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Original Article

Repeated bronchoconstriction attenuates the cough response to bronchoconstriction in naïve guinea pigs

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bronchoconstriction; CVA, cough variant

asthma; Cys-LTs, cysteinyl leukotrienes;

DI, deep inspiration; EB, eosinophilic

bronchitis; EIA, enzyme immunoassay;

MCh, methacholine; Penh, enhanced pause

ABSTRACT

Background: Cough variant asthma (CVA) is recognized as a precursor of bronchial asthma (BA). However, the cough response to bronchoconstriction differs between these similar diseases. Repeated bronchoconstriction and the resulting imbalance of endogenous lipid mediators may impact the cough response.

Methods: We investigated the influence of repeated bronchoconstriction on the cough response to bronchoconstriction using naïve guinea pigs. Bronchoconstriction was induced for 3 consecutive days and changes in the cough response and lipid mediators, such as PGE₂, PGI₂, and cysteinyl-LTs (Cys-LTs), in BAL fluid (BALF) were assessed. We investigated the effect of endogenous PGI₂ on the cough response by employing a PGI₂ receptor antagonist. In order to investigate the cough response over a longer period, we re-evaluated the cough response 2 weeks after repeated bronchoconstriction.

Results: The number of coughs induced by bronchoconstriction were significantly decreased by repeated bronchoconstriction. The levels of PGE₂, PGI₂, and Cys-LTs, and the ratio of PGI₂/PGE₂ were significantly increased, following repeated bronchoconstriction. This decrease in the cough response was suppressed by pretreatment with a PGI₂ receptor antagonist. Two weeks after repeated bronchoconstriction, the cough response returned to the same level as before repeated bronchoconstriction along with a concomitant return of lipid mediators, such as PGE₂, PGI₂, and Cys-LTs and the ratio of PGI₂/PGE₂.

Conclusions: Our results suggest that repeated bronchoconstriction and the resulting imbalance of endogenous lipid mediators contribute to the difference in cough responses to bronchoconstriction in CVA and BA.

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Introduction

Chronic cough is one of the most common complaints for which patients seek medical attention. In a recent Japanese survey, about 10% of the general population had symptoms of coughing, and

about 2% had a chronic cough, defined as a cough lasting for more than 8 weeks.¹ Although coughing is an important protective reflex to remove airway secretions and foreign matter present in airways, a persistent cough due to various diseases can markedly impair the quality of life.² Cough variant asthma (CVA) and bronchial asthma (BA) are important diseases in which coughing is triggered by bronchoconstriction. CVA is one of the most common causes of chronic non-productive cough^{3–5} and is recognized as a precursor or cough variant form of typical BA.⁶ However, in previous clinical studies, we have shown that the cough response to methacholine (MCh)-induced bronchoconstriction is quite different between CVA

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and BA.^{7,8} Specifically, the cough response to bronchoconstriction in CVA is heightened compared to healthy subjects, whereas this response is decreased in BA. It is unclear what mechanisms cause the difference in cough responses to bronchoconstriction between these two similar diseases.

BA is characterized by variable respiratory symptoms including wheezing, shortness of breath, chest tightness and coughing, which are based on chronic airway inflammation, AHR, and variable expiratory airflow limitation.⁹ CVA also displays chronic airway inflammation, often comorbid with mild AHR. However, CVA, in which coughing is the sole symptom, does not have the repeated excessive bronchoconstriction that is typically seen in asthmatic attacks. Considering this physiological difference between the 2 diseases, repeated excessive bronchoconstriction may be involved in the attenuation of the cough response to bronchoconstriction.

Lipid mediators, which are derived from arachidonic acid, play an important role in inflammatory airway diseases including BA and CVA.¹⁰ In previous studies using induced sputum, it was shown that the constitution of lipid mediators in the airway differs among diseases.^{11–14} We have reported that several lipid mediators, such as PGE₂, PGI₂ and cysteinyl-LTs (Cys-LTs), in bronchoalveolar lavage fluid (BALF) were elevated following MCh-induced bronchoconstriction, and that exogenous PGE₂ and PGI₂ affect the cough response to bronchoconstriction in guinea pigs.^{15,16} Although both PGE₂ and PGI₂ are known to be prostanoids with bronchodilating effects,^{17,18} their effect on the cough response to bronchoconstriction is quite different. That is, PGE₂ enhances the cough response, while PGI₂ attenuates the cough response.

In this study, we investigated whether repeated bronchoconstriction affected the cough response to bronchoconstriction using an MCh-induced bronchoconstriction guinea pig model. We hypothesized that repeated bronchoconstriction attenuates the cough response to bronchoconstriction, and that an imbalance of endogenous lipid mediators, such as PGE₂ and PGI₂, resulting from bronchoconstriction is the cause of this change in the cough response.

Methods

Study animals

Male albino Hartley guinea pigs weighing 400–600 g were obtained from Sankyo Laboratory Service (Toyama, Japan). They were quarantined in the Animal Research Center of Kanazawa University. All animal procedures in this study complied with the Guidelines for the Care and Use of Laboratory Animals at the Takara-machi Campus of Kanazawa University (approval number, AP-173844).

Inhalation

Saline alone or saline and increasing doses of MCh (100, 200, 400 µg/mL) were administered for 1 min via a Devilbiss 646 nebulizer (Devilbiss Co., Somerset, PA, USA) operated by compressed air (NE-C13, OMRON Health Care Co., Kyoto, Japan).

Measurement of bronchoconstriction

We measured enhanced pause (Penh) as an index of bronchoconstriction.¹⁹ The pressure signal is due to volume and pressure changes in the main chamber during the animal's respiratory cycle. From these pressure signals, the phases of the respiratory cycle, tidal volumes, and an index of airway caliber, Penh, can be calculated. Penh is a dimensionless value that reflects changes in the waveform of the pressure signal resulting from both inspiration

and expiration, combined with the timing comparison of early and late expiration. Penh was measured every minute for 10 min after inhalation of saline alone or saline and increasing doses of MCh. The average Penh during 10 min was defined as an index of bronchoconstriction by each MCh inhalation and was used for further analysis.

Measurement of the cough response to bronchoconstriction

Measurement of the cough response to bronchoconstriction was performed using previously described methods.²⁰ A trained observer carefully monitored the animal and counted the number of coughs characterized by high sound, with the mouth open, and a rapid abdominal movement. Coughs were also detected as a transient specific change in the flow in the body chamber (a rapid inspiration followed by rapid expiration). After inhalation of saline or saline and increasing doses of MCh for 1 min, the number of coughs was quantified every minute for 10 min, and the total number of coughs over 10 min was used for further analysis. Briefly, Penh and the number of coughs were measured over 40 min in total for each animal. A recurrent straight line was determined using Penh and the number of coughs, and the inclination of the regression line was defined as the index of the cough response to bronchoconstriction (CRB) of each animal.

Bronchoalveolar lavage (BAL)

BAL was performed immediately after completion of the measurement of Penh and the cough response using previously described methods.^{21,22} Briefly, the guinea pigs were anaesthetized with an intraperitoneal injection of 75 mg/kg sodium pentobarbital (Abbot Laboratories, North Chicago, IL, USA) and placed in a supine position. Lungs were lavaged twice with a tracheal cannula with 10 mL of saline (total: 20 mL).

Bronchoalveolar lavage fluid (BALF) analysis

The cells in BALF were stained with Turk solution and counted in duplicate with a hemocytometer (in a Burk chamber). Differential cell counts (analyzing 300 cells) were made on a smear prepared by cytocentrifugation and stained with Wright-Giemsa. BALF was centrifuged at 1500 rpm for 10 min at 4 °C and the supernatants were stored at –80 °C for late measurement of several mediators.

PGE₂, PGI₂, and Cys-LTs levels in the BALF

PGE₂, PGI₂, and Cys-LTs levels in BALF were measured using commercially available enzyme immunoassay (EIA) kits purchased from CAYMAN Chemical Co. (MI, USA): Prostaglandin E Metabolite EIA kit, Prostaglandin F_{1a} EIA kit, and Cysteinyl Leukotriene EIA kit.

Study design

Experimental protocol 1

Guinea pigs were assigned to the following 3 groups in order to investigate the effect of repeated bronchoconstriction on the cough response: the non-bronchoconstriction group (NC group: negative control), the 1-day bronchoconstriction group (PC1 group: positive control 1), and the 3-day repeated bronchoconstriction group (PC3 group: positive control 3) (n = 8 animals in the NC and PC1 groups, n = 12 animals in the PC3 group). Animals in the PC1 and PC3 groups inhaled saline and increasing concentrations of MCh solution. In the PC3 group, the inhalation procedure was repeated every 24 h for 3 days. Animals in the NC group inhaled saline alone repeatedly instead of MCh. Penh and the number of coughs were

measured and BAL was performed. Various cell types and the levels of lipid mediators in BALF from each animal were examined.

Experimental protocol 2

Guinea pigs were assigned to the following 2 groups in order to investigate the effect of endogenous PGI₂ on the cough response: vehicle group (Vehicle group; dimethyl sulfoxide) and the PGI₂ receptor antagonist group (IP group; CAY10441 4 mg/kg) (n = 7 animals in each group). CAY10441 was purchased from CAYMAN Chemical Co. (MI, USA). The dosage of CAY10441 was chosen based on our previous experiments.¹⁶ CAY10441 was dissolved in dimethyl sulfoxide. Animals in the IP group were intraperitoneally injected with CAY10441 just before measuring Penh and cough counts. Animals in the vehicle group were intraperitoneally injected with dimethyl sulfoxide instead of CAY10441. Intraperitoneal injections and measurement of Penh and cough counts were repeated every 24 h for 3 days.

Experimental protocol 3

We prepared naïve guinea pigs in order to investigate changes of the cough response and lipid mediators in BALF over a longer period. We used separate guinea pigs for measurement of the cough response and BALF lipid mediators (n = 6 animals in each measurement). The inhalation of MCh and measurement of Penh and cough counts were conducted using a protocol similar to the PC3 group (Experimental protocol 1), and cough responses to bronchoconstriction were re-evaluated 15 days after repeated bronchoconstriction. BAL was also performed 15 days after repeated bronchoconstriction (PC15 group: positive control 15), and inflammatory cell populations and lipid mediators in BALF were compared with the NC, PC1 and PC3 groups (evaluated in Experimental protocol 1).

Statistical analysis

All data are shown as means $\pm$ SD. The statistical differences between pairs of independent groups were analyzed by Mann–Whitney *U* tests or Kruskal–Wallis tests, and pairs of related groups were analyzed by Friedman tests. The relationship between Penh and the number of coughs induced by MCh inhalation was analyzed by simple regression analysis. The statistical differences in changes of Penh following MCh inhalation were analyzed by repeated Analysis of Variance. A *P*-value less than 0.05 was

considered statistically significant. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan),²³ which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics.

Results

Repeated bronchoconstriction induced AHR and attenuated the cough response to bronchoconstriction

The relationship between the concentration of inhaled MCh and Penh is shown in Fig 1A and B. In our experimental model using a whole-body plethysmograph, bronchoconstriction was induced by MCh inhalation, and we observed that Penh increased as MCh concentration increased. Penh was significantly increased in the PC3 group compared with the NC group on Day 1 (*P* < 0.01) (Fig. 1A). Furthermore, airway responsiveness increased following repeated bronchoconstriction, and bronchoconstriction was induced at lower concentrations of MCh inhalation on Days 2 and 3 compared with Day 1 in the PC3 group (*P* < 0.01) (Fig. 1B). Penh and the number of coughs were positively correlated, and the inclination of the regression line of each animal, i.e. CRB, was used for comparing the cough response to bronchoconstriction (Supplementary Fig. 1). The CRB was significantly decreased on Days 2 (1.3 ± 1.2) and 3 (0.5 ± 0.4) compared with Day 1 (5.2 ± 5.5) (*P* < 0.01) (Fig. 2A). The number of coughs was also significantly decreased on Days 2 (4.5 ± 3.3) and 3 (1.9 ± 1.5) compared with Day 1 (12.3 ± 8.1) (*P* < 0.05) (Fig. 2B). In the NC group, neither bronchoconstriction nor cough were induced (data not shown).

Repeated bronchoconstriction induced lipid mediators in BALF

The number of total cells and inflammatory cell populations in BALF were not significantly different between the NC, PC1 and the PC3 groups (Table 1). The concentrations of lipid mediators in BALF are shown in Fig. 3. PGE₂ and PGI₂ levels were significantly elevated in the PC1 and PC3 groups compared with the NC group (PGE₂: NC, 14.2 ± 3.4 pg/mL; PC1, 23.8 ± 8.8 pg/mL; PC3, 40.0 ± 16.3 pg/mL; PGI₂: NC, 12.8 ± 2.2 pg/mL; PC1, 43.1 ± 18.5 pg/mL; PC3, 57.8 ± 24.0 pg/mL, *P* < 0.01) (Fig. 3A, B). The ratios of PGI₂/PGE₂ were significantly increased in the PC1 and PC3 groups compared

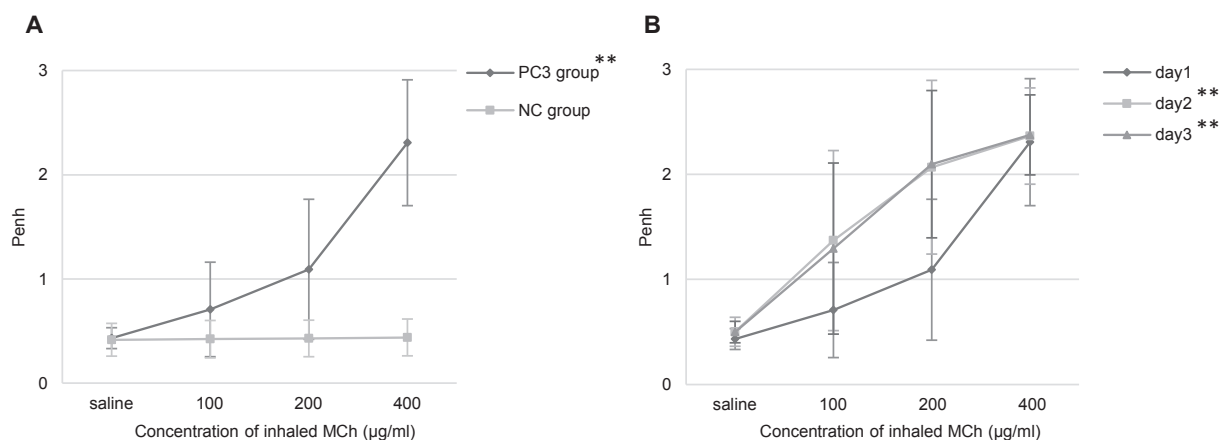


Fig. 1. Repeated bronchoconstriction induced AHR. Penh increased as MCh concentrations increased in the PC3 group. Penh was significantly increased in the PC3 group compared with the NC group on Day 1 (A). Airway responsiveness increased following repeated bronchoconstriction, and bronchoconstriction was induced at a lower concentration of inhaled MCh on Days 2 and 3 compared with Day 1 in the PC3 group (B). NC group, non-bronchoconstriction group; PC3 group, 3 days bronchoconstriction group. **, *P* < 0.01 compared with NC group; **, *P* < 0.01 compared with Day 1. n = 8 animals in the NC group; n = 12 animals in the PC3 group.

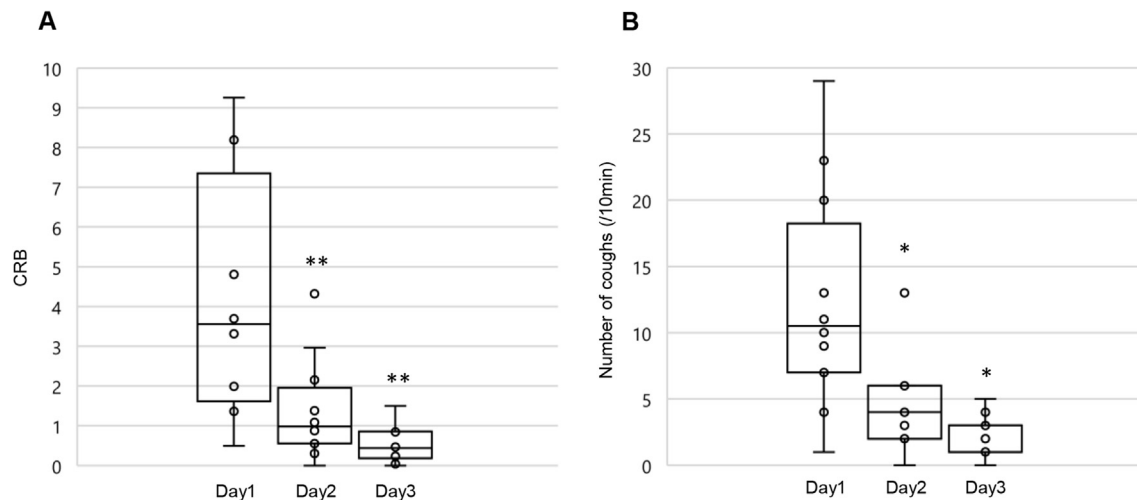


Fig. 2. Repeated bronchoconstriction influenced CRB and the number of coughs induced by MCh inhalation. CRB was significantly decreased on Days 2 and 3 compared with Day 1 (A). The number of coughs was also significantly decreased on Days 2 and 3 compared with Day 1 (B). *, $P < 0.05$ compared with Day 1; **, $P < 0.01$ compared with Day 1. $n = 12$ animals in each day.

Table 1
Number of total cells and inflammatory cells present in BALF.

Group	Total cells ($\times 10^4$ cells/ml)	Mac (%)	Neu (%)	Lym (%)	Eos (%)
NC	28.8 $\pm$ 10.7	82.4 $\pm$ 11.5	7.3 $\pm$ 7.3	1.5 $\pm$ 0.5	9.4 $\pm$ 7.7
PC1	27.4 $\pm$ 9.9	81.2 $\pm$ 19.0	9.1 $\pm$ 15.0	1.7 $\pm$ 1.4	8.1 $\pm$ 6.3
PC3	29.8 $\pm$ 8.4	83.6 $\pm$ 7.6	8.3 $\pm$ 7.1	1.4 $\pm$ 0.7	6.7 $\pm$ 3.9
PC15	27.4 $\pm$ 5.9	92.5 $\pm$ 3.3*	1.7 $\pm$ 2.6*	1.1 $\pm$ 0.7	4.7 $\pm$ 3.3

Data are shown as means $\pm$ SD; $n = 8$ animals in the NC and PC1 groups, $n = 12$ animals in the PC3 group, $n = 7$ animals in the PC15 group. NC group, non-bronchoconstriction group; PC1 group, 1 day bronchoconstriction group; PC3 group, 3 days repeated bronchoconstriction group; PC15 group, 15 days after repeated bronchoconstriction group; Mac, macrophage; Neu, neutrophil; Lym, lymphocyte; Eos, eosinophil. *, $P < 0.05$ compared with the NC, PC1 and PC3 groups.

with the NC group (NC 0.9 ± 0.3 , PC1 1.8 ± 0.5 , PC3 1.5 ± 0.4 , $P < 0.01$) (Fig. 3D). In other words, PGI₂, which has been shown to attenuate the cough response to bronchoconstriction, was relatively increased compared with PGE₂ by repeated bronchoconstriction. Cys-LTs levels were also significantly elevated in the PC3 group compared with the NC group (NC 141.9 ± 26.9 pg/mL, PC1 201.5 ± 49.3 pg/mL, PC3 347.6 ± 71.5 pg/mL, $P < 0.01$) (Fig. 3C).

PGI₂ produced by bronchoconstriction decreased the cough response to bronchoconstriction

We investigated the effect of endogenous PGI₂, which was relatively increased by bronchoconstriction, on the cough response by employing a PGI₂ receptor antagonist. In the vehicle group, CRB was significantly decreased on Days 2 (0.8 ± 0.8) and 3 (0.2 ± 0.3) compared with Day 1 (1.8 ± 1.2) ($P < 0.05$) (Fig. 4A). In the IP group, CRB tended to decrease on Day 3 (1.6 ± 0.9) compared with Day 1 (2.6 ± 1.4), although this effect did not reach statistical significance (Fig. 4B). Furthermore, on Days 2 and 3, the CRB of the IP group was significantly higher than that of the vehicle group (Day 1: vehicle, 1.8 ± 1.2 ; IP, 2.6 ± 1.4 ; Day 2: vehicle, 0.8 ± 0.8 ; IP, 2.6 ± 1.9 , $P < 0.05$; Day 3: vehicle, 0.2 ± 0.3 ; IP, 1.6 ± 0.9 , $P < 0.01$) (Fig. 4C, D, E).

AHR and the cough response improved two weeks after repeated bronchoconstriction

We investigated the change in the cough response over a longer period. Thus, we re-evaluated the cough response to

bronchoconstriction after 15 days of repeated bronchoconstriction. Fifteen days after repeated bronchoconstriction, AHR recovered to the same level as Day 1 (Fig. 5). Fifteen days after repeated bronchoconstriction, the attenuation of the cough response also recovered, and the CRB and number of coughs were the same as on Day 1 (CRB: Day 1, 2.1 ± 1.7 , Day 15, 2.0 ± 2.6 ; number of coughs: Day 1, 12.8 ± 9.1 , Day 15, 7.7 ± 6.4) (Fig. 6). The number of macrophages in BALF was increased and neutrophils in BALF were decreased in the PC15 group compared with the NC, PC1 and PC3 groups ($P < 0.05$), whereas the numbers of total cells and other inflammatory cell components in BALF were not significantly different among the 4 groups. The concentration of lipid mediators in BALF, such as PGE₂, PGI₂, and Cys-LTs, and the ratio of PGI₂/PGE₂ recovered to the same levels as the NC group (PGE₂: NC, 15.1 ± 2.7 pg/mL, PC15, 12.7 ± 16.3 pg/mL; PGI₂: NC, 11.9 ± 2.7 pg/mL, PC15, 10.9 ± 1.9 pg/mL; Cys-LTs: NC, 141.9 ± 26.9 pg/mL, PC15, 180.2 ± 42.8 pg/mL, PGI₂/PGE₂: NC, 0.9 ± 0.3 , PC15, 0.9 ± 0.3) (Fig. 7A, B, C, D).

Discussion

In the present study, we showed that repeated bronchoconstriction attenuated the cough response to bronchoconstriction in naïve guinea pigs. After repeated bronchoconstriction, the concentrations of PGE₂, PGI₂, and Cys-LTs in BALF, and the ratio of PGI₂/PGE₂ were significantly increased. In other words, the levels of PGI₂ in BALF were relatively increased following repeated bronchoconstriction. The attenuation of the cough response due to repeated bronchoconstriction was suppressed by pretreatment with a PGI₂ receptor antagonist. Furthermore, 15 days after repeated bronchoconstriction, the cough response had recovered to the same level as seen on Day 1 in parallel with the return of the concentrations of lipid mediators, such as PGE₂, PGI₂, and Cys-LTs, and the ratio of PGI₂/PGE₂. These results suggest that repeated bronchoconstriction and the resulting imbalance of endogenous lipid mediators, such as the relative increase of PGI₂, may be at least partly responsible for the difference of the cough response to bronchoconstriction between CVA and BA.

Previous animal studies have shown that bronchopulmonary C-fibers and A δ -fibers play an important role in the cough reflex. C-fibers densely innervate in and around the epithelium of whole airways.^{24,25} The receptors of C-fibers are directly activated by a

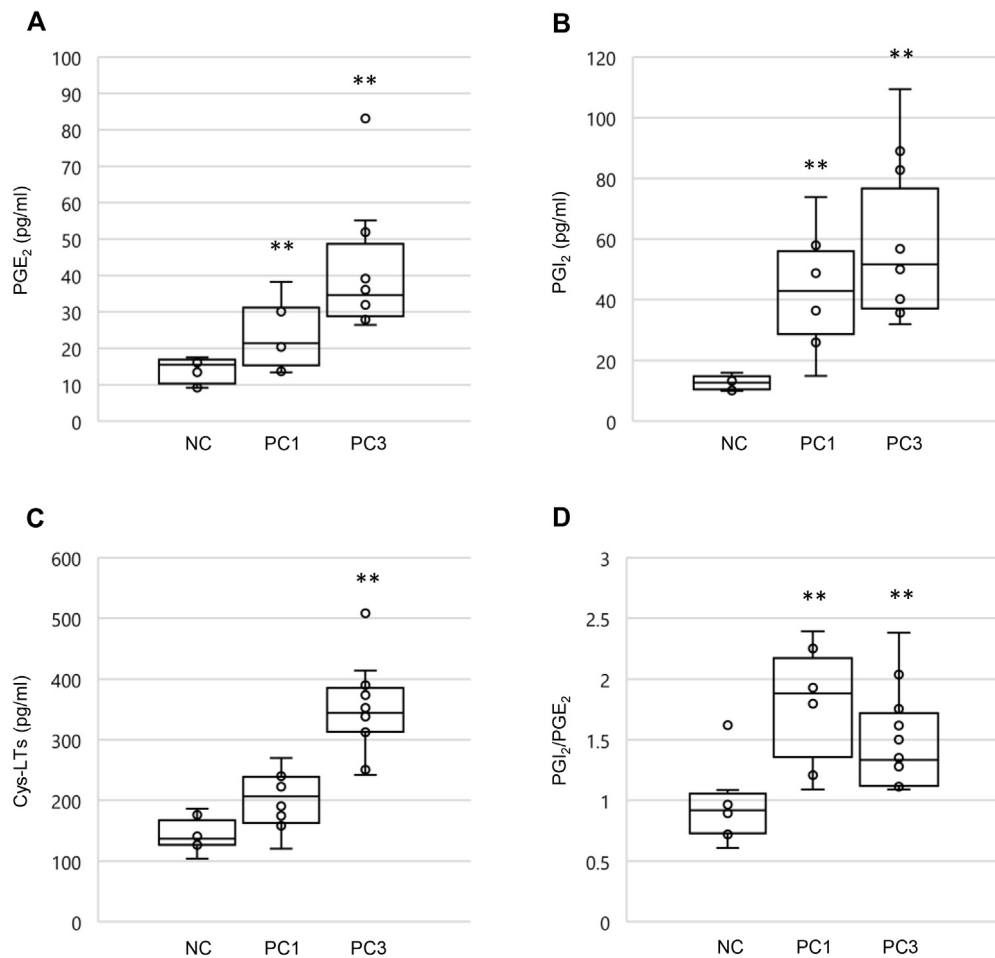


Fig. 3. Repeated bronchoconstriction influenced the concentration of lipid mediators in BALF. PGE₂ levels were significantly elevated in the PC1 and PC3 groups compared with the NC group (A). PGI₂ levels were significantly elevated in the PC1 and PC3 groups compared with the NC group (B). Cys-LTs levels were significantly elevated in the PC3 group compared with the NC group (C). The ratio of PGI₂/PGE₂ was significantly increased in the PC1 and PC3 groups compared with the NC group (D). NC group, non-bronchoconstriction group; PC1 group, 1 day bronchoconstriction group; PC3 group, 3 days bronchoconstriction group. **, $P < 0.01$ compared with NC group. $n = 8$ animals in the NC and PC1 groups; $n = 12$ animals in the PC3 group.

diverse range of chemical and environmental irritants²⁶ and are also indirectly activated by some inflammatory mediators, such as PGE₂ and bradykinin.²⁷ On the other hand, A δ -fibers sparsely innervate the space between the epithelium and smooth muscle in the proximal airways.²⁸ A δ -fibers are so-called “mechanoreceptors or cough receptors”, and they are insensitive to most chemical irritants. However, they are sensitive to punctate mechanical stimuli.²⁹ Rapidly adapting receptors and slowly adapting receptors are other important vagal afferent nerves and are recognized to regulate the respiratory cycle and bronchomotor tone, but do not directly impact the cough reflex.^{30,31} Our previous studies in naïve guinea pigs showed that bronchoconstriction triggers coughs via rapidly adapting receptors, but not C-fibers.²⁰ We speculate that rapidly adapting receptors interact with other subsets of the airway vagal afferent nerves, perhaps A δ -fibers (cough receptors), to synchronously regulate the bronchoconstriction-triggered cough reflex.

We showed that repeated bronchoconstriction attenuated the cough response to bronchoconstriction in naïve guinea pigs. Repeated bronchoconstriction induced by MCh promoted airway remodeling without exacerbation of eosinophilic airway inflammation in asthmatic patients,³² and airway inflammation, airway remodeling and AHR, even in non-sensitized groups, in animal experiments using rats.³³ These reports suggest that

bronchoconstriction itself induces some mediators in the airway that promote airway remodeling and AHR. In naïve guinea pigs, negative mechanical pressure stress, which imitated coughing, induced neutrophilic airway inflammation and cough reflex hypersensitivity to capsaicin,³⁴ and repeated coughing caused by citric acid induced airway remodeling and cough reflex hypersensitivity to citric acid.³⁵ The cough frequency highly correlated with the extent of airway remodeling.³⁵ These reports suggest that coughing itself has an effect of mechanical stress on the airway wall and that it induces some mediators that promote airway remodeling and cough reflex hypersensitivity to tussive agents. The effects of repeated bronchoconstriction and mechanical stress on the cough response to bronchoconstriction have not been investigated prior to this study. In this study, we showed for the first time that repeated bronchoconstriction attenuated the cough response to bronchoconstriction.

The total number of cells and inflammatory cells, such as eosinophils and neutrophils, in BALF was not increased by repeated bronchoconstriction. These results are consistent with previous studies,^{32,33} and suggest that repeated bronchoconstriction does not induce eosinophilic or neutrophilic airway inflammation. On the other hand, the levels of lipid mediators, such as PGE₂, PGI₂, and Cys-LTs in BALF, and the ratio of PGI₂/PGE₂ were significantly increased by repeated bronchoconstriction. Several reports have

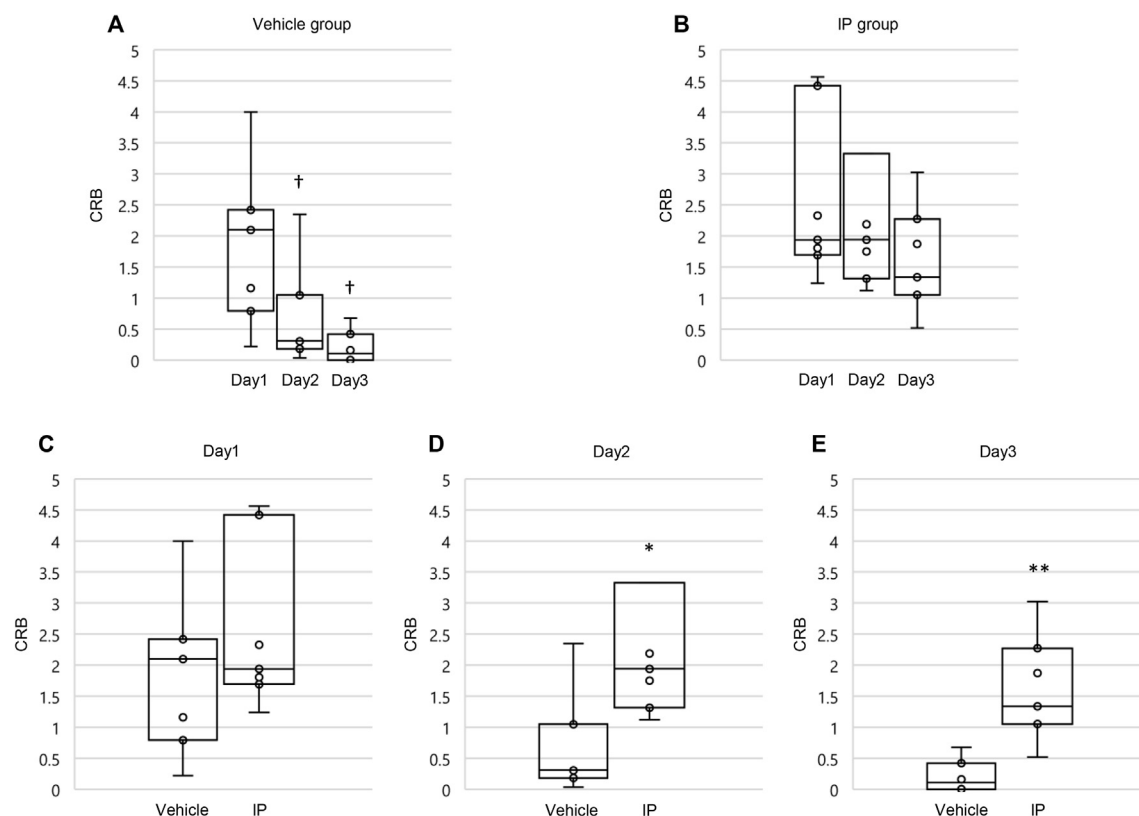


Fig. 4. PGI₂ contributed to the attenuation of the cough response due to repeated bronchoconstriction. In the vehicle group, CRB was significantly decreased on Days 2 and 3 compared with Day 1 (A). In the IP group, CRB was not different among the three days (B). On Days 2 and 3, the CRB of the IP group was significantly higher than that of the vehicle group (C–E). IP group, PGI₂ receptor antagonist group. *, P < 0.05 compared with vehicle group; **, P < 0.01 compared with vehicle group; †, P < 0.05 compared with Day 1. n = 7 animals in each group.

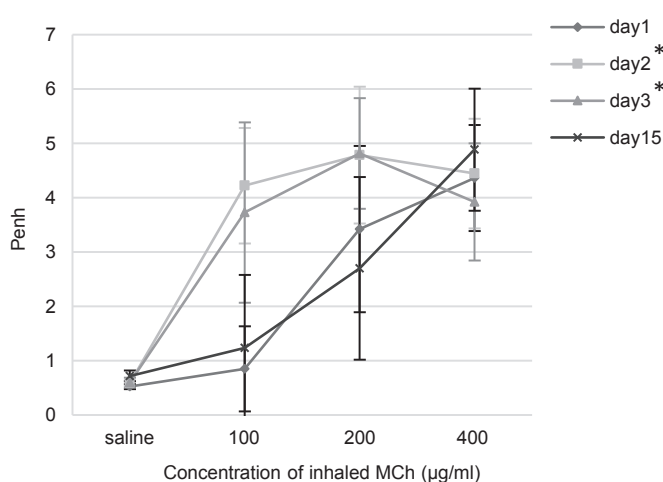


Fig. 5. AHR improved two weeks after repeated bronchoconstriction. Airway responsiveness was increased by repeated bronchoconstriction and bronchoconstriction was induced at a lower concentration of inhaled MCh on Days 2 and 3 compared with Day 1. Fifteen days after repeated bronchoconstriction, airway hyperresponsiveness improved to the same level as Day 1. *, P < 0.05 compared with Day 1. n = 6 animals in each day.

shown that the levels of Cys-LTs, LTC₄, PGD₂, PGE₂ and histamine in induced sputum are increased in BA, CVA and eosinophilic bronchitis (EB) when compared to normal subjects,^{11–14} and that the levels of PGD₂, PGE₂ and histamine in induced sputum were increased in EB compared to BA.^{12,14} Furthermore, the ratio of LTC₄/

PGE₂ in induced sputum is increased in BA compared to EB.¹⁴ Sputum Cys-LTs or the ratio of combined “contractile” prostanoids (PGD₂/PGF_{2α}/thromboxane B₂) to PGE₂ levels are increased compared to normal subjects and are associated with BA disease severity.^{11,36} These results suggest that the levels themselves or the imbalance of endogenous inflammatory mediators may be involved in the pathophysiology and disease severity of eosinophilic respiratory diseases such as BA, CVA, and EB. We previously investigated the role of PGE₂ and PGI₂ in bronchoconstriction-triggered coughing in naïve guinea pigs.^{15,16} Both PGE₂ and PGI₂ are known prostanoids with bronchodilating effects^{17,18}; however, their influences on the cough response to bronchoconstriction were completely different. That is, PGE₂ enhanced the cough response while PGI₂ attenuated the cough response. In the present study, PGI₂, which attenuates the cough response to bronchoconstriction, was relatively increased in the airway due to repeated bronchoconstriction. The attenuation of the cough response due to repeated bronchoconstriction was suppressed by pretreatment with an PGI₂ receptor antagonist. Furthermore, 15 days after repeated bronchoconstriction, the cough response had improved to the same level as Day 1 in parallel with the recovery of the levels of lipid mediators such as PGE₂ and PGI₂ and the ratio of PGI₂/PGE₂. These results suggest that an imbalance of endogenous lipid mediators in the airway due to repeated bronchoconstriction, as seen by a relative increase of PGI₂, contributes to an attenuated cough response to bronchoconstriction.

Nearly 30% of CVA patients eventually develop wheezing and progress to typical BA.^{6,37,38} Therefore, CVA is recognized as a precursor of typical BA. Duration of disease,³⁷ maximal airway response to MCh,³⁹ sputum eosinophilia⁴⁰ and sensitization to

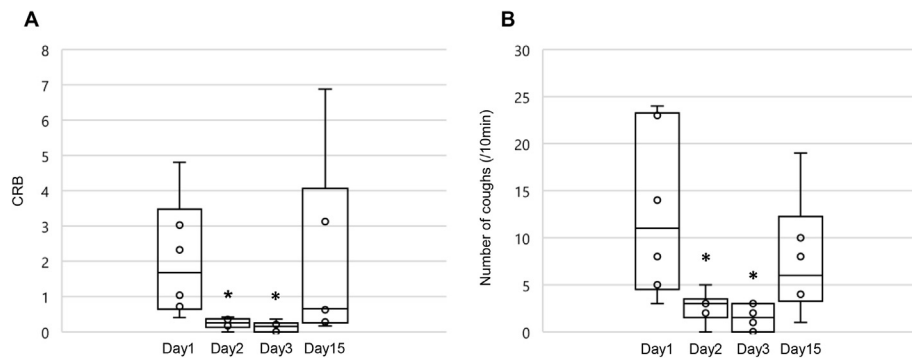


Fig. 6. CRB and the number of coughs improved 2 weeks after repeated bronchoconstriction. CRB was significantly decreased on Days 2 and 3 compared with Day 1 (A). The number of coughs was significantly decreased on Days 2 and 3 compared with Day 1 (B). Fifteen days after repeated bronchoconstriction, both CRB and the number of coughs were equal to Day 1 (A, B). *, $P < 0.05$ compared with Day 1. $n = 6$ animals in each day.

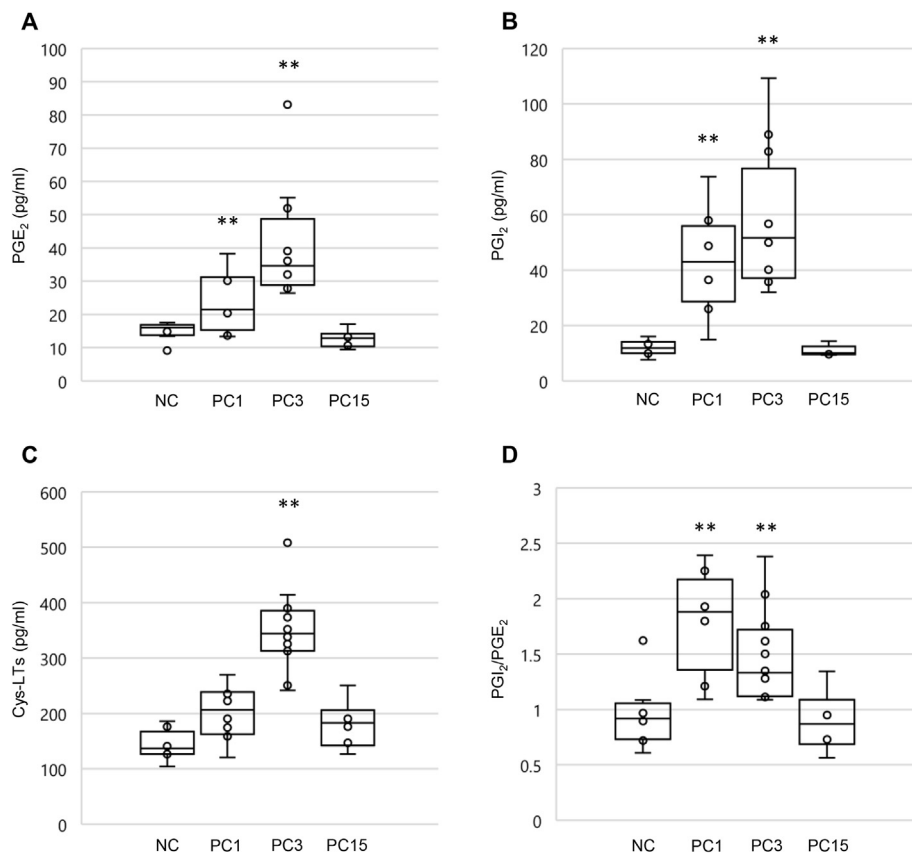


Fig. 7. The concentration of the lipid mediators in BALF improved two weeks after repeated bronchoconstriction. Fifteen days after repeated bronchoconstriction (PC15 group), the concentration of the lipid mediators, such as PGE₂, PGI₂, and Cys-LTs, and the ratio of PGI₂/PGE₂ improved to the same levels as Day 1 (A–D). NC group, non-bronchoconstriction group; PC1 group, 1 day bronchoconstriction group; PC3 group, 3 days bronchoconstriction group; PC15, 15 days after repeated bronchoconstriction group. $n = 8$ animals in the NC and PC1 groups; $n = 12$ animals in the PC3 group; $n = 6$ animals in the PC15 group. *, $P < 0.05$ compared with the NC group; **, $P < 0.01$ compared with the NC group.

allergens⁴¹ are considered to be contributing factors for the onset of typical BA following CVA. We previously showed that AHR was the most important risk factor for typical BA onset progressing from CVA,⁴² and that bronchoconstriction-triggered coughs were positively associated with the bronchodilatory effect of deep inspiration (DI) in healthy subjects.⁴³ Several groups have examined the role of DI in BA and CVA patients. Kapsali and coworkers reported that the loss of bronchodilation and a bronchoprotective effect of DI are important causes of AHR in BA.⁴⁴ Loughheed and coworkers also reported that BA and CVA are a continuum of airway disorders of airway responsiveness, that there was a bronchoprotective effect of

DI, and that preservation or loss of the bronchoprotective effect of DI may be the fundamental physiologic difference between BA and CVA.^{45–47} Moreover, the ratio of LTC₄ with bronchoconstriction and PGE₂ with bronchodilation in sputum increased in BA compared to EB.¹⁴ These results suggest that an imbalance of endogenous lipid mediators may be associated with the degree of DI. In the present study, repeated bronchoconstriction induced AHR and attenuated the cough response to bronchoconstriction, which are physiological features of BA, in naïve guinea pigs. Therefore, repeated bronchoconstriction is one of the factors that promote features of BA and one of the possible mechanisms for progression to typical BA from

CVA. This idea is consistent with our previous finding that AHR was the most important risk factor for the onset of typical BA from CVA.⁴²

There are several limitations in this study. First, we used naïve guinea pigs to investigate the effects of repeated bronchoconstriction on bronchoconstriction-triggered coughing, and it is unclear whether repeated bronchoconstriction similarly attenuates the cough response to bronchoconstriction in sensitized guinea pigs. In previous studies, we have shown that the fundamental physiological feature of CVA is a heightened cough response to bronchoconstriction.⁸ To our knowledge, there are no reports of a CVA animal model based on this feature. To clarify the mechanisms of different cough responses to bronchoconstriction between CVA and BA, more experiments using a CVA animal model are essential. Second, we have not investigated the effect of repeated bronchoconstriction on the airway structure using histology. It is known that bronchoconstriction induces airway remodeling^{32,33} and that the cough response correlates with the extent of airway remodeling.^{35,48} In our study, it is possible that airway remodeling was induced by repeated bronchoconstriction. However, 15 days after repeated bronchoconstriction, the attenuation of the cough response returned to the same level as seen on Day 1. This result suggests that the cough response to bronchoconstriction is not related to airway remodeling, which is known to be an irreversible structural change. Third, we showed that 3 days repeated bronchoconstriction attenuated the cough response to bronchoconstriction in naïve guinea pigs; however, it is unclear whether repeated bronchoconstriction of more many days induces similar results. In additional study, we obtained the following supplemental data. (1) The CRB and the number of coughs were significantly decreased after 5 days repeated bronchoconstriction (CRB: Day 1, 3.5 ± 3.8 , Day 5, 0.2 ± 0.2 ; number of coughs: Day 1, 17.4 ± 9.6 , Day 5, 1.9 ± 1.3 , $P < 0.01$) (Supplementary Fig. 2). (2) PGE₂, PGI₂, and Cys-LTs levels in BALF were significantly elevated after 5 days repeated bronchoconstriction (PC5 group) compared with the NC group (PGE₂: NC, 14.2 ± 3.4 pg/mL; PC5, 43.3 ± 8.9 pg/mL; PGI₂: NC, 12.8 ± 2.2 pg/mL; PC5, 70.8 ± 10.9 pg/mL; Cys-LTs: NC 141.9 ± 26.9 pg/mL, PC5 375.7 ± 60.1 pg/mL, $P < 0.01$). The ratios of PGI₂/PGE₂ were significantly increased in the PC5 group compared with the NC group (NC 0.9 ± 0.3 , PC5 1.7 ± 0.4 , $P < 0.01$) (Supplementary Fig. 3). These results suggest that repeated bronchoconstriction of more many days induces similar results as 3 days repeated bronchoconstriction. Fourth, we used Penh as an index of bronchoconstriction, but it is not appropriate for evaluating precise respiratory resistance. Measuring precise respiratory resistance requires anesthesia and airway intubation. In this study, we focused on the cough response to bronchoconstriction in conscious, unrestrained guinea pigs. Therefore, Penh was considered to be the most appropriate method for our experiments. Finally, the attenuation of the cough response due to repeated bronchoconstriction was significantly suppressed by pretreatment with a PGI₂ receptor antagonist; however, this group's cough response tended to decrease due to repeated bronchoconstriction. It is possible that other mediators produced by repeated bronchoconstriction or other mechanisms also impact the cough response. In this study, the level of Cys-LTs in BALF was significantly increased by repeated bronchoconstriction. Several previous reports have shown that leukotriene receptor antagonists have an antitussive effect in CVA patients.^{49–53} In our preliminary study using Cys-LT1/2 dual receptor antagonist, we obtained the following unpublished data. (1) The MCh-induced increase in Penh was suppressed by pretreatment with a Cys-LT1/2 dual receptor antagonist. (2) The number of coughs triggered by bronchoconstriction was decreased by pretreatment with a Cys-LT1/2 dual receptor antagonist. (3) The CRB was not decreased by pretreatment with a Cys-LT1/2 dual receptor

antagonist. These results suggest that leukotriene receptor antagonists provide an antitussive effect by a bronchodilating effect, and that Cys-LTs themselves do not affect the cough response to bronchoconstriction. Based on these results, we speculate that endogenous Cys-LTs produced by bronchoconstriction are not associated with the attenuation of the cough response in our experimental model. These speculations were consistent with our study that suggested that the antitussive effect of Montelukast, which is an antagonist of the cysteinyl leukotriene receptor, might result from relief of cysteinyl leukotrienes-mediated smooth muscle contraction in CVA patients.⁵¹

We conclude that repeated bronchoconstriction attenuates the cough response to bronchoconstriction. Endogenous lipid mediators produced by bronchoconstriction also impact the cough response. Repeated bronchoconstriction and the resulting imbalance of endogenous lipid mediators may be factors that contribute to the different cough responses to bronchoconstriction between CVA and BA. Furthermore, it may be a possible mechanism that contributes to progression from CVA to typical BA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2019.09.002>.

Conflict of interest

The authors have no conflict of interest to declare.

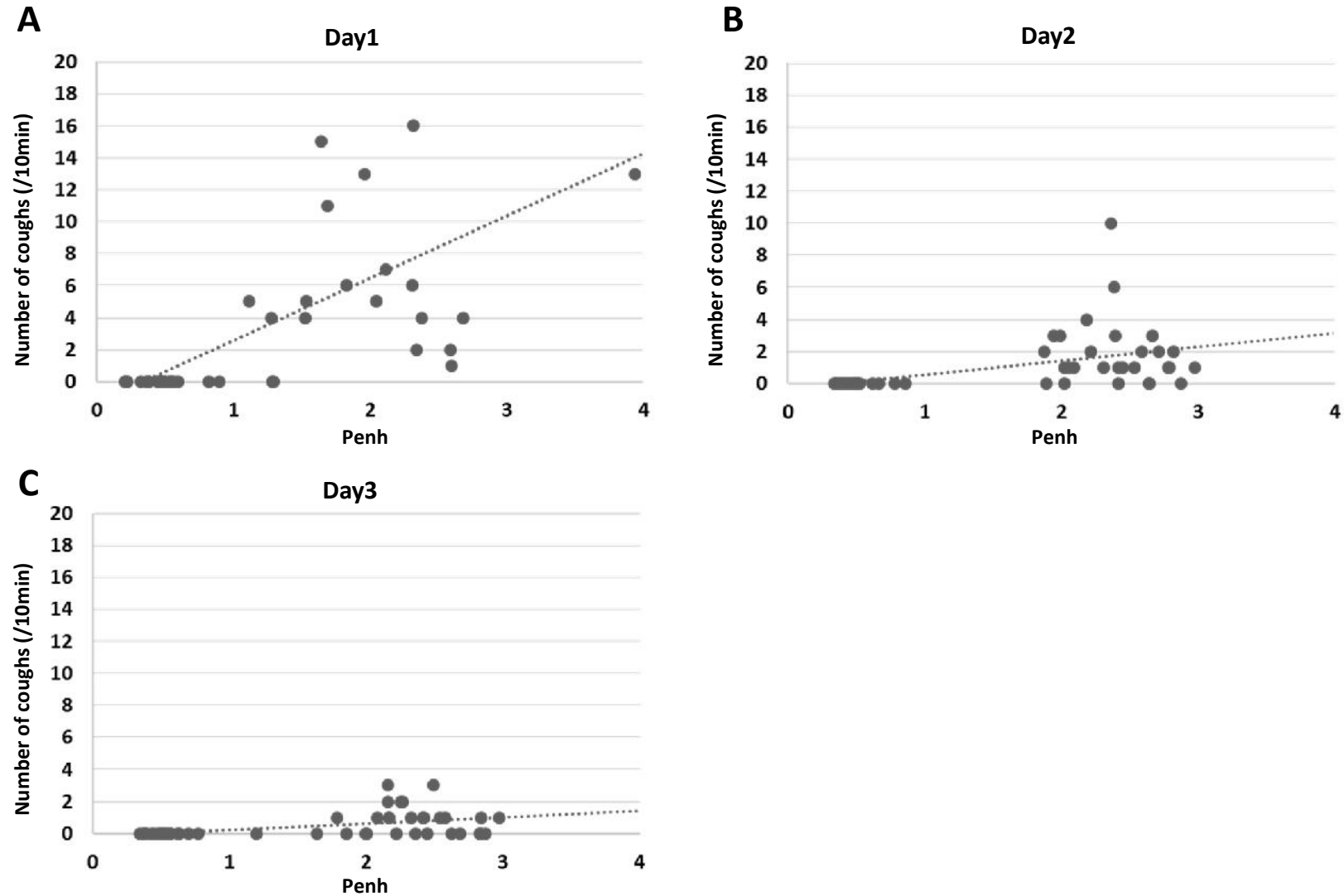
Authors' contributions

KY and JH designed the study and wrote the manuscript. TSa, NOh, MA, NOg, and AO contributed to data collection. TSa, HK, MF, SN, and KK performed the statistical analysis and interpretation of the results. All authors read and approved the final manuscript.

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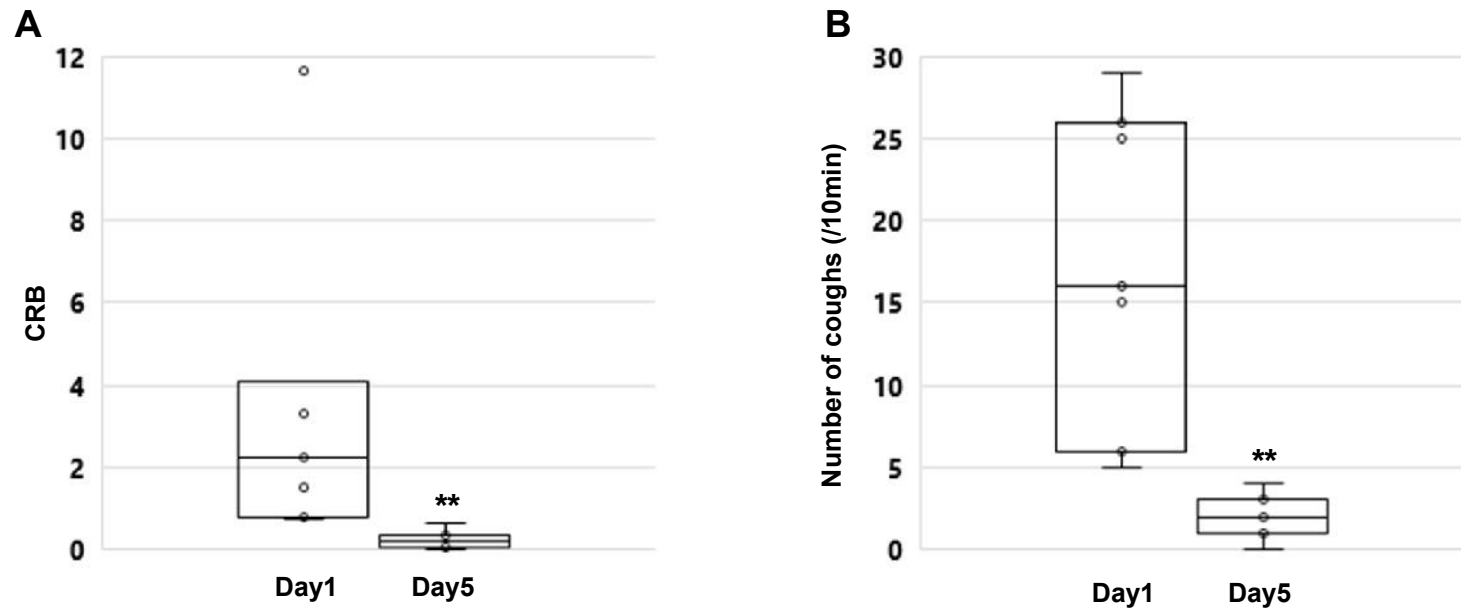
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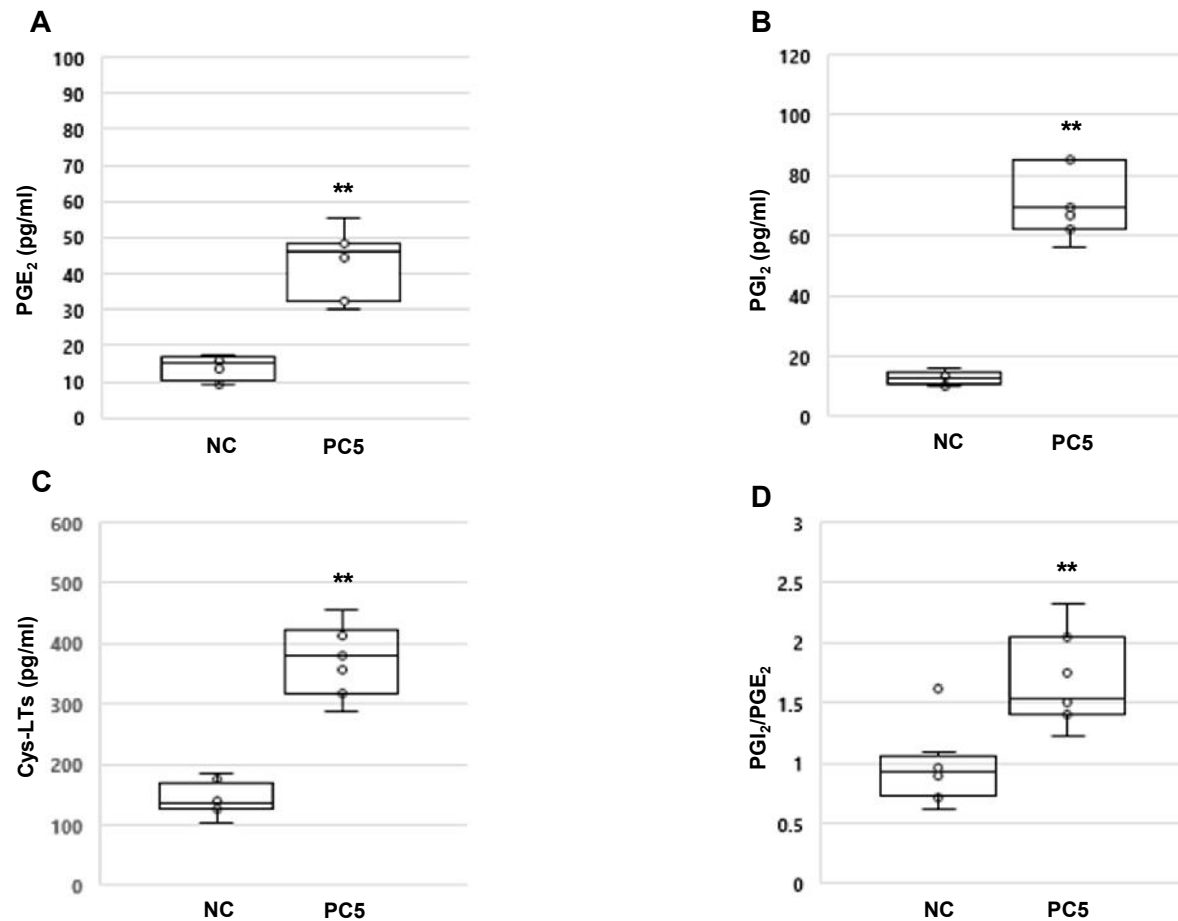
Supplementary Fig. 1. The relationship between Penh and the number of coughs induced by MCh inhalation.

There was a positive correlation between Penh and the number of coughs on Day 1 ($r = 0.59$, $P < 0.01$) (A), Day 2 ($r = 0.45$, $P < 0.01$) (B) and Day 3 ($r = 0.47$, $P < 0.01$) (C).



Supplementary Fig. 2. Five days repeated bronchoconstriction influenced CRB and the number of coughs induced by MCh inhalation.

CRB was significantly decreased on Day 5 compared with Day 1 (A). The number of coughs was also significantly decreased on Day 5 compared with Day 1 (B). **, $P < 0.01$ compared with Day 1. $n = 7$ animals in each day.



Supplementary Fig. 3. Five days repeated bronchoconstriction influenced the concentration of lipid mediators in BALF.

PGE₂ levels were significantly elevated in the PC5 group compared with the NC group (A). PGI₂ levels were significantly elevated in the PC5 group compared with the NC group (B). Cys-LTs levels were significantly elevated in the PC5 group compared with the NC group (C). The ratio of PGI₂/PGE₂ was significantly increased in the PC5 group compared with the NC group (D). NC group, non-bronchoconstriction group; PC5 group, 5 days bronchoconstriction group. **, P < 0.01 compared with NC group. n = 8 animals in the NC group; n = 7 animals in the PC5 group.