Role of P2X7R in eosinophilic and non-eosinophilic chronic rhinosinusitis with nasal polyps

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Abstract. Chronic rhinosinusitis with nasal polyps (CRSwNP) is an inflammation-mediated disease of the nasal mucosa. P2X7R has been reported to be a potential biomarker for inflammation. The aim of the present study was to explore the role of P2X7R in CRSwNP, and the interaction between P2X7R and the NLRP3 inflammasome in the development of CRSwNP. Firstly, the expression profiles of P2X7R in nasal mucosa were investigated using western blotting (WB), polymerase chain reaction (PCR) and immunofluorescence (IF) staining. Next, the effect of inflammatory stimulation with lipopolysaccharides (LPS) combined with BzATP, adenosine 5'-triphosphate triethylammonium salt (BzATP) on primary human nasal epithelial cells (HNECs) was determined. Then, the therapeutic effect of the selective P2X7R antagonist, A740003, on P3X7R, NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome and IL-1β alterations in HNECs was explored using enzyme-linked immunsorbent assay, WB and PCR. It was found that P2X7R was overexpressed in CRSwNP, especially in eosinophilic CRSwNP, the expression of P2X7R, NLRP3 and IL-1β were upregulated in HNECs after induction by LPS combined with BzATP; but the expression of NLRP3 and IL-1β were downregulated after stimulation with A740003. The aforementioned results indicate that P2X7R-mediated NLRP3 inflammasome activation may have a role in the pathogenesis of CRSwNP.

Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a nasal inflammatory disease, characterized by symptoms including nasal obstruction, drainage, smell loss and facial pain or pressure (1). CRSwNP affects a large proportion of the population world-wide and is associated with high cost of management and low quality of life (2). The prevalence of CRSwNP in Europe is estimated to be between 2.1 and 4.4%, while it is 4.2% in the United States and 1.1% in China (3). Lourijsen et al (4) found that the total direct costs were 1,501 Euros per year per patient with CRSwNP. CRS is currently classified into eosinophilic CRS (ECRS) and non-ECRS (NECRS) subtypes based on the presence or absence of tissue eosinophilic infiltration (1). Therefore, CRSwNP may also be subclassified into eosinophilic CRSwNP (ECRSwNP) and non-eosinophilic CRSwNP (NECRSwNP) (5), with the former having a higher recurrence rate and asthma incidence (6). Over the past 40 years, advances in functional endoscopic sinus surgery and pre- and postoperative drug therapy have greatly improved the cure rate of CRSwNP; however, disease recurrence ranges from 40 to 78.9% in CRSwNP, and the rate of revision surgery is as high as 36.8% (7,8). There is still a lack of effective mechanism-based treatments in clinical practice.

NOD-like receptor pyrin domain containing 3 (NLRP3) can assemble with apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1 to form a multimeric protein complex called the NLRP3 inflammasome. Recently, the NLRP3 inflammasome was demonstrated to be implicated in the pathogenesis of CRSwNP (9). It was found that the NLRP3 inflammasome was activated in nasal mucosa in a murine acute bacterial rhinosinusitis model (10). This suggested that the NLRP3 inflammasome contributed to nasal inflammation. Activated caspase-1, generated when pro-caspase-1 is cleaved by the NLRP3 inflammasome, proteolytically cleaves the inflammatory cytokines IL-1β and IL-18 into their mature forms (11). Elevated IL-1β and IL-18 levels are found in nasal polyps of CRSwNP patients (9,12). Furthermore, increased IL-18 levels was significantly associated with the radiological severity of sinusitis and local eosinophilia (12). Therefore, a mechanism for decreasing the levels of inflammatory cytokines may provide a clinical benefit. The formation of the NLRP3 inflammasome can be induced by several microbial and nonmicrobial stimuli.

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Nonmicrobial stimuli include substances which cause specific diseases such as uric acid, silica fibres and extracellular adenosine triphosphate (ATP) (13,14). The purinergic 2X7 receptor (P2X7R) can be triggered by ATP which then results in the assembly of the NLRP3 inflammasome (15).

The P2X7R protein expression in bronchial epithelial cell line BEAS-2B was examined. The expression of P2X7R in BEAS-2B cells was upregulated significantly upon treatment with ATP. This suggests that the activation of P2X7R could play a role in the development of CRSwNP.

**Materials and methods**

**Patients.** A total of 32 patients with CRSwNP (16 ECRSwNP and 16 NECRSwNP) and 16 control subjects were included in the present study. NP specimens were obtained from patients diagnosed with CRSwNP according to the criteria of the European Position Statement updated in 2020 (1). Human nasal mucosa of the middle turbinate were collected from patients that underwent neurosurgery, who had undergone surgery because of a pituitary tumour, as the control. The visual analogue scale and computed tomography scores were graded according to the method previously described (18). The clinical characteristics of patients are listed in Table I. Subjects who had used oral or nasal corticosteroids, anti-histamines, antibiotics or antileukotrienes within the preceding 4 weeks before sample collection were excluded. The collected samples were used for haematoxylin-eosin (HE) staining, western blotting (WB), immunofluorescence (IF) staining, and primary human nasal epithelial cell (HNECs) culture. The present experimental study was approved by the ethical committees of Tongji Medical College, Huazhong University of Science and Technology (permit no. S135). All participants of the study were informed and signed a consent form.

**Histological and immunofluorescence observation.** The NP tissues collected from patients were immediately fixed overnight in 4% formaldehyde-phosphate buffered saline solution, then dehydrated through a graded ethanol series (70, 80, 90, 95 and 100%) for 5 min each, before samples were embedding in paraffin, and finallysectioned at 5-μm thickness. In order to quantify the eosinophil infiltration of NP, haematoxylin and eosin staining was performed. The NP sections were observed at high power (HP) (magnification, x400; Leica GmbH; cat. no. DM2500) and then, 10 HP fields were randomly selected and eosinophil numbers were microscopically counted. A tissue eosinophil count of 10 or more eosinophils per high-power field (HPF) was defined as ECRSwNP (1,19). After deparaffinization and rehydration, the tissue sections underwent heat-induced epitope retrieval followed by blocking with 10% bovine serum albumin protein at room temperature for 30 min. The blocked sections were incubated with rabbit anti-human P2X7R (1:200; GeneTex; cat no. GTX104288) at 4˚C overnight. The next day, the sections were rinsed three times with PBS, and incubated with secondary anti-rabbit antibody (1:300; antGene; cat no. ANT032) in the dark at room temperature for 1 h and counterstained with DAPI (Beyotime Institute of Biotechnology; cat. no. C1005). Images were captured with a confocal laser scanning microscope (Nikon-AI-Si; Nikon Corporation).

**Cell culture.** HNECs isolated from seven patients with CRSwNP were cultured according to a previously reported method (9). In brief, the tissues were transferred and digested with 0.1% protease from Streptomyces griseus (cat no. 9036-06-0; Sigma-Aldrich; Merck KGaA) and 0.1 mg/ml deoxyribo-nuclease (cat no. D5025; Sigma-Aldrich; Merck KGaA). Separated epithelial cells were collected and seeded onto PureCol™ EZ Gel solution (Sigma-Aldrich; Merck KGaA; cat no. 5074)-coated 12-well culture plates, and cultured in PneumaCult™ Ex Plus Medium (Stemcell Technologies, Inc.; cat. no. #05040) at 37˚C in 5% CO₂. To induce inflammation, one part of the adherent epithelial cells were cultured under the following conditions: i) no additions (control); ii) 10 μg/ml LPS for 24 h (LPS from Pseudomonas aeruginosa; Sigma-Aldrich; Merck KGaA; cat no. L8643); iii) 10 μg/ml LPS for 24 h, 300 μM 2'(3')-O-(4- benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt (BzATP) (Sigma-Aldrich; Merck KGaA; cat no. B6396) was supplemented 1 h after LPS administration; iv) 20 μg/ml LPS for 24 h, 300 μM BzATP was supplemented 1 h after LPS administration. At the end of the incubation, the cells were collected, centrifuged and frozen at -80˚C until use. To confirm the role of the P2X7 receptor in the inflammatory response of HNECs, the other part of the adherent epithelial cells were treated with LPS and A740003 (P2X7 receptor blocker) were incubated under the following conditions: i) no additions (control); ii) 10 μg/ml LPS for 24 h, 300 μM BzATP was added 1 h after LPS administration; iii) 10 μg/ml LPS for 24 h, 300 μM BzATP was added 1 h after LPS administration, with supplementation with A740003 (10 μM; MedChemExpress; cat no. HY-50697) 15 min before BzATP stimulation. At the end of the incubation, the supernatants and cells were collected, centrifuged and frozen at -80˚C until use.

**WB.** NP specimens were collected and stored in liquid nitrogen at -80˚C until use. Total cellular protein was extracted from NP tissue and cultured cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) according to the manufacturer's instructions, and the protein concentration was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology). Then, 12% SDS-polyacrylamide gels were used to separate the protein (30 μg), after which the proteins were transferred onto polyvinylidenedifluoride membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked with 5% non-fat milk at 4˚C for 1 h and then incubated with the working dilution of primary antibodies: Rabbit anti-human P2X7R (1:1,000; GeneTex; cat no. GTX16827); rabbit anti-human NLRP3 (1:1,000; Abcam; cat no. ab260017); mouse anti-human IL-1β (1:5,000; Arigo Biolaboratories; cat. no. ARG66285); rabbit anti-human GAPDH (1:5,000; AmtGene; cat. no. ANT012)
at 4˚C overnight. Subsequently, the membranes were washed in Tris-buffered saline mixed with 0.1% Tween four times for 10 min each, and then incubated with the appropriate horse radish peroxidase-conjugated secondary antibody: HRP goat anti-rabbit IgG (H+L) (1:4,000; cat. no. ANT020; AntGene) and HRP goat anti-mouse IgG (H+L) (1:4,000; cat. no. ANT019; AntGene) for 1 h at room temperature. The immunolabelled proteins were detected using BeyoECL Plus (Beyotime Institute of Biotechnology). The gel images were analysed using Quantity One software (version 4.6; Bio-Rad Laboratories, Inc.) to estimate the relative quantitative density of the protein bands. GAPDH was used as an internal control.

Real-time PCR. The total mRNA of primary HNECs was extracted using an E.Z.N.A™ Total RNA kit (Omega Bio-tek, Inc.) according to the manufacturer's instructions. A PrimeScript™ RT Reagent kit with a gDNA Eraser (Takara Biotechnology Co., Ltd.) was used to conduct the reverse transcription reaction (37˚C for 15 min, 85˚C for 5 sec and stop at 4˚C) to obtain cDNA for real-time PCR analysis. Real-time PCR was performed using SYBR® Premix Ex Taq™ II (TliRNaseH Plus) (X2 concentration) X1 (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. Sequence-specific primers for P2X7R, IL-1β, and GAPDH were as follows: P2X7R, 5'-TCTGTACTTTCGAGCCAATCAGAC-3' (forward primer); P2X7R, 5'-CCAACTCTAGTGACCAAACCAGGA3' (reverse primer); IL-1β, 5'-CCAGGGCACAGGATATGGAGCAGA-3' (forward primer); IL-1β, 5'-TTCAACACGCGACAGGTACAG-3' (reverse primer); GAPDH, 5'-GCCCGCTCAAGGCTGAGAC-3' (forward primer), GAPDH, 5'-TGTTGAGACGCAGTGGGA-3' (reverse primer). The real-time PCR protocol was as follows: Denaturation at 95˚C for 30 sec, followed by 40 cycles of amplification at 95˚C for 5 sec, 60˚C for 30 sec and annealing at 60˚C for 30 sec. Relative mRNA level was determined by the 2−ΔΔCq method (20).

Enzyme-linked immunosorbent assay (ELISA). Human IL-1β was measured in cell culture supernatants from HNECs under different culture conditions using an ELISA kit (Arigo; cat. no. ARG80101) following the manufacturers' instructions. Samples were run at least in duplicate.

Statistical analysis. For continuous clinical variables, the data are expressed as median and interquartile ranges, or as box and whisker plots displaying medians and interquartile ranges, which were analysed by the Kruskal-Wallis H-test and Mann-Whitney U test. For dichotomous parameters, the χ² test or Fisher's exact test was performed to determine the difference between groups. Data were analysed using the Statistical Package for the Social Sciences (version 22.0; SPSS Inc.). For tissue samples and in vitro experiments, the data are expressed as mean ± standard deviation and were analysed by one-way ANOVA. Tukey's post hoc test was also performed. P<0.05 was considered to indicate a statistically significant difference.

Results

Histological changes and localisation of P2X7R in nasal mucosa. Overall, all patients, including 16 control subjects and 32 patients with CRSwNP, underwent H&E staining, which showed that numerous eosinophils infiltrated the nasal mucosa of patients with ECRSwNP. Furthermore, an intense oedematous stroma and subepithelial and perivascular inflammatory cell infiltration was also observed in CRSwNP (Fig. 1A). Immunofluorescence (Fig. 1B) showed that P2X7R was predominantly expressed in epithelial cells. It is worth noting that the level of receptor in the ECRSwNP group was higher compared with that of the other two groups.

Expression of P2X7R is higher in ECRSwNP compared with the control group. Protein content was analysed in the nasal mucosa of ECRSwNP, NECRSwNP and control groups. As shown by WB (Fig. 2A), P2X7R (Fig. 2B), NLRP3 (Fig. 2C) and IL-1β (Fig. 2D) were significantly overexpressed in CRSwNP (P<0.05), especially in the ECRSwNP group. The mRNA expression of P2X7R and IL-1β were elevated in the CRSwNP groups compared with the control group, and these increases were also found in the ECRSwNP group compared with the NECRSwNP group (Fig. 2E and F).
**IL-1β is upregulated after incubation with LPS combined with BzATP.** LPS induces a cellular inflammatory response in vitro. The expression of IL-1β was evaluated under different conditions by WB (Fig. 3). When treated with LPS alone, the expression of IL-1β increased relative to controls. When LPS was combined with BzATP, IL-1β markedly increased, and the increase was statistically significant compared with the control group (P<0.05). There was no significant difference in the expression of IL-1β between the 10 µg/ml LPS group and the 20 µg/ml group, thus, 10 µg/ml LPS combined with BzATP was chosen as the inflammatory stimulation condition.

Expression of P2X7R shows no significant change, but NLRP3 is downregulated after stimulation with A740003. A740003 significantly blocks the sustained phase of the BzATP-induced response. The expression levels of P2X7R and NLRP3 were evaluated by WB (Fig. 4). The expression of P2X7R (Fig. 4B) showed no significant change, while the expression of NLRP3...
(Fig. 4C) was significantly downregulated after A740003 treatment in HNECs after stimulation with LPS+BzATP. Similarly, after treatment of HNECs with LPS combined with BzATP, the expression of both P2X7R and IL-1β mRNA increased (P<0.05). After addition of the inhibitor A74003, compared with the inflammatory stimulation group, the expression of P2X7R decreased somewhat, but this was not statistically significant, while IL-1β mRNA significantly
discussion

The present study found that P2X7R, NLRP3 and IL-1β protein levels were significantly increased in the CRSwNP groups compared with the control group and expression was further significantly higher in the ECRSwNP group compared with the NECRSwNP group. Thus, the inflammatory form of ECRSwNP is more severe compared with NECRSwNP. There are obvious regional differences in the inflammatory characteristics of CRS. Approximately 80% of nasal polyps in Western patients are eosinophilic, while the rate in Asia is <50% (21). However, recent studies have shown that the incidence of ECRS in East Asian countries is increasing. A Chinese study showed that the proportion of eosinophilic CRSwNP significantly increased from 59.1 to 73.7% over 11 years (19). ECRS is considered a special and recalcitrant subtype of CRS (21).

ATP concentration is maintained at a low level in healthy tissues (22). Extracellular ATP is involved in the release of various pro-inflammatory cytokines including thymic stromal lymphopoietin, IL-25 and IL-33 in nasal mucosal inflammation (23,24). In pathological situations, cell injury leads to a substantial increase in extracellular ATP as a key danger alarmin that initiates inflammation and further amplifies immune responses (25). As the effects of extracellular ATP are mediated by P2 receptors, the role of P2X7 was investigated, which is the most involved of the P2 receptors in inflammation and infection and exhibits a high binding affinity for ATP (22).

In the present study, P2X7 was expressed in epithelial cells in control subjects; this result was consistent with previous reports (16). Furthermore, it was found that the expression of P2X7R was increased in ECRSwNP compared with controls and the protein was mainly located in epithelial cells. Since P2X7R has been demonstrated to be highly expressed in immune cells, the increase in P2X7R expression may be partly associated with enhanced infiltration of macrophages into nasal polyps (26). It was also found that LPS increased the expression level of IL-1β in a dose-dependent manner when combined with BzATP in primary human HNECs. LPS stimulation can result in the accumulation of cytoplasmic IL-1β through Toll-like receptor 4 (27). Meanwhile, LPS may cause pannexin-1 opening, allowing ATP release, which then triggers K+ efflux, to promote inflammasome-mediated caspase-1 activation. The activated NLRP3 inflammasome catalyses pro-IL-1β cleavage. NLRP3 inflammasomes have been proven to be activated in both ECRSwNP and NECRSwNP (9) and the present study findings confirmed this. The downstream inflammatory cytokines released after NLRP3 inflammasome assembly include IL-1β and IL-18, which were elevated in CRSwNP (9). Elevated IL-1β expression was found in HNECs after LPS inoculation. Further examination using the P2X7-selective antagonist A740003 confirmed that the LPS-induced effects are P2X7 specific. A740003 is a selective competitive antagonist of P2X7R (28). In addition, A740003 can block P2X7R-mediated calcium influx, pore formation and IL-1β release (29). This effect was also found in HNECs in the present study. This suggested that P2X7R may be implicated in the release of IL-1β in CRSwNP via the activation of the NLRP3 inflammasome (Fig. 5). P2X7R expression after antagonist administration in HNECs was slightly decreased compared with LPS alone. This effect may be associated with membrane internalization stimulated by P2X7R under inflammatory conditions. The administration of antagonists could decrease P2X7R activation and membrane internalization, which could induce receptor relocation, degradation and replenishment in the membrane, resulting in decreased receptor expression (30).

Nasal epithelial cells exist at the surface of the nasal mucosa and are capable of detecting microbial products and endogenous molecules associated with cellular damage via an assortment of pattern recognition receptors (31). NLRP3, a member of the NLR family, induces an increase in the expression of IL-1β, and can be activated by pathogens (9). The present study found that after blocking P2X7R, the expression of NLRP3 decreased, indicating that P2X7R is upstream of NLRP3 in nasal polyps. When persistent inflammation in nasal mucosa results in tissue damage, a variety of molecules are released that are normally sequestered intracellularly or

Figure 3. Expression profiles of IL-1β after LPS treatment for 24 h, followed by 300 µM BzATP 1 h after LPS administration. (A and B) IL-1β was found to be significantly increased in cultured human nasal epithelial cells after incubation with LPS+BzATP. Compared with the control group, IL-1β was significantly increased in the 10 µg/ml LPS group and the 20 µg/ml LPS combined with BzATP group, but there was no significant difference between the two groups. *P<0.05, n.s., not statistically significant; LPS, lipopolysaccharides; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt; IL-1β, interleukin-1β.
within the extracellular matrix. The nasal mucosa is exposed to harmful elements, such as pathogens and air pollutants, leading to an enhanced and sustained stream of danger signals (32,33). Studies examining the nasal mucosa of patients with CRSwNP have demonstrated the infiltration of immune cells including CD8\(^+\) T-cells, eosinophils, neutrophils and macrophages, and such inflammatory cells have been implicated in the pathogenesis of CRSwNP (3). Therefore, it was speculate that P2X7R may regulate the occurrence and development of nasal polyps by activating the NLRP3 inflammasome and mediating the release of IL-1\(\beta\).

There are some limitations to the present study, which should be pointed out. Firstly, the experiment only included 32 patients with CRSwNP and 16 control subjects, thus the
sample size is small. A larger sample is needed to confirm the present findings. Secondly, the experiment only compared patients with CRSwNP with a control group; consequently the role of P2X7R in the pathogenesis of CRSsNP and chronic refractory rhinosinusitis needs further research and analysis.

In summary, the present study found that the expression of P2X7R in the nasal mucosa of patients with CRSwNP and HNECs under inflammatory conditions was higher compared with that of the control group. P2X7R-NLRP3 inflammasome signaling pathway can be augmented by LPS combined with BzATP, but suppressed by A740003.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YW and SC performed the majority of the experiments, the statistical analysis and prepared the manuscript. WW and JC performed experiments and helped to draft the manuscript. WW, JC and Y JW analysed the data. WK and YJW participated in the conception and design of the study. YW and YJW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the ethical committees of Tong-ji Medical College, Huazhong University of Science and Technology (permit no. S135). All participants of the study were informed and signed a consent form.
The authors declare that they have no competing interests.

Patient consent for publication
Not applicable.

Competing interests
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