

## Original Article

# PRDX1 overexpression contributes to the protection on chondrocytes from IL-1 $\beta$ -induced apoptosis

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**Abstract:** Peroxiredoxin 1 (PRDX1) is a member of the peroxiredoxin family, which has been shown to act as an antioxidant, whose main function is to reduce reactive oxygen species in cells. The purpose of this article was to study the effect of PRDX1 overexpression on chondrocyte apoptosis in *in vitro* model (exposure to IL-1 $\beta$ ) and explore its mechanism. PRDX1 overexpression was constructed and infected chondrocytes for 24 h prior to the addition of 10  $\mu$ g/l IL-1 $\beta$  to isolated chondrocytes. 10  $\mu$ M SB203580 was added 30 min prior to the addition of IL-1 $\beta$  to isolated chondrocytes. CCK-8 and flow cytometry assay were used to detect the chondrocytes proliferation, apoptosis and ROS production. Real-time PCR and Western blot were performed to examine the expression of MMP-13, Bcl-2 and Bax, and the activation of p38 was measured by Western blot assay. In the current study, we demonstrated that IL-1 $\beta$  treatment inhibited osteoarthritic chondrocytes proliferation and induced apoptosis and ROS production. IL-1 $\beta$  treatment also increased p-p38/p38, MMP-13 and Bax/Bcl-2 expression. However, p38 inhibitor SB203580 and PRDX1 overexpression both inhibited osteoarthritic chondrocytes apoptosis and decreased ROS production in chondrocytes induced by IL-1 $\beta$  treatment. Furthermore, SB203580 and overexpression of PRDX1 also resulted in an altered expression of proteins associated with apoptosis. Collectively, these results demonstrated that the regulatory effects of PRDX1 can be partially attributed to p38 signaling.

**Keywords:** IL-1 $\beta$ , PRDX1, apoptosis, p38

## Introduction

Osteoarthritis (OA) is one of the most common chronic diseases characterized by extensive cartilage extracellular matrix degradation and loss of chondrocytes at its late stage. Chondrocyte apoptosis has been reported to be correlated with the severity of OA [1]. Pro-inflammatory cytokines, such as IL-1 $\beta$ , play an important role in promoting OA lesions by inducing chondrocytes to secrete matrix metalloproteinases (MMPs), which degrade the extracellular matrix [2] and facilitate chondrocyte apoptosis [3]. Recent studies showed that several different mechanisms may adjust apoptosis of chondrocytes, including Fas/CD95, p53, PI3K/AKT, p38, ubiquitin-proteasome protein degradation and mitochondrial pathway [4, 5]. Although chondrocyte apoptosis and apoptotic pathways in the pathology of OA have not yet clearly elucidated, chondrocyte apoptosis

understood to be important to the pathogenesis of OA and mechanism of cell death could be targeted for drug screening and new therapeutic strategies.

Nitric oxide (NO) and reactive oxygen species (ROS) also have been suggested to be key factors to mediate chondrocyte apoptosis [6, 7]. The NO is overproduced by iNOS, which is produced by chondrocytes, in response to stimulation such as IL-1 $\beta$ . Increased serum levels of NO have been observed in patients with OA [8]. Excessive ROS may lead to irreversible damage of mitochondrial DNA, resulting in mitochondrial dysfunction and ultimately cell death [9]. When ROS production exceeds the antioxidant capacities of the biological medium, an oxidant stress occurs that leads to structural and functional changes in chondrocytes and extracellular matrix [10]. All these changes are key features of cartilage degradation in OA. Although,

there are evidences reporting the pathological role of NO and ROS in cartilage degradation, little is known about the antioxidant defense system of chondrocytes.

Recently, a novel family of peroxidases, the per-oxiredoxins (Prdxs), was identified to act not only as important intracellular antioxidants but also as direct participants in redox signaling events through the relay of oxidizing equivalents to target proteins containing reactive thiols [11, 12]. Moreover, PRDX hyperoxidation, which was associated with inhibition of pro-survival IGF-1-Akt signaling and up-regulation of pro-death signaling pathways, was significantly higher in chondrocytes isolated from older adults [13]. PRDX1, a member of the per-oxiredoxin family contains two essential catalytic cysteine residues and abundantly expressed in all cells [14, 15], plays a critical role in the scavenging of ROS. Recent studies have found that PRDX1 and/or PRDX1-regulated ROS-dependent signaling pathways play an important role in the progression and metastasis of human tumors, particularly in breast, oesophageal, esophageal and lung cancers [16, 17]. PRDX5 is found to be elevated in OA cartilage and upregulated by IL-1 stimulation, suggesting it may play a protective role against oxidative stress involved in the pathogenesis of OA [18]. According to these insights, we wonder whether PRDX1 also associated with the development and progression of osteoarthritic chondrocytes, and the underlying mechanisms need fully characterized.

This study is designed to explore the mechanism by which PRDX1 overexpression protects rat articular chondrocytes against IL-1 $\beta$ -induced apoptosis. Our findings for the first time demonstrated that PRDX1 overexpression protects rat articular chondrocytes against IL-1 $\beta$ -induced apoptosis via scavenging ROS and inactivating p38 signaling.

### Materials and methods

#### *Animals*

Sprague-Dawley male rats (4 weeks old), weighing 180-200 g, were obtained from the Experimental Animal Center of Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, and were fed under standard conditions (temperature: 25°C; humidity: 55-60%)

with food and water continuously available. The care and use of animals followed the recommendations and guidelines of the National Institutes of Health, and were approved by the Henan Provincial People's Hospital, People's Hospital of Zhengzhou University Animal Care and Use Committee.

#### *Collection, isolation, and culture of rat knee articular chondrocytes*

Rat articular chondrocytes were isolated from the knee joints of 4-week-old rats by enzymatic digestion. The cartilage was harvested and minced before being digested with 0.25% trypsin (Gibco, Grand Island, NY, USA), and was washed with phosphate-buffered saline (PBS) three times, followed by digestion with 0.2% collagenase II (Gibco) in DMEM for 5 h at 37°C. The supernatant was collected and centrifuged at 1000 rpm for 5 min. The released cells were cultured in DMEM medium supplemented with 15% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Cells at 50-60% confluence were used for immunohistochemistry assay, after which DMEM-F12 medium supplemented with 10% FBS and 1-2% penicillin/streptomycin (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd, Hangzhou, China) and incubated with 5% CO<sub>2</sub> at 37°C. Then, chondrocytes were treated with different concentrations of IL-1 $\beta$  (1, 10, 50 and 100  $\mu$ g/l) for 24 h.

#### *Immunohistochemistry*

Chondrocytes were placed in 4% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. Antigen retrieval was performed using Citra Plus antigen retrieval solution for 5 min at 97°C in a microwave. All slides were incubated at 4°C overnight with the following antibodies: anti-Collagen type II (1:200, Abcam), and anti-SOX9 (1:200, Abcam). The slides were then stained with biotinylated goat anti-mouse IgG (1:500, OriGene Technologies Inc., China) for 30 min at 37°C, after being washed three times in PBS for 5 min each. Subsequently, the sections were treated by employing diaminobenzidine (DAB) as the chromogen, counterstained with hematoxylin and washed in water. Five random fields per section were selected under the microscope to calculate percentage of positive cells.

## PRDX1 and IL-1 $\beta$ -induced apoptosis in chondrocytes

### *Construction of lentivirus vector and overexpression of PRDX1*

The core plasmid pLKO.1-puro (Addgene, Cambridge, MA, USA) was used to construct the recombinant plasmid pLKO.1-puro-PRDX1 by the way of double enzymes restriction and ligation. The packaging plasmid psPAX2 and pMD2G together with the recombinant plasmid pLKO.1-puro-PRDX1 or non-carrier plasmid pLKO.1-puro (NC) were transiently transfected into 293T cells (ATCC, USA). pLKO.1-puro-PRDX1 was collected 48 h after transfection and used to infect chondrocytes.

### *MTT assay*

Cell viability was assessed with MTT assays. Briefly, chondrocytes were plated in a 96-well plate and cultured for 12 h in serum-free DMEM. After treatment, cells were incubated with MTT (Sigma, St. Louis, MO, USA) at a final concentration of 0.5 mg/ml at 37°C for 4 h. Absorbance at 490 nm was measured with a Dynatech MR5000 plate reader (Dynatech, Chantilly, VA, USA). Cells without any treatment were served as control group while triplicates were performed throughout all the procedures.

### *Flow cytometry assay*

The apoptotic rate was evaluated using an Annexin V-FITC apoptosis detection kit (Jingmei). Briefly, after treatment, the chondrocytes ( $1 \times 10^5$  cells/ml) were mixed with 5 ml Annexin V FITC and 10 ml propidium iodide (20 mg/ml) and incubated for 15 min at room temperature. The samples were analyzed using a FACSscan flow cytometer (BD Biosciences, San Jose, CA, USA).

For reactive oxygen species (ROS) assay, the chondrocytes ( $1 \times 10^5$  cells/ml) were incubated with 10  $\mu$ M DCFH-DA fluorescent probe (Beyotime Biotechnology, Shanghai, China) for 20 min in dark at 37°C. The fluorescence intensity was monitored at an excitation wavelength of 480 nm and an emission wavelength of 525 nm.

### *Biochemical analysis*

After treatment, the chondrocytes supernatant were collected and the production of LDH and NO was measured by Lacate dehydrogenase (LDH) assay kit (A020-1, Nanjing Jiancheng

Bioengineering Institute, Nanjing, China) and nitric oxide (NO) assay kit (Nitrate reductase method; A012, Nanjing Jiancheng Bioengineering Institute).

### *Caspase-3 activity assay*

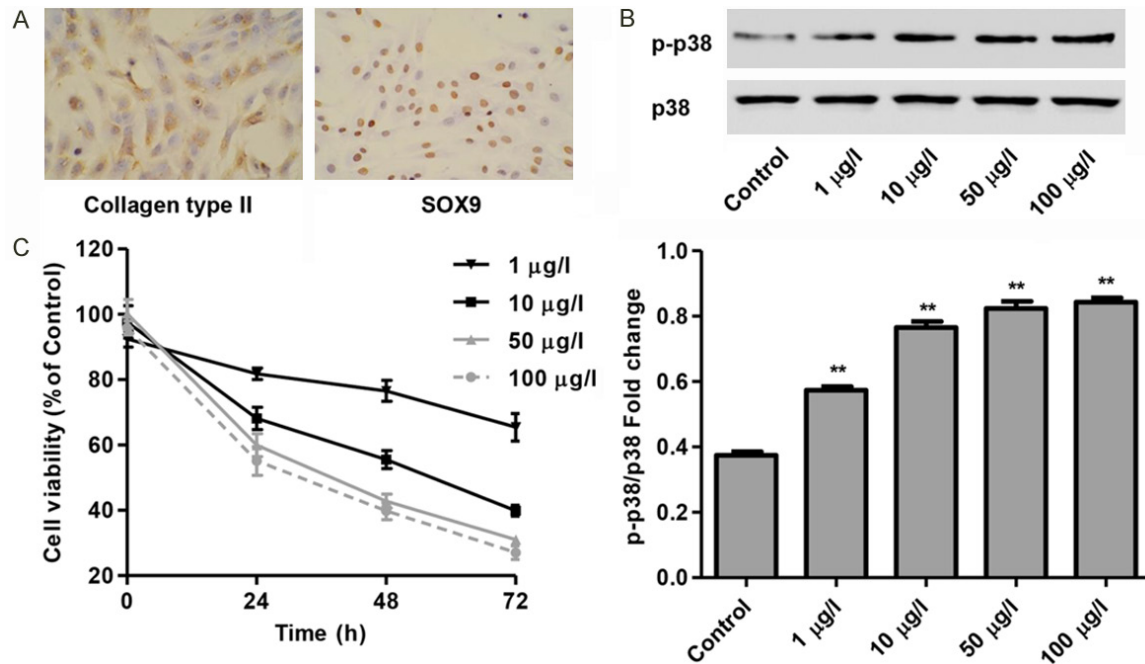
Caspase-3 activity was measured using a Caspase-3 colorimetric assay kit (KGA203; Kaiji Biological Engineering Materials Co., Ltd, Nanjing, China) according to the manufacturer's instructions. Briefly, cells were collected, resuspended in 50  $\mu$ l of chilled cell lysis buffer and incubated on ice for 10 min. After centrifugation for 1 min (at 400 $\times$ g), the supernatant was transferred to a fresh tube and then 100  $\mu$ g protein was diluted in 50  $\mu$ l cell lysis buffer for each assay. Samples were read at 405 nm in a plate reader Multiskan EX (Labsystems, Helsinki, Finland).

### *Real-time PCR*

Total RNA were extracted by Trizol Reagent (Invitrogen, USA), and the integrity of RNA was analyzed by the electrophoresis (Bio-Rad, USA). cDNA Reverse Transcriptase kit (Thermo, USA) was used here and a SYBR Green PCR kit (Thermo, USA) was used for the amplification. The primers of genes were as follow: PRDX1, F: 5'-GAAACCTGGCAGTGATAC-3', R: 5'-GTGGAAG-AAAGGCTAACC-3'; MMP-13, F: 5'-CAGACAGCAAGATAAAGAC-3', R: 5'-CAACATAAGCACAGTGTAAC-3'; Bcl-2, F: 5'-GGGATGCCTTTGTGGAAC-3', R: 5'-GTCTGCTGACCTCACTTG-3'; Bax, F: 5'-GG-ACGCATCCACCAAGAAG-3', R: 5'-CTGCCACACGG-AAGAAGAC-3'; GAPDH, F: 5'-GTCGGTGTGAACG-GATTG-3', R-TCCCATTCTCAGCCTTGAC-3'. The following PCR conditions were used: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 45 s. The mRNA level was analyzed by ABI-7300 (ABI, USA). The results were analyzed by ABI Prism 7300 SDS Software. Relative quantification of the gene expression was performed by normalization of the signals of different genes with the GAPDH signal. The  $\Delta\Delta$ Ct method for relative quantification of gene expression was used to determine mRNA expression levels.

### *Western blotting*

Chondrocytes were washed with PBS several times then added with lysis buffer premixed with proteinase and phosphatase inhibitor. The concentration of protein was quantified using



**Figure 1.** Effect of IL-1 $\beta$  on cell viability in rat chondrocytes. (A) The expression of collagen type II and SOX9 was measured by immunohistochemistry staining. After treatment of chondrocytes with different concentrations of IL-1 $\beta$  (0, 1, 10, 50 or 100 ng/ml), the cell viability was measured by CCK-8 assay (B) and the expression of p-p38 and p38 was measured by Western blot assay (C). \*\* $P < 0.01$  v.s. control.

BCA method (Thermo Fisher Scientific Inc., USA). Then equal amounts of proteins (25  $\mu$ g) were separated by the SDS/PAGE, blotted on polyvinylidenedifluoride (PVDF) membrane and probed with various primary antibodies (including p-p38, p38, MMP-13, Bcl-2, Bax and GAPDH), followed by incubation with the secondary antibodies and chemiluminescence detection. GAPDH antibody was used as an internal control. The blotting bands were quantified with ImageJ software.

#### Statistical analysis

Results were shown as mean  $\pm$  SD. All data were analyzed by SPSS 18.0 software (SPSS, Inc., Chicago, USA). Comparison was done with t test (unpaired), ANOVA and post hoc test. All  $P$  values presented were two-sided, and a  $P$  value of less than 0.05 was considered statistically significant.

#### Results

##### *IL-1 $\beta$ treatment inhibits chondrocytes proliferation and activates p-p38*

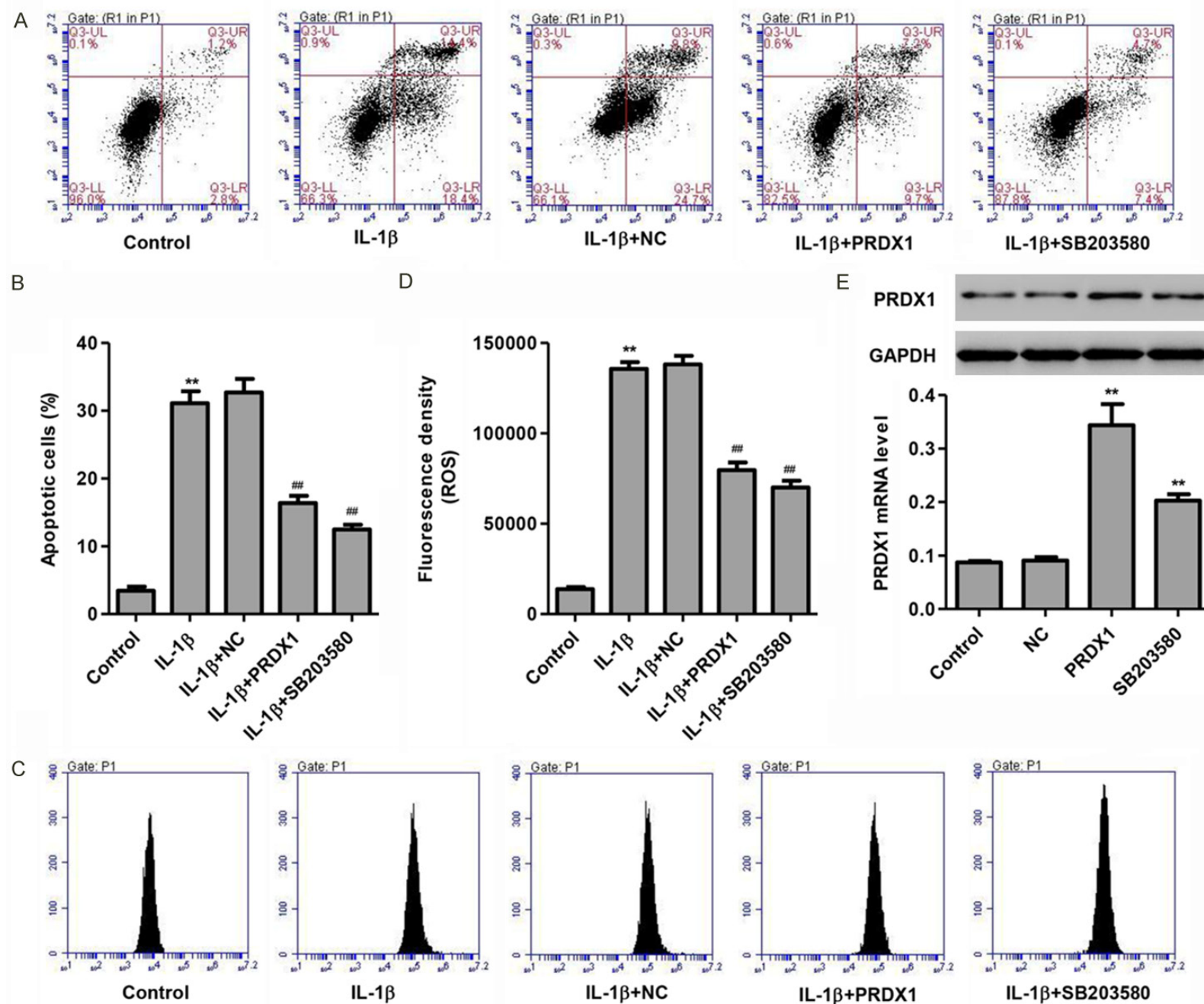
The chondrocyte-related genes: collagen type II and early chondrogenic transcription factor

SOX9 were analyzed by immunohistochemical staining in isolated chondrocytes. Collagen type II and SOX9 were shown markedly expressed in our cultured chondrocytes (**Figure 1A**). SOX9 is a member of the Sox (Sry-type HMG box) gene family, which is predominantly expressed in mesenchymal condensation and cartilage, and has been shown to activate collagen type II [19]. This indicates that a well chondrocytes system was established. Next, the effect of IL-1 $\beta$  treatment on chondrocytes proliferation was measured by CCK-8 assay. As shown in **Figure 1C**, the chondrocytes proliferation was significantly inhibited to 81.8%, 68.1%, 59.9% and 55.1% after IL-1 $\beta$  treatment (1, 10, 50 and 100  $\mu$ g/l) for 24 h in a time- and dose-dependent manner.

Moreover, we also determined the expression of p-p38 and p38 in IL-1 $\beta$ -treated chondrocytes. Our results showed that the expression ratio of p-p38/p38 was significantly increased by 53.1%, 113.3%, 120.3% and 135.1% after IL-1 $\beta$  treatment (1, 10, 50 and 100  $\mu$ g/l) for 24 h in a dose-dependent manner (**Figure 1B**). However, IL-1 $\beta$  treatment had no effect on the expression of p38 in chondrocytes. These results suggest that IL-1 $\beta$  treatment activates p38 signaling in chondrocytes.

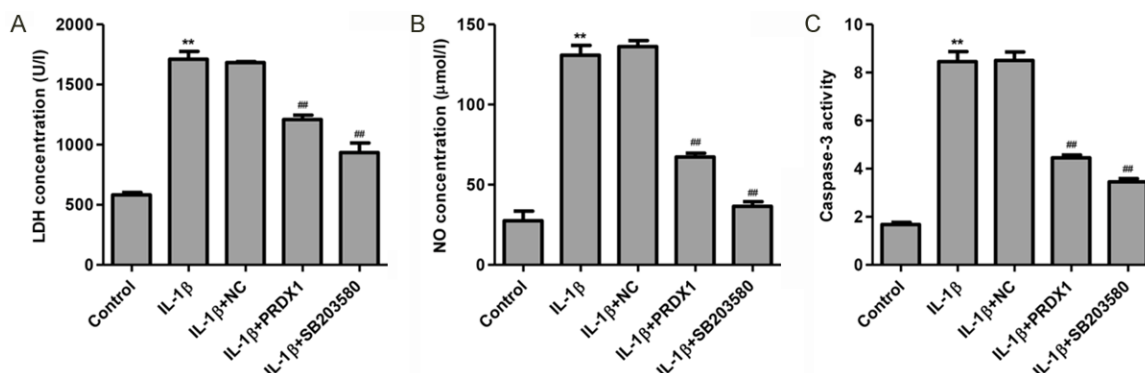


# PRDX1 and IL-1 $\beta$ -induced apoptosis in chondrocytes



## PRDX1 and IL-1 $\beta$ -induced apoptosis in chondrocytes

**Figure 2.** Effect of PRDX1 overexpression on cell apoptosis and ROS in IL-1 $\beta$ -induced rat chondrocytes. PRDX1 overexpression was constructed and infected chondrocytes for 24 h prior to the addition of 10  $\mu$ g/l IL-1 $\beta$  to isolated chondrocytes. 10  $\mu$ M SB203580 was added 30 min prior to the addition of IL-1 $\beta$  to isolated chondrocytes. The cell apoptosis (A, B) and ROS production (C, D) was measured by flow cytometry assay. The protein and mRNA expression of PRDX1 was measured by Western blot and Real-time PCR assay, respectively (E). \*\*P<0.01 v.s. control, ##P<0.01 v.s. IL-1 $\beta$  treatment.



**Figure 3.** Effect of PRDX1 overexpression on LDH, SOD and Caspase-3 level in IL-1 $\beta$ -induced rat chondrocytes. PRDX1 overexpression was constructed and infected chondrocytes for 24 h prior to the addition of 10  $\mu$ g/l IL-1 $\beta$  to isolated chondrocytes. 10  $\mu$ M SB203580 was added 30 min prior to the addition of IL-1 $\beta$  to isolated chondrocytes. The LDH (A), SOD (B) and Caspase-3 (C) level was measured by biochemical assay. \*\*P<0.01 v.s. control, ##P<0.01 v.s. IL-1 $\beta$  treatment.

### *PRDX1 overexpression inhibits apoptosis and ROS production in IL-1 $\beta$ -treated chondrocytes*

To investigate the effect of IL-1 $\beta$  on apoptosis and ROS production in chondrocytes, flow cytometry assay was performed. After 10  $\mu$ g/l of IL-1 $\beta$  treatment for 24 h, the apoptotic cells and ROS production in chondrocytes were significantly increased by 8.1-fold and 8.8-fold, respectively (**Figure 2A-D**). Additionally, the p38 inhibitor SB203580 significantly decreased the apoptotic cells and ROS production induced by IL-1 $\beta$ .

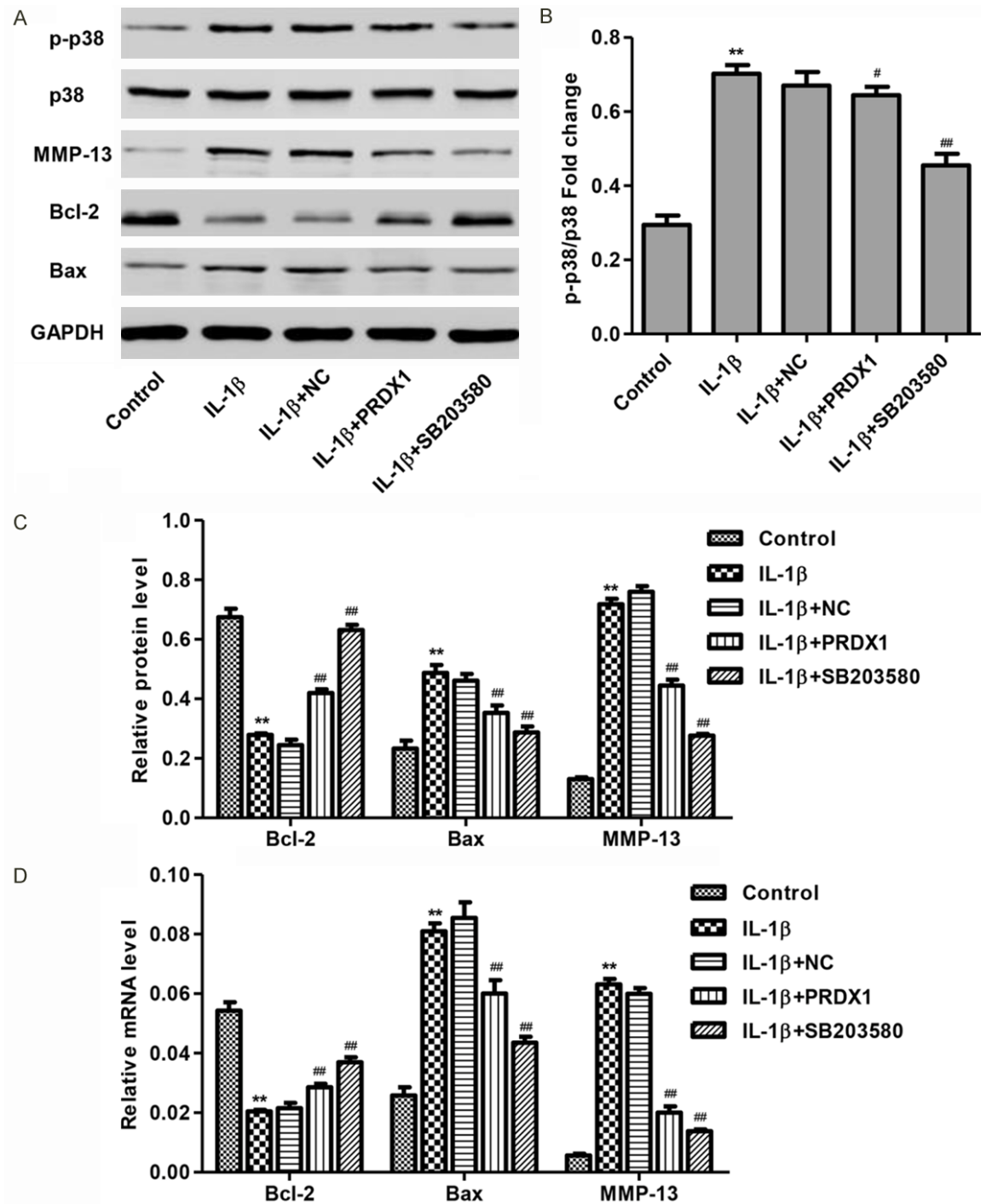
Recently, a novel family of peroxidases, the peroxiredoxins (Prdxs), has been suggested may play a protective role against oxidative stress involved in the pathogenesis of OA, and may have therapeutic value in the prevention and treatment of OA [18]. In the present study, we examined the role of PRDX1 in IL-1 $\beta$ -induced apoptosis and ROS production in chondrocytes. Transfection of pLKO.1-puro-PRDX1 into chondrocytes prior to IL-1 $\beta$  treatment for 24 h significantly increased the mRNA and protein expression of PRDX1 (**Figure 2E**). Interestingly, PRDX1 overexpression markedly reduced apoptotic cells and ROS production by 48.5% and 41.3%, respectively, in IL-1 $\beta$ -treated chondrocytes (**Figure 2A-D**).

### *PRDX1 overexpression reduced LDH, NO and Caspase-3 concentration in IL-1 $\beta$ -treated chondrocytes*

ELISA was used to analyze the concentrations of LDH, NO and Caspase-3 activity in supernatant. IL-1 $\beta$  treatment increased LDH, NO and Caspase-3 concentration in chondrocytes by 1.9-fold, 3.7-fold and 4.0-fold, respectively (**Figure 3A-C**). Importantly, SB203580 treatment and PRDX1 overexpression reduced the concentration of LDH by 45.4% and 29.3%, NO by 72.1% and 48.5%, and Caspase-3 by 59.2% and 47.3% induced by IL-1 $\beta$  (**Figure 3A-C**). These results indicate that SB203580 as well as PRDX1 overexpression reduced LDH, NO and Caspase-3 concentration in IL-1 $\beta$ -treated chondrocytes, and SB203580 is more sensitive than PRDX1 overexpression for IL-1 $\beta$ -treated chondrocytes.

### *PRDX1 overexpression reduced expressions of p-p38, MMP-13 and Bax/Bcl-2 in IL-1 $\beta$ -treated chondrocytes*

Similar to SB203580, PRDX1 overexpression also reduced the expression ratio of p-p38/p38 in chondrocytes induced by IL-1 $\beta$  treatment (**Figure 4A** and **4B**). Furthermore, the expression of MMP-13, Bcl-2 and Bax was also



**Figure 4.** Effect of PRDX1 overexpression on protein expressions in IL-1 $\beta$ -induced rat chondrocytes. PRDX1 overexpression was construed and infected chondrocytes for 24 h prior to the addition of 10  $\mu$ g/l IL-1 $\beta$  to isolated chondrocytes. 10  $\mu$ M SB203580 was added 30 min prior to the addition of IL-1 $\beta$  to isolated chondrocytes. The p-p38, p38 (A, B), MMP-13, Bax and Bcl-2 (A, C, D) expression was measured by Real-time PCR and Western blot assay. \*\*P<0.01 v.s. control, #P<0.05, ##P<0.01 v.s. IL-1 $\beta$  treatment.

measured by Real-time PCR and Western blot assay. As shown in **Figure 4A, 4C** and **4D**, the expression of MMP-13 and Bax was significantly increased by IL-1 $\beta$  treatment, but Bax was

decreased. However, SB203580 treatment and PRDX1 overexpression markedly reduced MMP-13 and Bax expression and increased Bcl-2 expression both at mRNA and protein levels.

## Discussion

These results of the present study support the notion that IL-1 $\beta$  induces apoptosis and ROS production in rat articular chondrocytes. Furthermore, this report for the first time demonstrated that PRDX1 overexpression prevents IL-1 $\beta$ -induced apoptosis by scavenging the IL-1 $\beta$ -triggered ROS production and inactivating p38 signaling.

In line with our findings that IL-1 $\beta$  induces chondrocytes apoptosis, previous studies observed that caspase-3 activity is significantly increased in IL-1 $\beta$ -induced chondrocyte apoptosis [20]. caspase-3 is activated by cytochrome c (Cyt c) that is induced by apoptotic signals [21]. Thus inhibition of apoptosis by Bcl-2 via binding to and inhibiting Bax could reduce IL-1 $\beta$ -induced chondrocyte apoptosis [22]. In the present study, we also found decreased proliferation of chondrocytes induced by different concentrations of IL-1 $\beta$  in time- and dose-dependent manner, which consistent with the increased apoptotic rate of chondrocytes.

ROS generation causes inflammation of articular cartilage, apoptosis of chondrocytes and finally results in arthritis [23]. In addition, ROS have been implicated as a second messenger in multiple signaling pathways and may be a cause or consequence of the mitochondria damaging [24]. In chondrocytes, NO can inhibit synthesis of collagens and proteoglycans and increase MMP activity [25]. From the inhibitory effect of NAC on SNP-induced apoptosis in rabbit chondrocytes, Nakagawa et al. [26] speculated that ROS acted as the downstream factor of NO to mediate SNP-induced apoptosis. Also, IL-1 $\beta$  has been shown to induce *in vitro* release of NO [3]. In the present study, both ROS and NO level were increased in IL-1 $\beta$ -induced chondrocytes.

Several previous studies have found that Prdxs are involved not only in oxidative stress protection mechanisms but also in cell differentiation, proliferation, apoptosis and signal transduction [27, 28]. Furthermore, to determine whether PRDX1 contributed to prevent IL-1 $\beta$ -induced osteoarthritic chondrocytes, we overexpressed the PRDX1 in osteoarthritic chondrocytes by pLKO.1-puro-PRDX1 infection. Overexpression of PRDX1 resulted in a decrease in apoptosis and ROS production in IL-1 $\beta$ -induced osteoar-

thritic chondrocytes. In agreement with our data, PRDX4 also showed regulation of ROS in oxidative stress and protected cells from apoptosis [29, 30]. Moreover, PRX1 protects the liver against ROS-mediated damage in the acute alcohol-induced liver injury model [14].

We also analyzed whether p38 activation was required for the apoptosis enhancing effect of IL-1 $\beta$ . Chondrocytes were stimulated with IL-1 $\beta$  in the presence or absence of the p38 inhibitor SB203580. 10  $\mu$ M SB203580 effectively abrogated IL-1 $\beta$ -induced increase of apoptosis, ROS production, and expression of Caspase-3 and Bax/Bcl-2. Similarly, application of SB203580 reduced the IL-1 $\beta$  induced nitrite and PGE2 release in a dose-dependent manner in the OA affected joint [4]. In our study, we also found that overexpression of PRDX1 resulted in inactivation of p38 in IL-1 $\beta$ -induced chondrocytes. In human chondrocytes apoptosis-enhancing effect of IL-1 $\beta$  is involved the activation of p38 and ROS [31]. These suggest that PRDX1 regulated apoptosis and oxidative stress may through targeting p38 signaling.

In conclusion, we discovered opposite effects of PRDX1 overexpression and SB203580 on chondrocyte apoptosis and ROS production induced by IL-1 $\beta$ . The pro-apoptotic capacity of IL-1 $\beta$  was mediated by p38. Apoptosis inhibition by PRDX1 overexpression was partially through inactivating p38 and thus might be considered as a promising therapeutic target during OA therapy.

## Disclosure of conflict of interest

None.

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