenase II for 4 h, followed by washing with PBS and then the isolated chondrocytes were cultured in DMEM/F12 medium. The chondrocytes at passages 1 to 3 were used for the subsequent experiments.

2.4. Chondrocytes viability assay

CCK-8 assay was used to examine the cell viability. Briefly, cells were seeded in 96-well plates at 5×10^5 cells/ml for 24 h and then cultured in various doses of PHI (0, 5, 20, 40, and 80 μM) for 24 h and 48 h. Next, 10 μl of the CCK-8 solution was added to each well and incubated at 37 °C for 2 h. Then, the OD values were measured at 450 nm using a microplate reader.

2.5. Western blotting assay

The total protein in the chondrocytes was isolated by the RIPA lysis buffer with 1 mM phenyl methane sulfonyl fluoride. Cytoplasmic and nuclear proteins were extracted by using Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology, Shanghai, China) based on the instructions provided. The protein concentration was quantified by the BCA protein assay kit (Beyotime). After separated on SDS-PAGE gels, the protein was transferred to a PVDF membrane, which was blocked with 5% skim milk for 2 h and incubated with primary antibodies overnight at 4 °C, followed by a subsequent incubation with respective secondary antibodies for 1 h at room temperature. The bands were washed three times and then visualized using an electrochemiluminescence reagent (Invitrogen), with ImageJ software (National Institutes of Health, Bethesda, MD, USA) used to quantify the band intensity.

2.6. Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from chondrocytes by the TRIZOL method (Thermo Fisher Scientific Inc., USA) according to its instruction. And 1 μg RNA was reverse transcribed using the reverse transcription kit (Accurate Biotechnology). Gene expressions were determined using RT-PCR with the relevant kits base on the manufacturer's instructions. Gene expression was expressed as the fold change, and was normalized to that of GAPDH, using the delta–delta Ct method. The mRNA primers for the listed genes were described in the other study [9].

2.7. NO, PGE2, TNF-α, and IL-6 measurement

The NO level in the supernatant was detected by Griess reagent. The content of PGE2, TNF-α, and IL-6 in cell culture supernatants was determined by ELISA kits (R&D Systems) according to the manufacturer's instructions.

2.8. Nrf2 shRNA

For Nrf2 silencing, the lentiviral construct encoding short hairpin RNA (shRNA) sequence of Nrf2 [9] was transduced to the primary murine chondrocytes ("sh-Nrf2"). Control cells were transduced with lentiviral scramble control shRNA ("sh-C"). The stable chondrocytes were established after puromycin selection. Nrf2 knockdown in the stable cells was verified by western blotting assay and RT-PCR assay.

2.9. Immunofluorescence staining

Chondrocytes were placed in a 6-well plate and treated with IL-1β (10 ng/ml) or combined with PHI (20 μM), followed by incubation overnight. After that, the samples were washed 3 times with PBS and fixed in 4% paraformaldehyde, followed by treatment with 0.1% Triton X-100 for 20 min. Then, the cells were blocked by 5% bovine serum albumin at 37 °C for 1 h, rinsed with PBS, and incubated with primary antibodies against Collagen II (1:100), MMP-13 (1:100), and HO-1 (1:100) at 4 °C overnight. Next, the cells were washed 3 times with PBS and incubated with Alexa Fluor®488-labeled secondary antibodies (1:400) at room temperature for 1 h. Then, they were counterstained with DAPI. Finally, five view fields were randomly selected and observed by a fluorescence microscope (Olympus, Tokyo, Japan).

2.10. Histopathological analysis

The mouse articular cartilage tissues of knee joints were fixed in 4% paraformaldehyde for 24 h. After decalcified, the samples were embedded in paraffin. Then, sections (5 μm) were prepared and stained with hematoxylin and eosin (HE) and safranin O/fast green (Sigma–Aldrich, Oakville, Ontario, Canada), followed by evaluation by three independent histology researchers in a blinded manner. The Osteoarthritis Research Society International (OARSI) scoring system was used to quantify the degeneration of cartilage as previously described [22]. Immunohistochemistry was further performed to analyze the protein expression of Nrf2 and p65 of knee joint sections. Primary antibodies against Nrf2 and p65 were incubated overnight at 4 °C. Then, a secondary antibody was added and incubated at room temperature for 1 h. Subsequently, a DAB solution kit was used for visualization. Immunofluorescence sections were sealed for observation after DAPI staining. The slides were visualized under a microscope (Olympus, Tokyo, Japan). The expressions of Nrf2 and p65 were evaluated by calculating the percentage of immunopositive cells.

2.11. Mitochondrial depolarization

In the presence of mitochondrial depolarization, JC-1 fluorescent dye aggregates in mitochondria to form green monomers [23]. And the detailed method for the JC-1 assay was described previously [24].

2.12. Measurement of reactive oxygen species (ROS) levels

The intracellular ROS was assessed using a cell-permeable fluorogenic probe, 2′, 7′-dichlorodihydrofluorescein diacetate (DCF-DA). Briefly, after the indicated treatments, cells were harvested and stained with 10 μM DCF-DA in the dark at room temperature for 20 min. The DCF fluorescence was tested by a fluorescence spectrofluorometer (Thermo Scientific, Shanghai, China) at 485 nm excitation and 525 nm emission.

2.13. Statistical analysis

All experiments were repeated in triplicate, with data presented as mean ± standard deviation (S.D.). Data analysis was performed by SPSS Statistics Version 19.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to analyze the data, and the Tukey test was used to perform comparisons between groups. The OARSI score was analyzed with a Kruskal–Wallis H test. And p value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of PHI on primary murine chondrocyte viability

The chemical structure of PHI was demonstrated in Fig. 1A. To examine the cytotoxic effect of PHI, several concentrations of PHI (0, 5, 10, 20, 40, 80 μM) were added to chondrocytes and cultured for 24 h and 48 h, and CCK-8 assay was used to detect cell viability. As demonstrated in Fig. 1B and C, PHI (≤40 μM) was nontoxic within 24 h and 48 h. Therefore, these concentrations of PHI were used for the following experiments. As shown in Fig. 1D, primary murine chondrocyte

morphology was observed via microscopy and the number of cells was reduced after IL-1β stimulation, while PHI (20 μM) treatment for 24 h reversed the effect.

3.2. PHI inhibited IL-1β-induced cell apoptosis and ROS production in chondrocytes

The effect of PHI (20 μM) on chondrocyte apoptosis and ROS production was examined. Our findings revealed that IL-1β increased the Annexin V-positive ratio (Fig. 2A and B), indicating apoptosis activation. Besides, mitochondrial depolarization (Fig. 2C and D) and ROS production (DCF-DA intensity increase, Fig. 2E and F) were potently enhanced in response to IL-1β stimulation. In contrast, these changes were distinctly mitigated by PHI treatment (Fig. 2A–F). The data implied that PHI showed effective chondrocyte protection against IL-1β-induced cell apoptosis and ROS production.

3.3. PHI ameliorates the productions of inflammatory cytokines in IL-1β-treated chondrocytes

The effect of PHI on IL-1β-triggered inflammation in chondrocytes were explored. Chondrocytes were treated with PHI (0, 5, 10, 20, 40 μM, 24 h) after IL-1β (10 ng/ml) stimulation. The RT-PCR (Fig. 3A–D) and western blotting (Fig. 3E) results showed that mRNA levels of Nos2, IL-6, Ptgs2, and Tnf, as well as protein levels of iNOS and COX-2 were increased after IL-1β stimulation, while PHI reversed these changes in a concentration-dependent manner. Furthermore, the NO concentration in the cell suspension was detected by the Griess reaction (Fig. 3F), and the

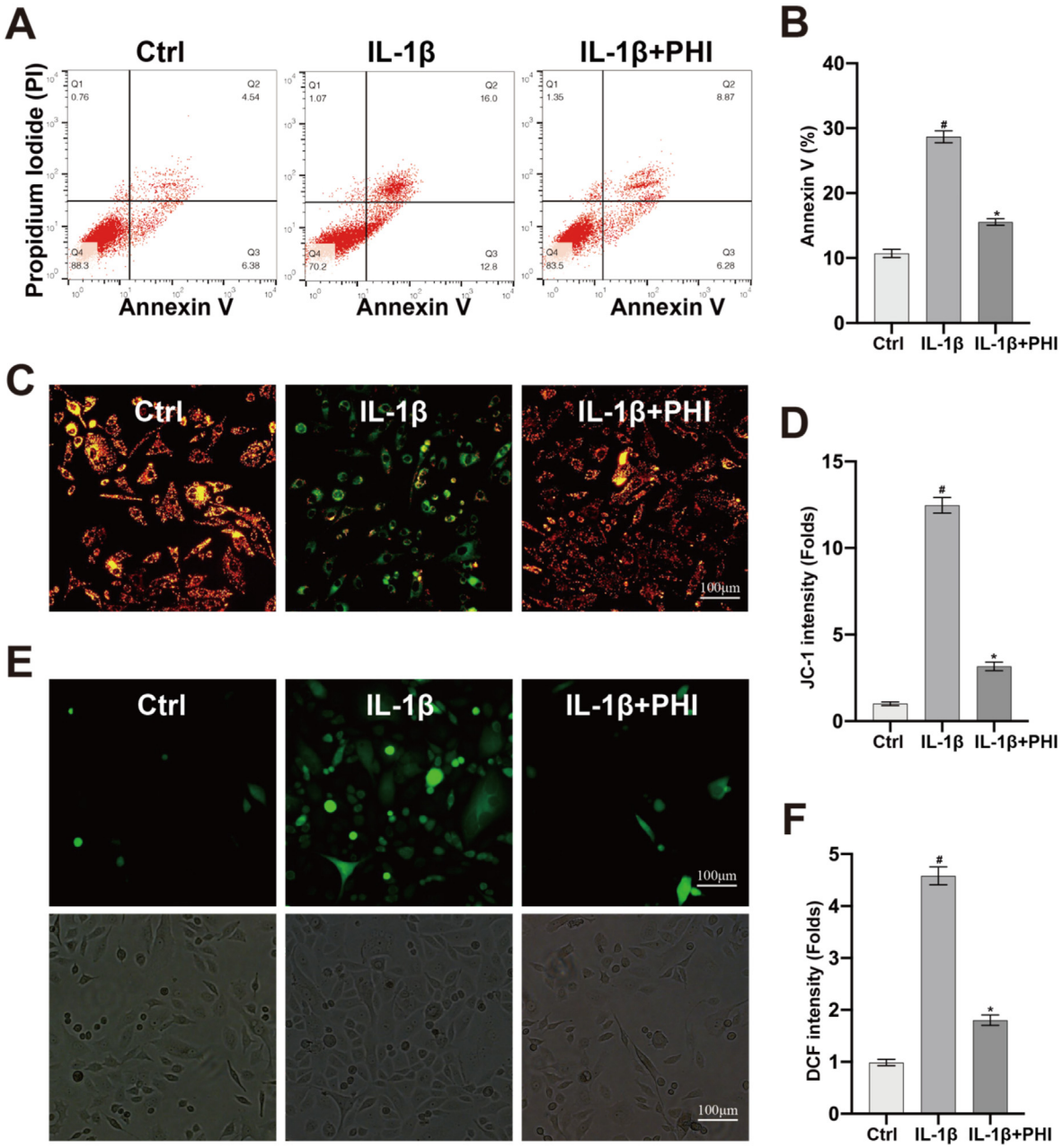

Figure 2. Effect of PHI (20 μM) on chondrocyte apoptosis and ROS production induced by IL-1β. Annexin V (A, B), mitochondrial depolarization (JC-1 intensity, C, D) and ROS production (E, F) were examined by the corresponding assays. The values are presented as mean ± S.D. Significant differences among different groups are indicated as $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$ vs. IL-1β group, n = 5.

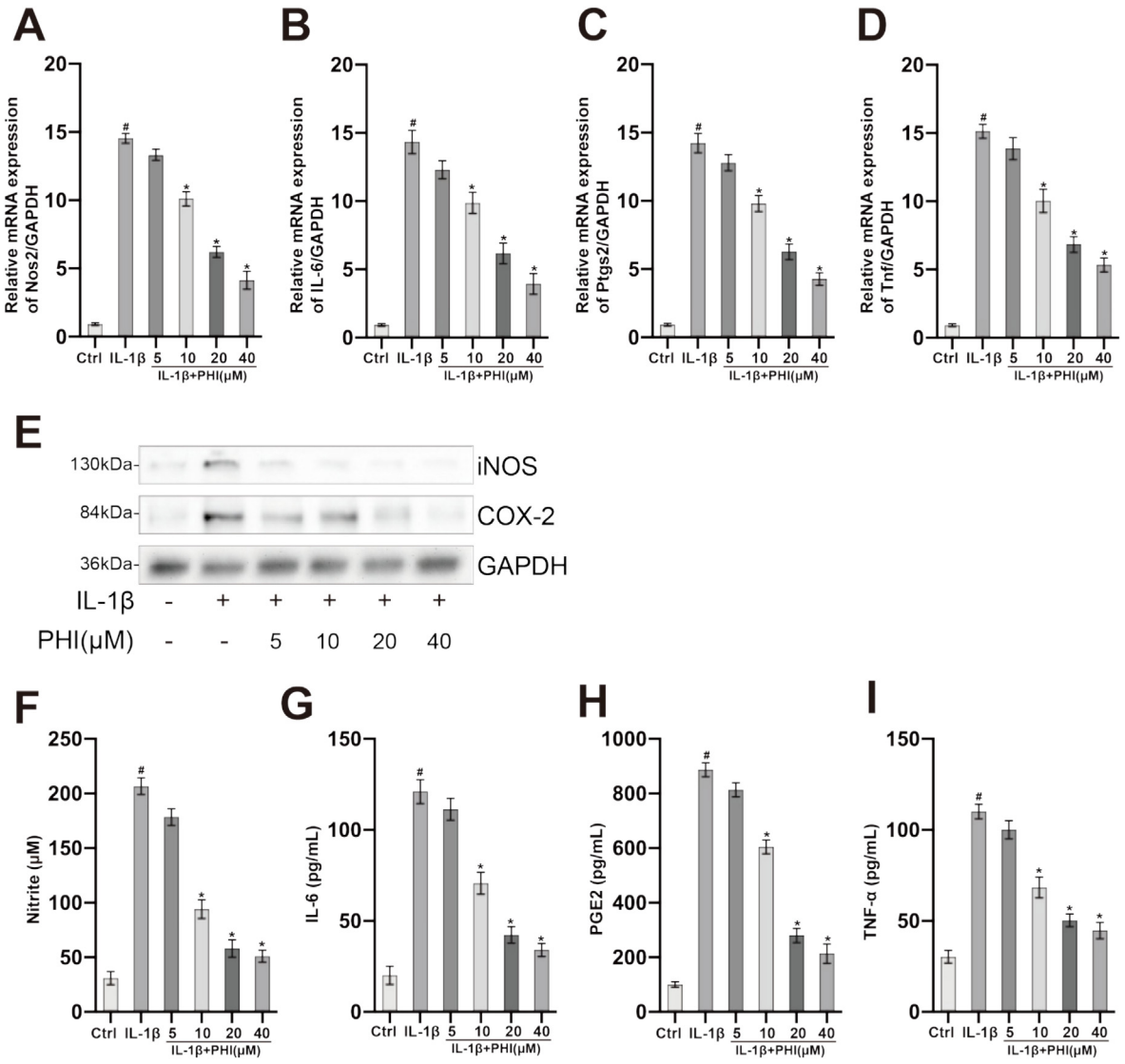

Figure 3. Effects of PHI on IL-1β-induced inflammation in primary murine chondrocyte. The mRNA expressions of Nos2, IL-6, Ptgs2, and Tnf by RT-PCR (A–D). Western blotting analysis of iNOS and COX-2 (E). The Griess reaction evaluated the level of nitrite in the medium (F). ELISA results of IL-6, PGE2, and TNF-α (G–I). The values are presented as mean ± S.D. Significant differences among different groups are indicated as $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$ vs. IL-1β group, n = 5.

levels of IL-6, PEG2, and TNF-α were measured via an ELISA kit (Fig. 3G–I). The data revealed that IL-1β induced upregulation of NO, IL-6, PEG2, and TNF-α, which was inhibited by PHI in a dose-dependent manner. Thus, our data unveiled that PHI could downregulate the IL-1β-stimulated expressions of the inflammatory cytokines in chondrocytes.

*3.4. PHI suppressed the **extracellular matrix** (ECM) degradation in IL-1β treated chondrocytes*

The effect of PHI on ECM metabolism was investigated. The western blotting results showed that IL-1β downregulated the production of Collagen II and Aggrecan, while upregulated ADAMTS5 and MMP-13. In contrast, PHI treatment concentration-dependently reversed the changes (Fig. 4A–E). Similar results were obtained for the immunofluorescence findings of Collagen II and MMP-13 (Fig. 4F and G, PHI 20 μM). Therefore, the findings suggested that PHI could suppress the IL-1β-triggered ECM degradation in chondrocytes.

3.5. PHI suppressed IL-1β-induced NF-κB pathway and activated Nrf2 pathway in chondrocytes

The effect of PHI on NF-κB pathway in primary mouse chondrocytes was explored. The western blotting results (Fig. 5A–C) showed that IL-1β dramatically promoted degradation of cytosol IκBα and upregulated the expression of nuclear p65. Nevertheless, PHI treatment significantly suppressed the above changes in a dose-dependent manner. The findings implied that PHI could inhibit the NF-κB pathway activated by IL-1β in mouse chondrocytes. In addition, a myriad of studies have focused on the importance of Nrf2 pathway on OA previously [25]. In this study, there were no significant changes in Nrf2 and HO-1 expressions under IL-1β stimulation in mouse chondrocytes, while PHI increased the Nrf2 nuclear translocation and HO-1 expressions in the cytoplasm (Fig. 5D–F). Besides, HO-1 immunofluorescence results (Fig. 5G, PHI 20 μM) showed no obvious change after IL-1β stimulation. However, it could be observed that HO-1 appeared high expression level in the cytoplasm, which was consistent with the western blotting results. The findings unveiled that PHI could boost Nrf2 signaling in mouse chondrocytes.

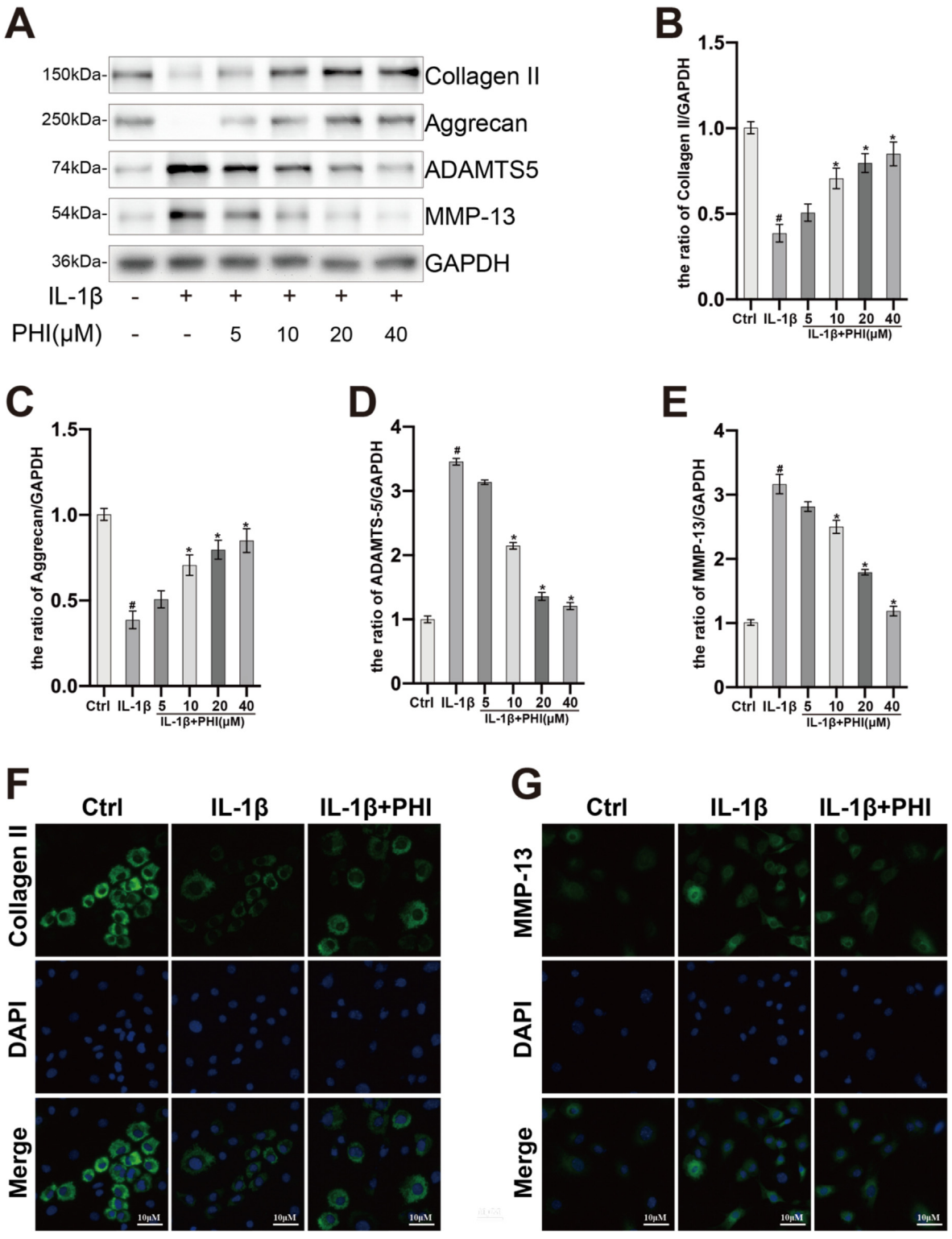

Figure 4. Effects of PHI on IL-1β-induced ECM degradation in primary murine chondrocytes. Western blotting (A) and quantitative analysis of protein expressions (B–E) of Collagen II, Aggrecan, ADAMTS5, and MMP13. Immunofluorescence analysis of Collagen II (F) and MMP13 (G). The values are presented as mean ± S.D. Significant differences among different groups are indicated as $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$ vs. IL-1β group, n = 5.

3.6. Nrf2 mediated the inhibition of NF-κB by PHI in chondrocytes

To examine whether Nrf2 could activate the NF-κB signaling, shRNA was employed to knock down the expression of Nrf2 in chondrocytes ("sh-Nrf2"), and control cells were transduced with lentiviral scramble control shRNA ("sh-C″). The western blotting results (Fig. 6A–D) revealed that the productions of nuclear Nrf2 and cytosol HO-1 were significantly suppressed by sh-Nrf2 transfection. In contrast, the p65 nuclear expression was dramatically upregulated following sh-Nrf2 transfection. Then, mitochondrial depolarization was examined and the

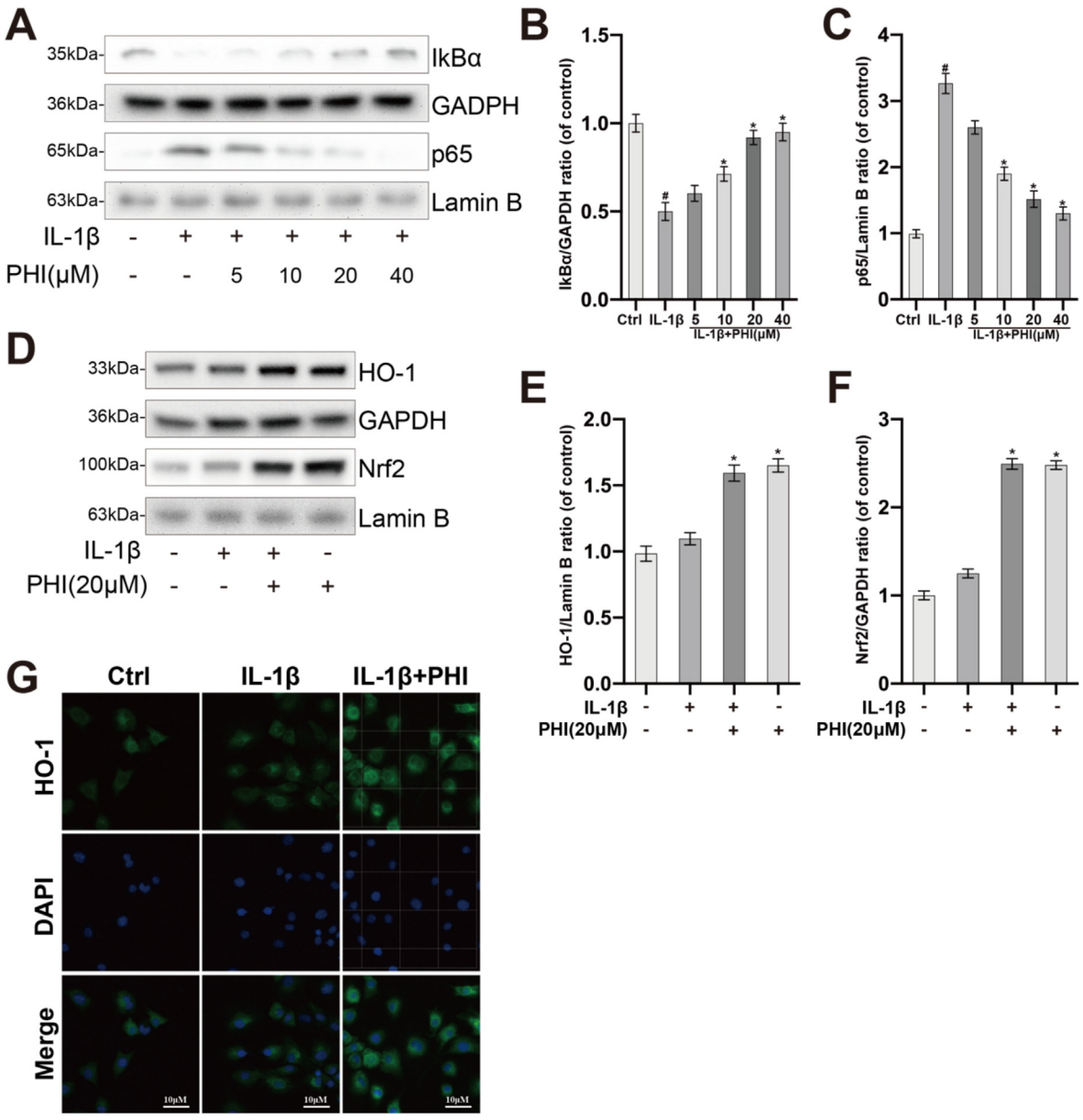

Figure 5. Effects of PHI on the NF-κB and Nrf2 pathways in chondrocytes. Primary murine chondrocytes were treated with the indicated concentration of PHI. Western blotting analysis of cytoplasmic IκBα and nucleic p65 in chondrocytes (A). Quantitative analysis the protein levels of IκBα and p65 (B, C). Western blotting analysis of cytosol HO-1 and nuclear Nrf2 in chondrocytes (D). Quantitative analysis the protein levels of HO-1 and Nrf2 (E, F). Immunofluorescence analysis of HO-1 (G). The values are presented as mean ± S.D. Significant differences among different groups are indicated as $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$ vs. IL-1β group, n = 5.

results (Fig. 6E and F) showed that PHI inhibited IL-1β-induced mitochondrial depolarization, while this phenomenon was offset by sh-Nrf2 transfection. Taken together, these results proved that Nrf2 mediated the PHI-induced inhibition of NF-κB signaling.

3.7. PHI attenuated OA development and boosted Nrf2 in the mouse DMM model

To explore whether PHI has protective effect on OA progression *in vivo* experiments, OA mouse model through DMM was established, and the mice were intragastrically administered a vehicle (0.5% carboxymethylcellulose alone) or 20 mg/kg PHI in 0.5% carboxymethylcellulose daily for 8 consecutive weeks. Histological analysis of OA was performed by HE and safranin O staining (Fig. 7A). The DMM group demonstrated severe cartilage erosion and fewer proteoglycan compared with the sham group. However, in the DMM + PHI group, the cartilage surface was smoother, and the proteoglycan was more than that in the DMM group. Consistent with the staining results, the DMM group showed higher OARSI scores than that of the sham group; while the OARSI scores were lower in DMM + PHI group comparing with the DMM group (Fig. 7B). Besides, immunohistochemical staining in Fig. 7C–E demonstrated increased expression of p65 in the DMM group than in the sham group, whereas the p65 was downregulated in the DMM + PHI group. In addition, the DMM + PHI group had dramatically higher Nrf2 expression and lower p65 expression compared to that in the DMM group (Fig. 7C–E). Further, Nrf2 in the cartilage were measured by

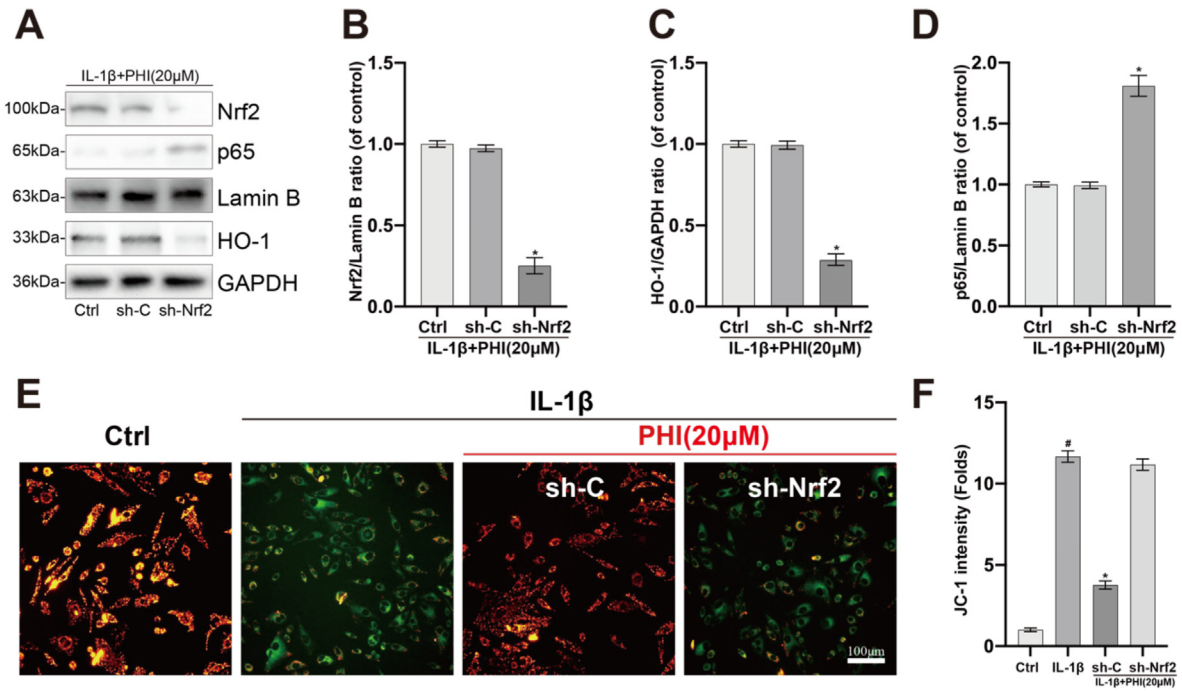

Figure 6. Nrf2 pathway was involved in the cytoprotection of PHI against IL-1β. Stable primary murine chondrocytes with the indicated Nrf2 shRNA (sh-Nrf2) and control cells with scramble control shRNA (sh-C) were established and cultured, and the expression of the indicated proteins was measured (A–D). Mitochondrial depolarization was examined (E, F). The values are presented as mean ± S.D. Significant differences among different groups are indicated as $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$ vs. IL-1β group, n = 5.

immunofluorescence staining (Fig. 7F and G), which showed that the DMM + PHI group exhibited significantly higher levels of Nrf2 in chondrocyte nucleus compared to that in the DMM group. Taken together, the results verified that PHI relieved OA development via Nrf2/NF-κB pathway. The schematic of the chondroprotective effect of PHI via the Nrf2/NF-κB pathway was depicted in Fig. 8.

4. Discussion

OA is a major cause of pain and disability around the globe, with unclear etiology and unavailable effective treatment currently [26,27]. Growing evidence has highlighted the involvement of several inflammatory factors in OA progression [28,29]. Although non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in clinic to ameliorate symptoms, they cannot prevent OA progression, and they are prone to cause several side effects [30,31]. Hence, safer and more effective agents to treat OA are urgently required. In this research, the data implied that PHI could inhibit NF-κB signaling via boosting Nrf2 to reverse the IL-1β-induced inflammation and ECM degradation in chondrocytes and ameliorate OA development in mouse models.

It is well accepted that the NF-κB signaling pathway regulates IL-1β-stimulated pro-inflammatory and catabolic factors and plays a prominent role in OA development [32–34]. Under normal conditions, p65 binds to IκBα in cytoplasm, making the NF-κB pathway inactive. Once induced by IL-1β, IκBα is phosphorylated and degraded, while p65 translocates to the nucleus, where p65 promotes the production of pro-inflammatory and catabolic mediators, such as PGE2, iNOS, COX-2, TNF-α and IL-6 [35]. NO, generated by the iNOS, upregulates the secretion of MMPs and inhibits the synthesis of Collagen II and Aggrecan, thus leading to ECM degradation [36]. COX-2 promotes the synthesis of PGE2, which enhances the expressions of MMPs and ADAMTS5 and contributes to ECM degradation [37,38]. Collagen II and Aggrecan are the major structural constituents of the ECM [39]. In MMPs family, MMP13 is a key mediator in Collagen II degradation, and ADAMTS5 is the major aggrecanase responsible for the cleavage of Aggrecan. Therefore, targeting ADAMTS5 and MMP13 could be a promising strategy for OA therapy.

In this research, our data indicated that PHI dramatically ameliorated the overproduction of NO, PGE2, IL-6, TNF-α, iNOS and COX-2. Besides, PHI ameliorated IL-1β-elicited inflammation by inhibiting NF-κB signaling in chondrocyte, which supported the recent study of the anti-inflammatory mechanism of PHI in RAW 264.7 macrophage cells by Deng et al. [40]. Furthermore, PHI mitigated the generation of MMP-13 and ADAMT5 and suppressed the breakdown of Aggrecan and Collagen II in chondrocytes. These findings suggested that PHI has the potential to treat OA.

Emerging evidence has implied that Nrf2 pathway plays a key role in maintaining cellular redox homeostasis [8,9], and its dysfunction has been shown to increase susceptibility to inflammatory disorders. In this study, the expressions of Nrf2 and HO-1 were dose-dependently upregulated by PHI in IL-1β-stimulated chondrocytes. Furthermore, in line with the previous researches showing that Nrf2 activation could suppress NF-κB [41], our data revealed that sh-Nrf2 offset the inhibitory effect of p65 nuclear translocation under the induction of PHI. Collectively, the findings indicated that the inhibitory effects of PHI on inflammatory mediators in IL-1β-stimulated chondrocytes was mediated by activating Nrf2 and inhibiting NF-κB signaling pathway.

Our DMM mouse model showed cartilage erosion, chondrocyte loss, and ECM degradation, however, these changes were improved significantly after PHI treatment, as evidenced by histological staining analysis and decreased OARSI scores. In addition, PHI mitigated OA progression via promoting the Nrf2 translocation to the nucleus, which was in line with the *in vitro* results. To sum up, this research indicated that PHI could be a potentially effective drug to treat OA.

5. Conclusion

In the current study, we revealed that PHI exerted cytoprotective effects against inflammation and ECM degradation in primary murine

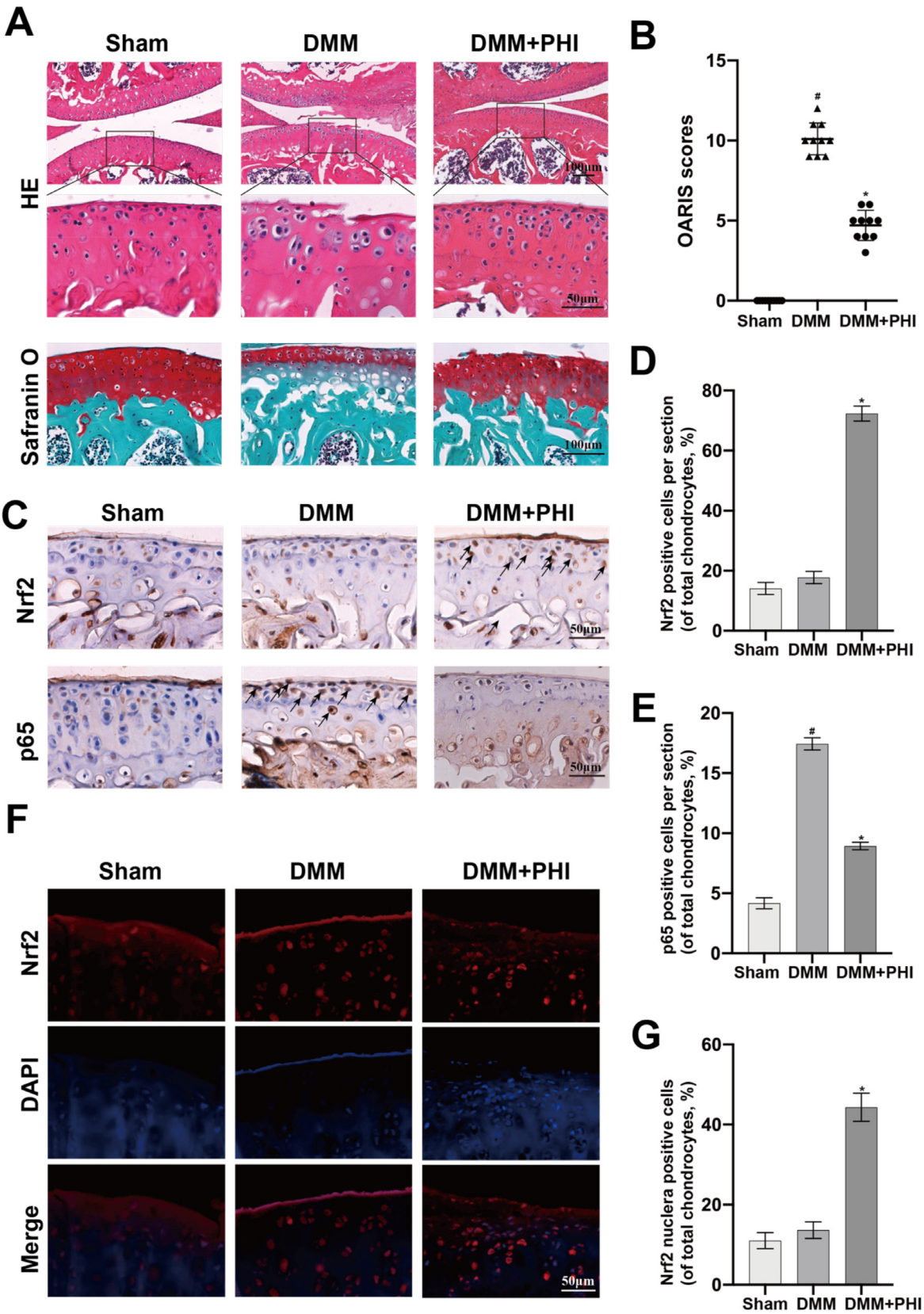

Figure 7. PHI ameliorated OA progression in the DMM mouse model. Histological analysis of OA was evaluated by HE staining and safranin O staining (A). Osteoarthritis Research Society International (OARSI) scores were calculated in the different experimental groups (B). The expression of Nrf2 and p65 in cartilage samples were examined by immunohistochemical staining (C). Quantitative analysis of Nrf2 and p65 positive expression in sections (D, E). Immunofluorescence analysis of Nrf2 (F, G). The values presented are the means ± SD. $^{\#}p < 0.05$ vs. the Sham group. $^{*}p < 0.05$ vs. the DMM group. Experiments were repeated three times, and similar results were obtained.

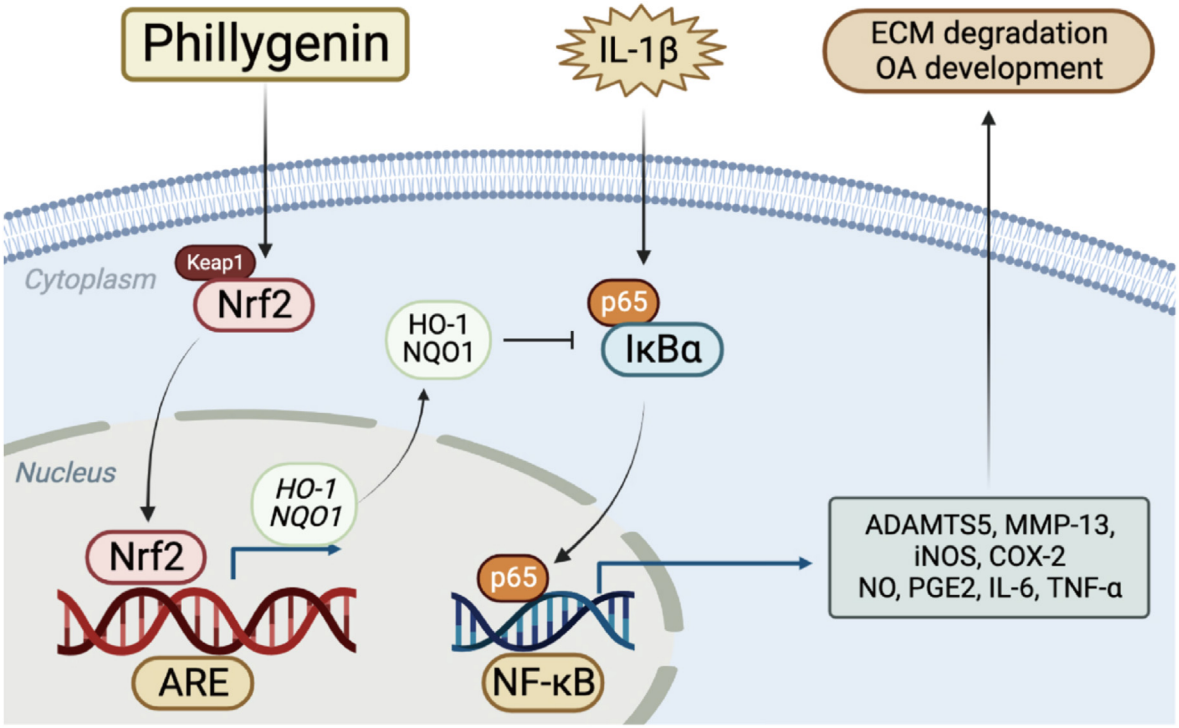

Figure 8. PHI protected chondrocytes against IL-1β-induced inflammation and ECM degradation by activating Nrf2 and inhibiting NF-κB pathway.

chondrocytes. Mechanically, PHI could inhibit NF-κB signaling via boosting Nrf2. Furthermore, PHI attenuated OA development in DMM mouse models (Fig. 8). Therefore, the results indicated that PHI might be a promising therapy for OA.

Credit author statement

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Ethics statement

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Animal Ethics Committee of the Second Affiliated Hospital of Soochow University.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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