

## Sustained exposure to prostaglandin D<sub>2</sub> augments the contraction induced by acetylcholine via a DP<sub>1</sub> receptor-mediated activation of p38 in bronchial smooth muscle of naive mice

Wataru SUTO<sup>1</sup>, Hiroyasu SAKAI<sup>2</sup> and Yoshihiko CHIBA<sup>1</sup>

<sup>1</sup>*Department of Physiology and Molecular Sciences, School of Pharmacy, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan*

<sup>2</sup>*Department of Biomolecular Pharmacology, School of Pharmacy, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan*

Submitted December 15, 2018; accepted in final form January 30, 2019

### Abstract

Prostaglandin D<sub>2</sub> (PGD<sub>2</sub>), one of the key lipid mediators of allergic airway inflammation, is increased in the airways of asthmatics. However, the role of PGD<sub>2</sub> in the pathogenesis of asthma is not fully understood. In the present study, effects of PGD<sub>2</sub> on smooth muscle contractility of the airways were determined to elucidate its role in the development of airway hyperresponsiveness (AHR). In a murine model of allergic asthma, antigen challenge to the sensitized animals caused a sustained increase in PGD<sub>2</sub> levels in bronchoalveolar lavage (BAL) fluids, indicating that smooth muscle cells of the airways are continually exposed to PGD<sub>2</sub> after the antigen exposure. In bronchial smooth muscles (BSMs) isolated from naive mice, a prolonged incubation with PGD<sub>2</sub> (10<sup>-5</sup> M, for 24 h) induced an augmentation of contraction induced by acetylcholine (ACh): the ACh concentration-response curve was significantly shifted upward by the 24-h incubation with PGD<sub>2</sub>. Application of PGD<sub>2</sub> caused phosphorylation of ERK1/2 and p38 in cultured BSM cells: both of the PGD<sub>2</sub>-induced events were abolished by laropiprant (a DP<sub>1</sub> receptor antagonist) but not by fevipiprant (a DP<sub>2</sub> receptor antagonist). In addition, the BSM hyperresponsiveness to ACh induced by the 24-h incubation with PGD<sub>2</sub> was significantly inhibited by co-incubation with SB203580 (a p38 inhibitor), whereas U0126 (a ERK1/2 inhibitor) had no effect on it. These findings suggest that prolonged exposure to PGD<sub>2</sub> causes the BSM hyperresponsiveness via the DP<sub>1</sub> receptor-mediated activation of p38. A sustained increase in PGD<sub>2</sub> in the airways might be a cause of the AHR in allergic asthmatics.

**Key words:** airway hyperresponsiveness, bronchial smooth muscle contractility, prostaglandin D<sub>2</sub> (PGD<sub>2</sub>), DP<sub>1</sub> receptor, p38

---

## Introduction

---

Heightened responsiveness to non-specific stimuli of the airways, called airway hyperresponsiveness (AHR), is a characteristic feature in patients with allergic asthma. It has been suggested that the augmented contraction of airway smooth muscle is one of the causes of the AHR (1–5). Clinical observation that the inhalation of short-acting  $\beta_2$ -stimulant is largely effective for airway limitation in asthma attack also suggests an increased airway smooth muscle contraction in the airway obstruction. It is thus important for development of asthma therapy to understand the disease-related changes in the contractile signaling of airway smooth muscle cells.

In allergic asthma, an involvement of prostaglandin D<sub>2</sub> (PGD<sub>2</sub>), one of the cyclooxygenase (COX) metabolites that released from mast cells in allergic reaction, has been suggested in its pathogenesis. In animal models of allergic asthma, the PGD<sub>2</sub> levels in bronchoalveolar lavage (BAL) fluids were increased (6–8). In people with asthma, an increase in PGD<sub>2</sub> in BAL fluids was observed after allergen challenge to the airways (9, 10). An involvement of PGD<sub>2</sub> in the allergic inflammation, including the airway inflammation in asthma, has also been suggested. In a DP<sub>1</sub> receptor (one of the receptors for PGD<sub>2</sub>) gene knockout mice, both cytokines increase in BAL fluids and airway eosinophilia induced by allergen challenge were weakened as compared to wild-type animals (11). In Th2 lymphocytes, PGD<sub>2</sub> also has an ability to cause cytokine release via an activation of DP<sub>2</sub> receptor (also called as CRTH2 receptor) (12). However, the functional role of PGD<sub>2</sub> on airway smooth muscle remains largely unclear.

We show here that PGD<sub>2</sub> levels in the airways were increased immediately after antigen challenge and were sustained during the development of antigen-induced AHR. To imitate the antigen-induced sustained increase in PGD<sub>2</sub> *in vitro*, the BSM tissues isolated from naive mice were incubated with PGD<sub>2</sub> for 24 h. Using the PGD<sub>2</sub>-treated BSM tissues, change in the BSM contractility was evaluated by measuring isometric contractions induced by acetylcholine (ACh) and high K<sup>+</sup> depolarization. The receptor subtypes and signaling molecules involved in the PGD<sub>2</sub>-induced BSM hyperresponsiveness were also identified pharmacologically.

---

## Methods

---

### Animals

Male BALB/c mice were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan) and housed in a pathogen-free facility. All animal experiments were approved by the Animal Care Committee of Hoshi University (Tokyo, Japan).

### Sensitization and antigenic challenge

Preparation of a murine model of allergic bronchial asthma, which has an *in vivo* AHR (13), was performed as described previously (14). In brief, BALB/c mice (8 weeks of age) were actively sensitized by intraperitoneal injections of 8  $\mu$ g ovalbumin (OA; Sigma-Aldrich, St. Louis, MO, USA) with 2 mg Imject Alum (Pierce Biotechnology, Inc., Rockford, IL, USA) on day 0 and day 5. The sensitized mice were challenged with aerosolized OA-saline solution (5 mg/ml) for 30 min on days 12, 16 and 20. A control group of mice received the same immunization procedure but inhaled saline aerosol instead of OA challenge. The aerosol was generated with a Mini Elite nebulizer (Philips Respironics, Amsterdam, Netherlands) and introduced to a Plexiglas chamber box (130  $\times$  200 mm, 100 mm height) in which the mice were placed. Mice were sacrificed 1, 3, 6, 12 and 24 h after the last challenge by exsanguination from abdominal aorta under urethane (1.6 g/kg, *i.p.*; Sigma, St. Louis, MO, USA) anesthesia.

**Table 1.** Primer sequences for RT-PCR used in the present study

Gene name	RefSeq Accession		Sequence	Amplicon size
mouse Pla2g4c	NM_001168504	Sense	5'-GGACCGTTGCGTTTTTGTGA-3'	150 bp
		Antisense	5'-GCAAAACCAGCATCCACCAG-3'	
mouse Ptgs2	NM_0011198	Sense	5'-CCGTGGGGAATGTATGAGCA-3'	128 bp
		Antisense	5'-GGGTGGGCTTCAGCAGTAAT-3'	
mouse Hpgds	NM_019455	Sense	5'-TTCCCATGGGCAGAGAAAGA-3'	143 bp
		Antisense	5'-GCCCAGGTTACATAATTGCCT-3'	
mouse Gapdh	NM_008084	Sense	5'-CCTCGTCCCGTAGACAAAATG-3'	100 bp
		Antisense	5'-TCTCCACTTTGCCACTGCAA-3'	

### *RNA extraction from bronchial smooth muscle (BSM) tissues*

Total RNA of the mouse BSM tissue was isolated from an aliquot of the tissue using NucleoSpin™ miRNA (TaKaRa Bio, Inc., Shiga, Japan) according to the manufacturer's instruction. cDNAs were prepared from the total RNA by using PrimeScript™ RT reagent Kit (TaKaRa) according to the manufacturer's instruction.

### *Real-time RT-PCR*

The mRNA levels were examined by real-time RT-PCR. cDNA was subjected to real-time PCR analyses using Fast SYBR Green Master Mix (Applied biosystems, Foster, CA, USA) according to the manufacturer's instruction. The PCR primer sets used are shown in Table 1, which was designed from published database, BLAST. The thermal cycle profile used was 1) denaturing for 3 s at 95 °C, 2) annealing and extending the primers for 30 s at 60 °C, and the reaction was run for 43 cycles.

### *Analyses of bronchoalveolar lavage fluids*

After the exsanguinations, the chest of each animal was opened and a 20-gauge blunt needle was tied into the proximal trachea. Bronchoalveolar lavage fluids (BALFs) was obtained by intratracheal instillation of 1 ml/animal of phosphate-buffered saline (PBS; pH7.5, room temperature) into the lung while it was kept located within the thoracic cavity. The lavage was reinfused into the lung twice before final collection. The resultant supernatants of the lavage fluids were subjected to protein analyses. The level of PGD<sub>2</sub> was measured by Prostaglandin D<sub>2</sub>-MOX, EIA Kit (Cayman Chemical Company, Ann Arbor, MI, USA), according to the manufacturer's instructions.

### *Determination of bronchial smooth muscle (BSM) responsiveness*

Mice were killed by exsanguination from abdominal aorta under urethane (1.6 g/kg, *i.p.*) anesthesia. Then the airway tissues under the larynx to lungs were immediately removed. About 3 mm length of the left main bronchus was isolated. The resultant tissue ring preparation was then suspended in a 5 ml-organ bath by two stainless-steel wires (0.2 mm diameter) passed through the lumen. For all tissues, one end was fixed to the bottom of the organ bath while the other was connected to a force-displacement transducer (TB-612T, Nihon Kohden) for the measurement of isometric force. A resting tension of 5.0 mN was applied. The buffer solution contained modified Krebs-Henseleit solution with the following composition (mM); NaCl 118.0, KCl 4.7, CaCl<sub>2</sub> 2.5, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25.0, KH<sub>2</sub>PO<sub>4</sub> 1.2 and glucose 10.0. The buffer solution was maintained at 37°C and oxygenated with 95% O<sub>2</sub>-5% CO<sub>2</sub>. After the equilibration period, the concentration-response curve to ACh (10<sup>-7</sup>-10<sup>-3</sup> M in final concentration) was constructed cumulatively. In another series of experiment, isotonic K<sup>+</sup>

solution (10–90 mM in final concentration) was cumulatively administered in the presence of atropine ( $10^{-6}$  M) to determine the BSM responsiveness to high  $K^+$ -depolarization.

### *Tissue culture of isolated bronchial smooth muscles (BSMs)*

After the determination of BSM responsiveness described above, the tissue preparations were immediately immersed in SmBM™ Medium (SmBM; Cambrex Bio Science Walkersville, Inc., MD, USA) containing 50 U/ml penicillin and 50 µg streptomycin (Invitrogen Corp., Grand Island, NY, USA) and cultured at 37 °C under a 5% CO<sub>2</sub> atmosphere in the presence or absence of PGD<sub>2</sub> (Cayman Chemical Company) for 24 h. After the incubation period, BSM responsiveness was determined as described above. In another series of experiments, the 24-h PGD<sub>2</sub> incubation was carried out in the presence or absence of pharmacological agent. The agents used in the present study were U0126, an ERK1/2 inhibitor (15), and SB203580, a p38 inhibitor (16).

### *Cell culture and sample collection*

Normal human BSM cells (hBSMCs; Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA) were maintained in SmBM medium supplemented with 5% fetal bovine serum, 0.5 ng/ml human epidermal growth factor (hEGF), 5 µg/ml insulin, 2 ng/ml human fibroblast growth factor-basic (hFGF-b), 50 µg/ml gentamicin and 50 ng/ml amphotericin B. Cells were maintained at 37 °C in a humidified atmosphere (5% CO<sub>2</sub>), fed every 48–72 h, and passaged when cells reached 90–95% confluence. Then the hBSMCs (passages 4–7) were seeded in 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at density of 3,500 cells/cm<sup>2</sup> and, when 80–85% confluence was observed, cells were cultured without serum for 24 h before the addition of PGD<sub>2</sub>. At the indicated time after the treatment with PGD<sub>2</sub>, hBSMCs were immediately collected and disrupted with 1× SDS sample buffer (150 µl/well), and used for Western blot analyses.

### *Western blot analyses*

Protein samples were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were then electrophoretically transferred to PVDF membrane. After blocking with EzBlock Chemi (ATTO, Japan), the PVDF membrane was incubated with the primary antibody. The primary antibodies used in the present study were monoclonal mouse anti-phospho-ERK1/2 (1:1,000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-ERK1/2 (1:1,000 dilution; Santa Cruz Biotechnology, Inc.) and monoclonal rabbit anti-phospho-p38 (1:10,000 dilution; Cell Signaling Technology Japan) antibodies. Then the membrane was incubated with HRP-linked anti-rabbit IgG (1:5,000 dilution; Cell Signaling Technology) or anti-mouse IgG (1:5,000 dilution; Santa Cruz Biotechnology, Inc.).

### *Statistical analyses*

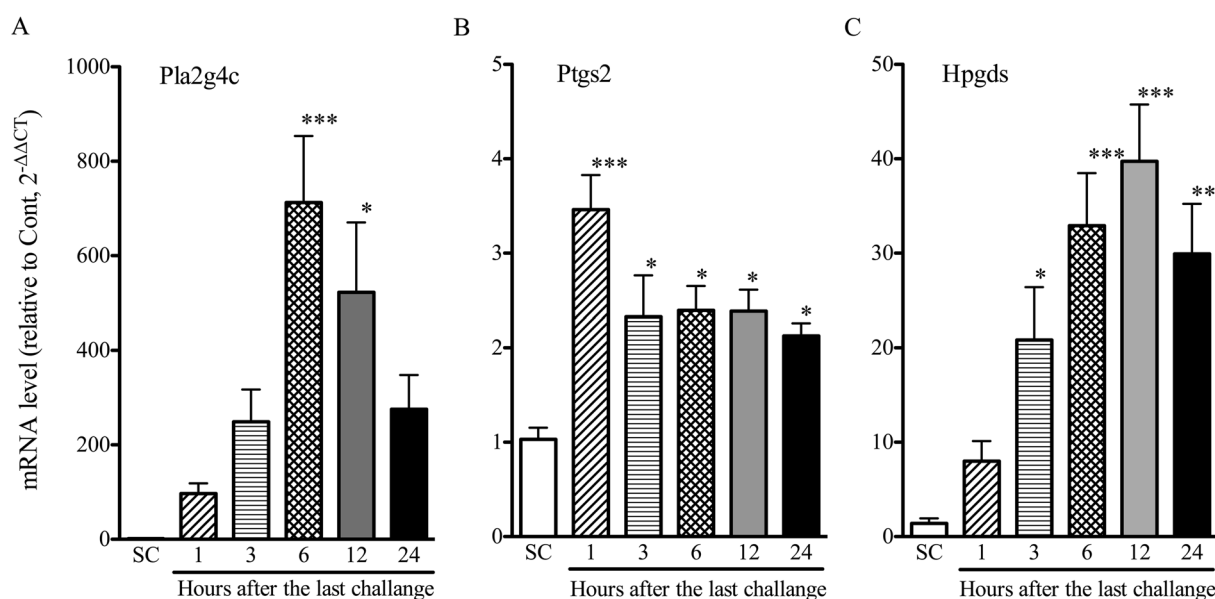
All the data are expressed as the mean ± S.E.M. Statistical significance of difference was determined by one-way analysis of variance (ANOVA) with post hoc Bonferroni/Dunn or two-way ANOVA (Prism™ 5 for Mac OS X; GraphPad Software, Inc., La Jolla, CA, USA). A value of  $P < 0.05$  was considered to be significant.

---

## Results

### *Change in prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) production in the airways after antigen exposure*

In the present study, we used our well-established murine model of allergic bronchial asthma (13, 14, 17–19). In this animal model of asthma, mRNAs for *Pla2g4c* (phospholipase A<sub>2</sub> group 4c), *Ptgs2* (cyclooxygen-

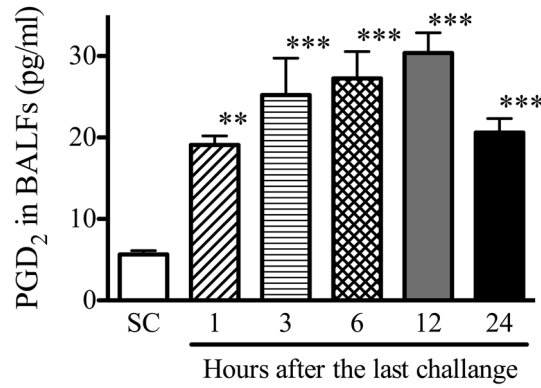


**Fig. 1.** Time-course changes in the mRNA levels for phospholipase A<sub>2</sub> group 4c (*Pla2g4c*: A), cyclooxygenase 2 (*Ptgs2*: B) and hematopoietic prostaglandin D synthase (*Hpgds*: C) in bronchial smooth muscle tissues of mice after the last antigen challenge determined by real-time reverse transcriptase-polymerase chain reaction. The relative gene expression to the sensitized control animals (SC) was calculated by the 2<sup>-ΔΔCT</sup> methods using *Gapdh* as an internal control. Results are presented as mean ± SEM from five animals, respectively. \**P*<0.05, \*\**P*<0.01 and \*\*\**P*<0.001 versus SC group by one-way ANOVA with post hoc Bonferroni/Dunnett's test.

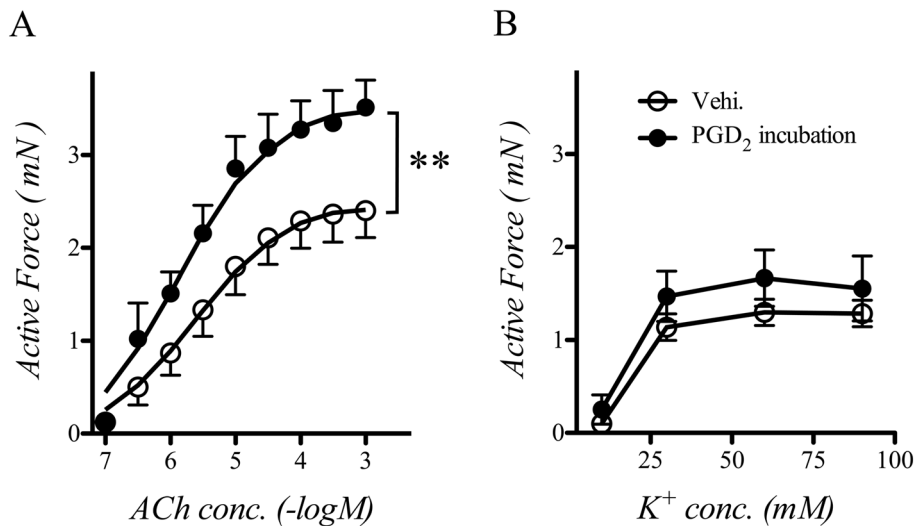
ase 2: COX2) and *Hpgds* (hematopoietic prostaglandin D synthase) were significantly increased in the BSM tissues: the peak increments were observed at 6, 1 and 12 h after the last antigen challenge, respectively (Fig. 1). The findings indicate that the AA metabolism is shifted toward PGD<sub>2</sub> production in the airways. So next, PGD<sub>2</sub> levels in bronchoalveolar lavage fluids (BALFs) were measured using the ELISA system. As shown in Fig. 2, the time-course analyses of PGD<sub>2</sub> levels in BALFs revealed that PGD<sub>2</sub> levels in the airways were increased immediately after antigen challenge and were sustained during the development of antigen-induced AHR. Interestingly, the time-course change of PGD<sub>2</sub> level (Fig. 2) was almost similar to that of *Hpgds* expression (Fig. 1C).

### ***Effects of a 24-h incubation with prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) on bronchial smooth muscle (BSM) contractility***

The findings that PGD<sub>2</sub> levels in BALFs sustainably increased (Fig. 2) indicate that smooth muscle cells of the airways are continually exposed to PGD<sub>2</sub>. To imitate the antigen-induced sustained increase in PGD<sub>2</sub> *in vitro*, the BSM tissues isolated from naive mice were incubated with PGD<sub>2</sub> for 24 h as described above. As shown in Fig. 3A, in the BSM tissues isolated from naive animals, the 24-h incubation with PGD<sub>2</sub> caused a hyperresponsiveness to acetylcholine (ACh): the ACh concentration-response curve was significantly shifted upward by the 24-h PGD<sub>2</sub> incubation (*P*<0.01 by two-way ANOVA; Fig. 3A). On the other hand, no significant change in the contractile responsiveness to high K<sup>+</sup> depolarization (in the presence of 10<sup>-6</sup> M atropine) was observed in the PGD<sub>2</sub>-incubated BSM tissues (Fig. 3B).



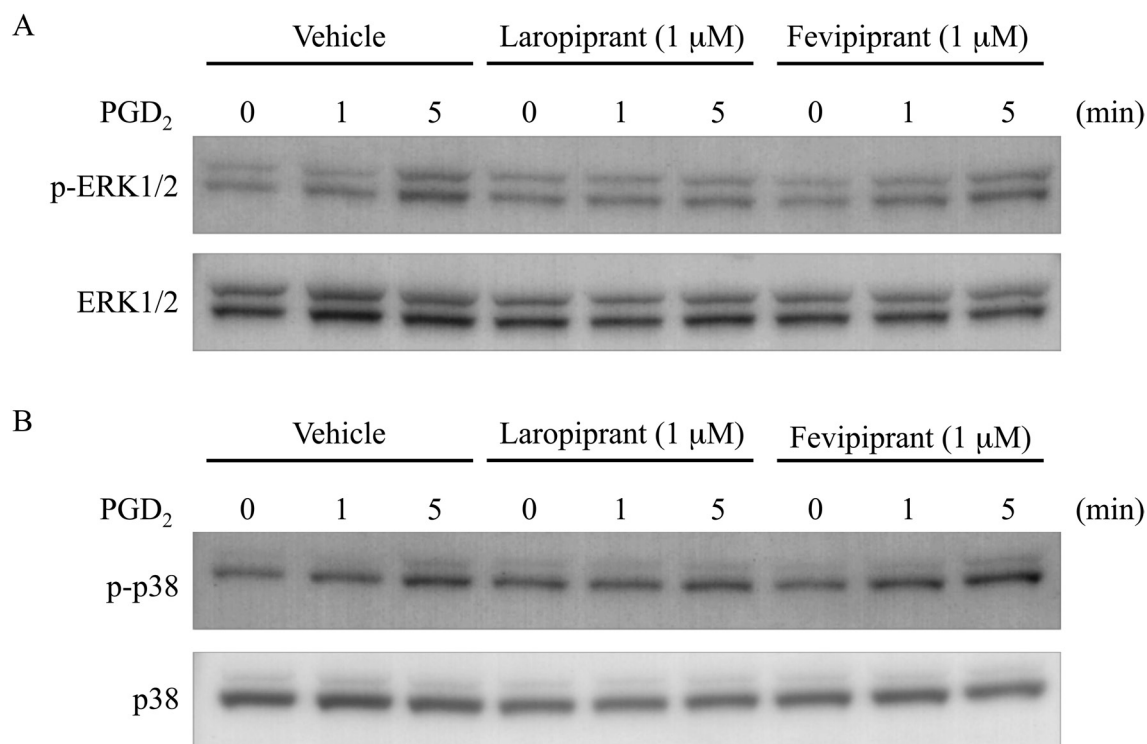
**Fig. 2.** Time-course changes in the prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) levels in bronchoalveolar lavage fluids (BALFs) after the last antigen challenge determined by ELISA. Results are presented as mean  $\pm$  S.E.M. from five animals, respectively. \*\* $P$ <0.01 and \*\*\* $P$ <0.001 versus sensitized control (SC) by one-way ANOVA with *post hoc* Bonferroni/Dunnett's test.



**Fig. 3.** Effects of *in vitro* incubation with prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) on bronchial smooth muscle (BSM) responsiveness to acetylcholine (ACh) in naive mice. The BSM tissues isolated from naive animals were incubated with PGD<sub>2</sub> (10<sup>-5</sup> M: *closed circles*) or its vehicle (0.1% DMSO: *open circles*) for 24 h. The BSM responsiveness to cumulatively applied acetylcholine (ACh: A) or K<sup>+</sup> (in the presence of 10<sup>-6</sup> M atropine: B) were measured as described in METHODS. The BSM responsiveness to ACh but not K<sup>+</sup> was significantly increased when the BSMs were incubated with PGD<sub>2</sub>. Results are presented as mean  $\pm$  S.E.M. from 8 animals, respectively. \*\* $P$ <0.01 versus vehicle by two-way ANOVA.

### Phosphorylation of ERK1/2 and p38 by prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) in bronchial smooth muscle (BSM) cells

To identify the signaling molecules activated by PGD<sub>2</sub> in the BSM cells, cultured BSM cells were stimulated with PGD<sub>2</sub>. As shown in Fig. 4, immunoblot analyses revealed that PGD<sub>2</sub> caused a distinct phosphorylation of ERK1/2 and p38, i.e., activation of these molecules, in the BSM cells. Both the PGD<sub>2</sub>-induced phos-



**Fig. 4.** Activation of ERK1/2 (A) and p38 (B) induced by prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) in cultured bronchial smooth muscle (BSM) cells. After a starvation period, the BSM cells were stimulated with prostaglandin D<sub>2</sub> (PGD<sub>2</sub>: 10<sup>-5</sup> M). At the indicated time after the stimulation, the protein expressions of ERK1/2 and p38 and their phosphorylated forms (p-ERK1/2 and p-p38) were determined from immunoblots. Cells were also pretreated with laropiprant (a DP<sub>1</sub> receptor antagonist), fevipiprant (a DP<sub>2</sub> receptor antagonist), or their vehicle (0.1% DMSO) 15 min before addition of PGD<sub>2</sub>.

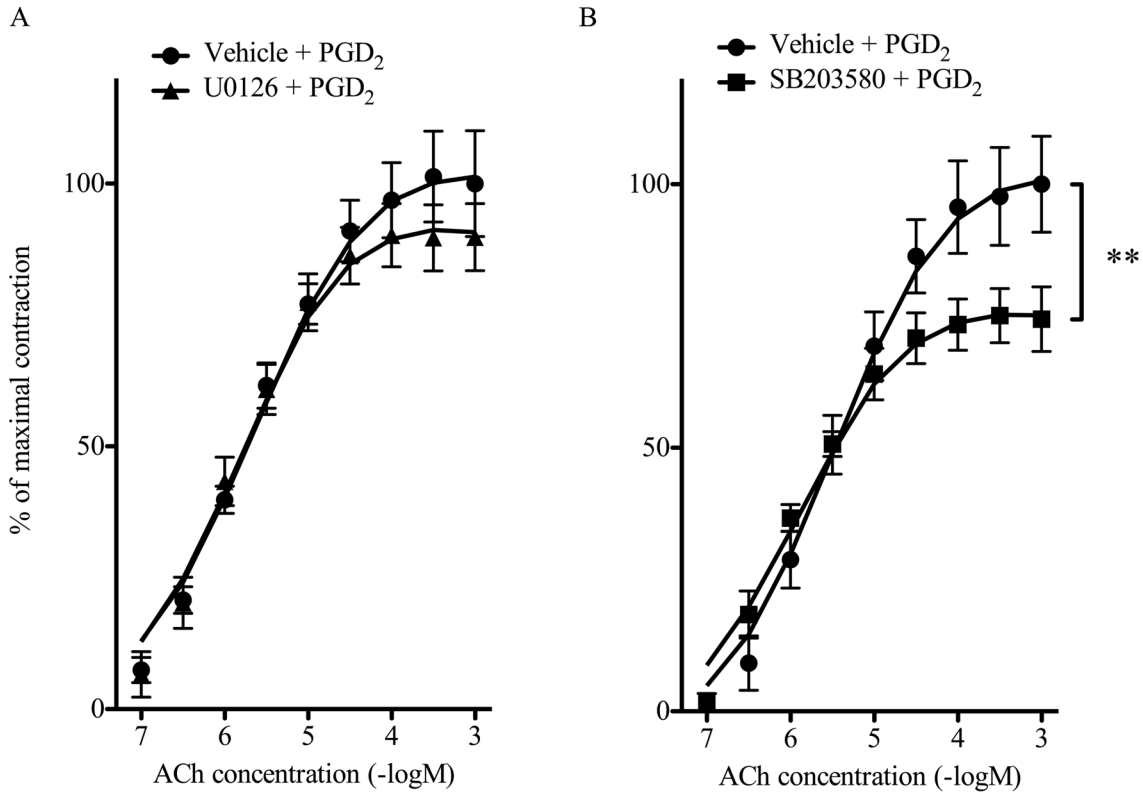
phorylation of ERK1/2 and p38 were abolished by laropiprant (a DP<sub>1</sub> receptor antagonist), whereas fevipiprant (a DP<sub>2</sub> receptor antagonist) had no effect on their phosphorylation (Fig. 4). On the other hand, in the BSM cells, PGD<sub>2</sub> did not cause activation of other molecules, such as JNK and STAT3 (data not shown), that were reportedly activated in various types of the cells (7, 20–23).

#### ***Effects of inhibitors for ERK1/2 and p38 on the bronchial smooth muscle (BSM) hyperresponsiveness induced by prostaglandin D<sub>2</sub> (PGD<sub>2</sub>)***

To determine the roles of ERK1/2 and p38 on the BSM hyperresponsiveness induced by PGD<sub>2</sub>, the BSM tissues isolated from naive mice were co-incubated with an ERK1/2 inhibitor U0126 or a p38 inhibitor SB203580 during the 24-h PGD<sub>2</sub> incubation period. As shown in Fig. 5A, co-incubation with U0126 had no significant effect on the ACh responsiveness in the 24-h PGD<sub>2</sub>-incubated BSMs. However, the BSM hyperresponsiveness to ACh induced by the 24-h PGD<sub>2</sub> incubation was attenuated by the co-incubation with SB203580 (Fig. 5B): the ACh concentration-response curve was significantly shifted downward when the BSM tissues were incubated in the presence of SB203580 ( $P < 0.01$  by two-way ANOVA; Fig. 5B).

## **Discussion**

Consistently with the previous report (8), the mRNA expressions for *Pla2g4c*, *Ptgs2* and *Hpgds* were significantly increased in the bronchial smooth muscle (BSM) tissues (Fig. 1), indicating that arachidonic acid



**Fig. 5.** Effects of co-incubation with U0126 (an ERK1/2 inhibitor: A) and SB203580 (a p38 inhibitor: B) on the prostaglandin D<sub>2</sub> (PGD<sub>2</sub>)-induced bronchial smooth muscle (BSM) hyperresponsiveness to acetylcholine (ACh) in mice. The BSM tissues isolated from naive BALB/c mice were incubated with PGD<sub>2</sub> (10<sup>-5</sup> M, for 24 h) in the absence or presence of respective inhibitors. After the incubation period, the BSM responsiveness to ACh was determined as described in METHODS. Results are presented as mean  $\pm$  S.E.M. from at least five animals. \* $P$ <0.05 and \*\* $P$ <0.01 versus control by two-way ANOVA.

metabolism is shifted towards prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) production in mice with allergic asthma. Indeed, a sustained increase in PGD<sub>2</sub> was observed in the airways after the antigen stimulation (Fig. 2). The BSM hyperresponsiveness reported in experimental asthma (14, 18) was replicated when the BSM tissues isolated from naive animals were incubated with PGD<sub>2</sub> for 24 h (Fig. 3). PGD<sub>2</sub> caused phosphorylation of ERK1/2 and p38 in the BSM cells via an activation of DP<sub>1</sub> receptor (Fig. 4). The BSM hyperresponsiveness induced by the 24-h PGD<sub>2</sub> incubation was inhibited by a p38 inhibitor SB203580, but not by an ERK1/2 inhibitor U0126 (Fig. 5). These findings suggest that a sustained increase in PGD<sub>2</sub> level in the airways is a cause of the AHR in asthma, and that the DP<sub>1</sub>-mediated activation of p38 is responsible for the BSM hyperresponsiveness induced by PGD<sub>2</sub>.

Our previous microarray analysis of differentially expressed genes (GEO accession No. GSE116504) demonstrated a significant change in the arachidonic acid (AA) metabolism pathway (KEGG map00590) in the BSM tissues of asthma (8). In particular, the AA metabolism was largely shifted towards PGD<sub>2</sub> production in the diseased BSMS (8). Currently, time-course studies of the gene expression revealed that *Pla2g4c*, *Ptgs2* and *Hpgds* were up-regulated in the relatively early period after antigen challenge (Fig. 1). Notably, time-course change of the PGD<sub>2</sub> level in BALF (Fig. 2) seems to be parallel with that of up-regulation of *Hpgds* (Fig. 1C), indicating that the hematopoietic prostaglandin D synthase (HPGDS) is responsible for the increased PGD<sub>2</sub> production in the diseased airways. The idea is also supported by the observation that the lipocalin-type PGDS (LPGDS), one of the two types of PGDS, expression was not changed in the diseased BSM tissues in the microarray analysis (GSE116504).



The current time-course study also revealed a sustained increase in PGD<sub>2</sub> in the airways: the PGD<sub>2</sub> levels in BALFs were already increased just after the antigen exposure (Fig. 2). The findings indicate that smooth muscle cells of the airways are continually exposed to PGD<sub>2</sub>, and remind us of an idea that the sustained increase in PGD<sub>2</sub> might be a cause of augmented BSM contractility at the AHR. Indeed, consistently with the previous report (8), the 24-h incubation with PGD<sub>2</sub> induced a hyperresponsiveness to ACh in the BSM tissues isolated from naive animals (Fig. 3A). PGD<sub>2</sub> had no effect on contractile responsiveness to high K<sup>+</sup> depolarization (in the presence of 10<sup>-6</sup> M atropine, to block the effect of endogenously released ACh: Fig. 3B). In the antigen-induced AHR mice, an augmented BSM contractility to ACh without alteration of high K<sup>+</sup> responsiveness has also been observed (14, 18). It is thus possible that PGD<sub>2</sub> is one of the factors that contribute to the altered property of BSMs in allergic asthma.

To identify the signaling molecule(s) activated by PGD<sub>2</sub> in BSM cells, cultured human BSM cells (hBSMCs) were stimulated with PGD<sub>2</sub>. Because the mouse BSM tissue contains various types of cells other than the BSM cells, the commercially available hBSMCs were used in the present study. Reportedly, PGD<sub>2</sub> has an ability to activate various signaling molecules, such as ERK1/2, p38, JNK, and STAT3 (7, 20–23). Among these molecules, the current immunoblot analyses revealed that PGD<sub>2</sub> caused a distinct activation of ERK1/2 and p38, i.e., phosphorylation of these proteins, in the hBSMCs (Fig. 4). The findings are consistent with the previous report, in which phosphorylation of these molecules induced by PGD<sub>2</sub> were demonstrated in a human BSM cell line, BSM2146 cells (7). To date, two distinct receptor subtypes for PGD<sub>2</sub>, termed DP<sub>1</sub> and DP<sub>2</sub> receptors, have been identified (24–26). To identify receptor subtype responsible for the phosphorylation of ERK1/2 and p38 induced by PGD<sub>2</sub>, the hBSMCs were pre-incubated with a DP<sub>1</sub> antagonist laropiprant (27) or a DP<sub>2</sub> antagonist fevipiprant (28) during the PGD<sub>2</sub> stimulation. As a result, both the PGD<sub>2</sub>-induced phosphorylation of ERK1/2 and p38 were abolished by laropiprant, whereas fevipiprant had no effect on their phosphorylation (Fig. 4). Thus, PGD<sub>2</sub> acts on DP<sub>1</sub> receptors to activate ERK1/2 and p38 in BSM cells.

The results that PGD<sub>2</sub> activated ERK1/2 and p38 in the BSM cells suggest an involvement of these signaling molecules in the augmented BSM responsiveness to ACh. Indeed, activation of these molecules could cause an augmentation of the contraction in airway smooth muscles (29–32). To determine the roles of ERK1/2 and p38 on the BSM hyperresponsiveness induced by PGD<sub>2</sub>, the BSM tissues isolated from naive mice were co-incubated with an ERK1/2 inhibitor U0126 (15) or a p38 inhibitor SB203580 (16) during the 24-h PGD<sub>2</sub> incubation period. As shown in Fig. 5, although U0126 had no significant effect (Fig. 5A), the BSM hyperresponsiveness to ACh induced by PGD<sub>2</sub> was attenuated by the co-incubation with SB203580 (Fig. 5B): the ACh concentration-response curve was significantly shifted downward when the BSM tissues were incubated in the presence of SB203580 (Fig. 5B). The findings strongly suggest that the PGD<sub>2</sub>-induced BSM hyperresponsiveness is mediated by an activation of p38.

The current study clearly demonstrated that the 24-h incubation with PGD<sub>2</sub> caused an augmented BSM contractility to ACh (Fig. 3). The results indicate that PGD<sub>2</sub> acts on DP<sub>1</sub> receptor on the BSMs to activate p38 (Figs. 4 and 5), resulting in the induction of BSM hyperresponsiveness. On the other hand, some of the PGD<sub>2</sub> metabolites may also be involved in the BSM hyperresponsiveness. PGD<sub>2</sub> is known to convert to PGJ<sub>2</sub> family of prostanoids (33), and their physiological and pathophysiological roles have also been suggested. For example, a PGD<sub>2</sub> metabolite 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15-deoxy PGJ<sub>2</sub>) has an ability to activate signaling molecules, such as peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) (34) and phosphoinositide 3-kinase (PI3 K)-Akt (35). Interestingly, exogenously applied 15-deoxy PGJ<sub>2</sub> could inhibit airway inflammation and hyperresponsiveness in murine models of allergic asthma (36). Further studies are needed to make clear the exact role of PGD<sub>2</sub> on the development of BSM hyperresponsiveness in allergic asthma.

Again, the current study revealed that PGD<sub>2</sub> caused a phosphorylation of p38 in the BSM cells via an activation of DP<sub>1</sub> receptor (Fig. 4B). The BSM hyperresponsiveness induced by the 24-h PGD<sub>2</sub> incubation was inhibited by a p38 inhibitor SB203580 (Fig. 5B). These findings indicate that the DP<sub>1</sub>-mediated activation of p38 is responsible for the BSM hyperresponsiveness induced by PGD<sub>2</sub>. However, it is well known that the DP<sub>1</sub> is a Gs protein-coupled receptor. In smooth muscle cells including the airways, the Gs protein activation causes an inhibition of the contraction via an increase in cAMP level (e.g., 37–39). Indeed, PGD<sub>2</sub> reportedly induced a relaxation via an activation of DP<sub>1</sub> receptor in the tracheal smooth muscles pre-contracted with carbachol (11). In contrast, PGD<sub>2</sub> also elicited a contraction of the airway smooth muscle (8, 40–42) probably via an activation of DP<sub>1</sub> receptor (42). Although the contrariety is not explainable currently, the DP<sub>1</sub> receptor stimulation with PGD<sub>2</sub> may primarily activate signaling molecules other than the cAMP-dependent relaxing ones, resulting in an augmentation of the BSM contraction.

A small GTPase RhoA is a key protein that mediates sensitization of smooth muscle contraction including the airways (43, 44). In the BSMs of experimental asthma, an augmentation of agonist-induced RhoA-mediated Ca<sup>2+</sup> sensitization has been demonstrated (14, 18, 45). No change in the contraction mediated solely by cytosolic Ca<sup>2+</sup>, such as the contraction induced by high K<sup>+</sup> depolarization, has also been demonstrated in the diseased BSMs (14, 18). The current findings that PGD<sub>2</sub> augmented the contraction induced by ACh, but not by high K<sup>+</sup> depolarization (Fig. 3), also support an idea that PGD<sub>2</sub> may augment the RhoA-mediated Ca<sup>2+</sup> sensitization of contraction. Indeed, our previous study demonstrated that PGD<sub>2</sub> has an ability to activate RhoA in the BSMs (42). However, in our preliminary study, the 24-h incubation with PGD<sub>2</sub> did not cause the RhoA up-regulation, that is often observed in BSMs of experimental asthma (14, 18, 45).

As described above, our previous study demonstrated that PGD<sub>2</sub> has an ability to activate RhoA/Rho-kinase signaling: the Ca<sup>2+</sup> sensitization of contraction was augmented in the presence of PGD<sub>2</sub> (42). Currently, the BSM tissues were incubated with PGD<sub>2</sub> for 24 h, and their contractile responses were then measured after washing out PGD<sub>2</sub>. Nevertheless, in the ABSENCE of PGD<sub>2</sub>, the BSM responsiveness to ACh, but not to high K<sup>+</sup> depolarization, was significantly augmented (Fig. 3). On the other hand, the 24-h PGD<sub>2</sub> incubation did not cause the RhoA up-regulation (*see above*). Taken together, the sustained exposure to PGD<sub>2</sub> may up-regulate signaling molecule(s) other than RhoA activated by the muscarinic receptor stimulation. Further studies are needed to identify the mechanism of BSM hyperresponsiveness induced by the sustained exposure to PGD<sub>2</sub>.

In conclusion, the current study demonstrated that the 24-h incubation with PGD<sub>2</sub> caused an augmentation of contraction in BSMs isolated from naive mice. The pharmacological study also revealed an involvement of DP<sub>1</sub> receptor-mediated activation of p38 in the BSM hyperresponsiveness induced by PGD<sub>2</sub>. Thus, a sustained increase in PGD<sub>2</sub> in the airways might be a cause of the AHR in allergic asthmatics.

---

## Funding

This work was partly supported by Grant-in-Aid for Scientific Research (C) Grant Number 15K08248 (YC) from the Japan Society for the Promotion of Science (JSPS).

---

## Conflict of Interest

None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

---

## Acknowledgment

---

We thank Shiho Kawashima for technical assistance. This work was partly supported by Grant-in-Aid for Scientific Research (C) Grant Number 15K08248 (YC) from the Japan Society for the Promotion of Science (JSPS).

---

## References

---

1. Seow CY, Schellenberg RR, Paré PD. Structural and functional changes in the airway smooth muscle of asthmatic subjects. *Am J Respir Crit Care Med.* 1998; 158(5 Pt 3): S179–86. [[Medline](#)] [[CrossRef](#)]
2. Martin JG, Duguet A, Eidelman DH. The contribution of airway smooth muscle to airway narrowing and airway hyperresponsiveness in disease. *Eur Respir J.* 2000; 16(2): 349–54. [[Medline](#)] [[CrossRef](#)]
3. Ma X, Cheng Z, Kong H, Wang Y, Unruh H, Stephens NL, Laviolette M. Changes in biophysical and biochemical properties of single bronchial smooth muscle cells from asthmatic subjects. *Am J Physiol Lung Cell Mol Physiol.* 2002; 283(6): L1181–9. [[Medline](#)] [[CrossRef](#)]
4. Björck T, Gustafsson LE, Dahlén SE. Isolated bronchi from asthmatics are hyperresponsive to adenosine, which apparently acts indirectly by liberation of leukotrienes and histamine. *Am Rev Respir Dis.* 1992; 145(5): 1087–91. [[Medline](#)] [[CrossRef](#)]
5. Tang DD. Critical role of actin-associated proteins in smooth muscle contraction, cell proliferation, airway hyperresponsiveness and airway remodeling. *Respir Res.* 2015; 16: 134. [[Medline](#)] [[CrossRef](#)]
6. Dworski R, Sheller JR, Wickersham NE, Oates JA, Brigham KL, Roberts LJ 2nd, Fitzgerald GA. Allergen-stimulated release of mediators into sheep bronchoalveolar lavage fluid. Effect of cyclooxygenase inhibition. *Am Rev Respir Dis.* 1989; 139(1): 46–51. [[Medline](#)] [[CrossRef](#)]
7. Mandal AK, Zhang Z, Ray R, Choi MS, Chowdhury B, Pattabiraman N, Mukherjee AB. Uteroglobin represses allergen-induced inflammatory response by blocking PGD<sub>2</sub> receptor-mediated functions. *J Exp Med.* 2004; 199(10): 1317–30. [[Medline](#)] [[CrossRef](#)]
8. Chiba Y, Suto W, Sakai H. Augmented Pla2g4c/Ptgs2/Hpgds axis in bronchial smooth muscle tissues of experimental asthma. *PLoS One.* 2018; 13(8): e0202623. [[Medline](#)] [[CrossRef](#)]
9. Chilton FH, Averill FJ, Hubbard WC, Fonteh AN, Triggiani M, Liu MC. Antigen-induced generation of lyso-phospholipids in human airways. *J Exp Med.* 1996; 183(5): 2235–45. [[Medline](#)] [[CrossRef](#)]
10. Hartert TV, Dworski RT, Mellen BG, Oates JA, Murray JJ, Sheller JR. Prostaglandin E(2) decreases allergen-stimulated release of prostaglandin D(2) in airways of subjects with asthma. *Am J Respir Crit Care Med.* 2000; 162(2 Pt 1): 637–40. [[Medline](#)] [[CrossRef](#)]
11. Matsuoka T, Hirata M, Tanaka H, Takahashi Y, Murata T, Kabashima K, Sugimoto Y, Kobayashi T, Ushikubi F, Aze Y, Eguchi N, Urade Y, Yoshida N, Kimura K, Mizoguchi A, Honda Y, Nagai H, Narumiya S. Prostaglandin D<sub>2</sub> as a mediator of allergic asthma. *Science.* 2000; 287(5460): 2013–7. [[Medline](#)] [[CrossRef](#)]
12. Xue L, Gyles SL, Wetley FR, Gazi L, Townsend E, Hunter MG, Pettipher R. Prostaglandin D<sub>2</sub> causes preferential induction of proinflammatory Th<sub>2</sub> cytokine production through an action on chemoattractant receptor-like molecule expressed on Th<sub>2</sub> cells. *J Immunol.* 2005; 175(10): 6531–6. [[Medline](#)] [[CrossRef](#)]
13. Kato Y, Manabe T, Tanaka Y, Mochizuki H. Effect of an orally active Th<sub>1</sub>/Th<sub>2</sub> balance modulator, M50367, on IgE production, eosinophilia, and airway hyperresponsiveness in mice. *J Immunol.* 1999; 162(12): 7470–9. [[Medline](#)]
14. Chiba Y, Ueno A, Shinozaki K, Takeyama H, Nakazawa S, Sakai H, Misawa M. Involvement of RhoA-mediated Ca<sup>2+</sup> sensitization in antigen-induced bronchial smooth muscle hyperresponsiveness in mice. *Respir Res.* 2005; 6: 4. [[Medline](#)] [[CrossRef](#)]
15. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl

- RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem.* 1998; 273(29): 18623–32. [[Medline](#)] [[CrossRef](#)]
16. Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* 1995; 364(2): 229–33. [[Medline](#)] [[CrossRef](#)]
  17. Chiba Y, Tanabe M, Goto K, Sakai H, Misawa M. Down-regulation of miR-133a contributes to up-regulation of RhoA in bronchial smooth muscle cells. *Am J Respir Crit Care Med.* 2009; 180(8): 713–9. [[Medline](#)] [[CrossRef](#)]
  18. Chiba Y, Nakazawa S, Todoroki M, Shinozaki K, Sakai H, Misawa M. Interleukin-13 augments bronchial smooth muscle contractility with an up-regulation of RhoA protein. *Am J Respir Cell Mol Biol.* 2009; 40(2): 159–67. [[Medline](#)] [[CrossRef](#)]
  19. Chiba Y, Kurotani R, Kusakabe T, Miura T, Link BW, Misawa M, Kimura S. Uteroglobin-related protein 1 expression suppresses allergic airway inflammation in mice. *Am J Respir Crit Care Med.* 2006; 173(9): 958–64. [[Medline](#)] [[CrossRef](#)]
  20. Wu EH, Lo RK, Wong YH. Regulation of STAT3 activity by G16-coupled receptors. *Biochem Biophys Res Commun.* 2003; 303(3): 920–5. [[Medline](#)] [[CrossRef](#)]
  21. Wetzker R, Böhmer FD. Transactivation joins multiple tracks to the ERK/MAPK cascade. *Nat Rev Mol Cell Biol.* 2003; 4(8): 651–7. [[Medline](#)] [[CrossRef](#)]
  22. Kainuma S, Tokuda H, Kuroyanagi G, Yamamoto N, Ohguchi R, Fujita K, Matsushima-Nishiwaki R, Kozawa O, Otsuka T. PGD2 stimulates osteoprotegerin synthesis via AMP-activated protein kinase in osteoblasts: Regulation of ERK and SAPK/JNK. *Prostaglandins Leukot Essent Fatty Acids.* 2015; 101: 23–9. [[Medline](#)] [[CrossRef](#)]
  23. Jeong KH, Jung JH, Kim JE, Kang H. Prostaglandin D2-Mediated DP2 and AKT Signal Regulate the Activation of Androgen Receptors in Human Dermal Papilla Cells. *Int J Mol Sci.* 2018; 19(2): E556. [[Medline](#)] [[CrossRef](#)]
  24. Boie Y, Sawyer N, Slipetz DM, Metters KM, Abramovitz M. Molecular cloning and characterization of the human prostanoid DP receptor. *J Biol Chem.* 1995; 270(32): 18910–6. [[Medline](#)] [[CrossRef](#)]
  25. Hirata M, Kakizuka A, Aizawa M, Ushikubi F, Narumiya S. Molecular characterization of a mouse prostaglandin D receptor and functional expression of the cloned gene. *Proc Natl Acad Sci USA.* 1994; 91(23): 11192–6. [[Medline](#)] [[CrossRef](#)]
  26. Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, Ichimasa M, Sugamura K, Nakamura M, Takano S, Nagata K. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J Exp Med.* 2001; 193(2): 255–61. [[Medline](#)] [[CrossRef](#)]
  27. Sturino CF, O'Neill G, Lachance N, Boyd M, Berthelette C, Labelle M, Li L, Roy B, Scheiget J, Tsou N, Aubin Y, Bateman KP, Chauret N, Day SH, Lévesque JF, Seto C, Silva JH, Trimble LA, Carriere MC, Denis D, Greig G, Kargman S, Lamontagne S, Mathieu MC, Sawyer N, Slipetz D, Abraham WM, Jones T, McAuliffe M, Piechuta H, Nicoll-Griffith DA, Wang Z, Zamboni R, Young RN, Metters KM. Discovery of a potent and selective prostaglandin D2 receptor antagonist, [(3R)-4-(4-chloro-benzyl)-7-fluoro-5-(methylsulfonyl)-1,2,3,4-tetrahydrocyclopenta[b]indol-3-yl]-acetic acid (MK-0524). *J Med Chem.* 2007; 50(4): 794–806. [[Medline](#)] [[CrossRef](#)]
  28. Sykes DA, Bradley ME, Riddy DM, Willard E, Reilly J, Miah A, Bauer C, Watson SJ, Sandham DA, Dubois G, Charlton SJ. Fevipiprant (QAW039), a Slowly Dissociating CRTh2 Antagonist with the Potential for Improved Clinical Efficacy. *Mol Pharmacol.* 2016; 89(5): 593–605. [[Medline](#)] [[CrossRef](#)]
  29. Pype JL, Xu H, Schuermans M, Dupont LJ, Wuyts W, Mak JC, Barnes PJ, Demedts MG, Verleden GM. Mechanisms of interleukin 1beta-induced human airway smooth muscle hyporesponsiveness to histamine. Involvement of p38 MAPK NF-kappaB. *Am J Respir Crit Care Med.* 2001; 163(4): 1010–7. [[Medline](#)] [[CrossRef](#)]

30. Sakai H, Nishizawa Y, Nishimura A, Chiba Y, Goto K, Hanazaki M, Misawa M. Angiotensin II induces hyperresponsiveness of bronchial smooth muscle via an activation of p42/44 ERK in rats. *Pflugers Arch.* 2010; 460(3): 645–55. [[Medline](#)] [[CrossRef](#)]
31. Li F, Zhang M, Hussain F, Triantaphyllopoulos K, Clark AR, Bhavsar PK, Zhou X, Chung KF. Inhibition of p38 MAPK-dependent bronchial contraction after ozone by corticosteroids. *Eur Respir J.* 2011; 37(4): 933–42. [[Medline](#)] [[CrossRef](#)]
32. Rogers NK, Clements D, Dongre A, Harrison TW, Shaw D, Johnson SR. Extra-cellular matrix proteins induce matrix metalloproteinase-1 (MMP-1) activity and increase airway smooth muscle contraction in asthma. *PLoS One.* 2014; 9(2): e90565. [[Medline](#)] [[CrossRef](#)]
33. Shibata T, Kondo M, Osawa T, Shibata N, Kobayashi M, Uchida K. 15-deoxy-delta 12,14-prostaglandin J<sub>2</sub>. A prostaglandin D<sub>2</sub> metabolite generated during inflammatory processes. *J Biol Chem.* 2002; 277(12): 10459–66. [[Medline](#)] [[CrossRef](#)]
34. Bie Q, Dong H, Jin C, Zhang H, Zhang B. 15d-PGJ<sub>2</sub> is a new hope for controlling tumor growth. *Am J Transl Res.* 2018; 10(3): 648–58. [[Medline](#)]
35. Suh J, Kim DH, Kim EH, Park SA, Park JM, Jang JH, Kim SJ, Na HK, Kim ND, Kim NJ, Suh YG, Surh YJ. 15-Deoxy-Δ<sup>12,14</sup>-prostaglandin J<sub>2</sub> activates PI3K-Akt signaling in human breast cancer cells through covalent modification of the tumor suppressor PTEN at cysteine 136. *Cancer Lett.* 2018; 424: 30–45. [[Medline](#)] [[CrossRef](#)]
36. Coutinho DS, Anjos-Valotta EA, do Nascimento CVMF, Pires ALA, Napimoga MH, Carvalho VF, Torres RC, E Silva PMR, Martins MA. 15-Deoxy-Delta-12,14-Prostaglandin J<sub>2</sub> Inhibits Lung Inflammation and Remodeling in Distinct Murine Models of Asthma. *Front Immunol.* 2017; 8: 740. [[Medline](#)] [[CrossRef](#)]
37. Morgan SJ, Deshpande DA, Tiegs BC, Misior AM, Yan H, Hershfeld AV, Rich TC, Panettieri RA, An SS, Penn RB. β-Agonist-mediated relaxation of airway smooth muscle is protein kinase A-dependent. *J Biol Chem.* 2014; 289(33): 23065–74. [[Medline](#)] [[CrossRef](#)]
38. Cheah EY, Mann TS, Burcham PC, Henry PJ. Influenza A infection attenuates relaxation responses of mouse tracheal smooth muscle evoked by acrolein. *Biochem Pharmacol.* 2015; 93(4): 519–26. [[Medline](#)] [[CrossRef](#)]
39. Donovan C, Bailey SR, Tran J, Haitsma G, Ibrahim ZA, Foster SR, Tang ML, Royce SG, Bourke JE. Rosiglitazone elicits in vitro relaxation in airways and precision cut lung slices from a mouse model of chronic allergic airways disease. *Am J Physiol Lung Cell Mol Physiol.* 2015; 309(10): L1219–28. [[Medline](#)] [[CrossRef](#)]
40. Tamaoki J, Sekizawa K, Graf PD, Nadel JA. Cholinergic neuromodulation by prostaglandin D<sub>2</sub> in canine airway smooth muscle. *J Appl Physiol* 1985. 1987; 63(4): 1396–400. [[Medline](#)] [[CrossRef](#)]
41. Underwood DC, Muccitelli RM, Luttmann MA, Hay DW, Torphy TJ, Wasserman MA. Differential antagonism of airway contractile responses to prostaglandin (PG)D<sub>2</sub> and 9 alpha, 11 beta-PGF<sub>2</sub> by atropine, SK&F 88046 and SQ 29,548 in the guinea pig. *J Pharmacol Exp Ther.* 1994; 268(1): 304–10. [[Medline](#)]
42. Suto W, Ando Y, Hirabayashi T, Takenoya F, Shioda S, Kamei J, Sakai H, Chiba Y. Prostaglandin D<sub>2</sub> Induces Ca<sup>2+</sup> Sensitization of Contraction without Affecting Cytosolic Ca<sup>2+</sup> Level in Bronchial Smooth Muscle. *Int J Mol Sci.* 2018; 19(10): E3036. [[Medline](#)] [[CrossRef](#)]
43. Kume H. RhoA/Rho-kinase as a therapeutic target in asthma. *Curr Med Chem.* 2008; 15(27): 2876–85. [[Medline](#)] [[CrossRef](#)]
44. Gerthoffer WT, Solway J, Camoretti-Mercado B. Emerging targets for novel therapy of asthma. *Curr Opin Pharmacol.* 2013; 13(3): 324–30. [[Medline](#)] [[CrossRef](#)]
45. Chiba Y, Takada Y, Miyamoto S, MitsuiSaito M, Karaki H, Misawa M. Augmented acetylcholine-induced, Rho-mediated Ca<sup>2+</sup> sensitization of bronchial smooth muscle contraction in antigen-induced airway hyperresponsive rats. *Br J Pharmacol.* 1999; 127(3): 597–600. [[Medline](#)] [[CrossRef](#)]