



Immunopharmacology and Inflammation

Prostanoid DP receptor antagonists suppress symptomatic asthma-like manifestation by distinct actions from a glucocorticoid in rats

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ABSTRACT

While inhaled glucocorticoids are the best treatment for the majority of chronic asthmatics, there is a small group who do not respond to these drugs or whose disease can only be controlled by high doses of oral glucocorticoids with risks of severe side effects. Therefore, a safe novel anti-asthmatic agent which has a different mechanism from that of glucocorticoids is needed for the management of asthma. We have previously shown that an orally active prostanoid DP receptor antagonist, S-5751, had potent anti-inflammatory effects in guinea pig and sheep asthma models. In this study, using a rat asthma like model, we found that lung neutrophilia and proinflammatory cytokine secretion as well as bronchial hyperresponsiveness and lung eosinophilia were induced by repeated antigen-inhalations after antigen-sensitization. These symptoms are similar to the pathogenesis of symptomatic asthma. Orally-administered prostanoid DP receptor antagonists S-5751 and pinagladin significantly suppressed not only bronchial hyperresponsiveness and lung eosinophilia but also neutrophilia and mucus secretion in the lung, while oral prednisolone inhibited only bronchial hyperresponsiveness and eosinophil infiltration. In addition, prostanoid DP receptor antagonists significantly suppressed interleukin (IL)-1 β , IL-6 and CXCL1 mRNA in contrast to suppression of IL-4 and CCL11 mRNA by prednisolone. The majority of prostanoid DP receptor-expressing cells in both rat and human asthmatic lungs are infiltrative macrophages and/or monocytes. These results suggest that prostanoid DP receptor antagonists utilize different mechanisms from glucocorticoids, and that they would be a novel alternative and/or combination drug for asthma therapy.

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1. Introduction

Prostaglandin D₂ (PGD₂), the major cyclooxygenase metabolite of arachidonic acid, is a chemical mediator released in large amounts by mast cells responding to various allergens (Lewis et al., 1982). Local allergen challenge in patients with bronchial asthma has been shown to induce rapid elevation of PGD₂ in bronchial fluids (Liu et al., 1991; Murray et al., 1986). Therefore, it is believed that PGD₂ plays an important role in asthma.

Two different functional receptors for PGD₂ have been reported, prostanoid DP receptor and chemoattractant receptor homologous molecule expressed on Th2 cells (CRTH2) (Hirai et al., 2001). In prostanoid DP receptor-deficient mice, ovalbumin-induced bronchial hyperresponsiveness, accumulation of inflammatory cells in lung and

production of Th2 cytokines are reported to be reduced (Matsuoka et al., 2000). It is also reported that PTGDR (prostanoid DP receptor coding gene) sequence variants are responsible for the genetic susceptibility to asthma in humans (Oguma et al., 2004). We have previously demonstrated that S-5751, a prostanoid DP receptor selective antagonist, inhibits bronchial infiltration of inflammatory cells and bronchial hyperresponsiveness in guinea pig and sheep asthma models (Arimura et al., 2001; Shichijo et al., 2009). These results suggest that prostanoid DP receptor antagonists could be used for the treatment of asthma.

Glucocorticoids are the most effective drugs available for the treatment of asthma. Indeed, inhaled glucocorticoids are the best controller with a low risk of side effects in the majority of chronic asthmatics. However, there is a small group (5–10%) of asthmatics who can only be controlled by high doses of inhaled glucocorticoids or oral glucocorticoids accompanied with some side effects or those who never respond to them (Barnes and Woolcock, 1998). This group disproportionately represents more than 50% of the overall medical costs for asthma (Antonicelli et al., 2004). Therefore, safe and novel

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anti-inflammatory agents which have a different mechanism from glucocorticoids are needed for the management of asthma.

It has been reported that dexamethasone does not suppress PGD₂ release from lung parenchyma and purified lung mast cells upon stimulation with anti-IgE antibodies (Schleimer et al., 1983). In addition, pretreatment for 3 days with oral prednisolone did not significantly inhibit the PGD₂ level in bronchial alveolar lavage fluids after antigen challenge of asthmatic subjects (Liu et al., 2001). These results suggest that the PGD₂ pathway in asthma is not attenuated by glucocorticoids. Therefore, PGD₂ antagonists might exert a therapeutic potency distinct from glucocorticoids. However, to address pharmacological and molecular biological differences of PGD₂ antagonists and glucocorticoids, comparable studies need to be conducted.

In the present study, we identified the differences in the pharmacological profile between prostanoid DP receptor selective antagonists and prednisolone using a rat experimental model with some asthma-like manifestations. We also compared the effect of prostanoid DP receptor antagonists and prednisolone on the molecular-basis of expression of mediators induced by antigen. In addition, we identified a major source of prostanoid DP receptor expression in rat and human asthmatic lungs as some of the cells targeted by prostanoid DP receptor antagonists.

2. Materials and methods

2.1. Compounds

S-5751 [((Z)-7-[(1R,2R,3S,5S)-2-(5-hydroxybenzo[b]thiophen-3-ylcarbonylamino)-10-norpinan-3-yl] hept-5-enoic acid)] and pinagladin [(Z)-7-[(1R,2R,3S,5S)-2-(benzo[b]thiophen-3-ylcarbonylamide)-10-norpinan-3-yl]hept-5-enoic acid] were synthesized in our laboratories. S-5751 and pinagladin are highly selective potent antagonists for the prostanoid DP receptor (Shichijo et al., 2009; Selectivity of pinagladin towards the prostanoid DP receptor is shown in Supplemental data). The structural difference of pinagladin in comparison with S-5751 is a hydroxyl substituent at the 5-position in the benzothiophen-3-carbonyl moiety. Comparative pharmacological activities in a guinea pig rhinitis model have been observed between S-5751 and pinagladin (Mitsumori et al., 2003). Prednisolone was obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Animals

Male Brown Norway (BN) rats weighing 240 g to 397 g were purchased from Japan Charles River (Yokohama, Japan). Five to ten animals in Figs. 1 to 3, four animals in Fig. 4, and three animals in Fig. 5

were used in each group. Each experiment in Figs. 1 to 3 was performed using same animals in each group. All animal studies were approved by the Institutional Animal Care and Use Committee at Shionogi & Co., Ltd.

2.3. Sensitization and challenge procedure in rats

All rats were immunized by intraperitoneal injection of 100 µg ovalbumin together with 1 mg alum on day 0. On days 12, 19, 26 and 33, antigen challenge was performed by inhalation of an aerosolized 1% ovalbumin for 30 min. For the negative control group, the animals were exposed to an aerosolized saline (0.9% sodium chloride; Otsuka Pharmaceutical Factory, Inc; Tokushima, Japan). The aerosols were generated with an ultrasonic nebulizer (NE-U17, Omron, Kyoto, Japan) and were inhaled by placing animals in exposure chambers. The measurement of bronchial responsiveness and the collection of bronchial alveolar lavage fluids were performed on 1 day (day 34) after the last antigen challenge.

2.4. Drug administration

S-5751 and pinagladin were orally administered to BN rats three times a day at a maximum dose of 100 mg/kg because of their poor pharmacodynamic profiles with low bioavailability (6.9% at 100 mg/kg of S-5751) and rapid elimination from blood (about 4 h at 100 mg/kg of S-5751) in rats. Indeed, the single administration of S-5751 at 100 mg/kg in rats showed a comparable plasma concentration in terms of C_{max} and the area under the curve (AUC) from 0 to 24 h to those at 10 mg/kg, the effective dose for allergic inflammation (Arimura et al., 2001) in guinea pigs (in preparation for publication). Prednisolone was orally administered at 3 mg/kg to the rats once a day, a dose which was chosen based on previous studies (Aoki et al., 2000; Takizawa et al., 2002). The administrations of the drugs were performed for 8 days (days 26–33) between the 3rd challenge and 1 day before measurement of various asthmatic parameters. On day 26 and day 33, the drugs were administered 1 h before antigen challenge.

2.5. Measurement of bronchial responsiveness for acetylcholine

At 24 h after the last antigen challenge, the animals were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and dose-response curves were constructed for acetylcholine (3.9–500 µg/kg, i.v.) as described previously (Arimura et al., 1994). The AUC calculated by the trapezoidal rule from the dose–response curve for acetylcholine was expressed as a magnitude of bronchial hyperresponsiveness, which is the increase in bronchial responsiveness for acetylcholine.

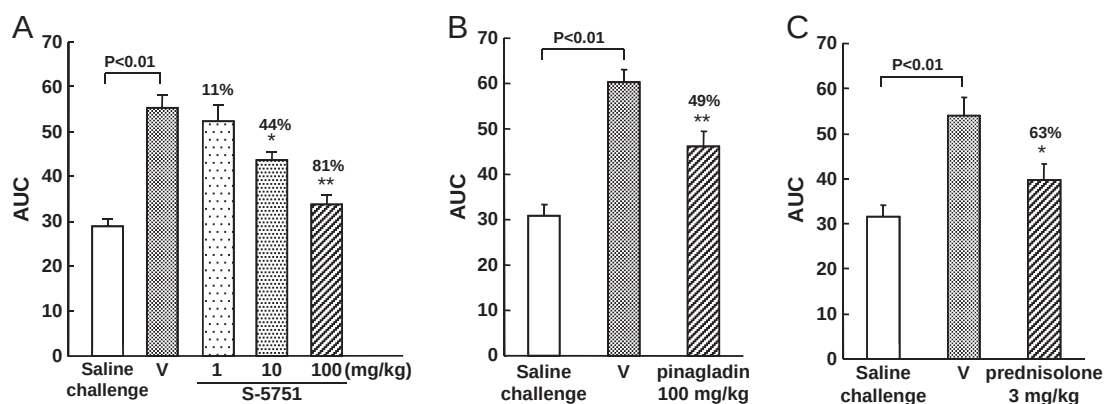


Fig. 1. Effect of prostanoid DP receptor antagonists and prednisolone on bronchial hyperresponsiveness to acetylcholine in a rat asthma model. The bronchial hyperresponsiveness was measured 24 h after last challenge and was expressed as the AUC. S-5751 (A) and pinagladin (B) were administered orally three times a day for 8 days. Prednisolone (C) was administered orally once a day for 8 days. Data represent the mean ± S.E.M. of 8 animals (S-5751), 5 (saline group)–7 animals (pinagladin) and 9 (saline group)–10 animals (prednisolone). V; vehicle control. Statistical significance: *P<0.05 and **P<0.01 for each drug treated group versus vehicle treated group (Dunnett's test; A; Welch's *t* test; B, C).

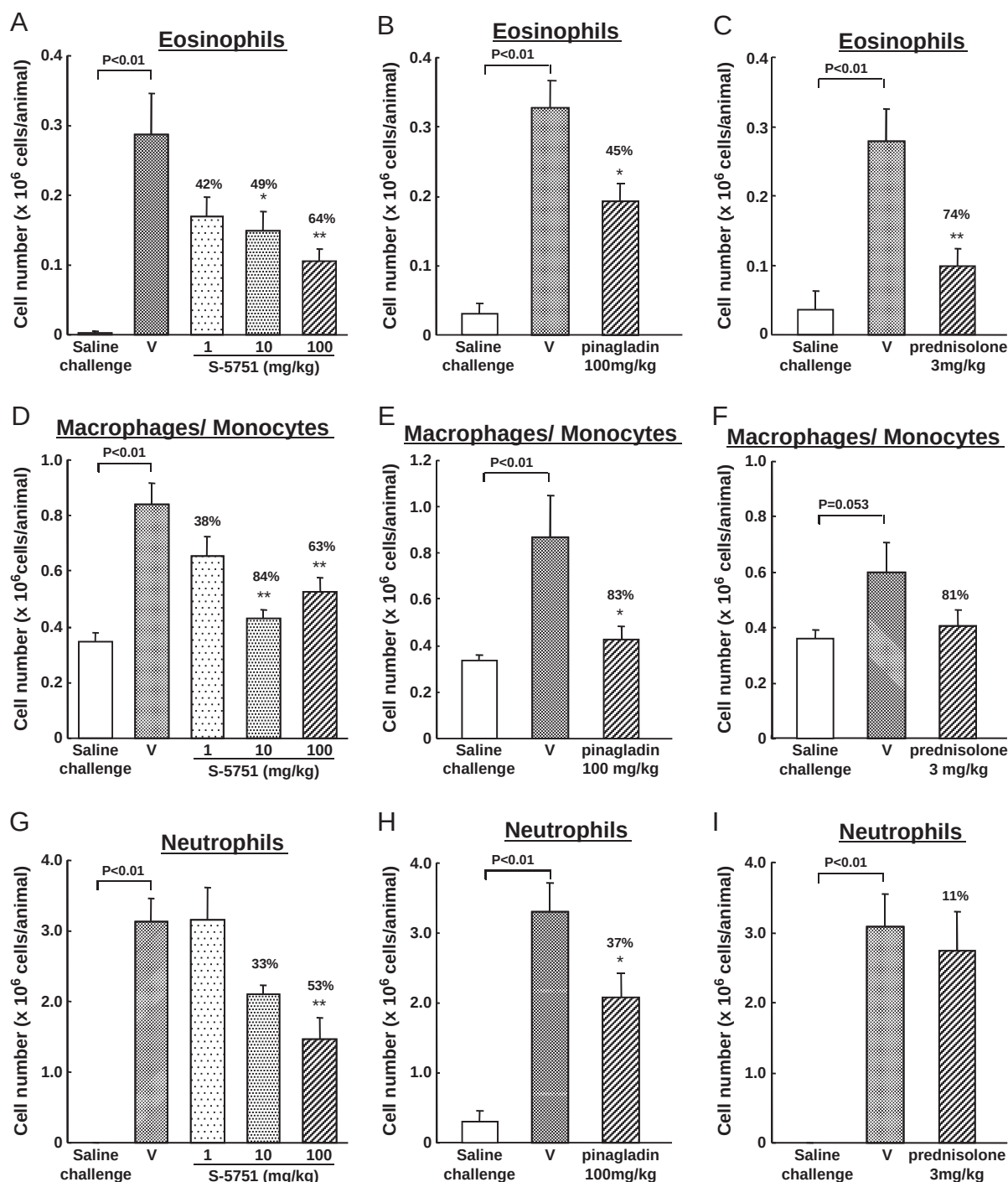


Fig. 2. Effect of prostanoïd DP receptor antagonists and prednisolone on infiltration of inflammatory cells in lung in a rat asthma model. The bronchial alveolar lavage fluids were collected 24 h after last challenge. The number of various cells in bronchial alveolar lavage fluids was microscopically counted. S-5751 (A, D, G) and pinagladin (B, E, H) were administered orally three times a day for 8 days. Prednisolone (C, F, I) was administered orally once a day for 8 days. (A, B, C) Number of eosinophils in bronchial alveolar lavage fluid. (D, E, F) Number of macrophages/monocytes in bronchial alveolar lavage fluid. (G, H, I) Number of neutrophils in bronchial alveolar lavage fluid. Data represent the mean $\pm$ S.E.M. of 8 animals (S-5751), 5 (saline group)–7 animals (pinagladin) and 9 (saline group)–10 animals (prednisolone). V; vehicle control. Statistical significance: * $P < 0.05$ and ** $P < 0.01$ for each drug treated group versus vehicle treated group (Dunnett's test: A, D, G; Welch's t test: B, C, E, F, H, I).

2.6. Collection of bronchial alveolar lavage fluids and evaluation of inflammatory cell infiltration

After measurement of bronchial responsiveness, the airway lumen was washed three times with 5 ml of saline through the tracheal cannula and the bronchial alveolar lavage fluids were obtained. The total

cell number in the bronchial alveolar lavage fluids was determined using a light microscope after Turk staining (Wako Pure Chemical Industries Ltd, Osaka, Japan). The preparations for differential cell count were done using Cytospin 3 (Thermo Fisher Scientific Inc., Waltham, MA). Differential cell counts were performed by counting 200 cells/sample using a light microscope after May–Grünwald Giemsa staining

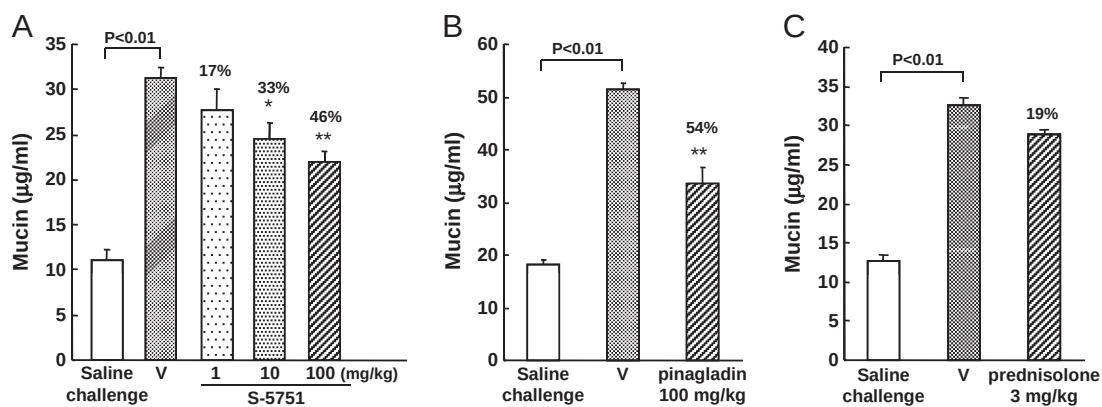


Fig. 3. Effect of prostanoid DP receptor antagonists and prednisolone on mucin secretion in lung in a rat asthma model. The bronchial alveolar lavage fluids were collected 24 h after last challenge. The volume of mucin in bronchial alveolar lavage fluids was measured by enzyme linked assay system. S-5751 (A) and pinagladin (B) were administered orally three times a day for 8 days. Prednisolone (C) was administered orally once a day for 8 days. Data represent the mean $\pm$ S.E.M. of 8 animals (S-5751), 5 (saline group)–7 animals (pinagladin) and 5 animals (prednisolone). V; vehicle control. Statistical significance: * $P < 0.05$ and ** $P < 0.01$ for each drug treated group versus vehicle treated group (Dunnett's test or Welch's t test).

(Merck, Darmstadt, Germany). The exact number of each cell type was calculated from the ratio and total cell number.

2.7. Measurement of mucin in bronchial alveolar lavage fluids

Mucus secretion was evaluated by measuring mucin levels in bronchial alveolar lavage fluids. Mucin levels were determined by an enzyme-linked assay system using the mucin-binding lectin, jacalin. Briefly, 96-well plates (Immulon IV; Thermo Fisher Scientific Inc, MA,

USA) were precoated with bronchial alveolar lavage fluid samples and mucin standard (Sigma-Aldrich, St. Louis, MO) for 2 h at 37 °C and then blocked with Block-Ace (Dainippon Pharmaceutical, Osaka, Japan). Plates were washed with PBS containing 0.05% Tween 20 and then incubated with 2.5 µg/ml biotinylated jacalin (Vector Laboratories, Burlingame, CA) for 1 h at 37 °C. Plates were washed and then incubated with a 1/1000 dilution of streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min at room temperature. After a final wash, a

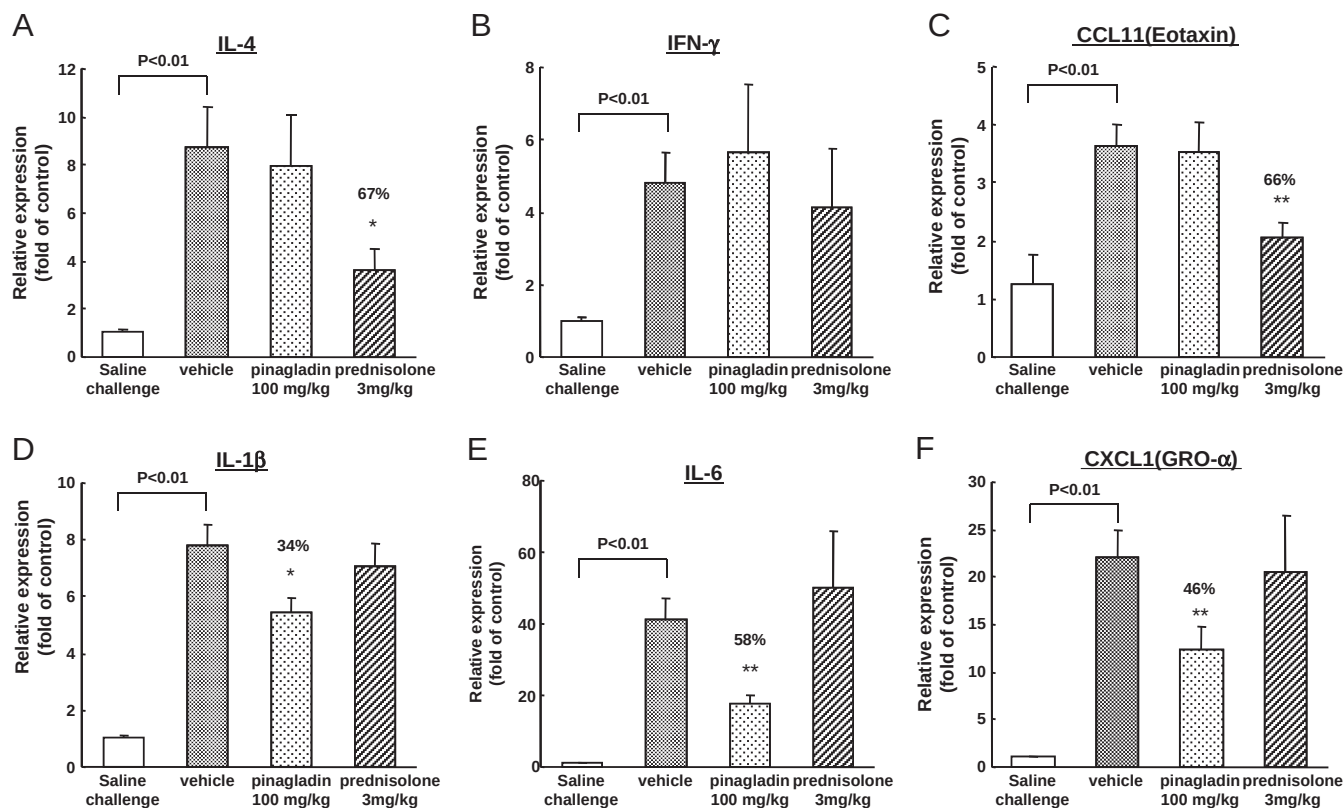


Fig. 4. Effect of pinagladin and prednisolone on production of cytokines and chemokines mRNA in lungs from a rat asthma model. The lung sections were isolated 6 h after last challenge. Cytokine and chemokine mRNAs were detected by a real-time PCR system. Pinagladin was administered orally three times a day for 8 days. Prednisolone was administered orally once a day for 8 days. Data show IL-4 (A), IFN-γ (B), CCL11 (C), IL-1β (D), IL-6 (E) and CXCL1 (F). Data represent the mean $\pm$ S.E.M. of 4 animals. Statistical significance: * $P < 0.05$ and ** $P < 0.01$ for each drug treated group versus vehicle treated group (Welch's t test).

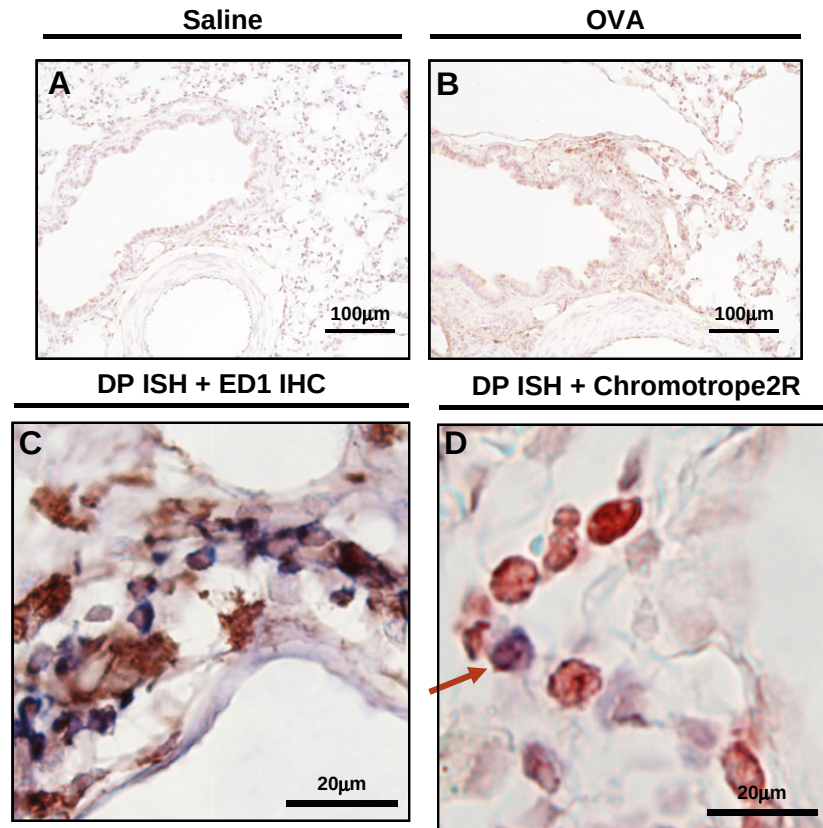


Fig. 5. Rat prostanoid DP receptor mRNA expression in ovalbumin-induced asthma and identification of rat prostanoid DP receptor mRNA expressing cells. Prostanoid DP receptor mRNA was performed by *in situ* hybridization. Representative lung images were obtained by applying antisense probe from three ovalbumin-exposed rats (B) and three normal control rats treated with saline (A). The dark-brownish color signals developed with diaminobenzidine were prostanoid DP receptor mRNA. To identify the type of cell expressing prostanoid DP receptor mRNA, ISH/IHC double staining or ISH/chromotrope 2R histochemistry were performed. The dark-blue color signals developed with NBT/BCIP were prostanoid DP receptor mRNA, and dark-brownish color signals developed with DAB substrate were macrophage/monocyte positive for ED1 antibody IHC (C) or eosinophils positive for chromotrope 2R histochemistry (D). Arrows indicate a typical double-positive cell with both the prostanoid DP receptor ISH and chromotrope 2R histochemistry.

colored reagent p-nitrophenyl phosphate liquid substrate system (Sigma-Aldrich, St. Louis, MO) was added to all wells. After stopping the reaction by adding 3 N NaOH, optical densities were measured at 405 nm with a multilabel counter.

2.8. Quantitative real-time PCR

The left lobes of the lung were isolated 6 h after the last challenge. The sections were immediately frozen by liquid nitrogen and stored in the freezer until the step of RNA extraction. Total RNA was extracted from rat lung using the TRIzol® Reagent (Invitrogen, CA) and RNeasy Mini Kit (Qiagen, Hilden, Germany). The first-strand cDNA was prepared with the SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen, CA). Real-time quantitative RT-PCR was carried out using an ABI PRISM® 7000 Real-Time PCR system (Applied Biosystems Japan, Tokyo, Japan) using SYBR® Premix ExTaq™ (Takara Bio, Shiga, Japan). The first-strand cDNA was synthesized from 2 µg total RNA in a 25 µl reaction mixture and was diluted by adding 80 µl sterilized double-distilled water. The amplifications were performed in 25 µl reaction volumes containing 2 µl diluted cDNA, SYBR® Premix ExTaq™, 400 nM of each primer and 0.5 µl ROX Reference Dye. The thermal cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Threshold cycles were averaged from duplicate reactions and the expression was determined relative to the mean value derived from the saline challenge group. The gene expression of samples was normalized with the internal reference gene (glyceraldehyde-3-phosphate dehydroge-

nase, GAPDH). The gene-specific primers used in this study are described in Table 1.

2.9. Tissue preparation

Twenty-four hours after ovalbumin final challenge, rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with PBS proceeded by 4% paraformaldehyde dissolved in PBS. The lungs of rats were removed and post-fixed at room temperature for 16 h. All steps were carried out with care to avoid contamination with RNase. Diethylpyrocarbonate (DEPC) treated distilled water was used at 0.1% to prepare each buffer. After washing

Table 1
Gene-specific primers for real-time quantitative RT-PCR.

Gene	Forward	Reverse
IL-4	5'-TGACCGAGATGTTTGTACCAGA-3'	5'-TTGCGAAGCACCTGGAAG-3'
IFN-γ	5'-AGGCCATCAGCAACAACATAA GTG-3'	5'-GCTAGATTCTGGTGACAGCTGG TG-3'
CCL11	5'-TGCTGCTTTACCATGACCAGTAA GA-3'	5'-TCCAGGTGCTTTGTGGCATC-3'
IL-1β	5'-GCTGTGGCAGCTACCTATGTCTTG-3'	5'-AGGTGCTCATCATCCACG AG-3'
IL-6	5'-CCACTTCACAAGTCGGAGGCTTA-3'	5'-GTGCATCATCGTGTTCATACAA TC-3'
CXCL1	5'-AGGCTTGCTTGACCTGAA-3'	5'-ACGCCATCGGTGAATCTATC-3'
GAPDH	5'-GACAACCTTGGCATCGTGGA-3'	5'-ATGCAGGGATGATGTTCTGG-3'

in PBS, the fixed lungs were dehydrated through graded alcohols and normal butanol, and then embedded in paraffin. The transversal tissue sections at 6 μ m in thickness were cut and mounted on slides coated with 3-aminopropyltriethoxysilane.

2.10. *In situ* hybridization (ISH)

Antisense and sense single-strand cRNA probes were *in vitro*-transcribed from a rat prostanoid DP receptor cDNA fragment. The rat prostanoid DP receptor cDNA fragment consisting of 399 bp (sequence nucleotides 60–458, GenBank accession no. NM_022241) was amplified from the QUICK-Clone cDNA of human whole brain (Clontech Laboratories, Inc., Mountain View, CA). The amplified fragment was sub-cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA). The template of the prostanoid DP receptor subunit was linearized with the restriction enzyme XhoI, and the labeled RNA probe was synthesized with SP6 RNA polymerase in the presence of digoxigenin (DIG)-labeled UTP (DIG Labeling Kit; Roche Diagnostics, Basel, Switzerland) for the antisense-probe, or it was linearized with the restriction enzyme SpeI, and the labeled RNA probe was synthesized with T7 RNA polymerase for the sense-probe. The probes were precipitated and DIG incorporation was assessed by dot blotting. After dewaxing, the lung sections were prepared following method in Section 2.9, the tissue sections were rinsed with PBS, permeabilized with 4 μ g/ml proteinase K (Roche) at 37 °C for 10 min, and then rinsed in 0.1 M triethanolamine containing 0.25% acetic anhydride. Hybridization was conducted by the method described previously (Lan et al., 1996). After hybridization, the samples were washed and blocked in 1.5% (v/v) normal rabbit serum (Vector Laboratories, Burlingame, CA) in PBS, and subsequently incubated in 1.5 μ g/ml of anti-DIG sheep polyclonal antibodies (Roche). After washing, tissues were processed using Vectastain Elite ABC peroxidase kit (Vector Laboratories) with 0.1% 3,3'-diaminobenzidine hydrochloride substrate dissolved in 50 mM Tris-HCl (pH 7.4) containing 0.05% H₂O₂. After a brownish color developed, the reaction was stopped by rinsing in PBS, and the target mRNA signals were visualized. The sections were counterstained with Mayer's Hematoxylin, dehydrated, and finally mounted in Entellan New (Merck, Darmstadt, Germany). For identification of pulmonary cell types, detection was done using the anti-digoxigenin alkaline phosphatase system (Roche, Basel, Switzerland). The staining procedure was performed in the dark for several hours using 5-bromo-4-chloro-3-indolyl phosphate, 4-toluidine salt and nitro blue tetrazolium chloride (Roche). To identify pulmonary cell types which were prostanoid DP receptor mRNA-positive, the alkaline phosphatase detection procedure for *in situ* hybridization was followed by macrophages/monocytes (ED1 antibody, BMA) immunohistochemical detection or histochemical eosinophil (chromotrope 2R staining) detection. For ED1 immunohistochemistry, donkey anti-mouse immunoglobulin conjugated to horseradish peroxidase was used as the secondary antibody. The peroxidase staining procedure was performed using DAB substrate (Roche). The ratios of both ED-1 and prostanoid DP receptor double positive cells or both chromotrope 2R and prostanoid DP receptor double positive cells were estimated under a microscope with a magnification of 320-fold (high power field). A total of 30 microscopic fields were observed per lung preparation. Fig. 5 shows the average lung images in the 180 fields/6 sections (2 sections/rat) in three ovalbumin-exposed rats and three normal control rats treated with saline.

2.11. Immunohistochemistry

Human lung preparations – two asthma (a 19 year-old male and a 79 year-old female) and two normal (both 75 year-old females) – were purchased in the form of paraffin embedded tissue blocks from Asterand Plc. (Royston, UK). Transversal tissue sections (8 μ m in thickness) were cut and mounted on slides freshly thin-coated with

3-aminopropyltriethoxysilane. Immunohistochemistry was performed after paraffin dewaxing with xylene. The tissue slides were incubated in methanol containing 0.3% H₂O₂ for 30 min and then treated with 5% normal mouse serum for 20 min. The slides were incubated with anti-prostanoid DP receptor monoclonal antibody (10 mg/ml) in PBS containing 0.1% BSA for 1 h at room temperature. After rinsing with PBS, they were incubated with biotin-conjugated goat anti-mouse IgG for 30 min followed by treatment with horseradish peroxidase avidin-biotin complex reagent (Vector Laboratories, Inc., Burlingame, CA). After washing, the peroxidase activity was visualized by 10 min incubation in 50 mM Tris-HCl (pH 7.6) containing 200 mg/ml of diaminobenzidine and 0.006% H₂O₂. After counterstaining the nuclei with 1.5% methyl green (Merck & Co. Inc., Munich Germany), the preparations were mounted in Entellan New medium (Merck & Co. Inc.).

Positive signals were detected as dark brown diaminobenzidine deposits. The specificity of prostanoid DP receptor signal was confirmed by the disappearance of the positive signal with the pretreatment of neutralizing antigen (data not shown). Other deparaffinized tissue slides were also stained with hematoxylin and eosin (Muto Kagaku Pure Chemicals, Tokyo Japan) to check the morphopathology.

2.12. Statistical analysis

Data were expressed as the mean $\pm$ S.E.M. Statistical significance of data was assessed by means of Dunnett's test for multiple comparisons, or Welch's *t* test for comparison between two groups (saline-exposed group versus antigen-exposed group).

3. Results

3.1. Effects of prostanoid DP receptor antagonists on antigen-induced bronchial hyperresponsiveness to acetylcholine

Repeated antigen provocation caused a significant increase in bronchial responsiveness detected after stimulation with acetylcholine in comparison with saline provocation (Fig. 1A, B, C). Prostanoid DP receptor antagonists S-5751 (10 and 100 mg/kg) and pinagladin (100 mg/kg) significantly suppressed the antigen-induced bronchial hyperresponsiveness (Fig. 1A, B). Prednisolone at a dose of 3 mg/kg also showed a significant suppression of bronchial hyperresponsiveness (Fig. 1C).

3.2. Effects of prostanoid DP receptor antagonists on antigen-induced infiltration of inflammatory cells

The number of eosinophils and neutrophils in bronchial alveolar lavage fluids was significantly increased 24 h after the final ovalbumin challenge when compared with saline challenge (Fig. 2A, B, C, G, H, I). The number of neutrophils was about 10 times more than the number of eosinophils in this rat model. S-5751 significantly suppressed the number of eosinophils at 10 and 100 mg/kg (Fig. 2A), and neutrophils at 100 mg/kg (Fig. 2G) in bronchial alveolar lavage fluids. Pinagladin also exhibited significant suppression of eosinophilia (Fig. 2B) and neutrophilia at 100 mg/kg (Fig. 2H). In addition, S-5751 at 10 mg/kg and 100 mg/kg (Fig. 2D) and pinagladin at 100 mg/kg (Fig. 2E) significantly inhibited infiltration of macrophages and/or monocytes. By contrast, prednisolone at 3 mg/kg had no effect on the number of neutrophils (Fig. 2I), although the eosinophil number was significantly decreased by prednisolone (Fig. 2C). In addition, prednisolone clearly inhibited the infiltration of macrophages/monocytes, although no significant increase of macrophage/monocyte was observed after ovalbumin challenge (Fig. 2F).

3.3. Effects of prostanoid DP receptor antagonists on mucus secretion in lung

When compared with the saline challenge group, ovalbumin challenge induced a significant increase in the production of lung mucin (Fig. 3A, B, C). As shown in Fig. 3A and B, the amount of lung mucin was significantly decreased by treatment with S-5751 (10 and 100 mg/kg) and pinagladin (100 mg/kg). However, prednisolone did not suppress lung mucin at 3 mg/kg (Fig. 3C).

3.4. Effects of prostanoid DP receptor antagonist on antigen-induced increase of cytokine and chemokine transcripts in rat lung

To compare the molecular mechanism of prostanoid DP receptor antagonist with that of prednisolone, we next determined the mRNA expression profile of cytokines and chemokines in rat lung 6 h after the last ovalbumin challenge. As shown in Fig. 4, ovalbumin inhalation induced a significant increase in the expression of mRNA for IL-4, Interferon (IFN)- γ , IL-1 β , IL-6, CCL11 (eotaxin) and CXCL1 (GRO- α) compared with saline inhalation at 6 h in lung. Expression of IL-4 and CCL11 mRNA was significantly suppressed by prednisolone at 3 mg/kg, while pinagladin had no effect (Fig. 4A, C). On the other hand, pinagladin but not prednisolone significantly suppressed the expression of IL-1 β , IL-6 and CXCL1 mRNA (Fig. 4D, E, F). Neither treatment affected the expression of IFN- γ mRNA (Fig. 4B).

3.5. Expression of prostanoid DP receptor mRNA in rat lung

To determine the localization of prostanoid DP receptor mRNA-positive cells in rat asthmatic lung, we performed ISH for detecting prostanoid DP receptor mRNA. There were distinct prostanoid DP receptor positive cells around blood vessels and bronchioles in the lung obtained 24 h after the final ovalbumin challenge (Fig. 5B), while the positive cells were hardly observed in the lung from the saline challenge (Fig. 5A). In contrast, no specific staining was observed in the lung from applying the sense probe (data not shown). We next attempted to identify the cell populations expressing prostanoid DP receptor by means of immunohistochemical or histochemical double staining (Fig. 5D, E). Most prostanoid DP receptor expressing cells were ED1 positive (approximately 86% of ISH positive 532 cells in 30 microscopic high-power 320-fold magnified fields) and were determined to be infiltrate macrophages and/or monocytes. In addition, a few prostanoid DP receptor expressing cells were chromotrope 2R positive cells (approximately 4.9% of ISH positive 631 cells in 30 microscopic high-power 320 fold magnified fields) which were identified as eosinophils. The ratio of prostanoid DP expressing cells in total ED1 positive cells was 18.2%, and the prostanoid DP expressing cells in total chromotrope 2R positive cells was 1.6%.

3.6. Localization of prostanoid DP receptor in lungs in patients with asthma

To examine whether prostanoid DP expressing cells were also detected in human lung, we performed immunohistochemical analysis using lungs obtained from patients with asthma. Airway narrowing, hypertrophy of tissue under the airway and infiltration of inflammatory cells were observed in the peribronchiolar regions in the subject with asthma (Fig. 6D) but not with normal lung (Fig. 6A). In the lung parenchyma of asthmatic lung but not normal lung there were also hypertrophy of parenchyma tissue and infiltration of inflammatory cells (Fig. 6B, E). The prostanoid DP receptor was localized on the inflammatory cells infiltrated in both the peribronchiolar (data not shown) and parenchyma (Fig. 6F) in tissue from a patient with asthma while no significant signal was observed in normal tissue (Fig. 6C). The majority of prostanoid DP receptor expressing cells was regarded as macrophage- and monocyte-like cells by their morphological features (Fig. 6F). The consistent focal

profile and pattern of prostanoid DP receptor localization were observed in another lung from an asthma patient (data not shown).

4. Discussion

In the present study, we compared the effects of prostanoid DP receptor antagonists to oral prednisolone in a rat asthma-like model. Oral glucocorticoids are mainly given to severe asthmatics who are not well-controlled by inhaled glucocorticoids or have acute exacerbations of chronic asthma. Although oral steroids can generally rapidly resolve airflow obstruction and reduce the rate of relapse for such asthma, it is known that they have a risk of severe side effects. In the present study, oral prednisolone at 3 mg/kg significantly suppressed age-dependent increases in body weight, whereas administration of vehicle or prostanoid DP receptor antagonists had no effect on the increase in body weight (data not shown). In addition, no noteworthy abnormalities in rats were observed in terms of clinical signs, body weight, food and water consumption up to 1000 mg/kg/day for one month repeated dosing of S-5751 (non-clinical GLP study). These results suggest that prostanoid DP receptor antagonists may prove to be safer anti-inflammatory drugs than oral glucocorticoids.

Interestingly, our rat asthma-like model showed potent infiltration of neutrophils rather than eosinophils in the lung after ovalbumin challenge, although the number of eosinophils markedly and significantly increased in lung after the challenge. In addition, the increase of transcripts for proinflammatory cytokines and chemokines such as IL-1 β , IL-6 and CXCL1, was observed as well as the Th2 cytokine such as IL-4 in lung. Thus potent neutrophilic inflammation with eosinophilia is a prominent feature of severe chronic asthma (Woodruff et al., 2001) and acute exacerbations of asthma (Lamblin et al., 1998). Besides, sputum neutrophilia increases with progression of asthma severity (Jatakanon et al., 1999). It has also been reported that proinflammatory cytokines such as IL-1 β , IL-6 and IL-8 in the sputum or bronchial alveolar lavage fluids of symptomatic patients were significantly higher than in asymptomatic subjects (Broide et al., 1992; Konno et al., 1996). These results suggest that our rat asthma-like model has some similar symptoms to subjects with symptomatic asthma. Glucocorticoids are reported to suppress bronchial hyperresponsiveness (Barnes, 1990; Bhagat and Grunstein, 1985), and infiltration of eosinophils and T cells (Liu et al., 2001), due to affecting the production of Th2 cytokines (Liu et al., 2001), and the survival of eosinophils (Meagher et al., 1996), although the neutrophil infiltration is resistant to glucocorticoids (Little et al., 2002). In the present study, oral administration of prednisolone at 3 mg/kg significantly inhibited ovalbumin-induced bronchial hyperresponsiveness, increase of eosinophils, and the expressions of IL-4 and CCL11 mRNA in the lung. However, the number of neutrophils was not significantly inhibited. Therefore, our present results with prednisolone could be also consistent with the results proposed in the above previous clinical reports. In contrast to glucocorticoids, the prostanoid DP receptor antagonists attenuated not only bronchial hyperresponsiveness and the eosinophilia but also neutrophilia in the present study.

It is reported that bronchial hyperresponsiveness to methacholine is associated with airway eosinophilia but not airway neutrophilia in chronic asthma (Little et al., 2002). It is also known that airflow obstruction expressed by lower FEV1 (Maximal forced expiratory volume in the first second) is independently associated with both airway eosinophilia and neutrophilia in chronic asthma, respectively (Little et al., 2002; Woodruff et al., 2001). These results suggest that prostanoid DP receptor antagonists may inhibit bronchial hyperresponsiveness mainly through the suppression of airway eosinophilia and airflow obstruction via the suppression of both airway eosinophilia and neutrophilia. In particular, several reports show that airway neutrophilia is not affected by glucocorticoid therapy (Basyigit et al., 2004; Little et al., 2002; Nguyen et al., 2005; Woodruff et al., 2001). In

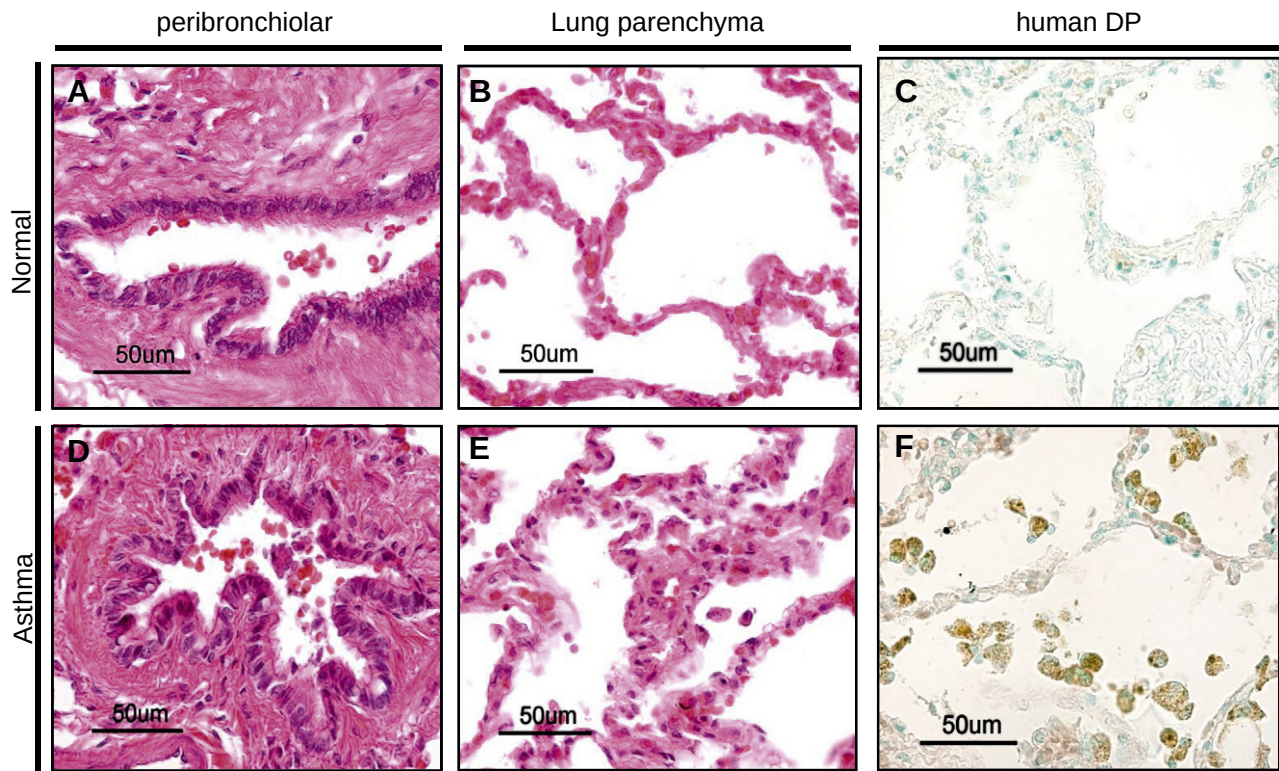


Fig. 6. Morphopathology/prostanoid DP receptor immunohistochemistry in human asthmatic lung. Peribronchiolar and parenchyma in normal (A, B) and asthmatic lungs (D, E) were stained by H&E. The lung tissues in normal (C) and asthmatic (F) were also immunostained with anti-prostanoid DP receptor monoclonal antibody. The positive signals were detected as diaminobenzidine deposits of dark-brownish color, and the nuclei were counterstained with methyl green. Bar indicates 50 μ m.

addition, Little et al. (2002) report that airflow obstruction is still associated with glucocorticoid resistant airway neutrophilia after glucocorticoid treatment. In the present study, the prostanoid DP receptor antagonists, but not prednisolone, attenuated neutrophilia. Therefore, prostanoid DP receptor antagonists might be useful in improving the airflow obstruction in symptomatic asthma as an alternative therapeutic benefit to glucocorticoids therapy. Further studies clinically or with animal models are needed to directly evaluate the anti-airflow obstruction action by prostanoid DP receptor antagonists.

Mucus secretion is also significantly inhibited by prostanoid DP receptor antagonists but not with prednisolone in the present study. Airway mucus hypersecretion is a feature observed in the airway of poorly-controlled asthma such as chronic asthma and fetal asthma. It is known that excess mucus production contributes not only to airflow obstruction by partial or complete mucus plugging but also to airway hyperresponsiveness (Rogers, 2004). Hence, the inhibitory effect of mucus secretion might be a therapeutic benefit of prostanoid DP receptor antagonists when compared with oral glucocorticoids.

The mechanism for the anti-inflammatory effects of prostanoid DP receptor antagonists remains unclear. The majority of prostanoid DP receptor localizing cells in asthmatic lungs both in rats and human was immunohistologically and morphologically identified to be macrophages and monocytes in the present study. It is known that macrophages and monocytes are a source of proinflammatory cytokines and chemokines such as IL-1 β , IL-6 and IL-8. In the present study, the prostanoid DP receptor antagonists, but not prednisolone, attenuated the increase of proinflammatory cytokine and chemokine transcripts such as IL-1 β , IL-6 and CXCL1. Although the role of IL-1 β , IL-6 and CXCL1 in asthma is unclear, CXCL1 is a strong chemoattractant of neutrophils and induction of IL-1 β expression in the lungs of adult mice causes pulmonary inflammation characterized by

neutrophil and macrophage infiltrates, and enhanced mucin production (Lappalainen et al., 2005). IL-6 has also been suggested to play a role in the biological significance of asthma using a mouse asthma model (Doganci et al., 2005). Hence, the direct and/or indirect suppression of these molecules produced by macrophages and/or monocytes might play a critical role in the anti-asthma effects of prostanoid DP receptor antagonists. In the rat model, prostanoid DP receptor was also partially expressed in eosinophils. Gervais et al. (2001) reported that survival of eosinophils is prolonged by the anti-apoptotic effect of PGD₂ via the prostanoid DP receptor. S-5751 completely suppressed the anti-apoptotic effect of eosinophils induced by the prostanoid DP receptor agonist BW245C in our preliminary study (data not shown). Therefore, the inhibition of eosinophil survival induced by lung PGD₂ might be also a possible mechanism for suppression of eosinophils in lung by prostanoid DP receptor antagonists.

In the present study, prednisolone also partially suppressed infiltration of macrophages/monocytes, although it had no effect on the mRNA expression of IL-1 β , IL-6 and CXCL1. Further studies are needed to evaluate the mechanisms of suppression of proinflammatory cytokines and chemokines, and infiltration of inflammatory cells by prostanoid DP receptor antagonists.

In summary, our results showed that prostanoid DP receptor antagonists had suppressive effects, without any side effects, on antigen-induced airway responses in rats with different mode of action from that of prednisolone, especially with regard to the infiltration of neutrophils, the expression of proinflammatory cytokines and neutrophil chemokine, and the production of mucin. These findings suggest that prostanoid DP receptor antagonists might provide a safe and alternative therapeutic choice from oral glucocorticoids, or added benefits in combination with glucocorticoids in asthma therapy.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ejphar.2011.05.003.

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