Abstract. P2X4 receptor (P2X4R) is the most widely expressed subtype of the P2XRs in the purinergic receptor family. Adenosine triphosphate (ATP), a ligand for this receptor, has been implicated in the pathogenesis of asthma. ATP-P2X4R signaling is involved in pulmonary vascular remodeling, and in the proliferation and differentiation of airway and alveolar epithelial cell lines. However, the role of P2X4R in asthma remains to be elucidated. This aim of the present study was to investigate the effects of P2X4R in a murine experimental asthma model. The asthmatic model was established by the inhalation of ovalbumin (OVA) in BALB/c mice. The mice were treated with P2X4R-specific agonists and antagonists to investigate the role of this receptor in vivo. Pathological changes in the bronchi and lung tissues were examined using hematoxylin and eosin staining, Masson’s trichrome staining and Alcian blue staining. The inflammatory cells in the bronchoalveolar lavage fluid were counted, and the expression levels of P2X4R, α-smooth muscle actin (α-SMA) and proliferating cell nuclear antigen (PCNA) were detected using western blotting.

In the OVA-challenged mice, inflammation, infiltration, collagen deposition, mucus production, and the expression levels of P2X4R and PCNA were all increased; however, the expression of α-SMA was decreased, compared with the mice in the control group. Whereas treatment with the P2X4R agonist, ATP, enhanced the allergic reaction, treatment with the P2X4R antagonist, 5-BDBD, attenuated the allergic reaction. The results suggested that ATP-P2X4R signaling may not only contribute to airway inflammation, but it may also contribute to airway remodeling in allergic asthma in mice.

Introduction

Asthma is a chronic inflammatory airway disease involving several different types of cell. It is considered an allergic disease with characteristic airway inflammation, airway hyper-responsiveness (AHR) and airway remodeling (1-3). At present, anti-inflammatories are the predominant therapeutic option for patients with asthma (4,5). Previous studies (6-8) have shown that airway remodeling results in the progressive loss of lung function. Airway remodeling includes epithelial denudation, subepithelial fibrosis, mucus gland hypertrophy, myofibroblast and smooth muscle cell proliferation, and angiogenesis (9-11). The expression levels of α-smooth muscle actin (α-SMA) (12,13) and proliferating cell nuclear antigen (PCNA) (14,15) are indicative of asthmatic cell proliferation.

Adenosine triphosphate (ATP), a key mediator of acute and chronic inflammation, can be released in substantial quantities from various cell types following cellular stress or tissue injury (16-19). Extracellular ATP exerts pro-inflammatory and immunomodulatory effects in the tumor microenvironment by binding to purinergic P2-receptors, which can be subdivided into two families: The G-protein-coupled P2YR (P2Y1-14) and the ligand-gated ion channel, P2X receptors (P2XR), which include P2X1-7 (20-22). Data from patient and animal models of allergic bronchial asthma indicate that ATP contributes to the pathophysiology of allergic airway inflammation (16). However, whether ATP-mediated P2X4R signaling is activated in allergic asthma remains to be elucidated.

Among the P2XR subtypes, P2X4R is the most widely expressed (23). P2X4 receptors are located in immune and non-immune cells, including alveolar epithelial cells, alveolar macrophages, platelets, lymphocytes and mast cells (24-26). P2X4R can affect T cell activation via Ca2+ influx. ATP-P2X4R signaling is involved in peripheral nerve injury inflammation, which includes the promotion of the expression levels of interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α via
mitogen-activated protein kinase activation; indicating that P2X4R is involved in the immune response to nerve inflammation (27). Several studies have demonstrated that P2X4 receptors are involved in hypersensitivity by acting on prostaglandin E2 (28-30). ATP-P2X4R signaling is also involved in pulmonary vascular remodeling, and in the proliferation and differentiation of airway and alveolar epithelial cell lines in pulmonary hypertension (31,32). However, whether P2X4R signaling is involved in allergic airway inflammation and airway remodeling requires further investigation.

The aim of the present study was to investigate the effects of P2X4R in a murine experimental asthma model and the effects of a P2X4R-specific antagonist, 5-BDBD, on airway inflammation and airway remodeling. Our findings may ultimately lead to the development of novel therapeutic targets for allergic asthma.

Materials and methods

Chemicals and reagents. Rabbit polyclonal anti-P2X4R primary antibody was purchased from Calbiochem (EMD Millipore, Billerica, MA, USA; cat. no. PC376) and used for western blotting. Mouse monoclonal anti-PCNA primary antibody (cat. no. BM0104) and anti-α-SMA primary antibody (cat. no. BM0002) were purchased from Boster Systems, Inc. (Wuhan, China). Mouse monoclonal anti-β-actin primary antibody (cat. no. TA-09) was purchased from ZSGB-Biotechnology, Inc. (Beijing, China). Mouse anti-rabbit (cat. no. sc-2357) and rabbit anti-mouse (cat. no. sc-358914) IgG-horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The P2X4R-specific antagonist, 5-BDBD, was obtained from Tocris Bioscience (Bristol, UK). The Masson's trichromatic staining kit and Alcian blue-periodic acid-schiff (AB-PAS) mucosa staining kit were purchased from Fuzhou Maixin Biotechnology, Co., Ltd. (Fuzhou, China). ATP and the P2XR1/2/3/5/7 antagonist, phenyl-isopropyl-amine dopes (PPAD), were all purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were obtained from common commercial sources.

Experimental animals. Pathogen-free female BALB/c mice, aged 6-8 weeks and weighing ~18-20 g, were purchased from the Laboratory Animal Research Center (Beijing, China). Mice were maintained under 12 h light/dark cycles at room temperature, with free access to food and water. All animal experiments and manipulation procedures were approved by the Ethics Committee for Animal Use and Care of Harbin Medical University-Daqing (Daqing, China).

Animal groups and allergic sensitization protocol. A total of 48 BALB/c mice were randomized into the following five groups: Phosphate-buffered saline (PBS) control group (n=8), OVA group (n=10), ATP group (n=10), PPAD group (n=10) and 5-BDBD group (n=10). In the OVA group, the mice were sensitized to OVA (20 µg per injection), absorbed to 2.0 mg per injection of aluminum hydroxide, by intraperitoneal injection. From day 23, the mice were exposed, using inhalation aerosols, of 4% OVA in PBS for 25-30 min, until the onset of bronchial obstruction, daily for 7 days consecutively, according to the methods described by Shen et al (31) and Vanacker et al (32) with modifications. The mice in the ATP, PPAD and 5-BDBD groups were also subjected to OVA sensitization and asthma induction, in the same manner. Intranasal application of 50 µl ATP (1 mM) (33), PPAD (0.1 mM) (34) and 5-BDBD (30 µmol) (35) were performed 3 h prior to each airway allergen challenge in the ATP, PPAD and 5-BDBD groups, respectively. All treatments were administered daily for 5 days consecutively. Mice in the control group were administered with PBS alone (0.5 ml) by injection, and were challenged with PBS (20 ml). All animals were humanely sacrificed within 24 h of the final OVA or PBS exposure.

Morphometric analysis. The lungs were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at a thickness of 5 µm. The sections from each animal were stained with hematoxylin and eosin for inflammation, Masson's trichrome staining for collagen-deposition and Alcian blue/periodic acid-Schiff (AB-PAS) staining for the analysis of goblet cell hyperplasia/mucus production. All staining procedures were performed in accordance with the manufacturer's protocol. Pathological changes in the lung and bronchial tissues were observed and images were captured using a BX-60 microscope (Olympus, Tokyo, Japan). The tissues were scored based on the presence of inflammatory cells, using the following scale: Absent (0), rare (1), mild (2), moderate (3) or severe (4). Scoring was performed by an observer in a blinded-manner. Positively stained areas of collagen and mucus in the lung and bronchial tissues were quantified using high-resolution images of individual vessels by image analysis, using a color-recognition algorithm, in Image Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). The results were expressed as the percentage of collagen area or mucus area, compared to the total measured area.

Bronchoalveolar lavage fluid (BALF). Samples of BALF were collected 24 h following the final challenge, according to previously described methods. The lungs were lavaged with 1.6 ml (0.8 ml per lavage) of ice-cold PBS via the tracheal cannula. The retrieved volume (~75-80%) of the instilled PBS was then centrifuged at 500 x g at 4°C for 5 min. The resultant pellet was washed twice with PBS and was resuspended in 1 ml PBS. The total numbers of cells were counted using a hemocytometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Smears of the BALF cells were stained with Wright and Giemsa staining fluid (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for differential cell counting. Using a BX-60 Olympus microscope, the numbers of cells were counted by two independent investigators, in a blinded-manner. A total of ~200 cells were counted in each of four randomly selected locations.

Western blotting. The lung tissues were homogenized with cold lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China), containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and 2 mM PMSF. Following
ultrasonication on ice for 5 sec using an FA‑25 (Fluko Equipment Shanghai Co., Ltd., Shanghai, China) and centrifugation at 1,250 x g at 4°C for 15 min, the supernatant was collected, and the protein concentration was determined using a Bicinchoninic Acid Protein Assay (Pierce Biotechnology, Inc., Rockford, IL, USA) with bovine serum albumin (Biosharp, Hefei, China) as a standard. Each sample, containing 20 µg protein, was separated on 8‑10% SDS‑PAGE gels and transferred onto nitrocellulose membranes (EMD Millipore). Following blocking with Tris-buffered saline, containing 20 mM Tris, 150 mM NaCl (pH 7.6) and 0.1% Tween 20, with 5% nonfat milk at room temperature, the membranes were incubated overnight at 4°C with specific antibodies against P2X4R (1:400 dilution), α‑SMA (1:500 dilution), PCNA (1:500 dilution) or β‑actin (1:1,000 dilution). This was followed by incubation for 2 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilution). All protein blots were visualized using enhanced chemiluminescence reagents (GE Healthcare Life Sciences). Integrated density values were analyzed using a computerized image analysis system (Fluor Chen 2.0; Olympus Corporation) and were normalized to those of β‑actin.

**Results**

**OVA‑challenge upregulates the protein expression of P2X4R in mice.** The present study performed western blotting to compare the protein expression levels of P2X4R in the lung tissues of the treatment groups (Fig. 1A and B). As shown in Fig. 1, OVA‑challenge significantly increased the expression of P2X4R, compared with the control mouse.

**P2X4R contributes to inflammation of the bronchi and lung tissues in allergic airway challenge in mice.** Histological analyses of the tissues of the mice in the OVA group showed more extensive infiltration of inflammatory cells around the bronchioles, blood vessels and alveoli. The airway mucosa showed edema, with bronchiolar wall thickening causing luminal stenosis, suggesting successful establishment of the asthmatic model. In the OVA‑challenged mice, treatment with ATP significantly increased OVA-induced inflammation, infiltration, and pathological changes of the bronchi and lung tissues. By contrast, treatment of OVA‑challenged mice with PPAD (a P2X1, 2, 3, 5 and 7R antagonist) (36) and 5‑BDBD (a P2X4R antagonist) (37) significantly reduced the extent of inflammation and cellular infiltration in the airway. In addition, the pathological changes of the bronchi and lung tissues were milder, compared with the changes found in the asthma group without treatment (Fig. 2A and B).

**Cell numbers and differential cell percentages in BALF.** The total cell numbers (Fig. 3A) and the percentages of eosinophils and lymphocytes (Fig. 3B) in the BALF from the asthmatic mice increased significantly, compared with those in the BALF from the control mice. The increased numbers of inflammatory cells, including eosinophils and lymphocytes, were significantly reduced by the administration of PPAD and 5‑BDBD in the asthmatic mice, compared with the numbers found in the untreated asthmatic mice. The changes in the percentages of macrophages showed the opposite results.

**P2X4R is required for collagen deposition in allergic airway challenge in mice.** Collagen deposition in airway remodeling was assessed using Masson's trichrome staining of lung histological sections. Collagenous matrix was identified by its characteristic blue color. No obvious collagen fibers were present in the lungs of mice in the control group. However, deposition of collagen fibers was increased around the bronchial airways and vessels in the lungs of the OVA‑challenged and ATP‑treated mice. By contrast, intervention with PPAD and 5‑BDBD significantly reduced collagen deposition (Fig. 4A and B).

**P2X4R is required for goblet cell hyperplasia and mucus production in allergic airway challenge in mice.** To assess mucus production and the hyperplasia of goblet cells, lung sections were stained with AB‑PAS. In the OVA‑challenged mice, bronchial epithelial mucus secretion and goblet cell hyperplasia were clearly observed, as a violet color in the bronchial airways, compared with the control group. The level of goblet cell hyperplasia in the OVA‑challenged mice treated with ATP was significantly increased, compared with that in the OVA group. Intervention by PPAD or 5‑BDBD significantly reduced goblet cell hyperplasia (Fig. 5A and B).

**P2X4R regulates the expression of α‑SMA and PCNA in the asthmatic mouse lung.** Western blotting was performed to examine the protein levels of α‑SMA (Fig. 6A and B) and PCNA (Fig. 7A and B) in each group. The expression levels
of α-SMA in the OVA-challenged mice and ATP-treated mice were significantly lower, compared with those in the control mice. In addition, the mice treated with 5-BDBD showed significantly increased protein expression levels of α-SMA, compared with the mice in the OVA and ATP groups. However, the mice treated with PPAD showed no significant differences in protein expression, compared with the OVA group. The expression level of PCNA in the OVA-challenged mice and the ATP-treated mice were significantly higher than the level in the control mice. In addition, the mice treated with 5-BDBD showed a significant reduction in the protein expression levels of PCNA, compared with the mice in the OVA and ATP groups. Treatment with PPAD had no significant effects.

Discussion

Allergic asthma is a chronic airway inflammatory-driven disease, which is characterized by airway...
Figure 4. P2X4R is required for collagen deposition in allergic airway challenge. (A) Masson's trichrome staining for examination of collagen deposition in the lungs. Collagen is stained blue. Black arrows indicate areas with collagen deposition. (B) Percentages of collagen, calculated as the % total area (n=3). Data are expressed as the mean ± standard error of the mean. *P<0.01, compared with the control group; **P<0.01, compared with the OVA group. ATP, adenosine triphosphate; PPAD, phenyl-isopropyl-amine dopes.

Figure 5. P2X4R is required for goblet cell hyperplasia and mucus production in allergic airway challenge. (A) Alcian blue/periodic acid-Schiff staining for mucus secretion (stained violet). Black arrows indicate areas with goblet cell hyperplasia and mucus production. (B) Percentage of mucus in the total mucus area measured (n=3). Data are expressed as the mean ± standard error of the mean. *P<0.01, compared with the control group; #P<0.05, compared with the OVA group. ATP, adenosine triphosphate; PPAD, phenyl-isopropyl-amine dopes.
hyper-responsiveness, airway inflammation and varying degrees of smooth muscle cell proliferation, termed airway remodeling (36-38). In the present study, airway inflammation and airway remodeling were examined in allergen-challenged mice. The pathophysiological mechanisms underlying airway remodeling remain to be fully elucidated. Compelling evidence indicates a pathophysiological relevance for ATP, acting via P2 purinergic receptors, in pain hypersensitivity and peripheral inflammation (39-41). Certain studies have suggested that ATP contributes to the pathophysiology of allergic airway inflammation (14,16,42).

In the present study, treatment of OVA-challenged mice with ATP significantly increased OVA-induced inflammation, infiltration, and pathological changes of the bronchi and lung tissues. Increased collagen deposition, goblet cell hyperplasia/mucus production, increased expression levels of PCNA expression, and decreased expression levels of α-SMA are indicative of airway remodeling (43-46), and were observed in mice belonging to the OVA-challenged and ATP-treated groups. These results indicated that ATP may enhance airway inflammation and airway remodeling in allergic asthma. However, whether P2X4R is activated in allergic asthma by ATP-mediated signaling has not been determined.

Among the P2XR subtypes, P2X4R is the most widely expressed, which is expressed in blood vessels, the lungs, kidneys and immune cells (47,48). Additionally, P2X4R is involved in regulating blood pressure and vascular remodeling by ATP-induced Ca2+ influx and flow-mediated vasodilatation (49,50). P2X4Rs are also involved in modulating right ventricular hypertrophy, which occurs due to pulmonary hypertension in hypobaric hypoxia (51). Asthma and pulmonary arterial hypertension exhibit three major pathological features: Inflammation, smooth muscle constriction and smooth muscle cell proliferation (52). In the present study, the results demonstrated that treatment with the P2X4R antagonist, 5-BDBD, prevented the development of the airway and pulmonary inflammation, and airway remodeling induced by OVA challenge and OVA challenge coupled with ATP treatment. By contrast, treatment with PPAD showed no significant effect on the expression levels of α-SMA or PCNA. These data indicate that ATP-mediated P2X4R activation, but not P2X1/2/3/5/7R activation, was involved in airway inflammation and airway remodeling in models of allergic asthma in mice. The exact mechanisms of ATP-P2X4R signaling responsible for asthma pathogenesis requires further investigation.

Figure 6. P2X4R regulates the expression of α-SMA in the asthmatic mouse lung. (A) Expression of α-SMA in the lung tissues. (B) Optical density values for α-SMA protein, relative to β-actin (n=4). Data are expressed as the mean ± standard error of the mean. *P<0.05, compared with the control group; **P<0.01, compared with the OVA group. ATP, adenosine triphosphate; PPAD, phenyl-isopropyl-amine dopes; α-SMA, α-smooth muscle actin.

Figure 7. P2X4R regulates the expression of PCNA in the asthmatic mouse lung. (A) Expression of PCNA in lung tissues. (B) Optical density values for PCNA protein, relative to β-actin (n=4). Data are expressed as the mean ± standard error of the mean. *P<0.05, compared with the control group; **P<0.05, compared with the OVA group. ATP, adenosine triphosphate; PPAD, phenyl-isopropyl-amine dopes; PCNA, proliferating cell nuclear antigen.
Acknowledgements

This study was supported by the Natural Science Foundation of China (grant no. 81200011) and the Postdoctoral Science Foundation of Heilongjiang Province in China (grant no. LBH-Z12213).

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