Interleukin-6 enhances acid-induced apoptosis via upregulating Acid-sensing ion channel 1a expression and function in rat articular chondrocytes

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Abstract

The inflammatory cytokine interleukin-6 (IL-6) is a causative agent of rheumatoid arthritis (RA), a chronic inflammatory disease complicated with degenerative arthritic cartilage. However, the precise mechanism of IL-6 on chondrocyte apoptosis is largely unclear. Acid-sensing ion channels (ASICs), a family of extracellular H⁺-activated cation channels, can be transiently activated by extracellular acid and play a pivotal role in acid-induced cell injury. In the present study, to investigate the role of IL-6 in regulating acid-induced articular chondrocyte apoptosis, primary rat articular chondrocytes were subjected to different treatments with or without IL-6 in the presence of acid. The results showed that the mRNA and protein expressions of ASIC1a were significantly increased in articular cartilage and chondrocytes of adjuvant arthritis (AA) rats. IL-6 could dramatically upregulate the level of ASIC1a in a time- and dose-dependent manner, and induce the activation of JAK2, STAT3, ERK, JNK and NF-κB in articular chondrocytes. Moreover, both the respective inhibitors of these signaling pathways and the specific antibody against IL-6 receptor (tocilizumab) could partially abrogate the ASIC1a upregulation induced by IL-6. Furthermore, IL-6 inhibited the cell viability and enhanced LDH release, [Ca²⁺]_i elevation, and apoptosis in acid-induced articular chondrocytes, and these changes could be reversed by using psalmotoxin 1(PcTX1), which is the specific antagonist of ASIC1a. In addition, pretreatment with PcTX1 could inhibit the downregulated expression of Bcl-2 and the upregulated expression of Bax induced by IL-6 in acid-induced articular chondrocytes. Taken together, these results indicated

that IL-6 could enhance acid-induced articular chondrocyte apoptosis, the mechanism of which might partially be involved with its ability of regulating the activation of ASIC1a-dependent JAK2/STAT3 and MAPK/ NF- κ B signaling pathways.

Keywords: IL-6; Acid-sensing ion channel 1a (ASIC1a); Rheumatoid arthritis; Articular chondrocytes; Apoptosis

Abbreviations:

ASICs, acid-sensing ion channels; ASIC1a, acid-sensing ion channel 1a; AA, adjuvant arthritis; IL-6, interleukin-6; RA, rheumatoid arthritis; JAK, janus kinase; STAT, signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor- κ B; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; H&E, hematoxylin and eosin; PBS, phosphate buffered saline; FBS, fetal bovine serum; DMEM/F-12, Dulbecco's modified Eagle's medium/Hams F-12; FLS, fibroblast like synoviocytes; LDH, lactate dehydrogenase; qRT-PCR, quantitative real-time PCR; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride film; PcTX1, psalmotoxin 1.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that results in chronic, progressive inflammatory disorders of the joints and surrounding tissues, finally leading to irreversible joint destruction [1]. It is widely accepted that the changes in the concentration of inflammatory cytokines was related to RA process, among which

interleukin-6 (IL-6) received much attention. Accumulating evidence has indicated that IL-6 is present at very high levels in the serum and synovial fluid of patients with RA, and increased IL-6 enhances the recruitment of inflammatory cells into the tissues and aggravates the joint destruction [2, 3]. Blocking IL-6 with tocilizumab, a recombinant humanized anti-human IL-6 receptor monoclonal antibody, is safe and effective in patients with active RA [4]. IL-6 also could regulate gene expression, cell proliferation, and differentiation via Janus kinase (JAK)/signal transducer and activator of transcription (STAT), mitogen-activated protein kinase (MAPK) and nuclear factor- κ B (NF- κ B) signaling pathways in articular chondrocytes [5-7]. Furthermore, results of recent studies have demonstrated that IL-6 could enhance susceptibility of malignant melanoma cells to TNF- α -induced apoptosis and promote hypoxic-ischemic encephalopathy (HIE)-induced neuronal apoptosis [8, 9]. However, the role of IL-6 in the articular chondrocyte apoptosis remains largely unknown.

Acid hydrarthrosis is another important pathological character in RA, and much attention has been paid to the role of acidosis-induced articular chondrocyte apoptosis in the pathogenesis of RA. Acid-sensing ion channels (ASICs) belong to the degenerin/epithelial sodium channel (DEG/ENaC) superfamily, and widely distributed in neurons of peripheral sensory and central nervous systems. So far, at least seven different ASIC subunit proteins (ASIC1a, ASIC1b, ASIC1b2, ASIC2a, ASIC2b, ASIC3, ASIC4) have been identified in mammalian [10-12], among which ASIC1a is unique due to its highly permeable to Ca^{2+} as well as Na^+ . By regulating intracellular Ca^{2+} accumulation, ASIC1a plays indispensable roles in controlling cell

behavior such as apoptosis [13], differentiation [14], activation [15] and autophagy [16]. Results of our previous studies showed that the articular chondrocytes isolated from rat knee joints could respond to a decrease pH value by increasing intracellular Ca^{2+} levels via ASIC1a [17], and the activation of ASIC1a was involved in the acid-induced articular chondrocyte injury, the mechanism of which was associated with the change of Ca^{2+} influx [18,19]. These findings indicated that ASIC1a-mediated articular chondrocyte apoptosis played important roles in the pathogenesis of RA.

Recently, many studies have focus on the relationship between ASICs and inflammatory cytokines in osteoarticular diseases. It has been reported that interleukin-1 β (IL-1 β) could increase the mRNA expression of ASIC3 in the fibroblast-like synoviocytes (FLS) [20], and nerve growth factor (NGF) could upregulate the expression of ASIC3 in nucleus pulposus cells of the intervertebral disc via regulation of p75NTR and ERK Signaling [21], while transforming growth factor- β (TGF- β) was reported to serve as a negative regulator of ASIC3 expression [22]. However, the relation of IL-6 to ASIC1a and their effect on the promoting articular chondrocyte apoptosis in RA have not been well clarified.

In the present study, we examined the expression of ASIC1a in the articular cartilage and chondrocytes from normal and adjuvant arthritis (AA) rats. We then investigated the effects of IL-6 on the expression of ASIC1a in primary articular chondrocytes from normal rats and determined whether the JAK2/STAT3, MAPK and NF- κ B pathways are implicated in this regulation. Furthermore, we evaluated

whether or not IL-6 is involved in ASIC1a mediated acid-induced articular chondrocyte apoptosis by increasing $[Ca^{2+}]_i$.

2. Materials and methods

2.1. Animals, rat model of adjuvant arthritis

Male Sprague–Dawley (160–180 g) rats were purchased from the Experimental Animal Center of Anhui Medical University (Hefei, China). The animal experimental procedures were reviewed and approved by the University Animal Care and Use Committee. Animals were allowed ad libitum access to water and standard pelleted food under a 12-hour light/dark cycle and a mean temperature of 22 ± 2 °C. All animals were acclimated for 6 days before the experiment began. The rats were divided into two groups: non-arthritic animals (n =20) and arthritic animals (n=20). Adjuvant arthritis was performed using a method we have previously described [23]. AA rats were induced by intradermal injection of a 100 μ l aliquot of Complete Freund's adjuvant (CFA, Sigma, St. Louis, MO, USA) containing 10 mg/ml of heat-inactivated BCG in liquid paraffin oil into the left hind metatarsal rat footpad.

2.2. Histological examination

After ether anesthesia on day 26, the hind paws were amputated above the knee joint and were fixed in 4% paraformaldehyde for 48 h, then decalcified in 10% ethylenediamine tetraacetic acid (EDTA) and embedded in paraffin for histopathological analysis. Serial paraffin sections (4 μ m) were stained with hematoxylin and eosin (H&E) and analyzed for synovial proliferation, cellular infiltration and cartilage erosion.

2.3. Immunostaining analysis

Sections from paraffin-embedded joints were deparaffinized by xylene and rehydrated with graded alcohols. Endogenous peroxidase activity was blocked by incubating tissue sections in 3 % H₂O₂ for 5 min. After washing with PBS, sections were blocked with 10% serum for 15 minutes at room temperature, and then were incubated for 1h at room temperature in primary antibody (ASIC1a, 1:100, alomone labs) for 1 h. The primary antibodies were replaced with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin as a negative control. Immunoreactivity was visualized using the Streptavidin/Peroxidase (SP) method (Zhongshan Golden bridge Biotechnology Co., LTD, Beijing, China) and diaminobenzidine (DAB) as the chromogen. Finally, the sections were counterstained with hematoxylin. Images were taken with a Nikon video microscope.

2.4. Isolation of articular chondrocytes and treatment

Rat articular chondrocytes were isolated using the method described previously [18]. Cartilage tissue from knee joint of three or four rats was pooled for each isolation. Cartilage from the knee joint was minced into small pieces (about 1 mm³) and digested initially with 0.2% type II collagenase (Sigma Chemical Co., St. Louis, MO, USA) in PBS. After digestion, isolated cells were centrifuged and washed three times with PBS. The freshly isolated chondrocytes were plated at a density of 2×10⁴ cells/well, which had been immersed previously in plastic dishes filled with Dulbecco's modified Eagle's medium/Hams F-12 (DMEM/F-12, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO,

USA), 100 IU/ml penicillin, and 100 µg/ml streptomycin. These cultures were maintained under sterile conditions at 37°C in a humidified 5% CO₂ incubator and used within the first three passages. Primary rat articular chondrocytes were subjected to different treatments with increased concentrations of IL-6 (1.25-20 ng/ml; Peprotech, Rocky Hill, NJ, USA) in serum-free medium for 24 h. After exposure to a normal extracellular solution (pH 7.4) with IL-6, chondrocytes were cultured in a low pH solution (pH 6.0) for 3 h. After treatment, the cells were washed three times with PBS and recultured in complete DMEM/F-12 containing 10% FBS for 4 h. For acidic stimulation, the extracellular pH was adjusted by the addition of an appropriate amount of HCl to achieve different pH values [13]. Chondrocytes cultured in the pH 7.4 solution without IL-6 were used as controls. To block IL-6 receptor, chondrocytes were pretreated with tocilizumab (10 µg/ml; Roche Co., Ltd, Shanghai, China) for 2 h. To investigate the effects of JAK2, STAT3, ERK, JNK and NF-κB signaling, cells were pretreated with the inhibitors for ERK (10 µM PD98059), JNK (10 µM SP600125), NF-κB (10 µM PDTC), or JAK2 (10 µM AG490) (Tocris Bioscience, Ellisville, Missouri) for 45 min. RNA was isolated for qRT-PCR analysis, and cell lysates were subjected to Western blot.

2.5. Immunofluorescence assay and Toluidine blue staining

After treatment as indicated earlier, the articular chondrocytes were fixed with ice-acetone for 15 min, permeabilized with 0.3% Triton X-100 in PBS for 15 min, and then blocked with PBS (Boster, Wuhan, China) containing 5% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) for 1 h. The cells were then incubated with

anti-ASIC1a (1:100, Alomone Labs, Israel), anti-collagen II (1:100; Boster, Wuhan, China), and anti-aggrecan (1:100; Boster, Wuhan, China) antibody overnight at 4 °C, followed by detection with a FITC-conjugated anti-rat IgG (Molecular Probes, Beijing, China) in the dark for 1 h at 37°C. Control sections were treated in parallel with a non-immune isotype-matched IgG substituting for the primary antibody. Nuclear staining was incubated with 4', 6-diamidino-2-phenylindole, dilactate (DAPI; Invitrogen, Carlsbad, CA, USA). Some slides were incubated for 30 min with 1% toluidine blue dye solution (Sangon Biotech, Shanghai, China), washed subsequently with aqua dest, 95% ethanol, propanol, and xylol, and mounted on microscopic slides with DePeX (Serva). Cells were washed and imaged using an inverted fluorescence microscope (Olympus, Tokyo, Japan).

2.6. Cell viability assay

Cells were seeded into 96-well plates (5×10^3 cells/well) and incubated for 24 h. After treatment, 20 μ l of MTT (Sigma, St. Louis, MO, USA) reagent solution (5 mg/ml) was added to each well, and the cells were incubated in complete DMEM/F-12 solution at 37°C for 4 h. At the end of incubation, MTT solution were removed, 150 μ l of dimethyl sulfoxide (DMSO) was added to each well and stirred to dissolve the water-insoluble formazan salt. Then, the optical density (OD) of each well was measured at 492 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Biotek, Vermont, USA), and the well without cells served as blank control. All experiments were performed in triplicate and should be repeated at least three times.

2.7. Lactate dehydrogenase (LDH) release assay

The degree of cell injury was assessed by quantitative measurement of LDH release. Cells were pretreated with IL-6 (10 ng/ml) for 24 h. Following acid incubation for 3 h, cells were washed and incubated in culture medium at 37°C. After 24 h, the supernatants were aspirated gently and saved for LDH determination. LDH release was detected in culture medium using Lactate dehydrogenase assay kit (Nanjing Jiancheng Biochemical Reagent Co., Nanjing, China). After reaction, each sample was detected and the absorbance was read at 450 nm. The maximum release of LDH was obtained in each well by 15 min incubation with 1% Triton X-100 at the end of each experiment. Data were expressed as a ratio of LDH released/ (LDH released + LDH Triton X-100 extracted). All experiments were performed in triplicate and should be repeated at least three times.

2.8. Flow cytometry

For apoptosis analysis, quantification of apoptotic cells was detected using Annexin-V-FITC Apoptosis Detection Kit (Bestbio, Shanghai, China) according to the manufacturer's instruction. In brief, the cells were harvested and washed twice with cold PBS, and then resuspended in 400 µl Annexin V binding buffer. Thereafter, 5 µl Annexin V-FITC and 10 µl PI were added and the cells were further incubated in the dark for 15 min at 4 °C. Cells were discriminated into viable cells, dead cells, early apoptotic cells, and apoptotic cells. Analyses were performed on BD FACSVerse (BD Biosciences, CA, USA). Apoptosis was analyzed with FACS using FlowJo data analysis software package (TreeStar, USA). The percentage of early apoptotic cells

were compared to control groups from each experiment. The experiments were repeated at least three times.

2.9. Observation of cell morphology by Hoechst 33258 Staining

Cells were stained with Hoechst 33258 (Beyotime, Shanghai, China) and examined under a fluorescence microscope. After induction by the extracellular solution (pH 6.0), the cells were washed twice with PBS and then stained with Hoechst 33258 according to the manufacturer's instructions in the dark for 15 min. Thereafter, cells were observed and photographed under an inverted fluorescence microscope (Olympus, Tokyo, Japan).

2.10. Laser scanning confocal microscopy

The intracellular calcium of isolated rat articular chondrocytes was monitored using fluorescence imaging [24]. Cells (2×10^5) on coverslips were washed three times with D-Hanks' solution and incubated with 4 μ M Fluo-3-AM and 0.02 % Pluronic F-127 (Biotium, Hayward, California, USA) for 30 min at 37 °C. After incubation, the cells were washed three times with Hank's solution to remove the extracellular Fluo3-AM. To eliminate the effects of voltage-gated Ca^{2+} channels from intracellular stores, nimodipine (5 μ M) and ω -Conotoxin MVIIC (3 μ M) were added to the extracellular fluid. Then cells were perfused initially with D-Hank's solution (pH6.0), and PcTX1 then with buffer containing IL-6 (10 ng/ml). The fluorescence of intracellular Fluo-3 was quantitated by confocal laser scanning fluorescence microscopy (Zeiss) with excitation at 488 nm and emission at 525 nm. Gray scale images with 0–255 steps were collected at different time points before and up to 10

min after fluorescence microscopy and archived as TIFF image files for later analysis. Digitized images were acquired, stored and analyzed on a computer controlled by Leica-sp5 LAS AF software. Images (340/380 ratio) were analyzed by averaging pixel ratio values in circumscribed regions of cells in the field of view. The values were exported from Axon Imaging Workbench software to SigmaPlot for additional analysis and plotting.

2.11. Quantitative real-time PCR analysis

Total RNA was extracted from articular tissues and chondrocytes using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA), then reverse transcribed into complementary DNA (cDNA) using First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR analyses for mRNA of ASIC1a, collagen II, aggrecan, Bcl-2, Bax and β -actin were performed by PIKO REAL RT-PCR kits (Thermo Fisher Scientific, Waltham, MA, USA). Standardization was performed with β -actin as the endogenous control. The primers were designed and synthesized by Shanghai Biology Engineering Corporation according to the serial number from Genbank, as shown in Table 1. PCR was performed at 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s with PIKO REAL 96. The fluorescent signals were collected during extension phase, Ct values of the sample were calculated, and transcript levels were analyzed by $2^{-\Delta\Delta Ct}$ method. The PCR was performed in triplicate and repeated at least three times.

2.12. Western blot analysis

Rat articular tissues and chondrocytes were lysed with RIPA lysis buffer

(Beyotime, Shanghai, China). The nuclear protein and cytoplasmic fractions were extracted using a nuclear and cytoplasmic protein extraction kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. Whole extracts were prepared, and the protein concentrations were determined using a BCA protein assay kit (Boster, Wuhan, China). Equal amounts of protein lysates (30 µg) were separated by SDS-PAGE (10%, 80 V for 30 min and then 120 V for 90 min). The proteins were transferred onto a PVDF membrane (Millipore Corp, Billerica, MA, USA). Then the PVDF membranes will be incubated in TBST containing 5% nonfat dry milk at 37 °C for 3 h. After blocking, the PVDF membranes were incubated with specific primary antibodies overnight at 4 °C. Guinea pig polyclonal anti-ASIC1a (Alomone Labs, Jerusalem, Israel) was used at 1:600, rabbit monoclonal antibodies against ERK, phospho-ERK, JNK, phospho-JNK, NF-κB, phospho-NF-κB (Cell Signaling Technology, USA) were used at 1:800, Bax and Bcl-2 (Boster, Wuhan, China) were diluted 1:200, mouse monoclonal anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, USA) was used at 1:500. Following incubation with primary antibodies, blots were washed four times in TBST before incubation at 37 °C for about 1 h in goat anti-mouse or goat anti-rabbit horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, USA) conjugate antibody at (1:8000-1:10000) dilution in TBS/Tween-20 containing 5 % nonfat dry milk. After extensive washing in TBS/Tween-20 for another four times, the membranes were developed with distilled water for detection of antigen using the enhanced chemiluminescence system. Proteins were visualized with ECL-chemiluminescent kit (ECL-plus, Thermo Fisher Scientific, Waltham, MA,

USA). Autoradiographs were scanned using a Image-Pro Plus Imaging analysis software (Media Cybernetics, MD Rockville, USA). All the experiments reported in this study were performed three times and the results were reproducible.

2.13. Statistics

Results were expressed as mean $\pm$ SE. The comparison among the different treatment groups was done using analysis of variance (ANOVA) and unpaired Student's *t*-test. For changes in mRNA or protein levels, ratios of mRNA (relative expression) and protein (densitometric values) to respective house-keeping controls were compared. A value of $P < 0.05$ was defined as significance.

3. Results

3.1. Upregulation of ASIC1a mRNA and protein in the articular cartilage tissues and chondrocytes from AA rats

To investigate the expression of ASIC1a in the articular cartilage tissues from AA rats, AA rats developed severe arthritis at day 26 after immunization and were determined by H&E staining. H&E staining showed the articular cartilage exhibited smooth surfaces with intact layers of flattened cells in the superficial zone in the normal control group, while the articular cartilage in AA rats exhibited very rough, uneven surfaces, and eroded (Fig. 1A). As shown in Fig. 1B, ASIC1a immunostaining signal was increased in the articular cartilage tissues from AA rats compared with the normal group. Compared with that in the normal control group, the ASIC1a mRNA was elevated 1.9-fold in AA rats (Fig. 1C), and the protein level of ASIC1a was also elevated (Fig. 1D).

The markers of articular chondrocyte phenotype for cells cultured *in vitro* were confirmed by toluidine blue staining of glycosaminoglycan and immunocytochemical staining of type II collagen (Fig. 1E). As shown in Fig. 1F, both qRT-PCR and western blot analysis demonstrated that the mRNA and protein expression of ASIC1a in articular chondrocytes were also elevated in AA rats.

3.2. IL-6 induces ASIC1a expression in articular chondrocytes in a time- and dose-dependent manner.

To investigate the effect of IL-6 on ASIC1a expression, we performed qRT-PCR, Western blot and immunofluorescence assays on articular chondrocytes from normal rats. As shown in Fig. 2A and C, treatment with IL-6 dose-dependent increased in the mRNA and protein levels of ASIC1a in rat chondrocytes. Likewise, Fig.2B showed that 6 h after IL-6 treatment ASIC1a mRNA expression increased by 1.99 fold. Longer time of IL-6 treatment resulted in higher expression of ASIC1a with the peak at 24 h after IL-6 treatment, at which ASIC1a mRNA expression was upregulated by 3.4 fold. In addition, ASIC1a protein level also was elevated in articular chondrocytes after treatment with IL-6 in a time-dependent manner (Fig.2D). Consistently, results of immunofluorescence (Fig. 2E) also showed that ASIC1a expression was increased in IL-6 treated cells (10 ng/ml IL-6 for 24 h). Furthermore, tocilizumab, an anti-IL-6-receptor antibody, was used to further confirm the role of IL-6 in regulating ASIC1a expression. As expected, the increased ASIC1a expression induced by IL-6 in articular chondrocytes was completely inhibited by tocilizumab (Fig. 2F and G).

3.3. Aggrecan and type II collagen expression are not induced by IL-6.

Aggrecan and type II collagen expression were also analysed in the articular chondrocytes after IL-6 treatment. No changes in the gene expression of aggrecan and type II collagen were detected in chondrocytes after IL-6 treatment with not only different amounts but also different time points (Fig.3A and B). Moreover, no induction on aggrecan and type II collagen proteins expression was observed in IL-6-treated chondrocytes by immunofluorescence (Fig.3C and D).

3.4. IL-6 induces the activation of JAK2/STAT3, ERK1/2, JNK and NF- κ B pathways.

Serum-starved articular chondrocytes were exposed to IL-6, and the phosphorylation levels of JAK2 and STAT3 were determined by Western blot. After treatment with IL-6, there was a rapid increase in p-JAK2 as well as p-STAT3 protein levels of chondrocytes (Fig. 4A). As expected, there was no appreciable change in the levels of total JAK2 and STAT3 during the treatment period. IL-6-treated cells were also analyzed for activation of ERK1/2, JNK, and p65. As shown in Fig. 4B, phosphorylation of ERK1/2, JNK, p65 and JAK2 were strongly induced in rat chondrocytes after stimulation with IL-6. Again, the levels of the respective total proteins remained generally unaffected. In addition, the effect of IL-6 on STAT3 and p65 nuclear translocation was also examined. Western blot showed that after IL-6 treatment, the expression of p65 and STAT3 were significantly increased in the nucleus, but decreased in the cytoplasm in articular chondrocytes (Fig. 4C and E). Furthermore, after 30 min of treatment, IL-6-stimulated chondrocytes showed a clear and positive labelling for activated p65 and STAT3 in nuclei and to a lesser extent in the cytoplasm of chondrocytes (Fig. 4D and F).

3.5. Inhibitors of JAK2/STAT3 and MAPK/NF- κ B downregulate IL-6-induced ASIC1a expression.

To further investigate the potential role of IL-6-induced activation of MAPK, NF- κ B and JAK2/STAT3 pathways in the upregulation of ASIC1a expression in articular chondrocytes, we used the inhibitors (PD098059, SP600125, PDTC and AG490) to selectively block these signaling pathways. Chondrocytes were pretreated with a specific pharmacological inhibitor of ERK pathway, PD98059, and then simultaneously exposed to IL-6. This inhibitor completely suppressed ERK1/2 phosphorylation without affecting the total ERK1/2 levels (Fig 5A). PD98059 repressed the IL-6 induction of ASIC1a mRNA (Fig.5F) and protein expression (Fig.5G). In addition, phosphorylation of JNK was attenuated by pretreatment of cells with SP600125, the JNK-specific inhibitor (Fig.5B). SP600125 revealed a partial inhibition of ASIC1a expression (Fig.5 F and G). Furthermore, the IL-6-induced phosphorylation of p65 was completely abolished by pretreatment with PDTC, the NF- κ B-specific inhibitor (Fig. 5C), and PDTC downregulated IL-6-induced expression of ASIC1a (Fig. 5F and G). Role of JAK2/STAT3 pathway in ASIC1a induction was investigated by pretreatment with the JAK2-specific inhibitor, AG490. This inhibitor blocked IL-6-stimulated phosphorylation of JAK2/STAT3 without affecting its total levels (Fig. 5D and E). Treatment of rat chondrocytes with AG490 resulted in partial suppression of ASIC1a mRNA and protein induction (Fig. 5F and G).

3.6. IL-6 promotes $[Ca^{2+}]_i$ elevation in acid-induced articular chondrocytes

In order to further investigate the roles of IL-6 in regulating ASIC1a function, we tested the effect of IL-6 and PcTX1, the ASIC1a-specific inhibitor, on the acid-induced articular chondrocytes. In all experiments, blockers of glutamate receptors (10 μ M MK801), voltage-gated Ca^{2+} channels (5 μ M nimodipine and 3 μ M ω -conotoxin MVIIC) and 1 μ M thapsigargin were added to all solutions to inhibit possible secondary activation of these channels and release of the internal Ca^{2+} stores. From the confocal micrographs and the representative traces, the results showed that IL-6 significantly increased $[\text{Ca}^{2+}]_i$ in acid-induced articular chondrocytes. Furthermore, blockade of ASIC1a with PcTX1 could significantly reduce the concentration of intracellular calcium by acid with or without IL-6 (Fig.6).

3.7. IL-6 inhibits cell viability of acid-induced articular chondrocytes

To investigate the effect of IL-6 on viability of acid-induced articular chondrocytes, experiments were first performed to determine the cell viability of cultures treated with IL-6 at the concentrations. MTT assays showed that these parameters were not significantly affected by a 24 h treatment with increased concentrations of IL-6, suggesting that IL-6 did not exert cytotoxic effects at these concentrations (Fig.7A). Then, the results revealed that when articular chondrocytes were treated with increased concentrations of IL-6 in the presence of acid (pH6.0), acid-induced death of articular chondrocytes was significantly enhanced and IL-6 restrained cell viability in a concentration-dependent manner (Fig.7B), which could be abolished by pretreated with tocilizumab (Fig.7C). Furthermore, to determine whether activation of ASIC1a is involved in acid-induced articular chondrocyte injury, we

tested the effect of PcTX1 on LDH release. Consistent with the MTT assay, LDH assay showed that IL-6 similar increased acid-induced cell injury. Inhibition of ASIC1a by PcTX1 at 100 μ M caused significant downregulation of LDH release by acid with or without IL-6 (Fig.7D).

3.8. IL-6 enhances acid-induced apoptosis in articular chondrocytes

Acid-induced apoptosis in articular chondrocytes was evaluated using flow cytometric analysis and Hoechst 33258 staining. The results in Fig. 8A and B showed that pretreatment with tocilizumab completely abrogated the enhanced effect of IL-6 on acid-induced apoptosis. Furthermore, in the pH6.0 group, cells undergoing apoptosis were observed, exhibiting bright blue nuclei and nuclear condensation (Fig. 8C). Cells pretreated with IL-6 showed more nuclear condensation or fragmentation compared to the pH6.0 group. As shown in Fig. 8D and E, the rate of apoptosis rose to 23.19 % with acid treatment (pH6.0 alone) for 3 h, which was higher than control ($P<0.01$). Moreover, pretreatment with IL-6 markedly promoted this acid-induced apoptosis ($P <0.01$), while IL-6 alone did not induce chondrocyte apoptosis. In addition, the results displayed that inhibition of ASIC1a by PcTX1 led to a significant downregulated the apoptotic ratio compared to the pH6.0 with or without IL-6 group. Of note, treatment of acid-induced chondrocytes with PcTX1 completely abrogated the upregulation of apoptotic ratio by IL-6. Then, the results also showed that pretreatment with IL-6 caused a significant downregulated Bcl-2 mRNA and protein levels in acid-induced chondrocytes compared with the acid-induced group. On the other hand, IL-6 pretreatment markedly increased Bax mRNA and protein levels in

acid-induced chondrocytes compared to the acid-induced group, which could also be reversed by using PcTX1 (Fig.8F and G).

4. Discussion

In this study, we explored the role of IL-6 in regulating acid-induced articular chondrocyte apoptosis. The results showed that IL-6 enhanced acid-induced apoptosis via increasing ASIC1a expression and function in rat articular chondrocytes. IL-6 treatment could upregulate the level of ASIC1a in a time- and dose-dependent manner, the mechanism of which might partially be involved with its ability of regulating the activation of JAK2/STAT3 and MAPK/NF- κ B signaling pathways in articular chondrocytes. Furthermore, IL-6 also reduced cell viability, but increase LDH release, $[Ca^{2+}]_i$ elevation and apoptosis of acid-induced articular chondrocytes, which could be reversed by the blockage of ASIC1a with PcTX1. Pretreatment with PcTX1 also reduced Bax expression levels and increased Bcl-2 expression levels in acid-induced articular chondrocytes.

Articular chondrocytes, represent 1% of the matrix volume, are the sole cell type present in cartilage and are responsible for producing the complex extracellular matrix (ECM), including type II collagen and aggrecan [25]. Although RA has a variety of complex causes, articular chondrocyte apoptosis, especially in the advanced stage, has been suggested as one of the primary causes of RA. ASIC1a is a proton-gated ion channel for Ca^{2+} transportation which is activated by extracellular acidosis, and the expression of ASIC1a in human skeleton and rat endplate chondrocytes has recently been reported [13, 26]. Our previous study also indicated that blockade of ASICs

attenuated articular cartilage destruction in rats with adjuvant arthritis, and led to the inhibition of apoptosis in articular chondrocytes by decreasing Ca^{2+} levels [18, 19, 27]. Consistently, the results of the present study showed that the expression levels of ASIC1a were significantly increased in articular cartilage and chondrocytes of AA rats compared with that in normal group. Therefore, these findings suggested that ASIC1a might play a certain role in the pathogenesis of RA.

It is well known that cytokines such as IL-1 β or IL-6 may have relevant roles in the induction of inflammation and bone erosion [1]. Gong et al. [20] has clearly demonstrated that IL-1 β significantly increases expression of ASIC3 in FLS. In the present study, our results revealed that the mRNA and protein expressions of ASIC1a in the articular chondrocytes were significantly increased in a dose- and time-dependent manner under the effect of IL-6. In line with previous research [28], the results of the present study showed that the expression of type II collagen as well as aggrecan did not change in the presence of IL-6. Moreover, our results also showed that IL-10 did not affect ASIC1a expression in articular chondrocytes (Supplement Fig. S1). Together with the findings that IL-6 is abundant in the synovial fluid and serum of RA patients, our results suggest that the IL-6-induced ASIC1a expression and activation in articular chondrocytes may participate in the cartilage destruction in RA.

Over the past few years, growing evidence has shown that transcription factors such as STAT3 and NF- κ B, play an important role in RA development [29, 30]. It has been well established that binding of IL-6 to its receptors could induce the tyrosine

phosphorylate gp130 and STAT3, then the phosphorylated STAT3 migrates from the cytoplasm to the nucleus, where they bind to specific DNA elements to regulate the downstream genes transcription [31], and activated STAT3 also can modify NF- κ B activation by binding p65 in the nucleus [32]. Consistent with many of the previous studies, our results displayed that JAK2/STAT3 and MAPK /NF- κ B signaling pathways were both significant activated following IL-6 stimulation in cultured articular chondrocytes, and the increased expression of p-JAK2, p-STAT3, p-JNK, p-ERK and p-p65 were inhibited by respective inhibitors of these pathways in IL-6 stimulated articular chondrocytes. In addition, these inhibitors could partially abrogate the ASIC1a upregulation by IL-6. Collectively, these results indicated that the activation of ERK1/2, JNK, p65 and JAK2/STAT3 was necessary for the upregulated expression of ASIC1a induced by IL-6.

It is widely accepted that Ca^{2+} plays an important role in apoptotic cell death. It has been suggested that extracellular acidosis enhances Ca^{2+} -permeable ASIC1a activity, which is responsible for cell injury and death [24]. In agreement with our previous research [17], our results also demonstrated that articular chondrocytes responded to a reduction in extracellular pH by increasing $[\text{Ca}^{2+}]_i$ levels, which was inhibited by ASIC1a blockade with PcTX1. Importantly, this study presented the first evidence that IL-6 could further increase the acid-induced elevation of intracellular calcium in articular chondrocytes and the increasing $[\text{Ca}^{2+}]_i$ levels could be downregulated by inhibition of ASIC1a. Furthermore, the results demonstrated that IL-6 promoted acid-induced cell injury by ASIC1a, as indicated by an increased LDH

release and decreased cell viability in the acid-induced articular chondrocytes.

Chondrocyte apoptosis is a feature of progressive cartilage degeneration in RA. Although IL-6 can inhibit or promote the apoptosis of various cells [8, 33], the role of IL-6 on acid-induced apoptosis in articular chondrocytes is mostly unknown. Our results showed that IL-6 dramatically increased apoptosis ratio in acid-induced articular chondrocytes. Furthermore, pretreatment with IL-6 significantly increased the level of Bax, and inhibited the level of Bcl-2 in acid-stimulated articular chondrocytes. In addition, blockade of ASIC1a with PcTX1 dramatically decreased the expression of pro-apoptotic proteins, while promoted the levels of Bcl-2. Taken together, these results indicated that the pro-apoptotic role of IL-6 in acid-stimulated articular chondrocytes was caused, at least in part, by its involvement in regulating ASIC1a expression and function. Future experiments are required to elucidate the effects of IL-6 on ASIC1a current in articular chondrocytes using the whole-cell patch clamp technique.

Although ASIC1a was mostly localized within cytoplasm rather than on the cell membrane, our investigation of PcTX1 showed that IL-6-enhanced acid-induced articular chondrocyte apoptosis is potently inhibited by PcTx1. Besides that a small part of ASIC1a was expressed in the membrane, it is possible that ASIC1a trafficking to the cell surface may also play a vital role for acid-induced articular chondrocyte apoptosis. Moreover, several studies showed that the forward trafficking of ASICs may be significant for acidosis-induced neuronal injury [34, 35]. In addition, recent findings showed that brain derived neurotrophic factor (BDNF) promoted forward

trafficking and surface expression of ASIC1a, and this was responsible for the upregulated channel function [36]. Based on our studies, we cannot exclude the possibility that IL-6 might promote forward trafficking and surface expression of ASIC1a in articular chondrocytes. It will be of future interest to test whether IL-6 enhanced acid-induced apoptosis by increasing ASIC1a surface trafficking.

In summary, our results confirmed the expression of ASIC1a was increased in rat articular cartilage tissues and chondrocytes from AA rats. Furthermore, IL-6 was able to promote ASIC1a expression through JAK2/STAT3 and MAPK/NF- κ B signaling pathways. Moreover, ASIC1a upregulation by IL-6 was involved in acid-induced apoptosis in articular chondrocytes. The blockade of ASIC1a was able to attenuate IL-6-enhanced acid-induced articular chondrocyte apoptosis (Fig.9), which may have therapeutic potential for the treatment of RA. Apart from the role of IL-6 in the inflammatory response, our results indicated that IL-6 could enhance acid-induced articular chondrocyte apoptosis by regulating ASIC1a expression and function. Together with the findings in our previous study, these results suggested that ASIC1a might exist as a potential therapeutic target in the treatment of RA.

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Fig.1. Upregulation of ASIC1a mRNA and protein in the articular cartilage of rats with AA. (A) Pathology observation of the experimental rat hind paw sections stained with hematoxylin and eosin (H&E) staining (original magnification, $\times 100$). (B) The level of the ASIC1a was analyzed by immunohistochemistry in rat articular cartilage. Representative views from each group are presented (original magnification, $\times 400$). (C and D) Total RNAs and proteins were isolated from the articular cartilage of AA rats or normal groups. The expression of ASIC1a was assessed by qRT-PCR and western blotting. (E) Culture and identification of rat articular chondrocytes *in vitro*. *i* Biosynthesis of glycosaminoglycan was evidenced by positive staining of toluidine blue. *ii* Immunohistochemical staining of type II collagen. (F) The mRNA and protein levels of the ASIC1a were analyzed by qRT-PCR and western blotting in primary articular chondrocytes isolated from AA rats or normal groups. Data were presented as mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$ versus normal group.

Fig.2. IL-6 induces the expression of ASIC1a in articular chondrocytes. (A and C) Concentration-dependent increases in ASIC1a mRNA and protein expressions stimulated by IL-6. Articular chondrocytes were treated without or with IL-6 (12 h or 24 h) at indicated concentrations (1.25–20 ng/ml). (B and D) Time-dependent increases in ASIC1a mRNA and protein expressions stimulated by IL-6. Articular chondrocytes were treated without or with IL-6 (10 ng/ml) for indicated time (3–48 h). The levels of ASIC1a mRNA were detected by qRT-PCR. The cell lysates were assayed by Western blot using ASIC1a antibodies. β -actin was chosen as an internal control. Data were presented as mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$ versus control group.

(E) Representative immunofluorescence analysis performed on articular chondrocytes using anti-ASIC1a (green) antibody after IL-6 treatment for 24 h. Nuclei were counterstained with DAPI. Merge contains the combined image of ASIC1a immunostaining and DAPI staining. Determinations were done in triplicate in three independent cell cultures (original magnification $\times 400$). (F and G) Tocilizumab inhibits the ASIC1a mRNA and protein expressions stimulated by IL-6. Data were presented as mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$ versus control group; # $p < 0.05$, ## $p < 0.01$ versus IL-6 group.

Fig.3. IL-6 does not modulate type II collagen and aggrecan expressions in articular chondrocytes. (A and B) Col2a1 and aggrecan mRNA levels were analysed by qRT-PCR after IL-6 treatment. Data were presented as mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$ versus control group. (C and D) Representative immunofluorescence analysis performed on articular chondrocytes using anti-collagen II (green) or anti-aggrecan (green) antibody after IL-6 treatment for 24 h. Nuclei were counterstained with DAPI. Merge contains the combined image of collagen II or aggrecan immunostaining and DAPI staining. Determinations were done in triplicate in three independent cell cultures (original magnification $\times 400$).

Fig.4. IL-6 induces activation of JAK2/STAT3 and MAPK/NF- κ B in a time-dependent manner. (A) Chondrocytes in serum-free confluent monolayer cultures were stimulated with or without 10ng/ml IL-6 for 0-120 minutes. (B) Chondrocytes were cultured with 10ng/ml IL-6 for 5, 15, 30, 60 min and 24 h, respectively. Then, the levels of phosphorylated and total JAK2, STAT3, ERK, JNK and p65 proteins were measured by Western blot. Data were presented as mean $\pm$ SE.

* $p < 0.05$, ** $p < 0.01$ versus 0 min. (C and E) Nuclear and cytoplasmic p65 as well as STAT3 proteins were isolated and determined by Western blot; Histone H3 was used as normalized control in nuclear protein. β -actin was chosen as normalized control in cytoplasmic protein. Data were presented as mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$ versus control group. (D and F) Representative immunofluorescence analysis performed on articular chondrocytes using anti-p65 (green) or anti-STAT3 (green) antibody after IL-6 treatment for 30 min. Nuclei were counterstained with DAPI. Merge contains the combined image of p65 or STAT3 immunostaining and DAPI staining. Determinations were done in triplicate in three independent cell cultures (original magnification $\times 400$).

Fig.5. PD 098059, SP600125, PDTC and AG 490 inhibit IL-6 modulation of ASIC1a expression. Cultured chondrocytes were pretreated with or without inhibitors for ERK (PD98059, 10 μ M), JNK (SP600125, 10 μ M), NF- κ B (PDTC, 10 μ M), and JAK2 (AG490, 10 μ M) for 45 minutes followed by stimulation with IL-6. (A-E) The levels of p-JAK2, p-STAT3, p-ERK, p-JNK, and p-p65 proteins were assessed by Western blot. (F and G) Western blot analysis indicates that treatment with these inhibitors partially abolished ASIC1a mRNA and protein induction by IL-6. Data were presented as mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$ versus control group.

Fig.6. Blockade of ASIC1a with PcTX1 reduces IL-6-induced elevation of $[Ca^{2+}]_i$ level in acid -mediated articular chondrocytes. Cellular confocal micrographs of the same scale showing the changes in the $[Ca^{2+}]_i$ concentration, as visualized by Fluo-3-AM, in articular chondrocytes. (A) Acid-induced elevation of $[Ca^{2+}]_i$ in

Ca²⁺-free extracellular solution. (B) Acid -induced elevation of [Ca²⁺]_i in extracellular Ca²⁺solution. (C) Acid-induced elevation of [Ca²⁺]_i in the presence of IL-6. (D) Acid-induced elevation of [Ca²⁺]_i in chondrocytes treated with PcTX1. (E) Acid-induced elevation of [Ca²⁺]_i in chondrocytes treated with PcTX1 in the presence of IL-6. The amplitude of [Ca²⁺]_i intensity in chondrocytes induced by acid and IL-6 treatment was quantified as the maximal rise of [Ca²⁺]_i above basal levels. *i* Before exposure to acid solution. *ii* Increased Ca²⁺ intensity when pH was decreased to 6.0. *iii* A few minutes after exposure to the acid solution (n = 6).

Fig.7. IL-6 promotes acid-induced cell injury by ASIC1a. Cell viability percentage was measured by MTT assay. (A) Chondrocytes were incubated with different concentrations of IL-6 for 24 h. (B) Chondrocytes were pretreated with various concentrations of IL-6 for 24 h before exposure to the extracellular solution (pH 6.0) for 3 h. The data are expressed as mean ± SE (n = 4), **p*<0.05, ***p*<0.01 versus control group. (C) Effects of tocilizumab on IL-6-inhibited cell viability of articular chondrocytes in the absence of extracellular solution (pH 6.0). (D) Effects of IL-6 and PcTX1 on LDH release from acid-induced chondrocytes. The data are expressed as mean ± SE (n = 6), **p*<0.05, ***p*<0.01 versus control group; #*p*<0.05, ##*p*<0.01 versus pH 6.0 alone; &*p*<0.05, &&*p*<0.01 versus pH 6.0 + IL-6 group.

Fig.8. Blockade of ASIC1a with PcTX1 reduced IL-6-enhanced acid-apoptosis in articular chondrocytes. (A and D) Cells were stained with Annexin V-FITC and PI, and analyzed by flow cytometry. (B and E) Histogram for statistical analysis shows the apoptotic rate in the different treatment groups. (C) Phase contrast microscopic

images of articular chondrocytes stimulated with different treatments and representative photomicrographs of articular chondrocytes stained with Hoechst 33258 (original magnification $\times 400$). Apoptotic cells were characterized as having condensed or fragmented nuclei (indicated by arrows). (F) The level of Bax and Bcl-2 mRNA was analyzed by qRT-PCR. (G) The level of Bax and Bcl-2 protein was assessed by Western blot. Data were presented as mean $\pm$ SE. $*p<0.05$, $**p<0.01$ versus control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ versus pH 6.0 alone; $^{\&}p<0.05$, $^{\&\&}p<0.01$ versus pH 6.0 + IL-6 group.

Fig.9. Schematic presentation of the signaling pathways involved in IL-6-induced apoptosis and ASIC1a expression of articular chondrocytes. IL-6 and IL-6 receptor interaction activates JAK2/STAT3, MAPK, and NF- κ B pathways, which leads to ASIC1a expression. Acid activates ASIC1a and increases pro-apoptotic proteins expression, which results in apoptosis of articular chondrocytes.

Figure.1

Figure.2

Figure.2

F

G

Figure.3

Figure.4

Figure.4

Figure.5

Figure.5

F

G

Figure.6

Figure.7

A

B

C

D

Figure.8

Figure.8

Figure.8

F

G

Figure.9

Table 1 Primer sequences for Real-time PCR

Gene	Forward primer	Reverse primer
ASIC1a	5'-GAGAAGCACAAAGGAGATGACACG-3'	5'-AGACAAAGGGCACCAGGATAGG-3'
ACAN	5'-CTAGCTGCTTAGCAGGGATAACG-3'	5'-TGACCCGCAGAGTCACAAAG-3'
Col2a1	5'-GAGTGGAAGAGCGGAGACTACTG-3'	5'-CTCCATGTTGCAGAAGACTTTCA-3'
Bcl-2	5'-ACTGAGTACCTGAACCGGCATC-3'	5'-GGAGAAATCAAACAGAGGTCGC-3'
Bax	5'-AGTGTCTCAGGCGAATTGGC-3'	5'-AGTGTCTCAGGCGAATTGGC-3'
β-actin	5'-CCCATCTATGAGGGTTACGC-3'	5'-TTTAATGTCACGCACGATTTC-3'