STAT3 inhibition decreases ATP-induced MUC8 gene expression in human airway epithelial cells

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Background: Contact between the human pulmonary system and bacteria, viruses, or other pathogens can induce airway diseases. Although pathogen-induced mucus oversecretion and hyperproduction are frequently observed in the human respiratory tract, the molecular mechanisms of pathogen-induced mucus hypersecretion and overproduction remain unclear. The objective of this study was to investigate the physiological signaling mechanism of adenosine triphosphate (ATP)-induced MUC8 gene expression in human airway epithelial cells.

Methods: Real-time reverse transcription polymerase chain reaction, a cytokine array, and a Ca²⁺ concentration assay were performed to investigate the ATP/P2Y2-induced MUC8 gene expression levels in human airway epithelial cells.

Results: The ATP/P2Y2 complex robustly secreted interleukin (IL)-6 in a time-dependent manner, whereas siRNA-P2Y2 did not. Moreover, ATP/P2Y2 induced MUC8 gene expression. IL-6 secreted by ATP strongly elevated ATP/P2Y2-induced MUC8 gene expression compared to ATP/P2Y2. Interestingly, a specific signal transducer and activator of transcription 3 (STAT3) inhibitor, 5,15-DPP, dramatically inhibited ATP/P2Y2/IL-6-induced STAT3 phosphorylation and resulted in an approximately 5-fold decrease in MUC8 gene expression.

Conclusion: We showed that IL-6-activated STAT6 is essential for ATP/P2Y2-induced MUC8 gene expression as part of inflammatory signaling by cytokines during airway inflammation. Our results provide a new molecular understanding of the signaling mechanism of MUC8 gene expression during airway inflammation.

Keywords: Airway; Inflammation; Adenosine triphosphate; Mucin 8; Purinergic P2Y2 receptor; STAT3 transcription factor

Introduction

Mucus has an essential function in the innate immune system against pathogens such as bacteria, virus, air pollutants, or fungus in the human respiratory track. Mucus hyperproduction and hypersecretion in airway track are frequently observed in a number of respiratory diseases, including asthma, chronic bronchitis, chronic obstructive pulmonary disease, and cystic fibrosis [1]. Various mucins have been named, but their exact physiological functions have yet to be identified. MUC8 has also been known to be expressed in the sinuses of chronic rhinosinusitis patients [2]. The partial cDNA sequence of MUC8 has been identified by Shankar et al. [3]. For this reason, the mechanisms by which MUC8 affect inflammation in the respiratory track are unknown. Recently, we reported that MUC8 acts as anti-inflammatory
mucin in reverse using the siRNA-MUC8 system [4]. In this study, we examined the signal mechanism of adenosine triphosphate (ATP)/P2Y2-induced MUC8 gene expression in human airway epithelial cells.

Purinergic receptors are ubiquitously expressed in many tissues and contribute to innate and adaptive immunity [5,6]. Of purinergic receptors, P2Y2 is expressed in the apical membrane of airway track cells [7] and is an important physiological receptor for airway inflammation [8]. P2Y2 is a Gαq-coupled receptor involved in intracellular signaling by heterotrimeric G proteins. This physiological mechanism has provided information on characterization of the receptor for ATP-induced MUC8 gene expression, its Gαq-protein coupling, and secondary messengers of the downstream process after P2Y2 receptor activation. Thus, understanding of intracellular signaling cascades that trigger mucus overproduction/hypersecretion is required for inflammatory control in an affected microenvironment.

Recently, a small molecule signal transducer and activator of transcription 3 (STAT3) inhibitor, C188-9, prevented house dust mite-induced airway remodeling, airway inflammation, and T helper type 2 and 17 (Th2/Th17) cells accumulation [9]. This was not surprising because local blockade of interleukin (IL)-6 decreased STAT3 activation, increased Th17 inflammation, and increased Th2 responses in mice [10,11]. However, the biochemical and physiological mechanisms by which ATP/P2Y2-induced MUC8 gene expression is affected by IL-6 in respiratory diseases remain unclear.

Methods

1. Materials and cell culture
ATP and 5,15-DPP were purchased from Merck (Darmstadt, Germany). The IL-6 ELISA kit was obtained from R&D Systems (Minneapolis, MN, USA). siRNA specifically targeting P2Y2 was synthesized by Bioneer (Daejeon, Korea). The siRNA sequences of P2Y2 were GAGGAAGGUGGCUUACCAA (dTdT). The human lung mucoepidermoid carcinoma cell line (NCI-H292) was obtained from the American Type Culture Collection (CRL-1848; Manassas, VA, USA). Cells were incubated in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) added with 10% fetal bovine serum in the presence of penicillin/streptomycin at 37°C in a humidified chamber with 5% CO2.

2. Real-time RT-PCR
Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed using a Bio-Rad iQ iCycler Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with iQ SYBR Green SuperMix. The following primers were used: MUC8, forward (5’-GACCTGCCGCCCATGGAC-3’) and reverse (5’-CAGGAGTTCCAGACCAGCT-3’). β2M, forward (5’-CGCTCCGTGGCCCTTAGC-3’) and reverse (5’-GAGTACGGTTGATACCTCCA-3’). Reactions were performed in a total volume of 20 µL, which included 10 µL of 2× SYBR Green PCR Master Mix (Thermo Fisher, Waltham, MA, USA), 300 nM of each primer, and 1 µL of previously reverse-transcribed cDNA template. Real-time RT-PCR was performed on a MiniOption Real-time RT-PCR Detection System (Bio-Rad Laboratories). The parameters were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. All reactions were performed in triplicate. The relative quantity of mRNA was obtained using the comparative cycle threshold method and was normalized using β2-microglobulin as an endogenous control [12].

3. Cytokine assay
Cytokine levels were quantified using a Human Cytokine Array Panel A kit (R&D Systems, ARY005B) according to the manufacturer’s instructions. Briefly, cells were plated in six-well plates 1 day before transfection with either a construct driving the expression of P2Y2 or P2Y2-specific siRNA using FuGENE 6 (Roche, Indianapolis, IN, USA). Twenty-four hours after transfection, serum-starved cells were treated with ATP for 4 hours. After treatment, supernatants were assayed for cytokine production according to the kit’s instructions [13].

4. Calcium colorimetric assay
Ca2+ concentration level was quantified using a Calcium Colorimetric assay kit (ab102505; Abcam, Cambridge, UK) according to the manufacturer’s instructions. Briefly, cells were plated in six-well plates 1 day before transfection with either a construct driving the expression of P2Y2 or P2Y2-specific siRNA using FuGENE 6 (Promega, Madison, WI, USA). Twenty-four hours after transfection, serum-starved cells were treated with ATP for 1 hour. The cells were assayed for Ca2+ concentration according to the kit’s instructions.
5. Statistical analysis
The data are presented as the mean±standard deviation of more than three independent experiments. When appropriate, statistical differences were measured using Wilcoxon Mann-Whitney tests. A *p*-value less than 0.05 was considered statistically significant.

Results

1. ATP activates P2Y2 purinergic receptor to induce *MUC8* gene expression in NCI-H292 cells
We performed real-time RT-PCR to determine whether P2Y2 receptor is essential for ATP-induced *MUC8* gene expression in human airway epithelial (NCI-H292) cells. After transfection with either wild-type P2Y2 or siRNA-P2Y2 construct, ATP was applied for 24 hours (Fig. 1A). The ATP/P2Y2 complex dramatically induced *MUC8* gene expression compared to ATP but not to siRNA-P2Y2. This result suggests that P2Y2 is critical for ATP-induced physiological functioning. ATP is an inducer of inflammation in many tissues in humans. We posit that ATP signaling can breakdown the secretagogues in cells to augment the inflammatory signal by secreting several cytokines/chemokines from the cells. To test that, cytokine array was performed with cell medium (Fig. 1B). Interestingly, IL-6 was secreted out of the cells by the ATP/P2Y2 complex but not by siRNA-P2Y2, suggesting that P2Y2 mediates ATP-induced IL-6 secretion. In addition, IL-6 secretion peaked at 6 hours after ATP treatment and then decreased. These results suggest that ATP/P2Y2 could result in extracellular secretion of IL-6 to induce an inflammatory condition in airway epithelial cells.

Fig. 1. ATP induces IL-6 secretion in a P2Y2-dependent manner in NCI-H292 cells. (A) Cells were transfected with either wild-type P2Y2 or a siRNA-P2Y2 construct and were then incubated with ATP for 24 hours before the generation of total cell lysates; *MUC8* transcripts were assessed by real-time RT-PCR. (B) A construct expressing wild-type P2Y2 or siRNA-P2Y2 was transiently transfected into NCI-H292 cells. The cells were washed, serum-starved overnight, and treated with ATP for 4 hours for a cytokine assay. The positive control was used as a loading control. (C) After transfection with a construct expressing wild-type P2Y2, NCI-H292 cells were incubated in a time-dependent manner. The supernatants were obtained to perform specific IL-6 ELISA. All figures are representative of three independent experiments. ATP, adenosine triphosphate; IL, interleukin; RT-PCR, reverse transcription polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay. *a)p*<0.05 compared to the control; *b)p*<0.05 compared to ATP only; *c)p*<0.05 compared to P2Y2-transfected cells.
2. Secreted IL-6 augments ATP/P2Y2-activated \( [\text{Ca}^{2+}] \) to increase \( MUC8 \) gene expression

To examine whether secreted IL-6 could affect ATP as an essential factor in P2Y2-induced \( MUC8 \) gene expression, extracellular ATP and purified IL-6 were applied to cells transfected with P2Y2 (Fig. 2A). Extracellular IL-6 treatment could strongly increase ATP/P2Y2-treated \( MUC8 \) gene expression compared to ATP/P2Y2 only. Because P2Y2 is a \( \text{G}_\alpha\text{q} \)-coupled receptor, intracellular calcium was measured after treatment with IL-6. As expected, intracellular calcium concentration was increased by extracellular IL-6 treatment (Fig. 2B). These results suggest that either/both autocrine or/and paracrine IL-6 can activate intracellular calcium concentration to increase ATP/P2Y2-induced \( MUC8 \) gene expression in NCI-H292 cells.

3. STAT3 is essential for ATP/P2Y2-induced \( MUC8 \) gene expression after cotreatment with IL-6

To investigate the signaling mechanism of ATP/P2Y2-induced \( MUC8 \) overproduction after cotreatment with IL-6, the specific STAT3 inhibitor 5,15-DPP was utilized (Fig. 3). Because STAT3 can be frequently activated by IL-6, it is a pivotal transcription factor for cell proliferation. Interestingly, 5,15-DPP significantly decreased ATP/P2Y2-induced \( MUC8 \) in a dose-dependent manner after cotreatment with IL-6. This is why IL-6 secretion is critical for airway inflammation in NCI-H292 cells. Although ATP activated \( \text{G}_\alpha\text{q} \)-coupled receptor and secondary messengers like intracellular calcium, STAT3 might play a critical role in this signal complex.

**Discussion**

We identified how ATP signaling could induce \( MUC8 \) gene expression via the P2Y2 G-protein coupled receptor receptor in human airway epithelial cells. Though ATP is an inflammation inducer, the precise signaling mechanism of ATP/P2Y2-induced \( MUC8 \) gene expression remains unclear. Interestingly, IL-6 could be secreted extracellularly by the ATP/P2Y2 complex in a time-dependent manner.
IL-6 is a pro-inflammatory cytokine that induces a number of physiological functions. The IL-6 secreted by the ATP complex was uptaken into nearby cells to transfer inflammatory signals. Surprisingly, IL-6 robustly induced ATP/P2Y2-induced MUC8 gene expression compared to ATP/P2Y2 treatment (Fig. 2A). Thus, we posited that ATP could transmit inflammatory signals to boost or magnify inflammatory signaling to nearby cells.

More interestingly, siRNA-P2Y2 did not induce this phenomenon, because P2Y2 is an essential ATP purinergic receptor in airway epithelial cells. In fact, many cytokines/chemokines were secreted in an ATP-time/dose-dependent manner, including regulated upon activation, normal T cell expressed, and secreted, IL-1α, and IL-1β; however, only IL-6 was affected by siRNA. Thus, we posit that IL-6 plays a critical role(s) in ATP signaling transduction in the airway.

Our previous study tested MUC8 as an anti-inflammatory mucin using siRNA study [4]. After 24 hours of ATP treatment, increased MUC8 significantly inhibited ATP/P2Y2-dependent inflammatory cytokine production for more than 72 hours. Additional information for full-length cDNA and promoter sequences of MUC8 is required to support the assumption that MUC8 is an anti-inflammatory mucin.

IL-6 is a small glycoprotein (21 KDa) produced by cells in the innate immune system [14]. Increased level of IL-6 has been frequently observed in asthmatic patients [15]. More importantly, a study examining IL-6 in bronchoalveolar lavage fluid has shown increased level of IL-6 in active asthmatic patients compared with the levels in healthy non-smoker, stable asthmatic, and non-asthmatic patients [16]. Thus, recently, scientists reported that inhibition of STAT3, a major downstream signal of IL-6, significantly decreased lung inflammation, airway remodeling, and inflammatory cell infiltration in asthma [9,17-19]. However, there is little information on the role of STAT3 in mucus hypersecretion and overproduction during airway inflammation.

In summary, our results showed that IL-6 is essential for ATP/P2Y2-induced MUC8 gene expression in human airway epithelial cells. In addition, activation of STAT3 is mainly related to MUC8 gene expression through induction of ATP/P2Y2. We suggest that the mucus hypersecretion/overproduction observed during airway inflammation is a consequence of ATP/P2Y2-induced increase in MUC8 gene expression within the inflamed microenvironment. Further analysis of the mucus hypersecretion/overproduction induced by various cytokines, bacterial products, or viruses might increase understanding of the pathogenesis of respiratory diseases.

### Article information

#### Conflicts of interest
Kyoung Seob Song is an editorial board member of the journal but was not involved in the peer reviewer selection, evaluation, or decision process of this article. No other potential conflicts of interest relevant to this article were reported.

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