Redox Biology 28 (2020) 101306

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Advanced oxidation protein products increase TNF-α and IL-1β expression in chondrocytes via NADPH oxidase 4 and accelerate cartilage degeneration in osteoarthritis progression

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ARTICLE INFO

Keywords:
Advanced oxidation protein products
Chondrocytes
TNF-α
IL-1β
NADPH oxidase 4
Osteoarthritis

ABSTRACT

Interleukin (IL)-1β and tumor necrosis factor (TNF)-α, in particular, control the degeneration of articular cartilage, making them prime targets for osteoarthritis (OA) therapeutic strategies. Advanced oxidation protein products (AOPPs) are prevalent in numerous diseases. Our previous work demonstrates that intra-articular injections of AOPPs accelerate regression of cartilage in OA models. Whether AOPPs exist in the course of OA and their effects on TNF-α and IL-1β expression in chondrocytes are still unclear. This study confirmed that AOPPs levels in human synovial fluid were positively associated with severity of OA. We also found AOPPs deposition in articular cartilage in anterior cruciate ligament transection (ACLT) induced rodent OA models. AOPPs increased expression of TNF-α and IL-1β in chondrocytes in vitro, which was inhibited by pre-treatment with SB202190 (p38-MAPK inhibitor) or apocynin (NADPH oxidase inhibitor) or NOX4 knockdown by siRNAs. Subsequently, we further verified in vivo that exogenous injection of AOPPs in OA mice up-regulated expression of TNF-α and IL-1β in cartilage, which was blocked by treatment with apocynin. In parallel, apocynin attenuated articular cartilage degeneration resulting in substantially lower OARSI scores. Specifically, apocynin reduced NOX4, p-P38, TNF-α and IL-1β and increased collagen II and glycosaminoglycan (GAG). This study demonstrated that AOPPs increased expression of TNF-α and IL-1β in chondrocytes via the NADPH oxidase4-dependent and p38-MAPK mediated pathway, and accelerated cartilage degeneration in OA progression. These findings suggest an endogenous pathogenic role of AOPPs in OA progression. Targeting AOPPs-triggered cellular mechanisms might be a promising therapeutic option for patients with OA.

1. Introduction

Osteoarthritis (OA) is a disease of joint degeneration characterized by articular cartilage loss, often leading to pain, joint stiffness, and disability. It has been recognized as a major cause for disability in elderly populations, incurring huge socioeconomic costs [1]. However, there are currently no effective disease-modifying therapies for it, and existing symptomatic treatment options are limited with undesirable side effects [2]. Lack of disease-modifying OA drugs results in progressive cartilage damage that eventually necessitates surgical intervention. Better understanding of OA pathogenesis and its molecular pathways is therefore crucial for identifying novel therapeutic targets [3].

Inflammation is believed to get involved in the development and progression of OA even in the early stages of the disease [4]. Inflammatory cytokines, like TNF-α, IL-1β, IL-6, IL-17 and IL-18, were generally recognized as the most important compounds controlling the progression of OA. It has been reported that inflammatory cytokines

Abbreviations: OA, osteoarthritis; AOPPs, Advanced oxidation protein products; NADPH, nicotinamide adenine dinucleotide phosphate; IL-1β, Interleukin (IL)-1β; TNF-α, tumor necrosis factor (TNF)-α

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https://doi.org/10.1016/j.redox.2019.101306
Received 20 February 2019; Received in revised form 18 August 2019; Accepted 20 August 2019
Available online 22 August 2019

have a destructive effect on cartilage by inhibiting anabolic activities of chondrocytes [5,6], activated chondrocytes to produce matrix metalloproteinase (MMPs), especially MMP-13 (a key regulator of cartilage destruction) [7]. Apart from their destructive effects, inflammatory cytokines also tend to induce apoptosis of chondrocytes [8,9]. TNF-α and IL-1β are considered as the key cytokines leading to pathogenesis of OA. Levels of TNF-α and IL-1β are remarkably increased in OA patients compared to healthy individuals [10]. The two cytokines, produced partly by chondrocytes in an OA joint, induce production of a number of inflammatory and catabolic factors [6,7]. Therefore, compounds that regulate cytokine synthesis might be favorable targets for OA therapy.

Advanced oxidation protein products (AOPPs) are a family of dityrosine-containing protein products forming during excessive production of reactive oxygen species (ROS). They are newly considered as a biomarker of oxidative stress, and believed to be involved in oxidation-associated diseases [11]. Recent studies have concluded that OA progression is closely associated with oxidative stress and ROS [12,13]. Our previous work demonstrates that intra-articular injections of AOPPs (exogenous injection) accelerate regression of cartilage in rabbit OA models [14]. We also confirmed that AOPPs were able to activate the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-mediated oxidative stress pathway to induce apoptosis of chondrocytes and upregulate the MMP-13 expression [15,16].

The pathogenic role and mechanism of AOPPs in OA disease need further research on the basis of previous extensive efforts into the complex disease before they can be completely elucidated. Specifically, the presence of AOPPs in the course of OA and their effects on TNF-α and IL-1β expression in chondrocytes have not been reported. This study was to investigate whether AOPPs exist in the course of OA and further illustrated whether AOPPs stimulate IL-1β and TNF-α expression in chondrocytes and the underlying molecular mechanisms as well.

2. Materials and methods

2.1. Preparation of AOPPs-MSA

As previously described, AOPPs-MSA were prepared with minor modifications. In brief, 20 mg/mL of mouse serum albumin (MSA, sigma, St.Louis, MO, USA) was added to 40 mM of HOCl for 30 min at room temperature and dialyzed for 24 h against PBS at 4 °C to remove free HOCl. Control incubation was performed in native MSA dissolved in PBS alone [17].

2.2. Human synovial fluid collection and preparation

Human synovial fluid samples were obtained from the knee joints of 68 Asian patients aged 14–86 years (41 female and 28 male Asians; mean (S.D.) age 56 [19] years) who underwent arthroscopic operation or total knee replacement at Nanfang Hospital, Southern Medical University, Guangzhou, China. All samples gained informed consent from the patients and approval by the ethic committee of Nanfang Hospital, Southern Medical University (NFEC-2015-014). All the experiments described were carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Kellgren-Lawrence Classification Scale for Osteoarthritis Severity (Table 2) was applied to access severity of knee osteoarthritis [18].

2.3. Animal and experimental design

30C57bl/6 mice (initial weight 18–20g, Animal Experiment Center, Southern Medical University, Guangzhou, China) were used in this study. All the mice were housed under a regulate light/dark cycle with food and water available ad libitum. They were acclimated for 7 days before any experimental procedures. All the animal experimental procedures were approved by the Laboratory Animal Care and Use Committee of Nanfang Hospital, Southern Medical University (NFYY-2015-68).

Table 1
Demographic data of the patients and the relative statistics.

			AOPPs(μmol/L)	Correlation p value
Age (yrs)		56.246 ± 19.073		
		N		
Sex	Male	28 (41%)		
	Female	41 (59%)		
K-L stage				
	0	15 (22%)	25.830 ± 12.443	
	I	6 (8%)	19.280 ± 2.657	
	II	29 (42%)	41.462 ± 8.970	
	III	15 (22%)	35.937 ± 10.818	
	IV	4 (6%)	44.100 ± 5.801	
Total		69	34.154 ± 12.763	< 0.001

Table 2
Kellgren-Lawrence classification of osteoarthritis.

Kellgren-Lawrence Classification Scale for Osteoarthritis Severity	
Grade	Description
0	No radiographic features of osteoarthritis
I	Possible joint space narrowing (normal joint space is at least 2 mm at the superior acetabulum) and osteophyte formation
II	Define osteophyte formation with possible joint space narrowing
III	Multiple osteophytes, define joint space narrowing, sclerosis and possible bony deformity
IV	Large osteophytes, marked joint space narrowing, severe sclerosis and definite bony deformity

Osteoarthritis mice models were established by knee anterior cruciate ligament transaction (ACLT). Mice were randomized into five groups: Group 1, sham-operated; Group 2, ACLT-operated and treated with PBS (pH = 7.4); Group 3, ACLT-operated and treated with AOPPs (50 mg/kg/d); Group 4, intraperitoneal injection of AOPPs-MSA (50 mg/kg/d) and intragastric administration of apocynin (a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, Sigma, USA) at 100 mg/kg/d dissolved in Tween-80; Group 5, intragastric administration of apocynin at 50 mg/kg/d dissolved in Tween-80.

2.4. Histological analysis

Following assessment of macroscopic changes, samples were decalcified for at least four weeks with Ethylene Diamine Tetraacetic Acid (EDTA). Samples were then dehydrated, paraffin embedded, sectioned to 4 μm. Then the slices were stained to assess cell morphology and matrix content via Safranin O-fast green and H&E staining. Slices were scored using the Osteoarthritis Research Society International (OARSI)-modified Mankin criteria by three blinded reviewers, and the scores were averaged across reviewers [19]. Alcian blue staining was used to evaluate the content of glycosaminoglycan (GAG) in cartilage.

2.5. Immunohistochemical staining

Paraffin-embedded cartilage issue was sliced into 4 μm-thick transverse sections. After treated with 0.3% hydrogen peroxide to reduce endogenous peroxidase activity, non-specific staining was blocked by incubation of the sections with 10% normal goat serum for 60 min. Then deparaffinized slices were incubated with primary antibodies against IL-1β (1:200 Abcam Cambridge, UK, Ab9722), TNF-α (1:100 Abcam Cambridge, UK, Ab6671), Type II collagen (1:50 Proteintech, China, 15,943-1-AP), NOX1 (1:500 Abcam Cambridge, UK, Ab131088), NOX2 (1:100 Abcam Cambridge, UK, Ab80508), NOX4 (1:200 Abcam Cambridge, UK, Ab154244), phospho-JNK (1:400, Cell Signaling Technology, Beverly, MA, USA, #4370), phospho-p38 (1:400, Cell

Signaling Technology, Beverly, MA, USA, #4511), and phosphor-ERK1/2 (1:50, Cell Signaling Technology, Beverly, MA, USA, #4668) at 4 °C overnight. The immunostaining was examined with a Leica DM5000B (Leica, Germany).

2.6. Immunofluorescence staining

Deparaffinized slices were blocked in 5% bovine serum albumin in PBS for 1 h at room temperature and then incubated overnight at 4 °C with the mouse anti-AOPPs (1:200, Department of immunology, Southern Medical University) overnight. After washing and incubated with FITC-conjugated anti-mouse Ig-G (1:100, Beyotime, China) for 1 h, the slices were stained by DAPI (Abcam, Cambridge, UK). Reacted slides were mounted and cover slipped. Images were captured with an Olympus FluoView FV10i self-contained confocal laser scanning microscope system (Olympus America Inc., PA, USA).

2.7. Mouse chondrocytes culture

Mouse chondrocytes were cultured according to our previous described procedure [15]. Briefly, we aseptically isolated fresh cartilage from both knee joints of newborn C57bl/6 mice. Then we minced and digested it in 0.25% trypsin (Gibco, Life Technologies, CA, USA) for 20 min. The remain tissues were further digested in a solution of 0.2% collagenase II (Sigma, St Louis, MO, USA) in DMEM/F12 (Gibco, Life Technologies, CA, USA) at 37 °C for 5 h then centrifuged at 1000 r/min for 5 min. The released chondrocytes were resuspended in DMEM containing 10% FBS (Gibco, Life Technologies, CA, USA) and antibiotics (100 IU/ml penicillin, 100 IU/ml streptomycin, Gibco, Life Technologies, CA, USA), then seeded onto a 25 cm^2 culture flask at 37 °C in a humidified atmosphere of 5% CO_2–95% air. Up to approximately 80–90% confluency, chondrocytes were passaged at a ratio of 1:3. To avoid the phenotype loss, only passage 1 chondrocytes were used in the study.

2.8. Small interfering RNA (siRNA) transfection

Nox4 siRNA, NC siRNA and scramble siRNA were purchased from Genomeditech (Shanghai, China). The sequence of Nox4 siRNA was UGGUAUUGUUCCUCAUGGU (5′-3′), NC siRNA was UUCUCCGAACG UGUCACGU (5′-3′)and scramble siRNA was GCCATTTCGTGTTAGT GTT. To introduce siRNA into mouse chondrocytes, the cells were plated on 6-well plates at 70% confluence before transfection. Lipofectamine 3000,siRNA and Opti-MEM were mixed and incubated at room temperature for 20 min. Lipofectamine 3000-siRNA complexes were added to cells for 24 h and the medium was replaced by fresh serum DMEM medium after transfection. Experiments were performed 24 h after transfection. Knockdown of Nox4 was assessed by western blot.

2.9. Western blot analysis

Cultured cells were homogenized in ice-cold RIPA buffer with 1 mM PMSF, protease, and phosphatase inhibitors and cleared by centrifugation (12,000 rpm, 4 °C, 10 min). The protein concentration and the supernatant were determined using the BCA Protein Assay Kit. The samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 8%–15% acrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked using 5% blocking buffer (5% MSA in Tris-buffered saline with 0.1% Tween 20) for 1 h at room temperature. The following primary antibodies were used to incubate with: rabbit anti-TNF-α (1:1000, Abcam Cambridge, UK, Ab6671), rabbit anti-IL-1β (1:1000, Abcam Cambridge, UK, Ab9722), MAPK Family Antibody Sampler Kit (1:1000, Cell Signaling Technology, Beverly, MA, USA, #8690, #4695, #9252), Phospho-MAPK Family Antibody Sampler Kit (1:1000, Cell Signaling Technology, Beverly, MA, USA, #4511, #4370, #4668) and rabbit anti-GAPDH (1:5000, Bioworld, China, AP0063). The membrane was washed 3 times with TBST for 10 min and followed by incubation with goat anti-rabbit and rabbit anti-goat IgG-horseradish peroxidase (HRP) secondary antibodies from Abcam (Abcam, Cambridge, UK). for 1 h and then washed again 3 times with TBST. Relative levels of immunoreactivity were quantified using the Kodak In-vivo Imaging System (Kodak, NY, USA).

2.10. Statistical analysis

All experiments were repeated at least three times and the data are expressed as the mean ± SD. All statistical analyses were performed using Statistical Packages for Social Sciences v13.0 software (SPSS, Chicago, IL). Continuous variables were expressed as mean ± standard deviation (SD). Comparisons were analyzed with student t-test (2 groups only) or ANOVA followed by the Bonferroni post hoc tests analysis. Spearman correlation analysis was used to assess the relationship between AOPPs levels and K-L assessment. Differences with $P \leq 0.05$ were considered significant.

3. Results

3.1. AOPPs accumulation in human synovial fluid was positively correlated to OA severity

To clarify the correlation between osteoarthritis and AOPPs accumulation, we collected knee synovial fluid separately from 68 patients undergoing arthroscopy or total knee replacement (Table 1). We determined the concentration of AOPPs by chloramines T method [20]. Assessment of knee osteoarthritis severity was done by two independent clinicians with Kallgren-Lawrence grading scale (Fig. 1A). Correlation analysis showed a moderate correlation between AOPPs concentration in knee synovial fluid and knee osteoarthritis severity (rs = 0.5499, $p < 0.001$) (Fig. 1B).

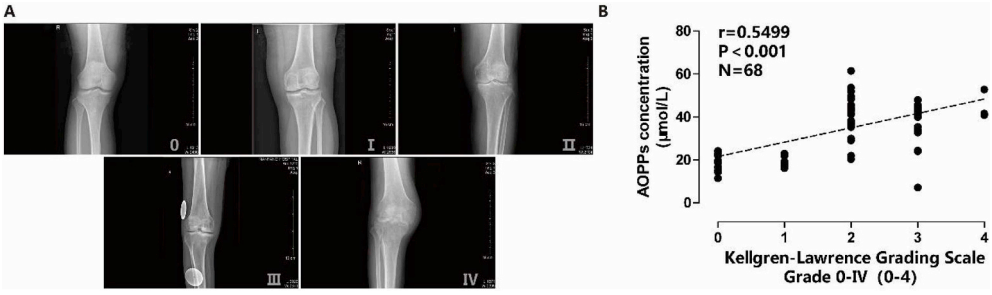

Fig. 1. (A) The representative image of various Kellgren-Lawrence grading scale. (B) Spearman correlation analysis result showed that the synovial fluid AOPPs concentration was in positive correlation with the Kellgren-Lawrence score grades (rs = 0.5499, 95% confidence interval 0.3521 to 0.7006). P (two-tailed) < 0.0001.

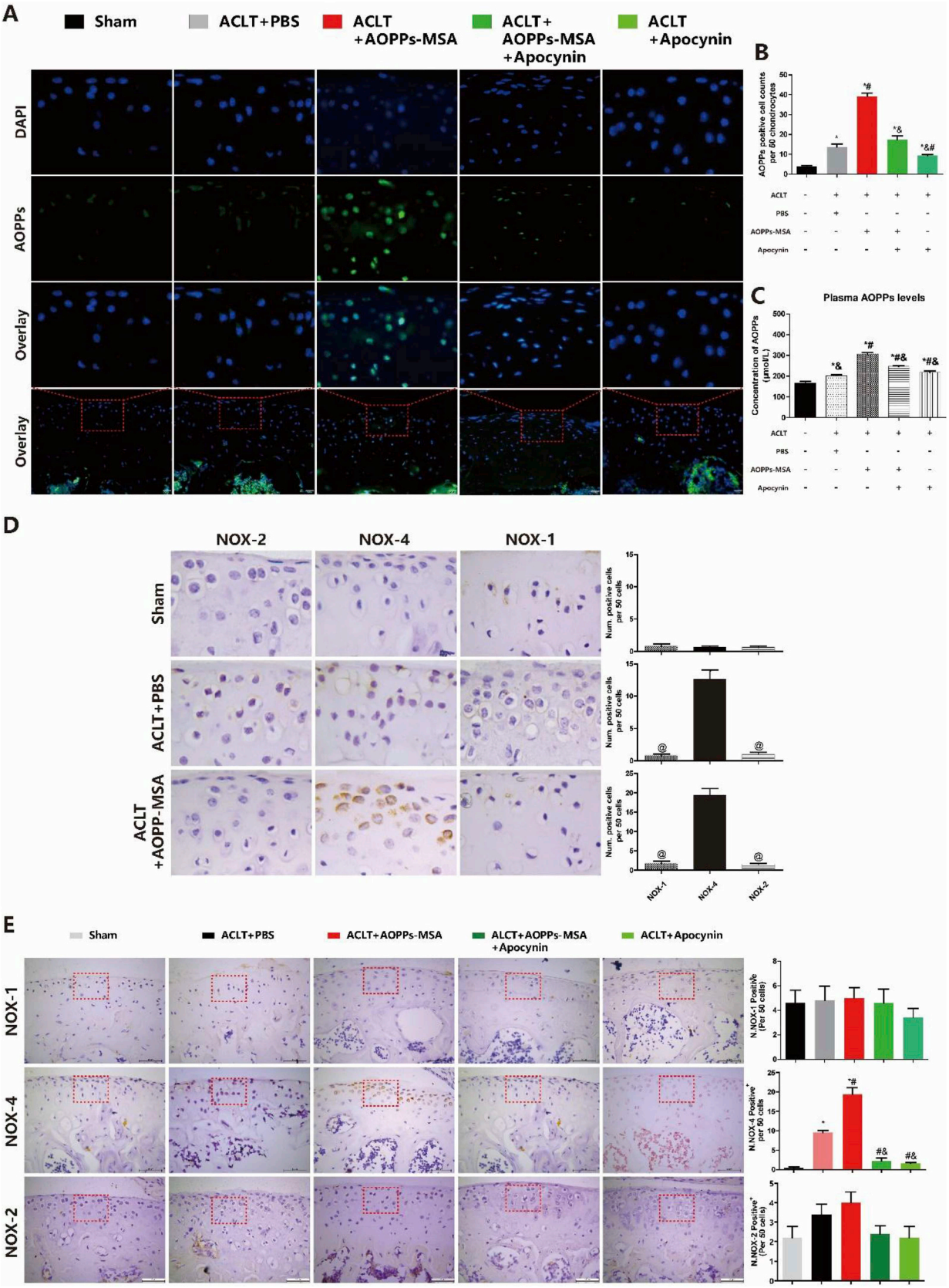

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Fig. 2. Chronic AOPPs overload rose NOX 4 expression in knee cartilage in ACLT induced osteoarthritic mice. (A) The expression of AOPPs in the chondrocytes was detected by immunofluorescence at 60 days post operation. Positive cell stain green while DAPI was blue. (B) Expression level of AOPPs was conducted. The ratio of AOPPs positive cells increased with ACLT and significantly increased with intraperitoneally injection of AOPPs (50 mg/kg). Meanwhile, intragastric administration of antioxidant apocynin (50 mg/kg) decreased the production of AOPPs. (C) The concentration of AOPPs in plasma was detected by chloramine-T method at 60 days post operation. Data represent mean ± SEM of at least 3 independent experiments. N = 6 per group. (D) Immunohistochemical staining of NOX1, NOX2 and NOX4 at 60 days post operation. The expression of NOX1, NOX2 and NOX4 in cartilage was pretty low in sham control group, and there was no significantly difference between them. (E) Immunohistochemical staining of NOX1, NOX4 and NOX2 expression in each group at 60 days post operation. ACLT increased NOX4 expression alone. ACLT + AOPPs increased more expression of NOX4. apocynin treatment significantly decreased the expression of NOX4.
Sham = sham-surgery. ACLT + AOPPs-MSA = ACLT-surgery treated with AOPPs-MSA. ACLT + AOPPs-MSA + apocynin = ACLT-surgery treated with AOPPs-MSA and intragastric administration of apocynin. ACLT + Apocynin = ACLT-surgery treated with intragastric administration of apocynin. N = 6 per group. *P < 0.05 versus Sham. #P < 0.05 versus ACLT + PBS. &P < 0.05 versus ACLT + AOPPs-MSA. @P < 0.05 versus NOX-4.

3.2. AOPPs accumulated in OA mice cartilage

To further investigate whether AOPPs might exist in OA joint, we conducted immunofluorescence staining of AOPPs in cartilage. Accumulation of AOPPs was increased in cartilage in the ACLT groups relative to the sham-operated group; in addition, accumulation of AOPPs was significantly increased in cartilage in the AOPPs-treated ACLT group compared to the ACLT and sham-operated groups, and was decreased significantly compared with the AOPPs + apocynin ACLT group (Fig. 2A).

3.3. Plasma AOPPs increased in OA mice

To investigate whether ACLT-induced osteoarthritis would increase accumulation of AOPPs, we also examined the concentration of AOPPs in plasma. The plasma AOPPs concentration was 1.2-fold in the ACLT mice and 1.8-fold in the chronic AOPPs-treated ACLT-induced mice in comparison with the sham group while intragastric administration in the apocynin groups led to a lower plasma concentration of AOPPs (Fig. 2C).

3.4. AOPPs upregulated NOX4 expression in OA mice cartilage

To investigate expression of NOX in cartilage, we conducted immunohistochemical staining. The expression levels of NOX1, NOX2 and NOX4 in cartilage were pretty low in the sham control group, and there was no significant difference between them; expression of NOX4 was increased significantly compared with that of NOX1 and NOX2 in the ACLT group and AOPPs-treated ACLT group (Fig. 2D). Expression of NOX4 was significantly increased in the ACLT group compare to the sham-operated group. Furthermore, NOX4 expression was significantly increased in the AOPPs-treated ACLT group compare to the ACLT group, and dramatically enhanced relative to the sham-operated group. There was no significant difference in expression of NOX1 or NOX2 in any group (Fig. 2E).

3.5. AOPPs increased TNF-α and IL-1β expression via NADPH oxidase 4/p38-MAPK pathway in vitro

The effect of AOPPs on expression of TNF-α and IL-1β in primary mouse chondrocytes was examined by western blot. In this study, chondrocytes treated with AOPPs-MSA showed significantly increased expression of TNF-α and IL-1β in a dose and time-dependent manner compared to those incubated with medium alone and with native MSA (Fig. 3A and B). Next, we evaluated the MAPK signal pathway relative protein expression in the chondrocytes stimulated with AOPPs-MSA. As shown in Fig. 3C, AOPPs-MSA significantly increased JNK, ERK1/2 and p38 phosphorylation in chondrocytes in a time-dependent manner compared to chondrocytes incubated with medium alone and with native MSA. AOPPs-MSA-induced increased expression of TNF-α and IL-1β was significantly suppressed by pretreatment of DPI (NADPH oxidase inhibitor), apocynin (NADPH oxidase inhibitor) and p38 inhibitor SB202190, indicating that these effects were mediated by NADPH oxidase and p38-MAPK (Fig. 3D). To further clarify the role of NADPH oxidase 4 in AOPPs-triggered cascades in chondrocytes, we preincubated chondrocytes with NOX4-siRNA before AOPPs stimulation. Eventually, AOPPs-induced increased expression of TNF-α and IL-1β and activation of p38-MAPK were significantly suppressed by NOX4-siRNA (Fig. 3E and F).

3.6. AOPPs increased TNF-α and IL-1β expression and accelerates progression of OA via NADPH oxidase in vivo

In order to investigate the effects of AOPPs on expression of TNF-α and IL-1β and on progression of OA, we administered AOPPs intraperitoneally in mice after ACLT. The optimal dose (50 mg/kg body weight) was indicated by previous researches. Expression of TNF-α, IL-1β, Phosphorylated P38, Phosphorylated ERK and Phosphorylated JNK was increased in the AOPPs-treated ACLT group compare to the ACLT group, and was decreased in the AOPPs + apocynin ACLT group compared with the AOPPs-treated ACLT group (Fig. 4A and B). The content of GAG and collagen II was decreased in the AOPPs-treated ACLT group compare to the ACLT group, and was increased in the AOPPs + apocynin ACLT group compared with the AOPPs-treated ACLT group (Fig. 4C). OARSI scores were increased in the AOPPs-treated ACLT group compare to the ACLT group, and were decreased in the AOPPs + apocynin ACLT group compared with the AOPPs-treated ACLT group (Fig. 4C)

3.7. Apocynin (NADPH oxidase inhibitor) decreased TNF-α and IL-1β expression and attenuated progression of OA

Expression of TNF-α, IL-1β, Phosphorylated P38, Phosphorylated ERK and Phosphorylated JNK was increased the in ACLT group compare to the sham-operated group, and was decreased in the apocynin + ACLT group compared with the ACLT group (Fig. 4A and B). The content of GAG and collagen II was decreased in the ACLT group compare to the sham-operated group, and was increased in the apocynin + ACLT group compared with the ACLT group (Fig. 4C). OARSI scores were increased in the ACLT group compare to the sham-operated group, and was decreased in the apocynin + ACLT group compared with the ACLT group (Fig. 4C).

4. Discussion

After the presence of AOPPs in human synovial fluid was confirmed, this study demonstrated that the content of AOPPs was positively associated with severity of OA. In vivo experiment found deposition of AOPPs in articular cartilage of OA mice. To verify whether AOPPs might regulate production of inflammatory factors in OA, this study demonstrated that AOPPs enhanced production of TNF-α and IL-1β in chondrocytes via the NADPH oxidase4-dependent, p38-MAPK mediated pathway. In addition, targeting at NADPH oxidase 4 activated by AOPPs in OA reduced NOX4, p-P38, TNF-α and IL-1β but increased collagen II and GAG in cartilage, ultimately resulting in substantially lower OARSI scores.

NADPH oxidase family enzymes are transmembrane proteins. There are seven NOX/Duox enzyme family members: NOX1-5, Duox1, and Duox2. NOX/Duox-isoform function is directly linked to the enzyme's structure conformation [21]. In contrast to all other isoforms, Nox4 is constitutively active and does not depend on cytosolic activator

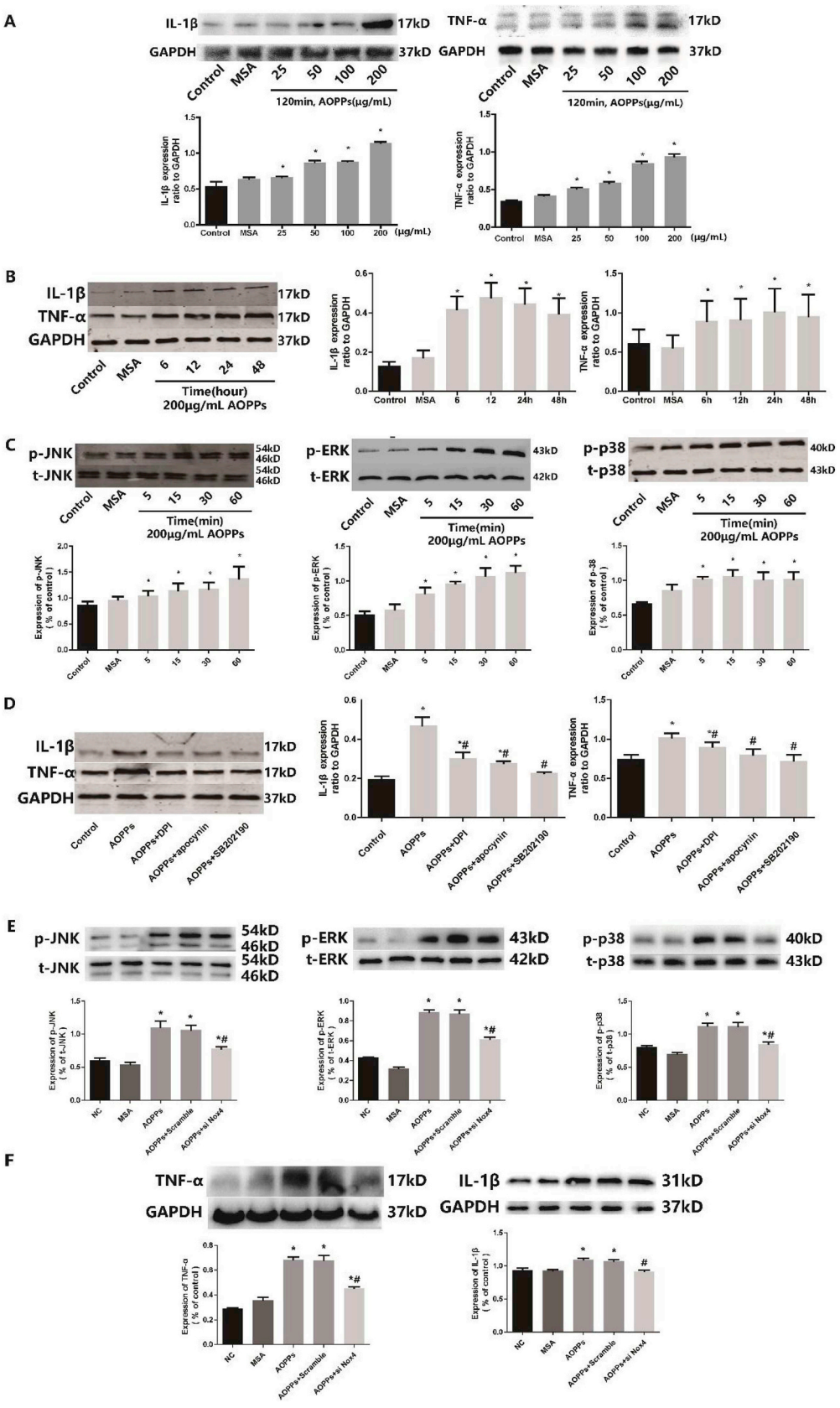

Fig. 3. AOPPs increased TNF-α and IL-1β expression in chondrocytes via NOX4/p38-MAPK pathway in vitro. (A)Expression of TNF-α and IL-1β in chondrocytes treated with AOPPs (0–200 μg/mL, 120min) induced TNF-α and IL-1β production in a concentration-dependent manner. (B) AOPPs (0–48 h, 200 μg/mL) induced TNF-α and IL-1β production in a time-dependent manner. (C)AOPPs significantly increased JNK, ERK1/2 and p38 phosphorylation of chondrocytes in a concentration-dependent manner. (D) Diphenyle neiodonium chloride (DPI, 10 μM), apocynin (100 μM) and p38 inhibitor SB202190 (10 μM) significantly decreased AOPPs-induced expression of TNF-α and IL-1β. (E) AOPPs–induced JNK, ERK1/2 and p38 phosphorylation was blocked by siNox4 pretreatment (F) TNF-α and IL-1β were decreased by siNox4 pretreatment compared with AOPPs treatment.
*P < 0.05 versus Control or NC.
#P < 0.05 versus AOPPs.

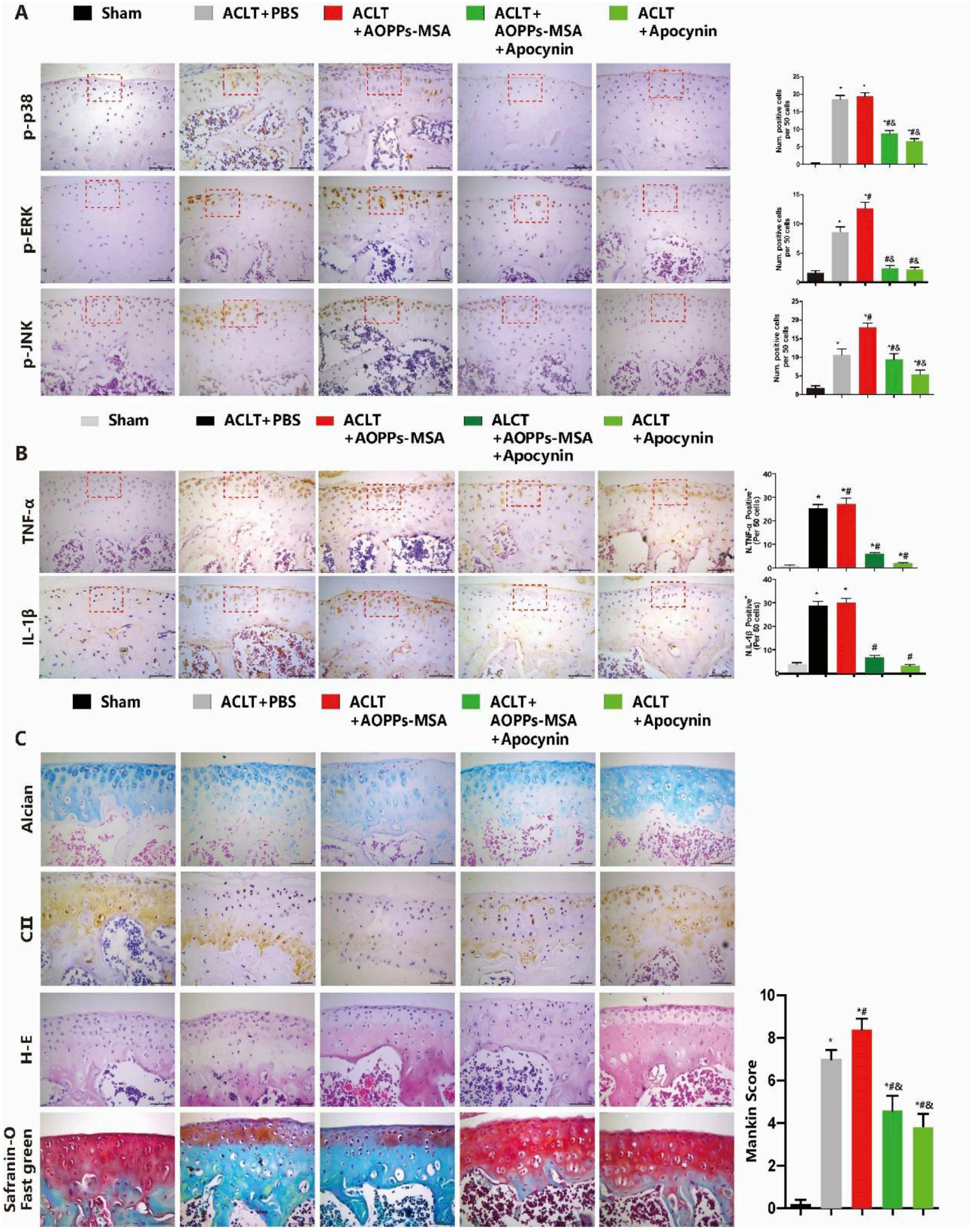

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Fig. 4. (A)Immunohistochemical staining of MAPKs relative protein JNK, ERK1/2 and p38 expression in each group at 60 days post operation. ACLT group presented medium-increase, AOPPs group presented more increase and intragastric administration of apocynin presented the decrease. (B)Immunohistochemical staining of pro-inflammatory cytokine TNF-α and IL-1β expression in each group at 60 days post operation. (C)AOPPs aggravated articular cartilage destruction after anterior cruciate ligament transection at 60 days and apocynin preserves it. Alcian blue staining (Left top). Type II collagen immunohistochemical staining (Left second line). Hematoxylin and Eosin (H&E) Staining (Left third line) and Safranin O and fast green staining (Left bottom). Osteoarthritis Research Society International–modified Mankin scores of articular cartilage at 60 days post operation. (Right).
Sham = sham-surgery. ACLT + AOPPs-MSA = ACLT-surgery treated with AOPPs-MSA. ACLT + AOPPs-MSA + apocynin = ACLT-surgery treated with AOPPs-MSA and intragastric administration of apocynin. ACLT + Apocynin = ACLT-surgery treated with intragastric administration of apocynin. N = 6 per group. *P < 0.05 versus Sham. #P < 0.05 versus ACLT + PBS. &P < 0.05 versus ACLT + AOPPs-MSA.

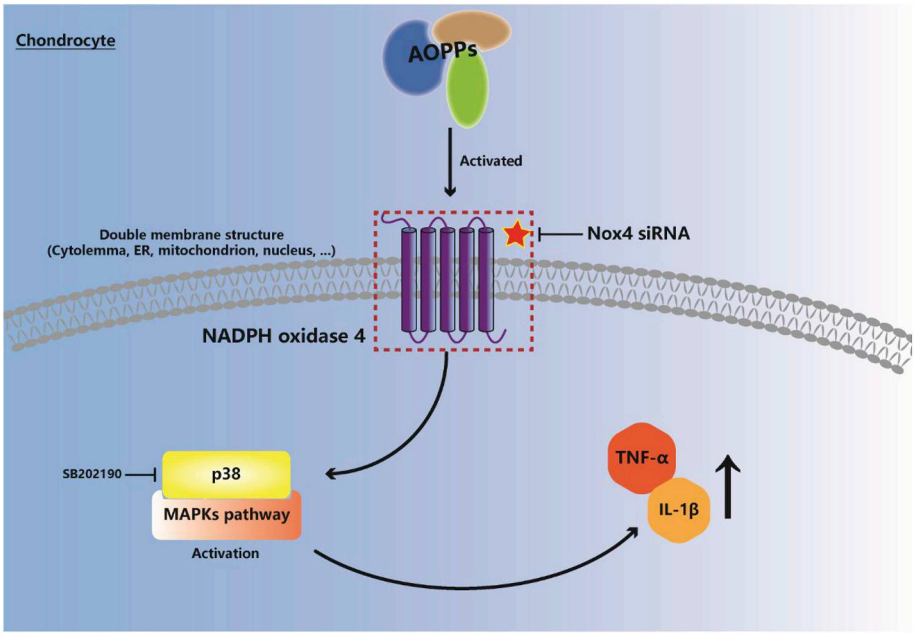

Fig. 5. Schematic representation of AOPPs-induced TNF-α and IL-1β expression.

proteins or regulatory domains [22,23]. NOX4 is highly expressed in human OA articular cartilage [24]. Primary chondrocytes derived from mouse embryos express NOX 1, 2 and 4 mRNA [25]. In this study we found that the expression of NOX 1, 2 and 4 was normally pretty low in mice cartilage. The up-regulated NOX4 expression was found in OA mice cartilage. In contrast, expression of the other NOXs (NOX 1, 2) was not elevated, pointing NOX4 as the sole NOX expressed in OA cartilage. NOX4 mainly catalyzes the formation of hydrogen peroxide [26]. On the one hand, hydrogen peroxide can up-regulate the expression of NOX4 [27]. on the other hand, hydrogen peroxide can form hypochlorite and hypochlorite products catalyzed by myeloperoxidase, and finally oxidize the protein to form AOPPs [11,26]. Our previous study has proven that up-regulated NOX4 expression was found in chondrocytes after AOPPs challenge in vitro [15]. This study further illustrated that AOPPs can up-regulate the expression of NOX4 in OA cartilage, suggesting that AOPPs/NOX4 can form positive feedback through ROS. Apocynin is an inhibitor of NOX, which can reduce the production of ROS in chondrocytes [15]. This experiment indicated that apocynin can block the positive feedback system of AOPPs/NOX4, finally reduce the level of AOPPs and NOX4. Taken together, we speculated that AOPPs might exert an adverse effect on chondrocytes in the cartilage through AOPPs/NADPH oxidase 4 pathway.

The MAPK cascades constituting three sequentially activated kinase complexes, p38 MAPK, c-Jun N-terminal kinase (JNK) and extracellular regulated kinase (ERK), are substrates for phosphorylation by MAPK kinases (MKKs) [28]. Our previous study reported that the MAPK pathway was involved in AOPPs signal transduction [17]. In this experiment all the three MAPK pathways were activated by AOPPs stimulation. Nevertheless, only p38-MAPK inhibitor could block AOPPs-induced expression of TNF-α and IL-1β in chondrocytes. However, JNK inhibitor and ERK inhibitor had no convincing effect on AOPPs-induced expression of TNF-α and IL-1β. Hence, p38-MAPK was firmly involved in AOPPs-triggered up-regulation of these two inflammatory factors but JNK-MAPK and ERK-MAPK were not for sure. This was consistent with a previous research finding that p38-MAPK may play a crucial role in regulating the biosynthesis of IL-1β and TNF-α [29]. By now, it is urgent to clarify the role of NADPH oxidase 4 in AOPPs-triggered cascades in chondrocytes. AOPPs-triggered activation of p38-MAPK pathway and consequent up-regulation of TNF-α and IL-1β in chondrocytes were attenuated by NOX4 knockdown, indicating that the cellular mechanisms involving in these effects were mediated by NADPH oxidase 4. In summary, we demonstrated that AOPPs up-regulated the expression of TNF-α and IL-1β in chondrocytes via the NADPH oxidase 4-dependent, p38-MAPK mediated pathway in vitro. It was established that IL-1β upregulate its own synthesis by activating NADPH oxidase 4 in chondrocytes [24]. Our data also suggest that AOPPs could be critical mediator involved in this loop.

Subsequently, we verified in vivo that exogenous AOPPs stimulation in OA mice could further activate the MAPK pathway and up-regulate expression of TNF-α and IL-1β in cartilage, which was blocked by NADPH inhibitor. In detail, ACLT (OA model) could lead to a significantly increase in expression of TNF-α and IL-1β in cartilage and exogenous injection of AOPPs slightly increased production of TNF-α and IL-1β in OA cartilage while NADPH oxidase inhibitor could largely decrease production of TNF-α and IL-1β in OA cartilage. Since AOPPs are continuously present during the course of OA and ACLT could induce formation of AOPPs in OA mice cartilage, AOPPs seem to be associated with the inflammatory cascade in OA cartilage via NADPH oxidase 4. Articular cartilage degeneration is a major characteristic of OA. The extracellular matrix (ECM) is mainly composed of crosslinked

triplehelical typeII collagen and GAG. A number of studies demonstrated that TNF-α and IL-1β might downregulate the synthesis of ECM components [5,30]. In this study, the expression of type IIcollagen and GAG was further downregulated in OA cartilage treated by exogenous AOPPs, which was blocked by NADPH oxidase inhibitor, indicating that AOPPs might magnify the inhibitory effect of inflammatory factors, or might have a direct effect, on cartilage anabolism. The direct effect of AOPPs on cartilage anabolism needs to be further clarified in future. Lastly, AOPPs might aggravate degeneration of articular cartilage in OA and NADPH oxidase inhibitor might attenuate articular cartilage degeneration, resulting in substantially lower OARSI scores. Taken together, the role of AOPPs/NADPH oxidase 4 to amplify and perpetuate articular cartilage degeneration in OA disease process might be performed partly by inducing production of TNF-α and IL-1β in cartilage chondrocytes or by its direct effect.

In conclusion, AOPPs which are present in OA joint may increase production of TNF-α and IL-1β in chondrocytes via the NADPH oxidase 4-dependent, p38-MAPK mediated pathway (see Fig. 5) and eventually accelerate degradation of OA cartilage. Targeting at the pathophysiological effects of AOPPs related cellular mechanisms can attenuate expression of TNF-α and IL-1β and articular cartilage degeneration in OA process. This study might provide a new clue to identification of a promising therapeutic option.

Conflicts of interest

The authors declare that they have no conflict of interests.

Acknowledgements

The authors thank Pro. Ning Fu, Dr. Zhao-Ming Zhong, Dr. Wen-Bin Ye, Dr. Jun Xiao and Dr. Jian Wang for their generous help during the project. This work was supported by the National Natural Science Foundation of China (No.81772395), the Natural Science Foundation of Guangdong Province (No.2016A030310404) and by the Director Foundation of NanFang Hospital (2017Z015).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101306.

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