Effects of macrolides on antigen-induced increases in cough reflex sensitivity in guinea pigs

<Title page>

Effects of Macrolides on Antigen-Induced Increases in Cough Reflex Sensitivity in Guinea Pigs

Akira Tokuda,[a] Noriyuki Ohkura,[a] Masaki Fujimura,[a] Shiho Furusho,[a] Miki Abo,[a] and Nobuyuki Katayama[a]

[a]Respiratory Medicine, Cellular Transplantation Biology, Kanazawa University Graduate School of Medical Science

postal address: 13-1, Takara-machi, Kanazawa-city, Japan

E-mail:

Akira Tokuda; tokuda@med3.m.kanazawa-u.ac.jp

Noriyuki Ohkura; nori@med3.m.kanazawa-u.ac.jp

Masaki Fujimura; fujimura@med3.m.kanazawa-u.ac.jp

Shiho Furusho; sfurusho@med3.m.kanazawa-u.ac.jp

Miki Abo; m-abo@katou-hosp.jp

Nobuyuki Katayama; katayama@med3.m.kanazawa-u.ac.jp

Corresponding author:

Akira Tokuda, MD

Respiratory Medicine, Cellular Transplantation Biology

School of Medicine, Kanazawa University

Address: 13-1, Takara-machi, Kanazawa-city, Ishikawa, Japan

Postal code: 920-8641

Telephone: +81-76-265-2274

Fax: +81-76-234-4252

E-mail: tokuda@med3.m.kanazawa-u.ac.jp

<Abstract>

Background: Macrolides are antibiotics that have anti-inflammatory activities. Hence, they are used for both acute and chronic inflammatory airway diseases. However, the effects of these agents on allergic airway disorders presenting with an isolated chronic cough, such as non-asthmatic eosinophilic bronchitis and eosinophilic tracheobronchitis with cough hypersensitivity (atopic cough), still remain to be elucidated.

Objective: To determine if macrolides are effective in the management of chronic cough caused by eosinophilic airway inflammation.

Methods: The cough reflex sensitivity to inhaled capsaicin was measured at 48 h after challenge with an aerosolized antigen in actively sensitized guinea pigs. The 14-, 15- or 16-membered macrolides (erythromycin, azythromycin, or josamycin, respectively) were given intraperitoneally every 12 h after the antigen challenge. Bronchoalveolar lavage and the resection of the tracheal tissue were performed immediately after the measurement of the cough response to capsaicin.

Results: The antigen-induced increase in the number of coughs elicited by capsaicin inhalation was significantly reduced by treatments with erythromycin and azythromycin, but not with josamycin. Erythromycin dose-dependently inhibited the increases in the substance P, prostaglandin E_2 and leukotriene B_4 levels, but not the histamine levels, in the bronchoalveolar lavage fluid. However, erythromycin did not influence the antigen-induced decrease in the neutral endopeptidase (NEP) activity in the tracheal tissue.

Conclusions: Both 14- and 15-membered, but not 16-membered, macrolides

could reduce the antigen-induced cough reflex hypersensitivity by inhibiting the antigen-induced release of the afferent sensory nerve sensitizers. These macrolides may be therapeutically useful for the treatment of isolated chronic cough based on cough reflex hypersensitivity in allergic airway diseases such as non-asthmatic eosinophilic bronchitis and atopic cough.

Key words

macrolide antibiotics, cough response, eosinophilic airway inflammation, substance P, prostaglandin E_2, capsaicin

1. Introduction

Chronic cough is one of the most common and distressing symptoms of respiratory diseases. One of the most representative airway disorders responsible for chronic cough is eosinophilic bronchitis [1], including bronchial asthma, non-asthmatic eosinophilic bronchitis (NAEB) [2], atopic cough (AC) [3], and cough variant asthma (CVA) [4]. Coughs are caused by cough reflex hypersensitivity in NAEB and AC. Moreover, the cough reflex hypersensitivity in these patients, which is suggested to be associated with sensory neuropeptides and chemical mediators such as arachidonic acid metabolites [5-7], histamine [8] and platelet activating factor (PAF), can become normalized after successful treatment [9], such as with corticosteroids and antihistamines. However, some of these patients experience persisting cough despite high levels of these medications, and therefore, they are severely impaired in their quality of life. Unfortunately, no effective therapy for such patients has yet been clearly established. Although affecting only a small proportion of all NAEB and AC sufferers, this problem is therefore crucial for clinicians.

Macrolide antibiotics (herein referred to as ‘macrolides’) are a well-characterized family of spectrum antimicrobials that are used to treat acute bacterial infections. Moreover, it has been shown that 14- and 15-membered, but not 16-membered, macrolides [10] possess immunomodulatory and anti-inflammatory activities to inhibit migration of neutrophils, production of reactive oxygen radicals and mucus hypersecretion. Such properties other than the antibiotic actions have been well studied and applied to the therapy for chronic neutrophilic inflammatory airway diseases, including diffuse

panbronchiolitis (DPB) [11] and sinobronchial syndrome (SBS) [12]. In addition, the effects of macrolides on bronchial asthma have been investigated in recent clinical studies [13-16]. These studies demonstrated that, in bronchial asthma, although the effects of macrolides on bronchcoconstriction remain controversial, the symptom score, including cough, was observed to improve [13, 15] by treatment with these agents. However, there have so far not been any reports concerning the effects of macrolides on the cough reflex hypersensitivity associated with the eosinophilic inflammatory airway diseases, such as NAEB and AC.

In this study, we investigated the effects of erythromycin (EM), azythromycin (AZM) and josamycin (JM), the representative agents for 14-, 15-, and 16-membered macrolides, respectively, on cough reflex sensitivity to inhaled capsaicin in an animal model of airway allergic inflammation with cough reflex hypersensitivity. We also examined the effects on the levels of chemical mediators and substance P (SP) in the bronchoalveolar lavage fluid (BALF) and neutral endopeptidase (NEP) activity in the tracheal tissue.

2. Methods

2.1 Animals

Male, albino, Hartley-strain guinea pigs weighing 200 to 220 g were obtained from the Sankyou Laboratory Service (Toyama, Japan). They were then quarantined in the Animal Research Center of Kanazawa University. All animal procedures carried out during this study complied with the standards set out in the Guidelines for the Care and Use of Laboratory Animals at the Takara-machi Campus of Kanazawa University. The total n value in this study was 144; namely, 40 for Experiment A, 24 for Experiment B, 24 for Experiment C, 40 for Experiment D and 16 for Experiment E.

2.2 Study design

Three experimental groups (including Experiments A, B and C) were set at different times, respectively. Guinea pigs in each experimental group were in turn assigned to negative control (NC) groups, positive control (PC) groups and macrolides (EM for Experiment A, AZM for Experiment B or JM for Experiment C) groups (n = 8 for each group). The animals in the PC and macrolides groups were then actively sensitized and exposed to aerosolized antigens. In addition, the animals in the NC groups were exposed to aerosolized saline.

Every 12 h after the antigen challenge (total; four times), 2.5 ml/kg of EM solution at concentrations of 0.2, 2 or 20 mg/ml (final dose: 0.5, 5 or 50 mg/kg, respectively), AZM solution at a concentration of 20 mg/ml (final dose: 50 mg/kg) or JM solution at a concentration of 20 mg/ml (final dose: 50 mg/kg) were intraperitoneally administered to the animals in the macrolides groups. The

animals in PC and NC groups were given the intraperitoneal vehicle (2.5 ml/kg of distilled water). At 48 h after the antigen or saline challenge, the cough reflex sensitivity to inhaled capsaicin was measured.

After the measurement of the cough reflex sensitivity, bronchoalveolar lavage (BAL) was performed, and then the tracheal segments (80 to 120 mg each) were resected and isolated from the animals. However, to investigate the effects of EM on the substance P (SP) levels in the BAL fluid (BALF), another experimental group (Experiment D) set-up was needed to avoid the influence of capsaicin inhalation. The NC, PC and macrolides group in Experiment D were performed under the same conditions as those in Experiment A, but they were not subjected to capsaicin provocation before the performance of BAL (n =8 for each group).

In addition, to investigate the effects of EM on the normal cough reflex sensitivity, Experiment E was also newly developed. The guinea pigs in Experiment E were not sensitized and they received either 2.5 ml/kg of EM solution at a concentration of 20 mg/ml (final dose: 50 mg/kg) or distilled water in the same manner as that used for experiment A (n = 8, respectively). At 48 h after the first administration of either EM or vehicle, the cough reflex sensitivity to inhaled capsaicin was then measured.

2.3 Active sensitization

The guinea pigs were actively sensitized to ovalbumin (OA) using a modification of the method described by Muraki et al. [17]. Briefly, each animal was pretreated with the intraperitoneal administration of 30 mg/kg of

cyclophosphamide. Two days later, the animals were immunized with 2.0 mg of OA and 100 mg of aluminum hydroxide [$Al(OH)_3$]. A booster injection of 0.01 mg of OA and 100 mg of $Al(OH)_3$ was then carried out three weeks after the primary immunization. The naïve guinea pigs that were kept in the same surroundings as the active sensitization animals that were used for NC.

2.4 Antigen challenge.

Three weeks after the booster injection, the actively sensitized guinea pigs were intraperitoneally administered 20 mg/kg of diphenhydramine to avoid the onset of acute anaphylactic respiratory distress. Thirty minutes later, the conscious guinea pigs were challenged for 90 seconds with 10 mg/ml OA aerosol by a method described previously [6, 18, 19]. The animals in the NC group were not injected with diphenhydramine, but they did inhale aerosolized saline by the same method as that described for the sensitized guinea pigs. In addition, the cough response to inhaled capsaicin was measured 48 h after the antigen challenge.

2.5 Measurement of cough reflex sensitivity

Cough reflex sensitivity was measured by a method as described previously [6]. Briefly, each conscious guinea pig was placed in an airtight custom-built transparent plastic box consisting of a head chamber (volume 1600 ml) isolated from the body chamber. The pressure in the body chamber was recorded. Coughs were detected as a transient specific change in the pressure (a rapid inspiration followed by rapid expiration). To disregard the motion- and

sneezing-related changes in the pressure, we visually monitored the movements of the guinea pigs. Progressively increasing concentrations of the capsaicin solution (10^{-6}, 10^{-4} M) were inhaled by the animals for 2 min from a Devilbiss 646 nebulizer (Devilbiss Co., Somerset, PA, USA) operated by compressed air at 1.6 l/min (Iwaki Air Pump AP-115AN, Iwaki Co. Ltd, Tokyo, Japan). The nebulizer output was 0.037 ml/min. Coughs were counted by a trained observer and recognized by both the transient change in the pressure within the box and the characteristic animal posture. The number of coughs was counted during a 2-minute inhalation of each capsaicin solution and for an additional one-minute observation (n=8 in each group). In addition, we examined the effect of EM on the cough reflex sensitivity in the naïve guinea pigs.

2.6 Bronchoalveolar lavage (BAL)

BAL was performed immediately after analyzing the cough reflex sensitivity to capsaicin by a method as described previously [18, 19]. Briefly, the guinea pigs were anaesthetized by the intraperitoneal injection of 75 mg/kg of sodium pentobarbital and they were placed in a supine position. Through the tracheal cannula the lungs were lavaged with 10 ml of saline solution (0.15 mM NaCl) 2 times (total: 20 ml).

2.7 Bronchoalveolar lavage fluid (BALF) analysis

The cells in the BALF were immediately stained with Turk solution and counted in duplicate with a hemocytometer (in a Burker chamber). Different cell counts (analyzing 300 cells) were made on a smear prepared by cytocentrifugation and

stained with Wright-Giemsa. Subsequently, the BALF was centrifuged (1500 rpm for 10 min) and the supernatants were stored at -80 °C. For measurement of the SP levels in the BALF, we performed BAL and treated the BALF in another experimental set-up in which the guinea pigs were not subjected to capsaicin provocation at 48 h after the antigen challenge.

2.8 Concentration of SP, prostaglandin E_2, histamine, leukotriene B_4 and thromboxane B_2 in BALF

SP, prostaglandin E_2 (PGE_2), histamine, and leukotriene B_4 (LTB_4) levels in the BALF were determined by using a commercial enzyme immunoassay (EIA) kit. Thromboxane B_2 (TXB_2) levels in the BALF were measured by a commercial TXB_2 [^{125}I] radioimmunoassay (RIA) kit.

2.9 Neutral endopeptidase (NEP) activity of tracheal tissue

The removed trachea was soaked in saline and homogenized by an ultrasonic homogenizer (SONIFIER 250, Branson Ultrasonics, Danbury, CT). The sample was centrifuged at 5000 rpm for 2 minutes. The supernatant was used as a tracheal sample.

The NEP activity in the tracheal sample was determined by a two-step reaction method as described previously [20], using the substratesuccinyl-alanyl-alanyl-phenylalanyl-*para*-nitroanilide (Suc-Ala-Ala-Phe-*p* NA). Briefly, a total of 100 µl of the tracheal samples were incubated with Suc-Ala-Ala-Phe-*p* NA (final concentration: 4 mmol/l in Tris-HCl [Sigma], pH 7.4) as the substrate in the presence or absence of

phosphoramidon (final concentration: 2 μmol/l). The reaction (total volume: 250 μl) was measured in duplicate in a 96-well microtiter plate at room temperature. The increase in specific absorbance at 405 nm (as a result of the accumulation of free *p*-nitroaniline) was determined at several time points (0-24 h) using a plate reader (EAR 340AT, SLT-Labinstruments, Grödiq, Austria). Several concentrations of aminopeptidase solution were used to construct the standard curve. The NEP activity was determined as the activity that could be inhibited by phosphoramidon and it was expressed as units/mg in reference to the standard curve. One unit represents the enzyme activity that is able to separate 1 μmol/min *p* NA from Suc-Ala-Ala-Phe-*p* NA at pH 7.4 at 25 °C.

2.10 Reagent

The following chemicals were used: ovalbumin (Sigma, St. Louis, MO), erythromycin (Abbott Japan Co., Ltd., Tokyo, Japan), azythromycin (LKT Laboratories, Inc. St. Paul, MN), josamycin (Astellas Pharm. Inc., Tokyo, Japan), cyclophosphamide (Shionogi Co., Ltd., Osaka, Japan), aluminum hydroxide (Wako Pure Chemical Ind. Osaka, Japan), diphenhydramine (Wako Pure Chemical Ind.), saline (Otsuka Pharmaceutical Co., Ltd., Osaka, Japan), capsaicin (Sigma), sodium pentobarbital (Abbott laboratories, North Chicago, IL), SP emzyme immunoassay (EIA) kit (CAYMAN chemical company, MI, USA), PGE_2 EIA kit (R&D Systems, Minneapolis, MN), histamine EIA kit (Immunotech a.s., Praha, CR), LTB_4 EIA kit (CAYMAN chemical company), TXB_2 [^{125}I] RIA kit (NEN Life Science Products, Inc., Boston, USA), Suc-Ala-Ala-Phe-*p* NA (Sigma), Tris-HCl (Sigma), phosphoramidon (Sigma), and aminopeptidase (Sigma).

2.11 Statistical analysis

All the data are shown as the mean ± S.E.M. The differences among all groups for each experiment were analyzed using the Kruskal Wallis test. If the Kruskal Wallis test was positive ($P < 0.05$), then the differences between any pair of groups in each experiment were analyzed using the Mann-Whitney *U* test (StatView 5.0 software program, SAS Institute Inc., Cary, NC), with the level of significance set at $P < 0.05$.

3. Results

3.1 Effects of macrolides on cough reflex sensitivity

The number of coughs, induced by 1.0×10^{4} M of capsaicin, was significantly ($P < 0.01$) increased in the sensitized group that was administered distilled water (PC group) in comparison to the NC group (see Figure 1A, 1C and 1D). EM both significantly and dose-dependently inhibited the antigen-induced increase in the number of coughs ($P < 0.01$) (see Figure 1A). Although AZM also inhibited the antigen-induced increase in the number of coughs ($P < 0.05$) (see Figure 1C), JM showed no such inhibition (see Figure 1D).

The number of coughs induced by 1.0×10^{6} M of capsaicin did not significantly differ between the NC, PC and macrolides groups (EM, AZM, JM, respectively) (see Figure 1A, 1C and 1D).

EM did not affect the number of capsaicin-induced coughs in the naïve guinea-pigs (see Figure 1B).

3.2 Effects of macrolides on BALF cells

The total number of cells and the percentage of neutrophils and eosinophils in the BALF increased significantly in the PC group in comparison to the NC group ($P < 0.01$ and $P < 0.01$, respectively). EM inhibited the increase in the total number of cells dose-dependently ($P < 0.01$), whereas the percentage of neutrophils or eosinophils was unchanged (see Table 1A). Likewise, AZM also inhibited the increase in the total number of cells ($P < 0.05$), and did not influence the proportion of leukocytes in the BALF (see Table 1B). JM did not affect either the total number of cells or the proportion of leukocytes in the BALF

(see Table 1C).

3.3 Effects of EM on the concentrations of SP, PGE_2, LTB_4, histamine, and TXB_2 in the BALF

The concentration of SP in the BALF increased greatly in the PC group in comparison to the NC group ($P < 0.01$). EM significantly inhibited the increase in the SP levels in a dose dependent manner ($P < 0.05$) (see Figure 2A).

The PGE_2 levels also increased significantly in the BALF of the PC group in comparison to the NC group ($P < 0.05$). EM inhibited the antigen-induced PGE_2 increase in the BALF in a dose dependent manner ($P < 0.01$) (see Figure 2B).

The LTB_4 levels in the BALF increased significantly in the PC group in comparison to the NC group ($P < 0.05$). EM inhibited the antigen-induced LTB_4 increase in the BALF in a dose dependent manner ($P < 0.05$) (see Figure 2C).

The histamine levels also increased significantly in the BALF of PC group in comparison to the NC group ($P < 0.05$). EM did not significantly affect the antigen-induced histamine increase in the BALF (see Figure 2D).

The TXB_2 levels in the BALF did not increase in the PC group in comparison to the NC group, and EM did not alter the TXB_2 levels in the BALF (data not shown).

3.4 Effect of EM on NEP activity

NEP activity decreased significantly in the PC group in comparison to the NC group ($P < 0.05$). EM did not prevent the decrease in the NEP activity of the tracheal tissue following the antigen challenge (see Figure 3).

4. Discussion

Macrolides, especially 14- and 15-membered macrolides [10], have separate and distinct antibiotic and anti-inflammatory actions. Several reports have shown that 14- and 15-membered macrolides also affect the arachidonic acid cascade [21, 22], which is associated with an increase in the cough reflex sensitivity. However, the effects of macrolides on coughing resulting from cough reflex hypersensitivity, which is characteristic of NAEB and AC patients, have never been investigated. Therefore, in this study, we tested macrolides in an established experimental model of airway eosinophilic inflammation with cough reflex hypersensitivity [6]. The absence of the involvement of bronchoconstriction in the cough response to inhaled capsaicin in our study has been confirmed in our previous study which showed that the β2-adenoceptor agonist procaterol did not alter the number of coughs induced by inhaled capsaicin, while completely inhibiting the capsaicin-induced increase in airway resistance 24 h after an antigen challenge in this model [6].

In this study, the doses of macrolides were found to be significantly greater than those used to treat human diseases. These doses were required to define whether or not macrolides have the potential to inhibit cough reflex hypersensitivity, because they were the maximal doses of macrolides systemically administrated among previous *in vivo* studies [23-25] we searched. The present study showed for the first time that EM, a 14-membered macrolide, dose-dependently inhibited the increase in the cough reflex sensitivity to inhaled capsaicin and the total number of cells in the BALF that developed 48 h after antigen challenge in the actively sensitized guinea pigs, but not in the naïve

animals. Likewise, AZM, a 15-membered macrolide, also reduced the antigen-induced increase in the cough reflex sensitivity and the total number of cells in the BALF. In contrast, JM, a 16-membered macrolide, did not influence either the antigen-induced increase in the cough reflex sensitivity or the total number of cells in the BALF. The analysis of the BALF demonstrated that EM dose-dependently reduced the antigen-induced increase in SP, PGE_2 and LTB_4, but not the TXB_2 or histamine, levels in the BALF. Moreover, the inhibitory effect of EM on the antigen-induced increase in the SP levels in BALF was not followed by the restoration of the antigen-induced decrease in NEP, a major SP-degrading enzyme, activity in the tracheal tissue. This result indicates that EM reduced the release of SP, while not recovering the ability of SP degradation.

Coughing is initiated when the afferent nerve impulse activity is increased. Several findings suggest that the capsaicin-induced cough is mediated mainly by the activation of the transient receptor potential vanilloid type channel-1 (TRPV1), a capsaicin receptor on the terminal of the nonmyelinated C-fibers which subsequently release the sensory neuropeptides, such as tachykinins (SP, neurokinin A, and neurokinin B). These tachykinins have been known to act on NK receptors on the afferent nerves, denoted NK_1, NK_2 and NK_3, which have the highest affinity for SP, neurokinin A and neurokinin B, respectively, to mediate many airway responses, including gland secretion and increased vascular permeability. However, the precise role of SP in the peripheral initiation of the cough reflex still remains controversial. Several studies have shown that the exposure of guinea pigs to SP or NEP inhibitor induced a cough response [26, 27], while other controversial data have been obtained about the influence of

aerosolized SP, which induced no direct effect on cough reflex sensitivity of guinea pigs [28] or pigs [29]. On the other hand, Katsumata and coworkers [30] have reported that inhaled SP induced cough in patients with upper airway infection, but not in healthy subjects. In addition, it has been shown that both the NK_1 receptor antagonist FK888 and the dual NK_1 and NK_2 peptide receptor antagonists FK224 significantly inhibited the increase in cough response to inhaled capsaicin in different types of guinea pig models of cough with allergic airway inflammation [31-33]. In our laboratory, we previously showed in the actively sensitized guinea pigs that the increase in the cough reflex sensitivity after an antigen challenge is correlated with increase in the SP levels in the BALF [18] and the decrease in the NEP activity in the tracheal tissue [19], which are consistent with the results in the present study. Taken together, these findings suggest that the role of peripheral SP in cough reflex sensitivity may be different between species and/or employed experimental systems, especially presence of airway inflammation. We believe that at least the antigen-induced increase in the cough response to capsaicin is associated with the results from the increase of SP. Furthermore, the findings that EM reduced SP in BALF and the increase of the cough reflex sensitivity following antigen challenge are therefore considered to support our standpoints.

In addition, we also found in this study that EM dose-dependently inhibited the antigen-induced release of PGE_2 in the BALF (as is shown in a previous study [34]) and this was correlated with the decrease in cough reflex sensitivity. PGE_2 is a cyclooxygenase (COX)-2 metabolite of arachidonic acid released from the airway epithelium and smooth muscles during airway inflammatory reaction

including NAEB [7]. There are several reports demonstrating that the number of capsaicin-induced coughs is increased in the presence of prostaglandins in the airway [35, 36], furthermore, several other lines of evidence indicate that PGE_2 in the airway affects the cough reflex sensitivity via enhancing the sensitivity of the C-fibers to capsaicin, but does not directly stimulate the afferent fibers to induce cough [5, 37, 38]. The results that EM reduced the PGE_2 levels in BALF and cough reflex sensitivity simultaneously support the sensitizing effect of PGE_2 on C-fibers to capsaicin.

The increased vascular permeability and the subsequent interstitial airway edema evoked by the released SP and PGE_2 in the airways [39] may also stimulate and activate the afferent sensory nerves [40]. Our result, in which the antigen-induced increase in the total number of cells, but not cell components, in the BALF was decreased by the treatment with EM, suggests the suppressive effects of EM on the antigen-induced increase of vascular permeability via SP or PGE_2.

However, in this study, even though EM had a maximal anti-tussive effect at 5 mg/kg, there was no difference between the animals treated at this dose of EM and PC group with respect to SP or PGE_2, with the only difference in LTB_4. LTB_4 is a lipoxygenase metabolite of arachidonic acid, which is also released during airway inflammation similar to PGE_2. Although the effects of EM on LTB_4 in neutrophilic inflammatory reaction have been shown in several studies, in the present study we showed for the first time that EM inhibited the increase of LTB_4 in the eosinophilic inflammatory reaction, while the observed reduction of LTB4 by EM was correlated with the number of coughs. This result suggests that LTB_4

rather than SP and PGE_2 may thus be involved in the effect of EM on the cough reflex sensitivity in this model. A recent study showed that LTB_4 directly activates TRPV1 and promotes the release of SP from the C-fibers *in vitro* [41]. However, as far as we could determine based on an extensive literature search, there are no reports concerning the role of LTB_4 in the cough reflex hypersensitivity associated with airway eosinophilic inflammation. Further studies are necessary to elucidate the relationship between LTB_4 and cough reflex sensitivity.

On the other hand, we also measured the histamine levels in BALF. Histamine is a biogenic amin that is mainly released from mast cells and basophils. Moreover, it is considered to be a potent and direct activator of the afferent sensory nerves [8] and, therefore, the use of antihistamines has been recommended for the treatment of AC, thus resulting in the normalization of the cough reflex sensitivity. Our results showed that the antigen-induced increase in the histamine levels in the BALF was not affected by the administration of EM, despite a normalization of the cough reflex sensitivity. These results suggest that EM and antihistamine normalize the cough reflex sensitivity by different mechanisms. Therefore, the additional administration of EM may be effective in the treatment of atopic cough when the antihistamines alone are insufficient. Further studies are necessary before this possibility can be established with greater certainty.

5. Conclusions

The present study clearly showed that 14- and 15-membered, but not 16-membered, macrolides have a potent antitussive effect in antigen-induced

eosinophilic airway inflammation. This antitussive action of EM may result from the suppressive effect of EM on the afferent sensory nerves by inhibiting antigen-induced release of afferent sensory nerve sensitizers, such as LTB_4 and partly SP and PGE_2 in the airways and by decreasing the subsequent vascular permeability. These results suggest that 14- and 15-membered macrolides may provide a therapeutic benefit in patients with allergic airway disorders, in which the cough reflex sensitivity is observed to be increased, such as non-asthmatic eosinophilic bronchitis and atopic cough.

<Acknowledgement>

This study was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports Science and Technology – Japan (No. 20590916).

<References>

1. Brightling CE, Ward R, Wardlaw AJ, Pavord ID, Airway inflammation, airway responsiveness and cough before and after inhaled budesonide in patients with eosinophilic bronchitis. Eur Respir J 2000;15: 682-6.
2. Gibson PG, Dolovich J, Denburg J, Ramsdale EH, Hargreave FE, Chronic cough: eosinophilic bronchitis without asthma. Lancet 1989;1: 1346-8.
3. Fujimura M, Ogawa H, Nishizawa Y, Nishi K, Comparison of atopic cough with cough variant asthma: is atopic cough a precursor of asthma? Thorax 2003;58: 14-8.
4. Corrao WM, Braman SS, Irwin RS, Chronic cough as the sole presenting manifestation of bronchial asthma. N Engl J Med 1979;300: 633-7.
5. Choudry NB, Fuller RW, Pride NB, Sensitivity of the human cough reflex: effect of inflammatory mediators prostaglandin E2, bradykinin, and histamine. Am Rev Respir Dis 1989;140: 137-41.
6. Liu Q, Fujimura M, Tachibana H, Myou S, Kasahara K, Yasui M, Characterization of increased cough sensitivity after antigen challenge in guinea pigs. Clin Exp Allergy 2001;31: 474-84.
7. Sastre B, Fernandez-Nieto M, Molla R, Lopez E, Lahoz C, Sastre J, del Pozo V, Quirce S, Increased prostaglandin E2 levels in the airway of patients with eosinophilic bronchitis. Allergy 2008;63: 58-66.
8. Kamei J, Takahashi Y, Involvement of ionotropic purinergic receptors in the histamine-induced enhancement of the cough reflex sensitivity in guinea pigs. Eur J Pharmacol 2006;547: 160-4.
9. Fujimura M, Kamio Y, Hashimoto T, Matsuda T, Cough receptor sensitivity

and bronchial responsiveness in patients with only chronic nonproductive cough: in view of effect of bronchodilator therapy. J Asthma 1994;31: 463-72.

10. Rubin BK, Immunomodulatory properties of macrolides: overview and historical perspective. Am J Med 2004;117 Suppl 9A: 2S-4S.

11. Kudoh S, Uetake T, Hagiwara K, Hirayama M, Hus LH, Kimura H, Sugiyama Y, [Clinical effects of low-dose long-term erythromycin chemotherapy on diffuse panbronchiolitis]. Nihon Kyobu Shikkan Gakkai Zasshi 1987;25: 632-42.

12. Fujimura M, Ishiura Y, Saito M, Shibata K, Nomura M, Nakatsumi Y, Matsuda T, Additive effect of continuous low dose ofloxacin on erythromycin therapy for sinobronchial syndrome. Drugs 1995;49 Suppl 2: 409-11.

13. Amayasu H, Yoshida S, Ebana S, Yamamoto Y, Nishikawa T, Shoji T, Nakagawa H, Hasegawa H, Nakabayashi M, Ishizaki Y, Clarithromycin suppresses bronchial hyperresponsiveness associated with eosinophilic inflammation in patients with asthma. Ann Allergy Asthma Immunol 2000;84: 594-8.

14. Kostadima E, Tsiodras S, Alexopoulos EI, Kaditis AG, Mavrou I, Georgatou N, Papamichalopoulos A, Clarithromycin reduces the severity of bronchial hyperresponsiveness in patients with asthma. Eur Respir J 2004;23: 714-7.

15. Shoji T, Yoshida S, Sakamoto H, Hasegawa H, Nakagawa H, Amayasu H, Anti-inflammatory effect of roxithromycin in patients with aspirin-intolerant

asthma. Clin Exp Allergy 1999;29: 950-6.

16. Simpson JL, Powell H, Boyle MJ, Scott RJ, Gibson PG, Clarithromycin targets neutrophilic airway inflammation in refractory asthma. Am J Respir Crit Care Med 2008;177: 148-55.

17. Muraki M, Tohda Y, Sugihara R, Nagasaka Y, Nakajima S, The effect of TYB-2285 on dual phase bronchoconstriction and airway hypersensitivity in guinea-pigs actively sensitized with ovalbumin. J Pharm Pharmacol 1994;46: 883-6.

18. Hara J, Fujimura M, Myou S, Oribe Y, Furusho S, Kita T, Katayama N, Abo M, Ohkura N, Herai Y, Hori A, Ishiura Y, Nobata K, Ogawa H, Yasui M, Kasahara K, Nakao S, Comparison of cough reflex sensitivity after an inhaled antigen challenge between actively and passively sensitized guinea pigs. Cough 2005;1: 6.

19. Katayama N, Fujimura M, Ueda A, Kita T, Abo M, Tachibana H, Myou S, Kurashima K, Effects of carbocysteine on antigen-induced increases in cough sensitivity and bronchial responsiveness in guinea pigs. J Pharmacol Exp Ther 2001;297: 975-80.

20. Van Der Velden VH, Naber BA, Van Hal PT, Overbeek SE, Hoogsteden HC, Versnel MA, Peptidase activities in serum and bronchoalveolar lavage fluid from allergic asthmatics--comparison with healthy non-smokers and smokers and effects of inhaled glucocorticoids. Clin Exp Allergy 1999;29: 813-23.

21. Ianaro A, Ialenti A, Maffia P, Sautebin L, Rombola L, Carnuccio R, Iuvone T, D'Acquisto F, Di Rosa M, Anti-inflammatory activity of macrolide

antibiotics. J Pharmacol Exp Ther 2000;292: 156-63.

22. Tamaoki J, Kadota J, Takizawa H, Clinical implications of the immunomodulatory effects of macrolides. Am J Med 2004;117 Suppl 9A: 5S-11S.

23. Azoulay-Dupuis E, Mohler J, Bedos JP, Barau C, Fantin B, Efficacy of cethromycin, a new ketolide, against Streptococcus pneumoniae susceptible or resistant to erythromycin in a murine pneumonia model. Antimicrob Agents Chemother 2006;50: 3033-8.

24. Yanagihara K, Morinaga Y, Nakamura S, Seki M, Izumikawa K, Kakeya H, Yamamoto Y, Yamada Y, Kamihira S, Kohno S, Subinhibitory concentrations of telithromycin, clarithromycin and azithromycin reduce methicillin-resistant Staphylococcus aureus coagulase in vitro and in vivo. J Antimicrob Chemother 2008;61: 647-50.

25. Yanagihara K, Tomono K, Imamura Y, Kaneko Y, Kuroki M, Sawai T, Miyazaki Y, Hirakata Y, Mukae H, Kadota J, Kohno S, Effect of clarithromycin on chronic respiratory infection caused by Pseudomonas aeruginosa with biofilm formation in an experimental murine model. J Antimicrob Chemother 2002;49: 867-70.

26. Kohrogi H, Graf PD, Sekizawa K, Borson DB, Nadel JA, Neutral endopeptidase inhibitors potentiate substance P- and capsaicin-induced cough in awake guinea pigs. J Clin Invest 1988;82: 2063-8.

27. Takahama K, Fuchikami J, Isohama Y, Kai H, Miyata T, Neurokinin A, but not neurokinin B and substance P, induces codeine-resistant coughs in awake guinea-pigs. Regul Pept 1993;46: 236-7.

28. El-Hashim AZ, Amine SA, The role of substance P and bradykinin in the cough reflex and bronchoconstriction in guinea-pigs. Eur J Pharmacol 2005;513: 125-33.

29. Moreaux B, Nemmar A, Vincke G, Halloy D, Beerens D, Advenier C, Gustin P, Role of substance P and tachykinin receptor antagonists in citric acid-induced cough in pigs. Eur J Pharmacol 2000;408: 305-12.

30. Katsumata U, Sekizawa K, Inoue H, Sasaki H, Takishima T, Inhibitory actions of procaterol, a beta-2 stimulant, on substance P-induced cough in normal subjects during upper respiratory tract infection. Tohoku J Exp Med 1989;158: 105-6.

31. Ogawa H, Fujimura M, Saito M, Matsuda T, Akao N, Kondo K, The effect of the neurokinin antagonist FK-224 on the cough response to inhaled capsaicin in a new model of guinea-pig eosinophilic bronchitis induced by intranasal polymyxin B. Clin Auton Res 1994;4: 19-28.

32. Sekizawa K, Ebihara T, Sasaki H, Role of substance P in cough during bronchoconstriction in awake guinea pigs. Am J Respir Crit Care Med 1995;151: 815-21.

33. Xiang A, Uchida Y, Nomura A, Iijima H, Dong F, Zhang MJ, Hasegawa S, Effects of airway inflammation on cough response in the guinea pig. J Appl Physiol 1998;85: 1847-54.

34. Tamaoki J, Isono K, Sakai N, Kanemura T, Konno K, Erythromycin inhibits Cl secretion across canine tracheal epithelial cells. Eur Respir J 1992;5: 234-8.

35. Fujimura M, Kamio Y, Kasahara K, Bando T, Hashimoto T, Matsuda T,

Prostanoids and cough response to capsaicin in asthma and chronic bronchitis. Eur Respir J 1995;8: 1499-505.

36. Shinagawa K, Kojima M, Ichikawa K, Hiratochi M, Aoyagi S, Akahane M, Participation of thromboxane A(2) in the cough response in guinea-pigs: antitussive effect of ozagrel. Br J Pharmacol 2000;131: 266-70.

37. Jia Y, Lee LY, Role of TRPV receptors in respiratory diseases. Biochim Biophys Acta 2007;1772: 915-27.

38. Kwong K, Lee LY, PGE(2) sensitizes cultured pulmonary vagal sensory neurons to chemical and electrical stimuli. J Appl Physiol 2002;93: 1419-28.

39. McDonald DM, Mitchell RA, Gabella G, Haskell A, Neurogenic inflammation in the rat trachea. II. Identity and distribution of nerves mediating the increase in vascular permeability. J Neurocytol 1988;17: 605-28.

40. Bonham AC, Kott KS, Ravi K, Kappagoda CT, Joad JP, Substance P contributes to rapidly adapting receptor responses to pulmonary venous congestion in rabbits. J Physiol 1996;493 (Pt 1): 229-38.

41. Hwang SW, Cho H, Kwak J, Lee SY, Kang CJ, Jung J, Cho S, Min KH, Suh YG, Kim D, Oh U, Direct activation of capsaicin receptors by products of lipoxygenases: endogenous capsaicin-like substances. Proc Natl Acad Sci U S A 2000;97: 6155-60.

Table 1 Effects of EM (A), AZM (B) and JM (C) on cellular accumulation in BALF 48 h after challenge with an antigen in actively sensitized guinea pigs.

	Total cells ($\times 10^4$ cells/mL)	Percentage (%)			
		Mac	Neu	Lym	Eos
A					
Negative control	18.1 ± 0.3	84.6 ± 3.9	0.3 ± 0.2	3.1 ± 0.3	12.1 ± 3.9
Positive control	112.9 ±1.0$^{\#}$	27.5 ± 2.0$^{\#}$	6.0 ± 0.9$^{\#}$	2.7 ± 0.7	63.8 ± 2.6$^{\#}$
EM 0.5 mg/kg	80.8 ± 0.9†	31.3 ± 3.2	10.4 ± 1.6†	2.4 ±0.6	55.8 ± 4.2
EM 5 mg/kg	73.1 ± 0.7‡	30.8 ± 3.2	8.3 ± 1.0	2.0 ± 0.4	58.8 ± 3.7
EM 50 mg/kg	50.6 ± 0.8‡	27.8 ± 1.2	6.3 ± 1.0	1.7 ± 0.3	64.2 ± 1.9
B					
Negative control	16.5 ± 0.3	89.8 ± 2.4	0.1 ± 0.1	3.2 ± 0.3	7.0 ± 2.6
Positive control	91.5 ± 1.5$^{\#}$	33.8 ± 3.7$^{\#}$	7.2 ± 2.1$^{\#}$	2.8 ± 0.7	56.2 ± 3.8$^{\#}$
AZM 50 mg/kg	31.1 ± 0.5‡	40.5 ± 3.9	6.4 ± 1.0	1.9 ± 0.6	51.2 ± 3.8
C					
Negative control	18.1 ± 0.3	84.3 ± 5.0	0.4 ± 0.3	3.1 ± 0.3	12.3 ± 5.0
Positive control	67.2 ± 0.7$^{\#}$	27.9 ± 2.4$^{\#}$	3.9 ± 0.6$^{\#}$	2.9 ± 0.5	65.2 ± 2.5$^{\#}$
JM 50 mg/kg	71.2 ± 0.7	26.5 ± 2.3	6.0 ± 1.7	1.4 ± 0.3†	66.5 ± 3.0

Negative control, the animals received a challenge with saline and were intraperitoneally administered saline; Positive control, the animals received a challenge with OA and were intraperitoneally administered saline; Mac, macrophages; Neu, neutrophils; Lym, lymphocytes; Eos, eosinophils; $^{\#}P < 0.01$ vs. negative control; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ vs. positive control, respectively. Experiments A, B and C are

independent experiments, respectively.

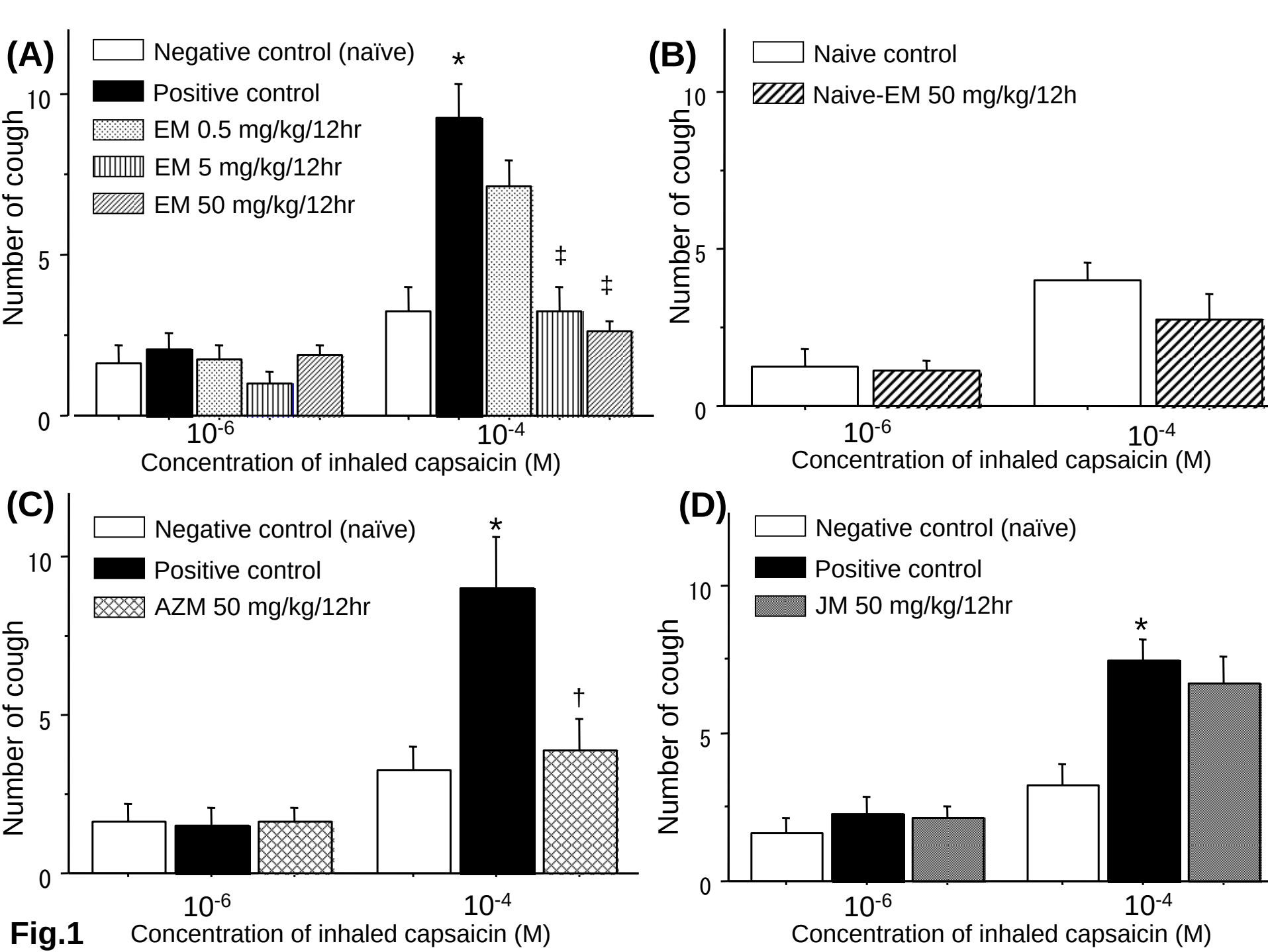
(A)
Negative control (naïve)
Positive control
EM 0.5 mg/kg/12hr
EM 5 mg/kg/12hr
EM 50 mg/kg/12hr
*
‡
‡
Number of cough
10
5
0
10-6
10-4
Concentration of inhaled capsaicin (M)
(B)
Naive control
Naive-EM 50 mg/kg/12h
Number of cough
10
5
0
10-6
10-4
Concentration of inhaled capsaicin (M)
(C)
Negative control (naïve)
Positive control
AZM 50 mg/kg/12hr
*
†
Number of cough
10
5
0
10-6
10-4
Concentration of inhaled capsaicin (M)
(D)
Negative control (naïve)
Positive control
JM 50 mg/kg/12hr
*
Number of cough
10
5
0
10-6
10-4
Concentration of inhaled capsaicin (M)

Fig.1

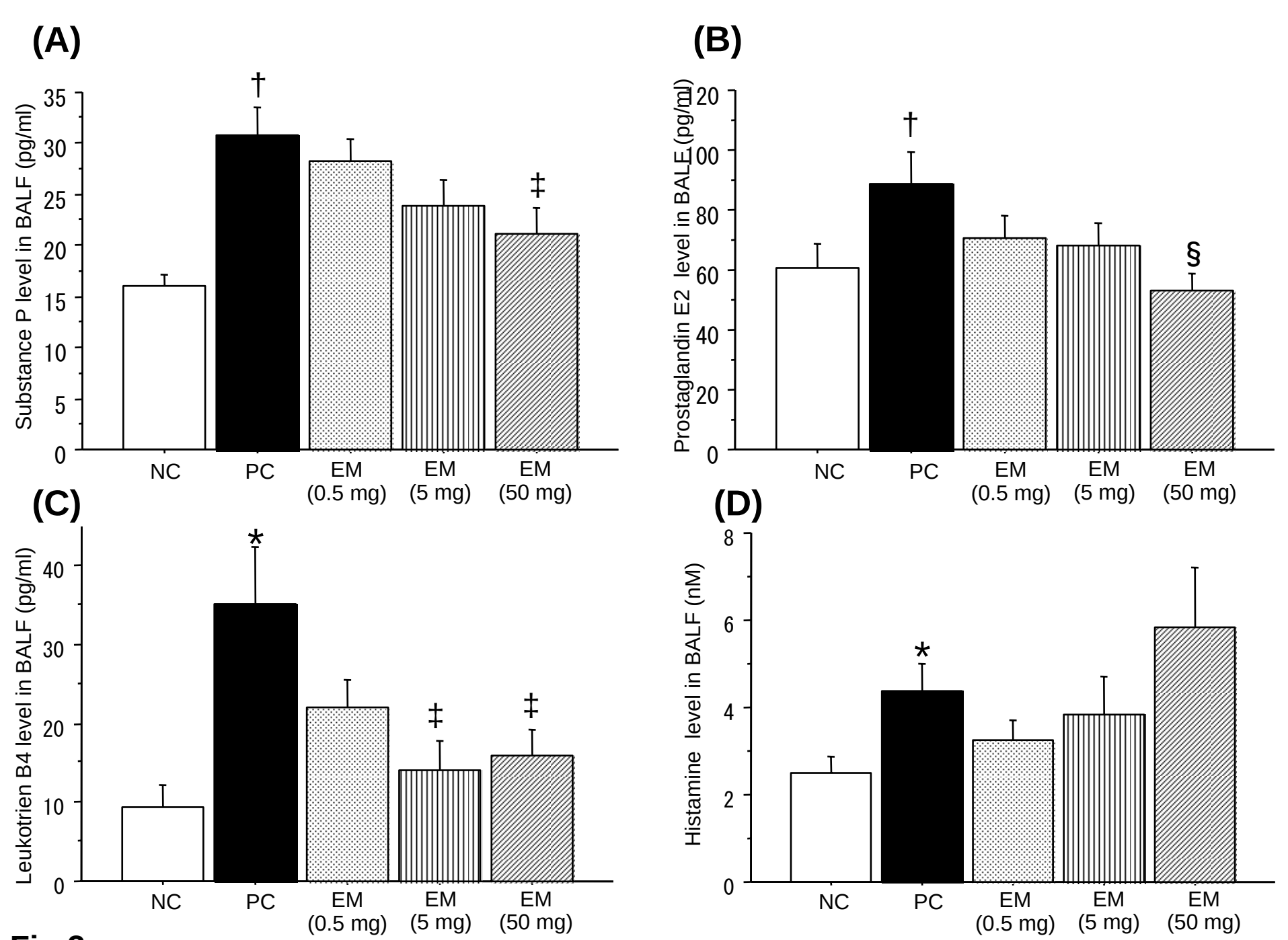
(A)
Substance P level in BALF (pg/ml)
35
30
25
20
15
10
5
0
†
‡
NC
PC
EM (0.5 mg)
EM (5 mg)
EM (50 mg)
(B)
Prostaglandin E2 level in BALF (pg/ml)
120
100
80
60
40
20
0
†
§
NC
PC
EM (0.5 mg)
EM (5 mg)
EM (50 mg)
(C)
Leukotrien B4 level in BALF (pg/ml)
40
30
20
10
0
*
‡
‡
NC
PC
EM (0.5 mg)
EM (5 mg)
EM (50 mg)
(D)
Histamine level in BALF (nM)
8
6
4
2
0
*
NC
PC
EM (0.5 mg)
EM (5 mg)
EM (50 mg)

Fig.2

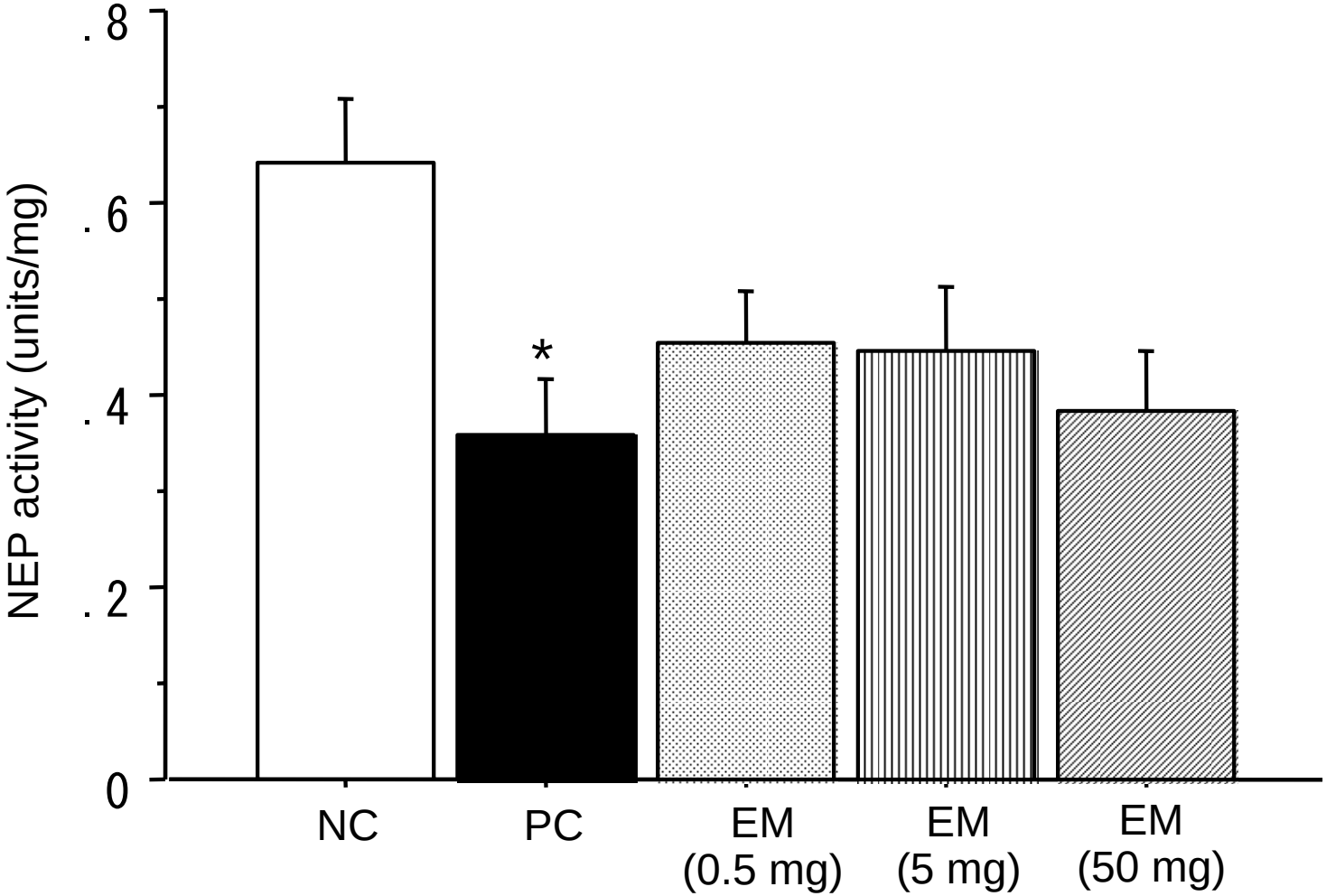

Fig.3