P2X4 promotes interleukin-1β production in osteoarthritis via NLRP1

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Abstract. Interleukin-1β (IL-1β) has a significant role in osteoarthritis (OA). The purinergic receptor, P2X4, has previously been implicated in IL-1β secretion. The NLRP1 inflammasome mediates the production of IL-1β in inflammatory disorders. However, it is unknown whether P2X4 modulates NLRP1-mediated IL-1β release. In the present study, the correlation between the P2X4 receptor and NLRP1 was investigated in OA fibroblast-like synoviocytes (OAFLS). The expression of P2X4 and NLRP1 was detected in the OAFLS. The OAFLS were stimulated with P2X4 and the levels of IL-1β and matrix metalloproteinases (MMPs) were measured. To determine whether P2X4 is involved in NLRP1-triggered IL-1β production, NLRP1 small interfering RNA (siRNA) was used. In the OAFLS, a markedly higher expression of P2X4 and NLRP1 was revealed compared with that in the normal FLS. OAFLS stimulated by P2X4 resulted in concentration-dependent increases in the production of IL-1β, MMP-3 and MMP-9. Furthermore, P2X4-mediated IL-1β production was attenuated by the NLRP1 siRNA. The results of the present study indicate that P2X4 induced IL-1β, MMP-3 and MMP-9 production in the OAFLS. IL-1β induced by P2X4 is mediated via NLRP1. P2X4/NLRP1 may be important in the pathogenesis of OA and may represent a novel therapeutic target.

Introduction

As the most common form of arthritis, osteoarthritis (OA) is one of the most significant causes of disability in older adults (1), yet its exact etiology remains unknown (2). The diagnosis and evaluation of joint damage are predominantly based on clinical and radiological findings. With medical advances, molecular markers are likely to become promising indicators for evaluating local inflammation, joint alterations and cartilage damage (3).

Fibroblast-like synoviocytes (FLS) have been accepted to be key in OA inflammation and joint destruction, primarily through their secretion of a wide range of proinflammatory mediators (4,5). In response to the proinflammatory cytokines, including interleukin-1β (IL-1β), the OAFLS produce chemokines that promote inflammation, neovascularization and cartilage degradation via activation of matrix-degrading enzymes, including matrix metalloproteinases (MMPs) (5). IL-1β is a multifunctional cytokine that contributes to the pathogenesis of OA (6). Investigations into the IL-1β signaling pathway led to the identification of novel potential drugs for the treatment of OA (7). However, current treatment with these drugs remains unsatisfactory, and further research is required to achieve the desired therapeutic goals (7).

The inflammasome is a multi-protein complex that mediates the activation of caspase-1, which in turn produces the proinflammatory cytokines, IL-1β and IL-18 (8). The human NLRP1 inflammasome was the first caspase-1-activating protein complex to be identified (9). Recent studies have indicated that NLRP3 is involved in the genetic predisposition and pathogenesis of crystal arthritis, including gouty and rheumatoid arthritis (10,11). Recently, a study has revealed that the expression of the P2X4 receptor is required for IL-1β and IL-18 release in mouse bone marrow-derived dendritic cells (12). However, it is unknown whether the P2X4 receptor modulates the NLRP1-mediated release of IL-1β. In the present study, the correlation between the P2X4 receptor and NLRP1 was investigated in FLS.

Materials and methods

Patients and synovial samples. Human synovial samples were collected with informed consent in line with the Declaration of Helsinki (2000 revision). The study protocol was approved by the local Ethics Committee of Shandong University (Jinan, China). The diagnosis of OA was based on clinical and radiological evidence of degenerative changes during surgery. Synovial tissues were obtained under aseptic conditions from 30 OA patients undergoing total knee replacement surgery, and samples of non-arthritic synovial tissues were obtained at arthroscopy following trauma/joint derangement.

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All the patients (n=30; F/M, 19/11; age, 62.9±4.6 years) in this study were enrolled from the Department of Orthopedic Surgery, The General Hospital of Jinan Military Command (Jinan, China) and from the Department of Orthopedic Surgery, The Third Hospital of Jinan (Jinan, China). Data from a medical history, physical examination, electrocardiogram and routine blood test were compiled for each patient. The mean (± SD) disease duration was 7.2±1.6 years. A total of 75% of the enrolled patients were receiving non-steroidal anti-inflammatory drugs and 25% were not on medication.

Cell isolation and culture. Synovial tissues were homogenized in Dulbecco's modified Eagle's medium and then incubated overnight at 37˚C with 1 mg/ml type I collagenase (Sigma-Aldrich, St. Louis, MO, USA). Following cell dissociation, the samples were filtered through a cell strainer. The cell suspensions and cultures were conducted as described previously (13). The cell confluence and morphology were assessed throughout the experiments by phase-contrast microscopy (DMI3000B; Leica, Wetzlar, Germany) (14). All the functional experiments were conducted using primary synovial cultured cells from passages 3 to 6.

Small interfering RNA (siRNA). To inhibit the NLRP1 expression in the FLS, commercially available NLRP1 siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used. The cells were transfected using transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. siRNA was diluted in transfection reagent and culture medium, and the cells were incubated with 20 nM siRNA for 12 h.

Quantitative PCR (qPCR). For qPCR, the primers were designed as follows: P2X4, (forward) 5'-CTACCAGGAAACT GACTCCGT-3' and (reverse) 5'-GGTATCACATAATCCGC CACAT-3'; IL-1β, (forward) 5'-ATGATGGCTTATTACAGTG GCAA-3' and (reverse) 5'-GTCGGAGATTCGTAGCTGGA-3'; NLRP1, (forward) 5'-GCAGTGCTAATGCCCTGGAT-3' and (reverse) 5'-GAGCTTGGTAGAGGAGTGAGG-3'; MMP-3, (forward) 5'-CTGGACTCCGACACTCTGGA-3' and (reverse) 5'-CAGGAAAGGTTCTGAAGTGACC-3'; MMP-9, (forward) 5'-GGGACGCAGACATCGTCATC-3' and (reverse) 5'-TCGT CATCGTCGAAATGGGC-3'; GAPDH, (forward) 5'-ACAACTTTGGTATCGTGGAAGG-3' and (reverse) 5'-GCCATCACGCCACAGTTTC-3'. The total RNA was isolated using the total RNA isolation kit (Qiagen). The total RNA (20 ng) was reverse transcribed for all targets. The temperature profile for PCR included reverse transcription at 50˚C for 30 min, hot start Taq (1.25 units/sample; Thermo Fisher Scientific, Waltham, MA, USA) activation for 15 min at 95˚C, 28 cycles of denaturation for 15 sec at 94˚C, annealing for 30 sec at 56˚C and extension for 30 sec at 72˚C. SYBR-Green fluorescence was acquired at the end of the extension cycle or at 79˚C. A melting curve analysis was performed at the end of each run to verify the single product formation for each reaction. The relative expression of the target genes was determined by comparison with GAPDH using the CT method.

Western blotting. Human FLS were washed and then lysed in cell lysis buffer, and the protein concentration was determined using a bicinchoninic acid assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Aliquots of 60 μg total protein sample were analyzed using monoclonal rabbit antibodies specific for human P2X4 and NLRP1 (Cell Signaling Technology, Inc., Danvers, MA, USA). The filters were washed and incubated overnight at 4˚C with secondary antibody (1:2,000; Cell Signaling Technology, Inc.). Western blotting assays were revealed with enhanced chemiluminescence (Pierce, Rockford, IL, USA) and normalized against the internal control, GAPDH.

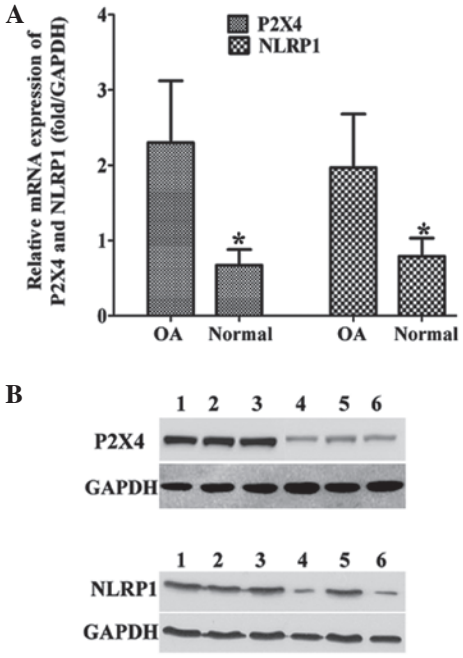

Figure 1. Increased expression levels of P2X4 and NLRP1 in human osteoarthritis fibroblast-like synoviocytes (OAFLS). The total RNA or proteins were extracted from normal FLS and OAFLS, and subjected to (A) qPCR and (B) western blotting. The results are expressed as the mean ± SD. *P<0.05 compared with OAFLS. Lanes 1-3, OAFLS; lanes 4-6, normal FLS. qPCR, quantitative PCR.

Measurements of IL-1β and MMPs in the medium. Human FLS were cultured in 24-well plates. Subsequent to reaching 80% confluence, the cells were treated with NLRP1 and then incubated in a humidified incubator at 37˚C for 12 h. The cells were pretreated with NLRP1 siRNA. Following incubation, the medium was removed and stored at -80˚C until the assay was performed. The IL-1β and MMPs in the medium were assayed using an ELISA (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Statistical analysis. The data are expressed as the mean ± SD. The statistical analysis was performed with Graphpad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). An analysis of variance and unpaired two-tailed Student's t-test were used to determine the significant differences between the means. P<0.05 was used to indicate a statistically significant difference.

Results

Expression of P2X4 and NLRP1 is upregulated in OAFLS. The expression levels of P2X4 and NLRP1 were examined

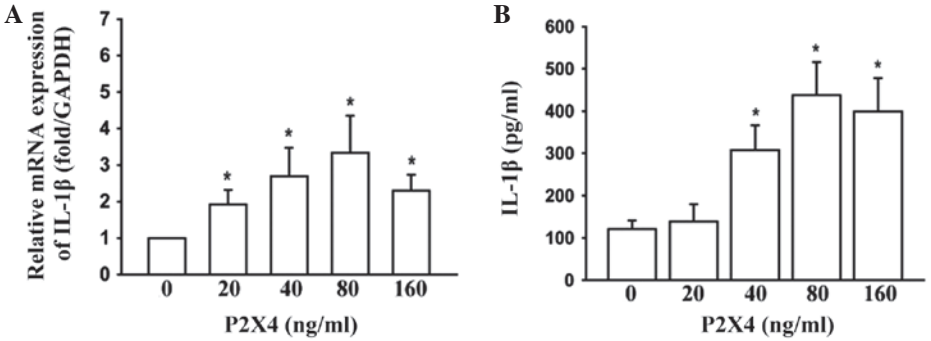

Figure 2. OAFLS were incubated with various concentrations of P2X4 for 12 h. The IL-1β expression was examined by (A) qPCR and (B) ELISA. The results are expressed as the mean ± SD. *P<0.05 compared with the basal level. OAFLS, osteoarthritis fibroblast-like synoviocytes; qPCR, quantitative PCR; IL-1β, interleukin-1β.

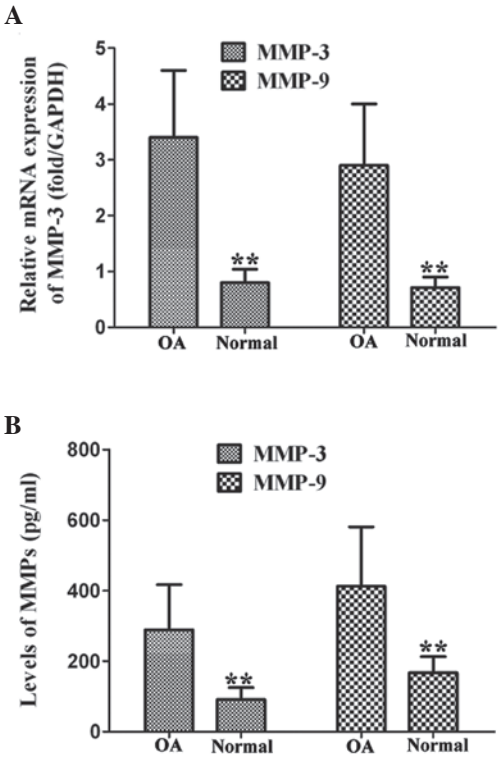

Figure 3. OAFLS were incubated with various concentrations of P2X4 for 12 h. The expression of MMP-3 and MMP-9 was examined by (A) qPCR and (B) ELISA. The results are expressed as the mean ± SD. **P<0.01, compared with the basal level. MMP, matrix metalloproteinase; qPCR, quantitative PCR; OAFLS, osteoarthritis fibroblast-like synoviocytes.

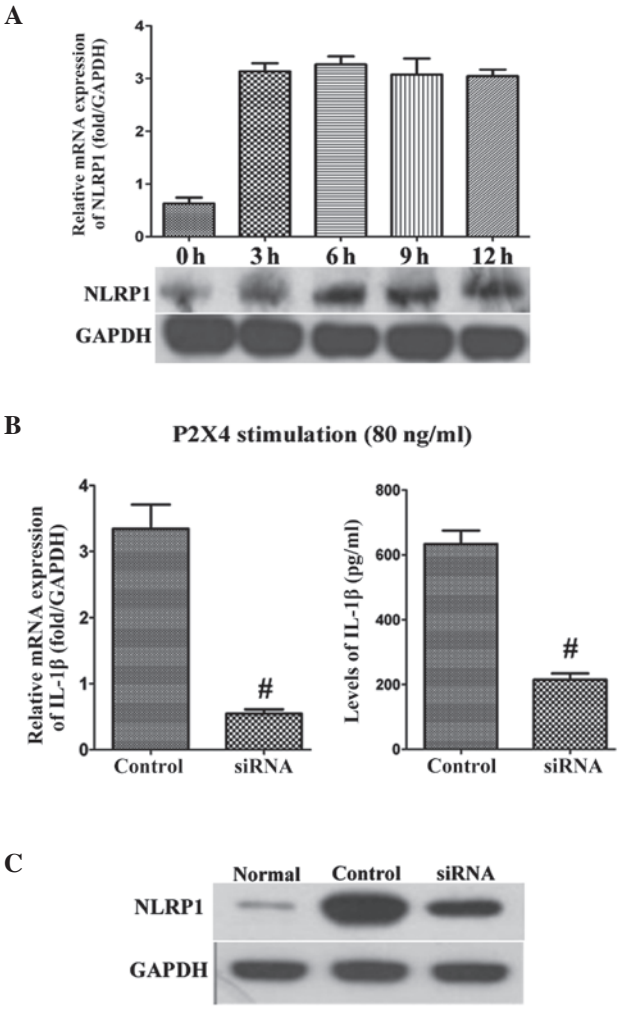

Figure 4. (A) P2X4 at 80 ng/ml significantly upregulated the expression of NLRP1 at the mRNA and protein levels in the OAFLS. (B) NLRP1 siRNA at 20 nM blocked the production of IL-1β induced by P2X4 at the mRNA and protein levels compared with the control scrambled siRNA. (C) The silencing effect of NLRP1 siRNA was also confirmed by western blotting. The results are expressed as the mean ± SD. #P<0.01 compared with the control siRNA. siRNA, small interfering RNA; IL-1β, interleukin-1β.

in the samples from the patients with OA. The expression of P2X4 and NLRP1 in the human OAFLS was demonstrated to be significantly higher than in the normal FLS at the mRNA and protein levels (Fig. 1A-B), indicating the potential roles of P2X4 and NLRP1 in OA.

P2X4 induces expression of IL-1β and MMPs in OAFLS. The effects of P2X4 stimulation on IL-1β and the MMPs were examined in the OAFLS. P2X4 (Abnova, Walnut, CA, USA) was applied directly to the OAFLS. The treatment of the OAFLS with P2X4 at concentrations of 0, 20, 40, 80 and160 ng/ml for 12 h was demonstrated to induce IL-1β mRNA (Fig.2A) and protein expression (Fig. 2B) in a dose-dependent manner. According to these results, P2X4 at 80 ng/ml was most effective, and this concentration was selected in all the subsequent experiments. In addition, the levels of MMP-3 and MMP-9 in the OAFLS were significantly higher than in the normal FLS, as observed by qPCR and ELISA, following 12 h stimulation of P2X4 at 80 ng/ml (Fig. 3). These data indicated that P2X4

is a critical factor in the production of IL-1β and MMPs in OAFLS.

P2X4-induced IL-1β in OAFLS is mediated via NLRP1. NLRP1 is involved in the production of IL-1β (12). P2X4 at 80 ng/ml was identified to significantly upregulate the expression of NLRP1 at the mRNA and protein levels in the OAFLS (Fig. 4A). Furthermore, the NLRP1 siRNA at 20 nM was added in combination with P2X4 at 80 ng/ml into the culture system. The NLRP1 siRNA was observed to block the production of IL-1β induced by P2X4 at the mRNA and protein levels compared with the control scrambled siRNA (Fig. 4B). The silencing effect of the NLRP1 siRNA was also confirmed by western blotting (Fig. 4C). The normal FLS were used as a normal control. These data reveal that NLRP1 is a crucial determinant in P2X4-mediated IL-1β release.

Discussion

The P2X4 receptor is expressed in human osteoblast-like cells (15). P2X4 has only recently been revealed to have a functional role in the induction of brain-derived neurotrophic factor expression from OAFLS (16). In the present study, the expression of P2X4 and NLRP1 was confirmed to be significantly higher in patients with OAFLS compared with those with normal FLS. P2X4 induced the expression of IL-1β and MMPs in the OAFLS. Furthermore, IL-1β was identified as a target protein for the P2X4 signaling pathway that regulates the inflammatory response. In addition, secretion of IL-1β by the OAFLS was shown to require NLRP1. These findings indicate that P2X4 acts as an important inducer of inflammatory cytokines, including IL-1β and MMPs, in OA.

A previous study revealed that P2X4R-deficient mice exhibit reduced inflammatory pain behaviors and an impaired production of prostaglandin E2 (PGE2) in the peripheral tissue in response to inflammatory challenges (17). Ulmann *et al* (17) identified paw-resident macrophages as the predominant cell type responsible for P2X4R-evoked PGE2 production. In the present study, it was revealed that in the OAFLS, P2X4R activation triggered the necessary intracellular signals leading to the activation of IL-1β synthesis, indicating P2X4 to be the key receptor mediating the inflammatory response in OA inflammation. In addition, P2X4 induced the expression of MMP-3 and MMP-9. MMPs have been demonstrated to be involved in joint destruction via degradation of the articular cartilage (18). The data have revealed significantly increased concentrations of plasma MMP-3 and MMP-9 in early OA, and a positive correlation of plasma MMP-3 and MMP-9 with the severity of clinical symptoms in early OA has also been reported (19). In the present study, P2X4 stimulation was observed to result in increased levels of MMPs, indicating that P2X4 may be a potential therapeutic target for healing joint damage.

Another significant finding in the present study is that IL-1β induced by P2X4 is mediated by NLRP1. NLRP1 is a regulator of the innate immune response and is expressed in a number of immunocompetent cell types (20). The assembly of the NLRP1 inflammasome and the subsequent activation of caspase-1 cleaves the inactive IL-1β precursor to the mature bioactive IL-1β, thereby stimulating downstream inflammatory responses (21). A dominant activating mutation in mouse NLRP1 has recently been demonstrated to result in a severe systemic inflammatory phenotype associated with a greatly elevated release of active IL-1β (22). The NLRP1/IL-1β axis has been reported to be associated with autoimmunity (20). NLRP1 polymorphisms are involved in the predisposition to systemic lupus erythematosus and rheumatoid arthritis (23,24). The results of the present study are concordant with previous studies and add novel findings to the current understanding of NLRP1 in arthritis. In conclusion, the results of the present study demonstrated that P2X4 mediates the inflammatory response of OAFLS by inducing the NLRP1/IL-1β axis. These results also indicate that the P2X4/NLRP1 pathway may be a promising treatment target in human OA.

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