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ORIGINAL ARTICLE

P2X4R promotes airway remodeling by acting on the phenotype switching of bronchial smooth muscle cells in rats

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Abstract

The P2X4 receptor (P2X4R) contributes to airway inflammation and airway remodeling in mice with allergic asthma. However, the molecular mechanism by which P2X4R affects the airway remodeling in allergic asthma remains largely unknown. We established an allergic asthma model by ovalbumin (OVA) inhalation in BALB/c mice. Compared with the mice in the control group, the expression of proliferating cell nuclear antigen (PCNA) increased and that of alpha-smooth muscle actin (α-SMA) decreased in the OVA-challenged mice. 5-BDBD, a P2X4R antagonist, alleviated the OVA-induced changes. To clarify the role of P2X4R in the phenotype switching of the bronchial smooth muscle, bronchial smooth muscle contractility and p38MAPK expression were investigated. Platelet-derived growth factor-BB (PDGF-BB) was used to activate the proliferation of primary-cultured rat bronchial smooth muscle cells (BSMCs). P2X4R, p38MAPK, and phenotype markers were evaluated using Western blotting or immunofluorescence. PDGF-BB administration increased the P2X4R and phospho-p38MAPK expression in BSMCs, and the increased phospho-p38MAPK expression was downregulated by silencing of the P2X4R mRNA. PDGF-BB stimulated the proliferation and synthetic phenotype of BSMCs, which was aggravated by a P2X4R agonist and alleviated by a P2X4R antagonist or silencing the P2X4R mRNA. The decreased contractile phenotype induced by PDGF-BB was alleviated by a P2X4R antagonist or by silencing the P2X4R mRNA. SB203580, p38MAPK inhibitor, inhibited the PDGF-BB-induced increasing of synthetic phenotype and the proliferation of BSMCs. These findings indicate that P2X4R acts directly on the phenotype switching of BSMCs. Inhibiting P2X4R can promote the contractile differentiation of BSMCs via p38MAPK signaling. Thus, the effect of P2X4R on airway remodeling indicates that this receptor could be a target for future drug candidates.

Keywords P2X4R · Contractile differentiation · Bronchial smooth muscle cells

Introduction

Airway remodeling and airway hyperresponsiveness play central roles in bronchial asthma. Hypercontraction of the airway smooth muscle cells is one manifestation of airway hyperresponsiveness [1, 2]. Moreover, the proliferation of airway smooth muscle cells (ASMCs) has a strong effect on airway remodeling [3]. The accumulated evidence indicates that ASMCs are the main effectors of the pathological remodeling of airways [4, 5]. It is generally accepted that phenotype switching of vascular smooth muscle cells is the main molecular mechanism for many cardiovascular diseases, and it has been reported that ASMCs play a similar role in asthma [6]. Thus, it is necessary to understand the molecular mechanisms that modulate the contractile function of airway smooth muscle and investigate more options for effective therapy of asthma. Increased bronchial smooth muscle mass is a central characteristic in airway remodeling during the development of lung diseases, including asthma. Abnormal ASMC proliferation is directly involved in the pathophysiology of ASMC hyperplasia. The pathogenesis of restricting ASMC proliferation is not very clear [3, 7]. Phenotype switching of ASMCs and proliferating cell nuclear antigen (PCNA) are signs of non-severe asthma [8–11].

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Compelling evidence demonstrates that adenosine triphosphate (ATP) is released from the intracellular to the extracellular space by various cell types [12, 13]. Extracellular ATP can modulate the migration, maturation, and function of various inflammatory cells such as macrophages, dendritic cells, and T cells by the activation of the P2Y- and P2X-family [14, 15]. It has been reported that ATP contributes to allergic airway inflammation [16, 17]. The P2X4 receptor (P2X4R) is a ligand-gated cation channel that is usually activated by extracellular ATP [18]. P2X4R is expressed in structural cells, such as alveolar epithelial and smooth muscle cells [15]. Studies have shown that the ATP-activated P2X4R pathway is associated with the vascular remodeling of the lung and the proliferation and differentiation of airway and alveolar epithelial cell in pulmonary hypertension [19]. We have previously shown that both airway inflammation and airway remodeling are connected to the ATP-mediated P2X4R pathway in ovalbumin (OVA)-sensitized mice [20]. However, the effect of P2X4R on the contractile differentiation of bronchial smooth muscle cells (BSMCs) remains unclear.

Here, we studied the roles of P2X4R in the proliferation and phenotype switching of BSMCs. Our data suggest that P2X4R participates in the phenotype switching of BSMCs and that inhibiting P2X4R can promote the contractile differentiation of BSMCs. Thus, our study may shed light on exploring novel approaches in the airway remodeling of lung diseases.

Materials and methods

Reagents and antibodies

We purchased grade V OVA from Sigma-Aldrich Corp. (St. Louis, Mo, USA). We obtained recombinant rat Platelet-derived growth factor-BB (PDGF-BB) from R&D Systems (Minneapolis, MN, USA). Inhibitor SB203580 was obtained from Sigma-Aldrich (St. Louis, MO). Antibodies were purchased from the following companies: P2X4R (EMD Millipore, Billerica, MA, USA), PCNA, calponin (CNN1), osteopontin (OPN), and alpha-smooth muscle actin (α-SMA) (Boster, Wuhan, China), p38MAPK, phospho-p38MAPK (p-p38MAPK), and β-actin (Santa Cruz, CA, USA). Alexa Fluor 488-Conjugated AffiniPure goat anti-rabbit IgG was obtained from ZSGB Biotechnology, Inc. (Beijing, China). ATP was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The 5-BDBD was bought from Tocris Bioscience (Bristol, UK). We obtained TRIzol reagent from Invitrogen Life Technologies (Carlsbad, CA, USA), and the SYBR Green system from Bio-Rad Laboratories Ltd. (Hercules, CA, USA).

Sensitization and airway challenge

BALB/c mice were obtained from the Laboratory Animal Research Center in Beijing, China. The mice (6- to 8-week-old females) were raised in pathogen-free conditions, provided with a 12 h light-dark cycle, and given food and water at room temperature. The mice were divided into three groups ($n = 7$): the control group, the OVA group, and the 5-BDBD group. OVA-induced asthma was established as described in our previous report with a minor modification (Fig. 1) [21]. Briefly, the mice in the OVA group were sensitized with a mixture containing 20 μg of OVA absorbed to 2.0 mg of aluminum hydroxide by intraperitoneal injection on the 1st day. Then, mice were again sensitized with 10 μg of OVA absorbed to 1.0 mg of aluminum hydroxide by intraperitoneal injection on the 8th, the 15th, and the 22nd day. Beginning on the 23th day, the mice were challenged by exposure to an aerosol of 4% OVA in phosphate-buffered saline (PBS) for 25 to 30 min (until the onset of bronchial obstruction) once a day for 7 successive days. The 5-BDBD group mice were sensitized and challenged with OVA in the same manner. The mice in the 5-BDBD group underwent intranasal application of 5-BDBD (30 μmol) [20] 3 h before each airway challenge. The mice of the control group were injected and challenged with PBS.

Immunohistochemical detection

Sections of left lung were deparaffinized, rehydrated, and subjected to antigen retrieval before blocking the endogenous peroxidase with 1% H_2O_2 in methanol for 10 min at room temperature. The sections were blocked with 5% bovine serum albumin and then incubated with mouse monoclonal anti-a-SMA antibody (1:200 dilution) or mouse monoclonal anti-PCNA antibody (1:200 dilution) at 4 °C overnight. The sections were then washed with PBS and incubated with anti-mouse secondary antibodies (1:200) for 30 min at room temperature. We observed the slides and captured the images using a microscope (Olympus, Tokyo, Japan).

Cell culture

BSMCs were isolated as previously described [22, 23] with some modifications. Briefly, the isolated bronchial rings from fresh rat lung were denuded of endothelium by rubbing the luminal surface with a cotton swab and then digested with 1.5 mg/ml of collagenase (type II, Worthington, Shanghai, China) and bovine serum albumin (1.5 mg/ml) dissolved in PBS for 2 h at 37 °C. The cells were cultured with DMEM containing 20% fetal bovine serum (FBS). The cells were stimulated with or without rat PDGF-BB at a final concentration of 20 ng/ml [7] after incubation with serum-free-DMEM for 24 h. Then, some cells were treated with siRNA plus

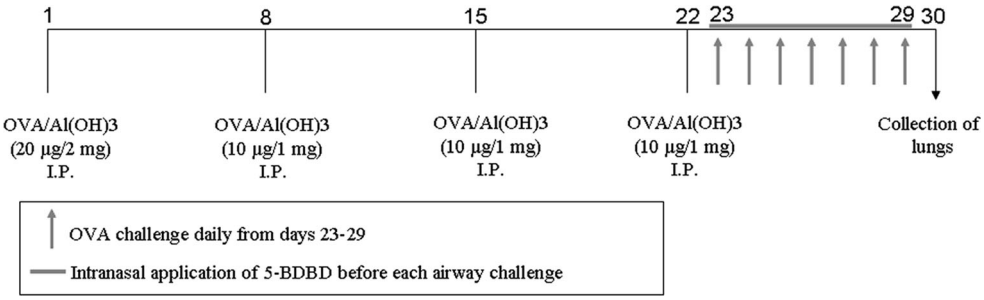

Fig. 1 Sensitization and airway challenge protocol

PDGF-BB in 5% FBS-DMEM. The cells cultured in complete medium were treated with vehicle (VEC) (4 mM HCl), PDGF-BB, PDGF-BB plus ATP (0.5 μmol) [24] or PDGF-BB plus 5-BDBD (10 μmol) [25].

Immunofluorescence analysis

BSMCs were grown up to 80% confluence on coverslips and subsequently serum-starved. After treatment, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.01% Triton X-100, and blocked with 5% bovine serum. Then, the cells were incubated with an anti-P2X4R antibody (1:100) overnight at 4 °C. The cells were washed three times with PBS, then incubated with Alexa Fluor 488-conjugated goat anti-rabbit antibody at 37 °C for 2 h and incubated with DAPI, protected from light. The slides were then examined using a microscope (Olympus, Japan).

siRNA transfections

The siRNA sequences that targeted rat P2X4Rs (siP2X4R) and non-specific targeted siRNA (nsRNA) was purchased from Santa Cruz. siPORT™ Amine (Ambion, Austin, TX) was applied to transfect siRNA following the manufacturer's instruction. The cells were grown to 60–80% confluence and were treated with 100 nM siRNA in 2% FBS media. The cells were used as required after incubation for 72 h.

Western blotting analysis

BSMCs were homogenized with cold lysis buffer (Beyotime, Shanghai, China) that contained 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 2 mM PMSF. After protein quantification with the Enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China), samples containing 30 μg total protein were separated on 10% SDS-polyacrylamide gels. Then, the samples were transferred to a nitrocellulose membrane. The membranes were incubated with anti-P2X4R antibody (1:400 dilution), anti-PCNA antibody (1:500 dilution), anti-α-SMA antibody (1:500 dilution), anti-CNN1 antibody (1:500 dilution), anti-OPN antibody (1:500 dilution), anti-p38MAPK antibody (1:500 dilution), anti-p-p38MAPK antibody (1:500 dilution), or anti-β-actin antibody (1:1000 dilution) overnight at 4 °C. Then, they were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution) for 2 h at room temperature. Using enhanced chemiluminescence reagents (GE Healthcare Life Sciences), we visualized the protein band. We analyzed the integrated density values with a computerized image analysis system (Fluor Chen 2.0; Olympus Corporation).

MTT

BMSCs were cultured in 96-well plates at 1×10^4 cells/well and, then, treated with or without PDGF-BB or SB203580. At 36 h after indicated treatments in the incubation, MTT (5 mg/mL) was added into each well and incubated for 4 h at 37 °C. After removing medium, DMSO was used to terminate the reaction. The absorbance value was measured at 490 nm.

Statistical analysis

SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA) was used to analyze the data, which were presented as the means ± SEM. We analyzed the data with *t* tests and one-way analysis of variance. Statistical difference was defined as $p < 0.05$.

Results

5-BDBD increased α-SMA expression and inhibited PCNA expression in the lungs of mice in allergic asthma

a-SMA expression (Fig. 2a) decreased in the bronchial walls of lung tissue in OVA-sensitized mice compared with the control group. The decreasing α-SMA expression was increased in the OVA-sensitized mice that had been treated with 5-BDBD. PCNA expression was increased in the bronchial walls of the lungs in the OVA-sensitized mice compared with those of the control group (Fig. 2b). 5-BDBD administration suppressed the expression of PCNA in the bronchial walls of the lungs in OVA-sensitized mice. Consistent with these observations, the Western blotting results demonstrated that 5-

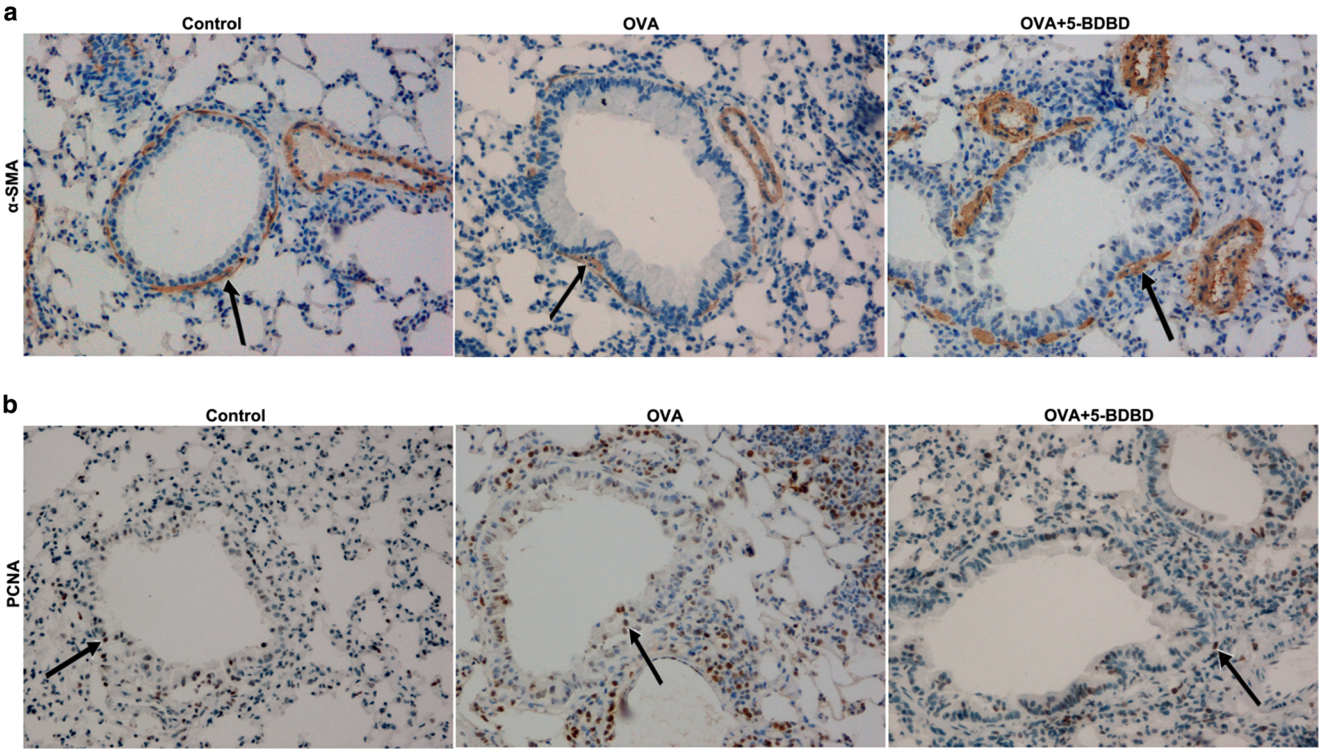

Fig. 2 Immunohistochemical staining for a-SMA (**a**) and PCNA (**b**) in lung sections (original magnification × 200). a-SMA level in the bronchial wall of the lung was increased by 5-BDBD in OVA-sensitized mice, but that of PCNA was decreased ($n = 3$). Arrows indicate positive staining

BDBD abolished the OVA-induced downregulation of α-SMA and upregulation of PCNA in lung extracts [20].

PDGF-BB promoted P2X4R expression in BSMCs

Expression of P2X4R in the BSMCs was evaluated at the protein level. The P2X4R expression in the BSMCs was examined by immunofluorescence (Fig. 3a) and Western blotting (Fig. 3b) after vehicle and PDGF-BB treatment. These data indicated that P2X4R was expressed in the BSMC membrane and cytoplasm. The P2X4R level was much higher in the PDGF-BB treatment group than in the VEC group ($p < 0.05$).

P2X4R was associated with the cell proliferation induced by PDGF-BB in BSMCs

To explore the influence of P2X4R on the proliferation of BSMCs, the PCNA levels were examined using Western blotting. The data indicated that PDGF-BB increased the PCNA levels ($p < 0.05$), and this effect was exacerbated by ATP and alleviated by 5-BDBD ($p < 0.05$) (Fig. 4a). The enhancement of the PCNA expression induced by PDGF-BB was decreased by silencing the P2X4R mRNA ($p < 0.05$) (Fig. 4b, c). Thus, ATP-mediated P2X4R signaling may participate in BSMC proliferation induced by PDGF-BB.

P2X4R was associated with the increased contractile phenotype induced by PDGF-BB in BSMCs

Western blotting was performed to examine the contractile phenotype of BSMCs as indicated by the levels of α-SMA (Fig. 5a, b) and CNN1 (Fig. 5c, d) in each group. The data showed that α-SMA and CNN1 expression decreased following activation with PDGF-BB ($p < 0.05$). However, the decreased α-SMA and CNN1 expression increased in the 5-BDBD-treated cultures ($p < 0.01$ or $p < 0.05$). In addition, silencing mRNA directed toward P2X4R could increase the contractile phenotype transformation of the BSMCs compared with the PDGF-BB group ($p < 0.05$ or $p < 0.01$).

P2X4R was associated with the increased synthetic phenotype induced by PDGF-BB in BSMCs

Next, we examined the role of P2X4R in the synthetic phenotype of BSMCs (Fig. 6a, b). OPN expression in the BSMCs was upregulated after treatment with PDGF-BB. The effect of PDGF-BB on OPN expression was strengthened by ATP, but the effect was not significant. Moreover, the OPN expression that was enhanced by PDGF-BB was decreased by the P2X4R antagonist (5-BDBD) ($p < 0.01$) or by silencing the P2X4R mRNA ($p < 0.05$).

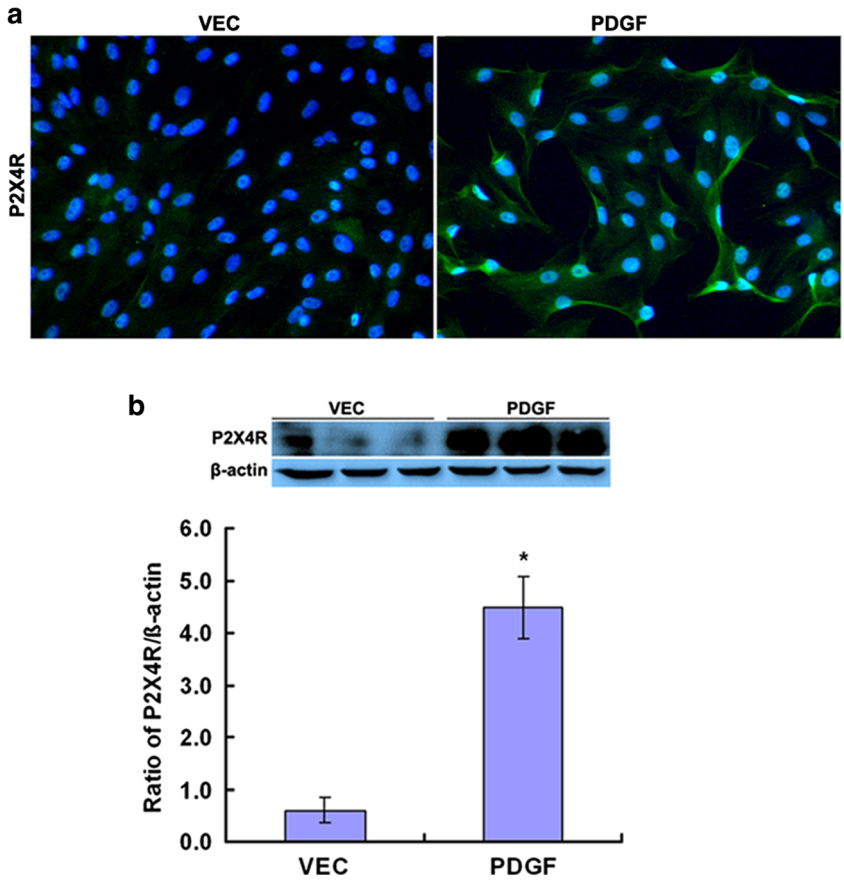

Fig. 3 PDGF-BB promoted P2X4R expression in BSMCs. **a** P2X4R expression in BSMCs by immunofluorescence ($n = 3$). P2X4R was mainly expressed in the membrane and cytoplasm of BSMCs, and the green fluorescence was more intense in the PDGF-BB group than in the VEC group (original magnification, fluorescence microscopy, × 200). P2X4R staining (green), nuclear staining (in blue). **b** P2X4R expression was measured in BSMCs via Western blotting ($n = 3$). *$p < 0.05$ versus VEC group

P2X4R regulated PDGF-BB-mediated BSMCs remodeling via p38MAPK

Expression of p-p38MAPK/p38MAPK in BSMCs was evaluated using Western blotting (Fig. 7a). PDGF-BB treatment significantly increased p-p38MAPK in BSMCs, whereas silencing the P2X4R mRNA significantly decreased the PDGF-BB-induced upregulation of p-p38MAPK/p38MAPK in BSMCs ($p < 0.01$). SB203580, p38MAPK inhibitor, inhibited the PDGF-BB-induced decreasing of α-SMA ($p < 0.01$), and SB203580 inhibited the PDGF-BB-induced increasing of OPN ($p < 0.05$) (Fig. 7b). Moreover, MTT results showed that SB203580 inhibited the PDGF-BB-induced proliferation of BSMCs ($p < 0.01$) (Fig. 7c).

Discussion

Airway remodeling refers to the epithelial denudation, subepithelial fibrosis, mucous cell hypertrophy, smooth muscle cell proliferation, and angiogenesis in the airway walls in asthma [26]. As the disease progresses, the remodeling that is characterized by the changes in the airway structure is gradually aggravated, which can lead to various degrees of irreversible airway obliteration [27, 28]. Thus, airway remodeling is closely related to the prognosis of asthmatic patients. Early diagnosis and prevention of airway remodeling has the potential to decrease disease severity. The etiology of airway remodeling and the potential therapies deserve further investigation. The relationship between structural changes and clinical and functional abnormalities clearly deserves further investigation [26]. Moreover, airway remodeling can occur at the early stage in childhood asthma [29]. These studies indicate that airway remodeling is not an end-stage manifestation but rather an active process in this disease.

Airway remodeling participates in the cyclical process of damage and repair. The increase of the airway smooth muscle mass caused by a dysregulation of ASMC proliferation is related to airway remodeling in asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis [30]. Airway smooth muscle proliferation and contraction are major determinants of airway remodeling in asthma and COPD [1, 31]. The airway smooth muscle mass is the strongest predictor of airflow limitation [27], and it is related to asthma severity [4, 32, 33]. Illustrating the molecular mechanisms that underlie BSMC proliferation and phenotypic switching may shed light onto a new idea for curing certain lung diseases that are associated with airway remodeling. We demonstrated that 5-BDBD increased α-SMA expression and inhibited PCNA expression in the lungs of mice in allergic asthma, which indicated that P2X4R may play roles in airway remodeling via phenotype switching of BSMCs.

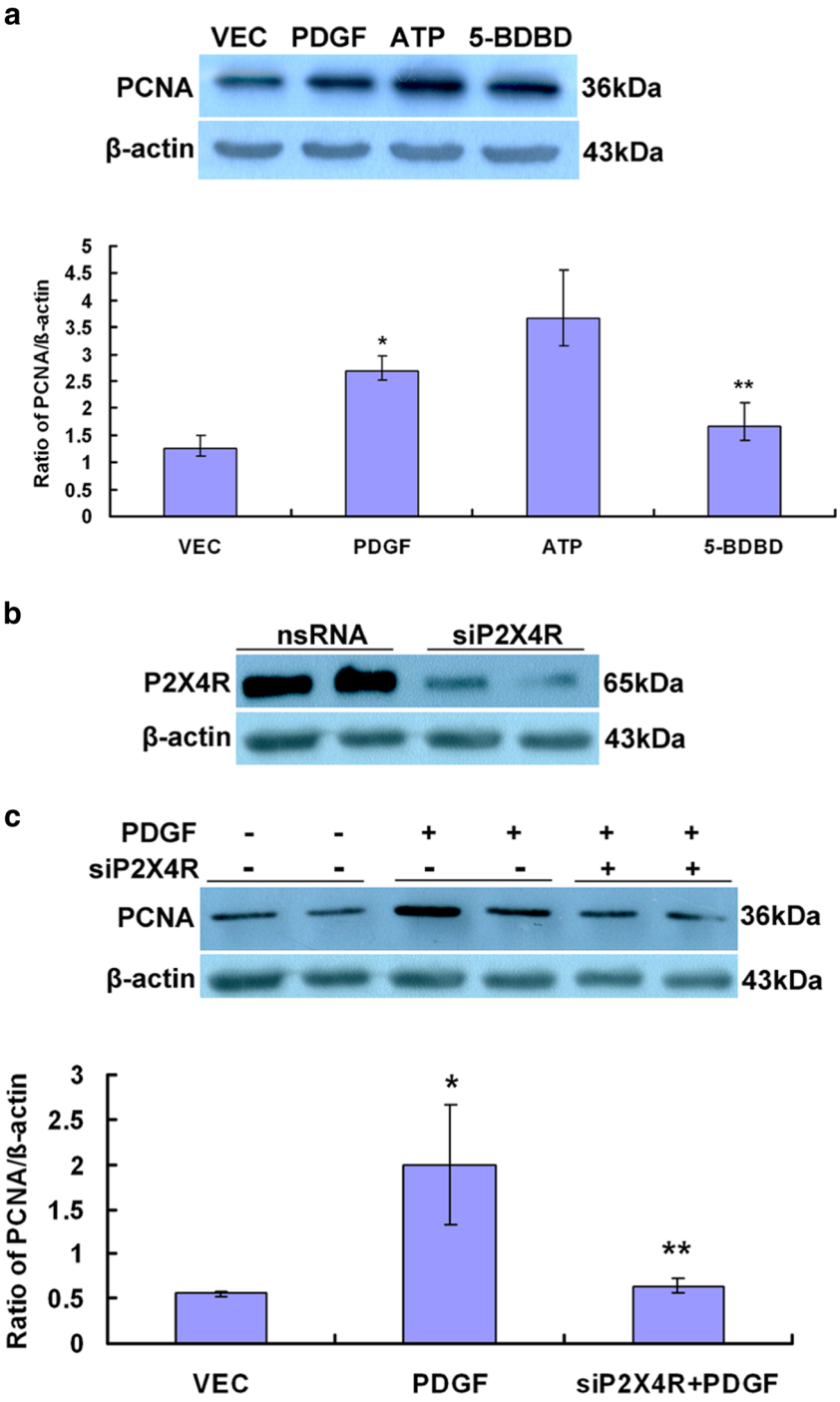

Fig. 4 P2X4R was involved in PDGF-BB-induced cell proliferation in BSMCs. **a** Expression of PCNA in BSMCs was evaluated after treatment with 0.5 μmol ATP and 10 μmol 5-BDBD ($n = 4$). *$p < 0.05$ versus the VEC group; **$p < 0.05$ versus the PDGF-BB group. **b** BSMCs were transfected with non-specific siRNA (nsRNA) and siRNA specific for P2X4R (siP2X4R). **c** PDGF-BB administration increased the PCNA level, which was decreased via siP2X4R ($n = 4$). *$p < 0.05$ versus the VEC group; **$p < 0.05$ versus the PDGF-BB group

P2X4R is a purinergic P2X receptor subtype that is a ligand-gated cation channel activated by extracellular ATP [12]. P2X4R is found in many types of immune and non-immune cells, including alveolar cells, lymphocytes, and mast cells [34]. It has been demonstrated that extracellular ATP regulates cochlear lateral blood flow through P2X4R activation in endothelial cells [35]. Furthermore, P2X4R is also widely distributed in the brain. In cerebral arteries, heteromeric P2X1/4 receptors mediate the sustained vasoconstrictions evoked by ATP [36]. Our previous report has demonstrated that the ATP-activated P2X4R pathway is involved in allergic asthma in mice [20]. Moreover, the proliferation and differentiation of airway and alveolar endotheliocytes as well as pulmonary vascular remodeling are associated with the ATP-activated P2X4R pathway in pulmonary hypertension [8]. However, to our knowledge, the effect of P2X4R on proliferation and phenotype switching in BSMCs has not yet been addressed.

Here, we revealed that P2X4R is expressed in the membrane and cytoplasm of BSMCs. Moreover, PDGF-BB could stimulate the production of P2X4R in BSMCs. We further confirmed the

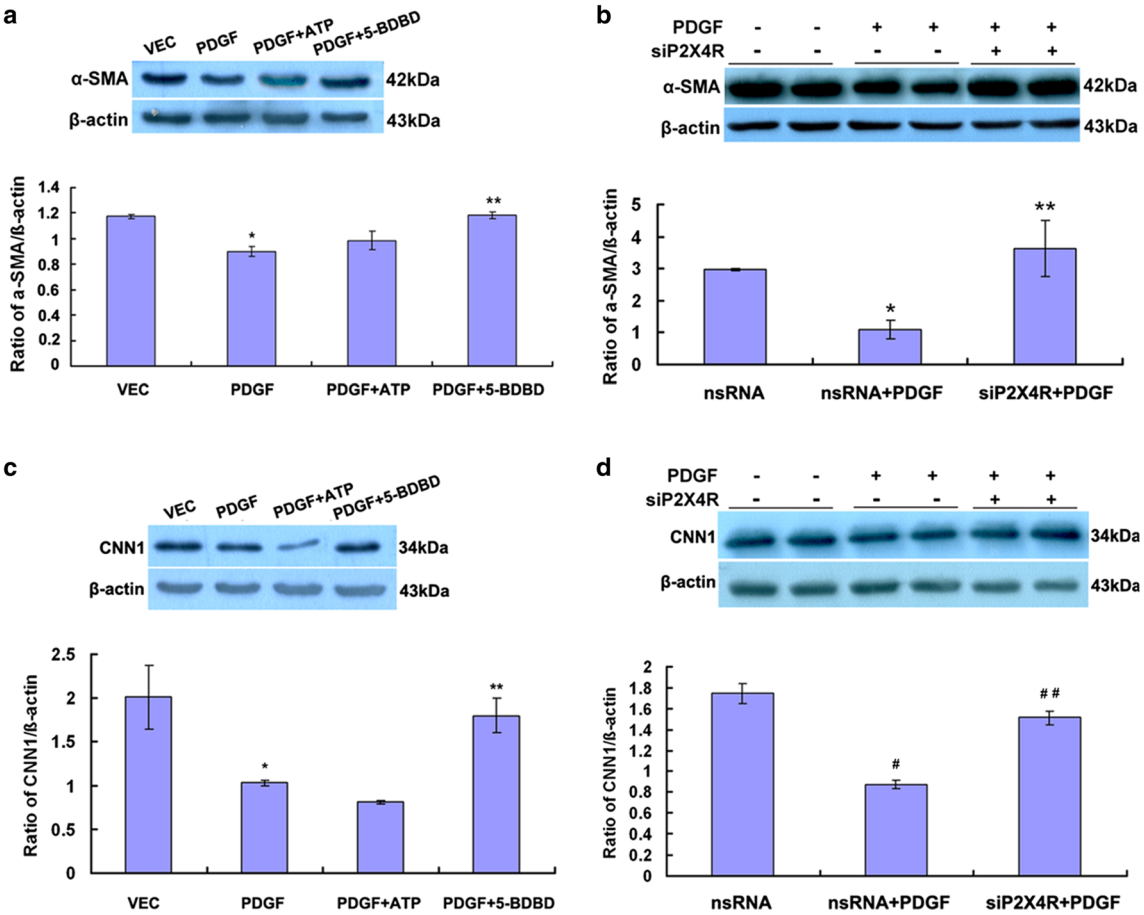

Fig. 5 P2X4R was involved in the PDGF-BB-induced decrease of the contractile phenotype in BSMCs. **a**, **c** The expression of α-SMA and CNN1 was detected after treatment with 0.5 μmol ATP and 10 μmol 5-BDBD in BSMCs ($n = 4$). *$p < 0.05$ versus the VEC group; **$p < 0.05$ versus the PDGF-BB group. **b**, **d** PDGF-BB administration reduced the α-SMA and CNN1 levels that were increased following treatment with siP2X4R ($n = 4$). *$p < 0.05$ or $^{\#}p < 0.01$ versus the VEC group; **$p < 0.05$ or $^{\#\#}p < 0.01$ versus the PDGF-BB group

effect of P2X4R on the phenotype switching of BSMCs. In our results, PDGF-BB promoted the proliferation and synthetic phenotype of BSMCs, which was aggravated by a P2X4R agonist and alleviated by a P2X4R antagonist, whereas the opposite effect of P2X4R antagonist was observed on the contractile phenotype. Silencing the P2X4R mRNA had the same effect as inhibiting P2X4R. The results indicate a new mechanism for airway remodeling that involves phenotype switching via the

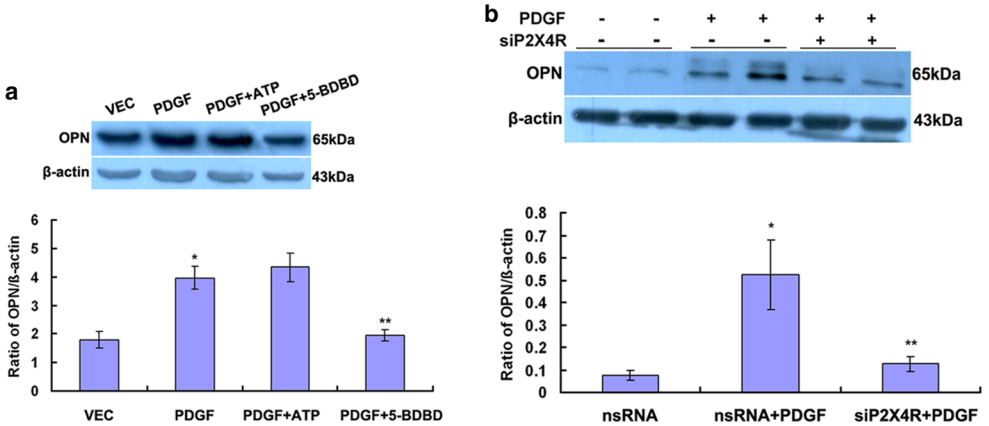

Fig. 6 P2X4R was involved in the PDGF-BB-induced increase of the synthetic phenotype in BSMCs. **a** Expression of OPN was examined after treatment with 0.5 μmol ATP and 10 μmol 5-BDBD in BSMCs ($n = 4$). *$p < 0.01$ versus the VEC group; **$p < 0.01$ versus the PDGF-BB group. **b** siP2X4R blocked the expression of OPN that was increased by the PDGF-BB treatment ($n = 4$). *$p < 0.01$ versus the nsRNA group; **$p < 0.05$ versus the nsRNA + PDGF-BB group

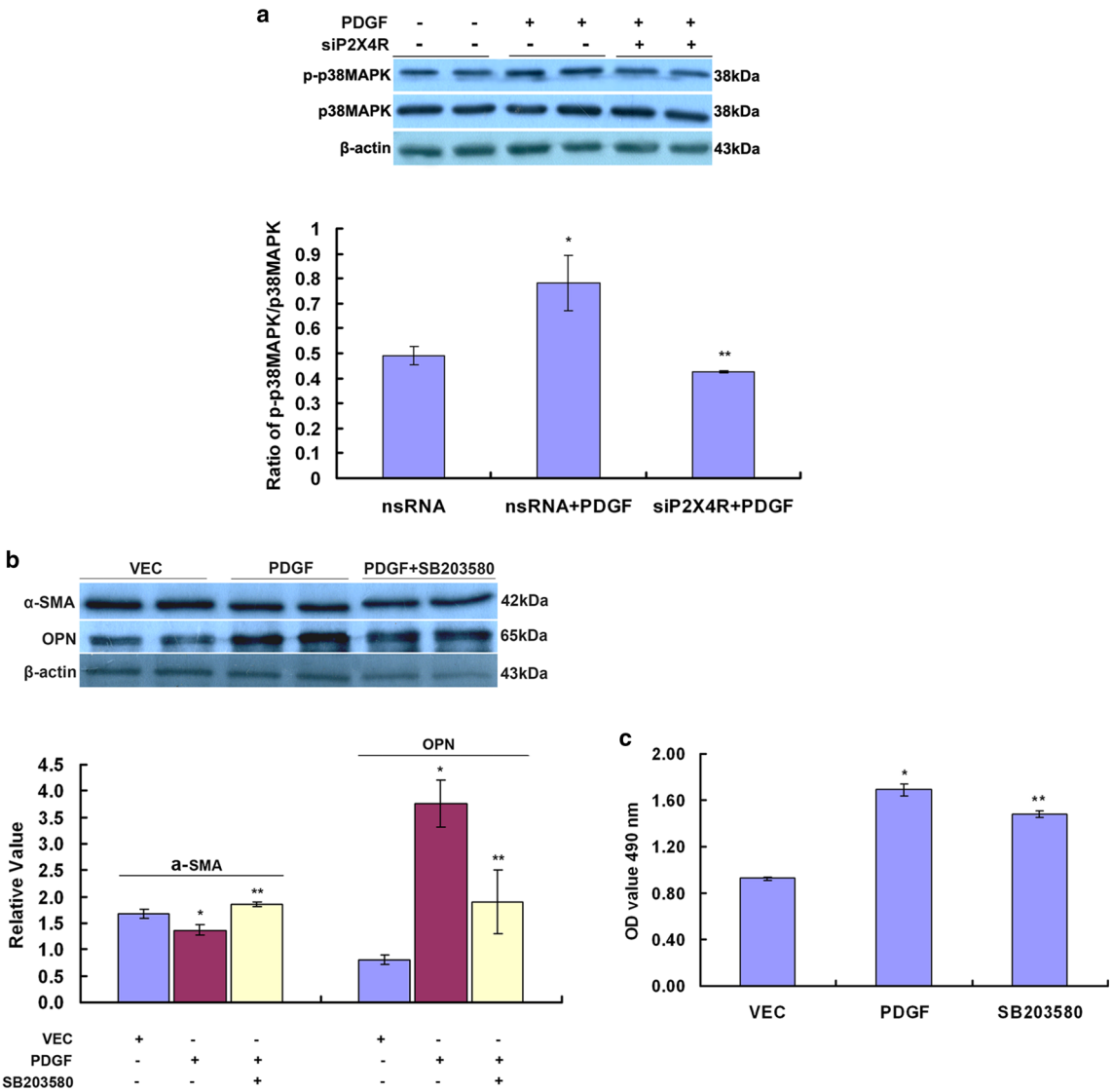

Fig. 7 P2X4R regulated the PDGF-BB-mediated BSMC remodeling via p38MAPK. **a** siP2X4R decreased the PDGF-BB-induced upregulation of p-p38MAPK/p38MAPK in BSMCs ($n = 4$). *$p < 0.05$ versus the nsRNA group; **$p < 0.01$ versus the nsRNA + PDGF-BB group. **b** SB203580 (30 μmol) inhibited the PDGF-BB-induced decreasing of α-SMA ($n = 3$). *$p < 0.05$ versus the VEC group; **$p < 0.01$ versus the PDGF-BB group. SB203580 inhibited the PDGF-BB-induced increasing of OPN ($n = 3$). *$p < 0.01$ versus the VEC group; **$p < 0.05$ versus the PDGF-BB group. **c** SB203580 inhibited the PDGF-BB-induced proliferation of BSMCs by MTT ($n = 3$). *$p < 0.01$ versus the VEC group; **$p < 0.01$ versus the PDGF-BB group

ATP-P2X4R axis in BSMCs. Studies have demonstrated that the p38MAPK pathway is associated with cell proliferation and differentiation [37, 38]. P2X4R-mediated p38MAPK signaling contributes to cancer-induced bone pain by activating Toll-like receptor 4 in the spinal cord [39]. In addition, the role of the p38MAPK pathway is significant in the inflammatory response, the airway hyperresponsiveness, and the airway remodeling [40]. In our study, silencing the P2X4R mRNA significantly decreased the PDGF-BB-induced upregulation of p-p38MAPK/p38MAPK in BSMCs. SB203580 inhibited the PDGF-BB-induced remodeling of BSMCs. The results indicated that P2X4R may contribute to synthetic differentiation of BSMCs via p38MAPK signaling.

Conclusion

The study indicates that P2X4R may be a new molecule that can modulate BSMCs proliferation and phenotype switching via p38MAPK signaling. The results provide us with the concept that inhibiting P2X4R may be a novel approach to relieve BSMCs proliferation and phenotype switching, then in turn may minimize airway remodeling.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests. The authors alone are responsible for the content and writing of this paper.

Ethical approval The experimental protocols were approved by the Animal Care and Protection Committee of Harbin Medical University-Daqing. Our use of animals conformed to our Institution's and country's animal welfare laws, and our studies were approved.

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