

Characterization of increased cough sensitivity after antigen challenge in guinea pigs

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Summary

Background Increased sensitivity of cough reflex is a fundamental feature of bronchodilator resistant non-productive cough associated with eosinophilic tracheobronchitis. Our hypothesis is that cough sensitivity is increased by airway allergic reaction characterized by airway eosinophilic inflammation. The aim of this study was to elucidate the hypothesis and clarify the characteristics of the increased cough sensitivity.

Materials and methods Number of coughs elicited by inhalation of increasing concentrations of capsaicin (10^{-8} , 10^{-6} and 10^{-4} M) was counted 24 h after an aerosolized antigen or saline in actively sensitized or non-sensitized (naïve) conscious guinea pigs and then bronchoalveolar lavage was performed. The cough response was also measured 1 day before and 1, 2, 3, 5 and 7 days after an aerosolized antigen challenge in sensitized or naïve animals. In addition, effect of procaterol (0.1 mg/kg), atropine (1 or 10 mg/kg), phosphoramidon (2.5 mg/kg) given intraperitoneally 30 min before the capsaicin challenge or capsaicin desensitization on the cough response was examined. Furthermore, the thromboxane A₂ (TXA₂) receptor antagonist S-1452 in a dose of 0.01 or 0.1 mg/kg or vehicle (saline) was given intraperitoneally at 24 and 1 h before the measurement of cough response.

Results Number of coughs caused by capsaicin was extremely increased 24 h after an antigen challenge in sensitized guinea pigs compared with a saline or an antigen challenge in naïve animals or a saline challenge in sensitized animals. The increased cough response disappeared at 3–7 days after the antigen challenge. Eosinophils in bronchoalveolar lavage fluid obtained after the measurement of capsaicin-induced coughs, which was performed 24 h after the antigen challenge, were significantly increased in sensitized guinea pigs. The eosinophil count was significantly correlated to the number of capsaicin-induced coughs. Procaterol or atropine did not alter the antigen-induced increase of cough sensitivity, whereas atropine did reduce the cough response in naïve animals. Phosphoramidon increased the number of capsaicin-induced coughs in naïve guinea pigs but not in sensitized and antigen-challenged animals. Capsaicin desensitization decreased the cough response in both antigen-challenged sensitized guinea pigs and naïve animals. S-1452 reduced the antigen-induced increase of cough response in sensitized guinea pigs, but not in naïve animals.

Answer to the question Airway allergy accompanied with airway eosinophilia induces transient increase in cough sensitivity, which is not mediated by bronchoconstriction. The increased cough sensitivity may result in part from inactivation of neutral endopeptidase and TXA₂, one of the inflammatory mediators.

Keywords: airway allergy, capsaicin desensitization, cough sensitivity, guinea pigs, phosphoramidon, thromboxane A₂

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Introduction

Increased cough sensitivity is a fundamental pathophysiology of some conditions presenting with non-productive cough, such as cough induced by inhibitors of angiotensin-converting enzyme [1], idiopathic dry, unproductive cough [1], and gastroesophageal reflux-associated cough [2,3]. Furthermore, our series of clinical studies [4–6] have proposed another clinical entity presenting with chronic bronchodilator-resistant non-productive cough in which eosinophilic tracheobronchitis and increased cough sensitivity are the pathological and physiological fundamental features, respectively. Recently we [7–9] reported that a single inhalation challenge with environmental fungal antigen extract caused non-productive cough and increased cough sensitivity to inhaled capsaicin in patients with the eosinophilic tracheobronchitis. From these findings we hypothesized that a single antigen challenge could cause an increase in cough sensitivity associated with airway eosinophilic inflammation.

Xiang *et al.* [10] reported that multiple antigen challenge increased cough response to capsaicin in sensitized guinea pigs. However, it has been unknown whether the antigen-induced increase in cough response results from increased cough sensitivity or increased bronchial responsiveness to inhaled capsaicin. In addition, the time course of the cough response after the antigen challenge is unknown. A single antigen challenge is useful to elucidate the time course of cough sensitivity.

For the above mentioned reasons we designed the present study to characterize cough sensitivity after a single aerosolized antigen challenge. We examined the time course of cough response and the effect of bronchodilators, the β_2 -adrenoceptor agonist procaterol and the anticholinergic drug atropine, on the cough response to inhaled capsaicin after a single antigen challenge in actively sensitized guinea pigs in comparison with non-sensitized (naïve) animals. We also studied the effects of the neutral endopeptidase inhibitor phosphoramidon and capsaicin desensitization on the cough response to elucidate the role of neutral endopeptidase and sensory neuropeptides in the antigen-induced increase in cough response. Furthermore, we investigated the effect of the thromboxane A₂ (TXA₂) antagonist S-1452 on the cough response to examine the involvement of TXA₂, which is one of inflammatory mediators.

Materials and methods

Animals

Male, albino, Hartley strain guinea pigs weighing 200–250 g were obtained from Sankyo Laboratory Service

(Toyama, Japan). They were quarantined in the Animal Research Center of Kanazawa University for 1 week before the study. All the animal procedures in this study complied with the standards set out in the Guidelines for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University.

Sensitization of animals

Guinea pigs were actively sensitized by the method reported by Muraki *et al.* [11]. Guinea pigs were intraperitoneally administered 2.0 mg of ovalbumin (OA) and 100 mg of aluminium hydroxide (AL(OH)₃) 2 days after an intraperitoneal administration of 30 mg/kg of cyclophosphamide. Boosting was carried out by intraperitoneal administration of 0.01 mg of OA and 100 mg of AL(OH)₃ 3 weeks later.

Antigen challenge

Actively sensitized or naïve guinea pigs were challenged with an aerosolized OA or physiological saline solution for 60 s under spontaneous breathing 3 weeks after the boosting. Conscious guinea pigs were placed in a dual chamber plethysmograph (head chamber volume, 1520 mL) (Model PMUA + SAR, Buxco Electronics, Sharon, CT, USA). Animals were challenged with a 10-mg/mL OA or saline aerosol (head chamber only, 60 s, 0.08 mL/min output). The aerosol was generated by a Devilbiss 646 nebulizer (Devilbiss Co., Somerset, Pennsylvania, USA) operated by compressed air at 7.57 L/min (Minipon 54B-588, Origin Medical Industry Co. Ltd, Tokyo, Japan).

Measurement of cough response

Each conscious guinea pig was placed in an airtight transparent plastic box consisting of a head chamber (volume 1600 mL) isolated from a body chamber and pressure in the body chamber was recorded. Coughs were detected as a transient change in the pressure (a rapid inspiration followed by rapid expiration). To disregard the motion- and sneezing-related changes in the pressure, movements of the guinea pigs were visually monitored.

Capsaicin (30.5 mg) was dissolved in Tween 80 (1 mL) and ethanol (1 mL) and then dissolved in physiological saline (8 mL) to make a stock solution of 10^{-2} M, which was stored at -20°C . This solution was diluted with physiological saline to make solutions of 10^{-8} , 10^{-6} and $>10^{-4}$ M. Each conscious guinea pig was exposed to a nebulized control solution of physiological saline followed by progressively increasing concentrations of the capsaicin solution. Solutions were inhaled for 2 min every 8 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. The number of coughs was counted during a 2-min inhalation of each capsaicin solution and during a subsequent 1-min observation time.

Measurement of bronchoconstrictor response

Animals were placed in a whole body, double chamber plethysmograph to allow measurement of specific airway resistance ($sRaw$ = airway resistance $\times$ thoracic gas volume; units = $\text{cmH}_2\text{O}\cdot\text{s}$) with a modified version of the method described by Pennock et al. [12]. A respiratory mechanics analyser (Model PMUA + SAR, Buxco Electronics, Troy, NY) was used to measure the phase shift between nasal and thoracic air flows and compute $sRaw$ on a breath by breath basis. A bias flow of air (3 L/min) was maintained through the nasal chamber to ensure a constant supply of fresh air to the animals. This flow was subtracted by the respiratory mechanics analyser from the calculations.

In a preliminary study to examine the time course of capsaicin-induced bronchoconstrictor response, conscious sensitized guinea pigs were placed in the dual chamber plethysmograph for measurement of $sRaw$ before and immediately after completion of a 2-min inhalation of 10^{-4} M capsaicin solution using the above mentioned apparatus for measurement of cough response which was carried out 24 h after an OA challenge. $sRaw$ was measured before and 3–20 min after starting the capsaicin provocation. As the peak response was seen 5 min after the starting of capsaicin provocation or 2 min after the completion of measurement of cough response, $sRaw$ was measured 1–5 min after the completion of measurement of cough response in this study. Namely, conscious animals were placed in the dual chamber plethysmograph for non-invasive measurement of $sRaw$ before and 25 min after administration of each tested drug and after the completion of measurement of capsaicin-induced cough following the drug administration. The $sRaw$ was measured before and 30 min after the drug administration and 1–5 min after completion of the measurement of cough response under spontaneous breathing. Peak $sRaw$ value between 1 and 5 min after the capsaicin provocation was used as an index of capsaicin-induced bronchoconstrictor response.

Increased cough response after antigen challenge

Progressively increasing concentrations of capsaicin solution were inhaled to actively sensitized or naïve guinea pigs 24 h after the OA or saline challenge and the number of coughs caused by each capsaicin solution was counted and recorded ($n = 10$ in each group).

Bronchoalveolar lavage (BAL) was performed after the measurement of $sRaw$ following the capsaicin provocation to confirm antigen-induced airway eosinophilic inflammation. Each guinea pig was anaesthetized with an intraperitoneal injection of 75 mg/kg of sodium pentobarbital and the trachea was cannulated with a polyethylene tube (outside diameter 2.5 mm, inside diameter 2.1 mm). Then, the guinea pig was artificially ventilated by a small animal respiratory pump (Model 1680, Harvard Apparatus Co., Inc., South Natick, MA, USA) adjusted to a tidal volume of 10 mL/kg at a rate of 60 strokes/min. The lower airways were lavaged via the tracheal cannula using two 10-mL aliquots of physiological saline solution at 37 °C. The fluid was recovered by gentle aspiration with a disposable syringe. The BAL fluid was immediately centrifuged at 800 r.p.m. (70 g) for 8 min.

After discarding the supernatant, the cells were washed twice in Hank's solution and resuspended in 1 mL of Hank's solution. They were then counted manually in a Burkner Chamber. Cyto-centrifuged preparations (Cytospin 2, Shandon Southern Products Ltd, Cheshier, UK) were stained with May Giemsa and a differential cell count was performed on 500 cells according to the standard morphologic criteria.

Time course of increased cough response after antigen challenge

Capsaicin-induced coughs were measured 1 day before and 1, 2, 3, 5 and 7 days after the antigen challenge in sensitized guinea pigs and naïve guinea pigs ($n = 7$ for each day and each group, a total of 84 animals). Different animals were used for each measurement to avoid possibility of tachyphylaxis to repeated inhalation of capsaicin.

Effect of procaterol on cough response

The β_2 -adrenoceptor agonist procaterol in a dose of 0.1 mg/kg or vehicle (saline) was intraperitoneally administered to sensitized or naïve guinea pigs 30 min before the measurement of cough response to capsaicin ($n = 10$ in each group).

Effect of atropine on cough response

The anticholinergic agent atropine sulphate in a dose of 1 or 10 mg/kg or vehicle (saline) was intraperitoneally administered to sensitized ($n = 6$ in each group) or naïve guinea pigs ($n = 5$ in each group) 30 min before the measurement of cough response.

Effect of capsaicin desensitization on cough response

Guinea pigs were pre-treated subcutaneously with 25, 50 and 100 mg/kg of capsaicin dissolved with 10% Tween 80 and 10% ethanol in saline ($n = 7$) or the vehicle ($n = 7$) 3 days, 2 days and 1 day before the antigen challenge in sensitized animals, respectively. The same pre-treatment with capsaicin ($n = 8$) or the vehicle ($n = 8$) was carried out in naive animals. Each capsaicin pre-treatment was performed after intraperitoneal administration of 0.1 mg/kg procaterol in order to avoid death due to respiratory distress. Cough response to capsaicin was measured 2 days after the last administration of capsaicin.

Effect of phosphoramidon on cough response

The neutral endopeptidase inhibitor phosphoramidon in a dose of 2.5 mg/kg ($n = 8$) or vehicle (saline) ($n = 8$) was administered intravenously 30 min before the measurement of capsaicin-induced cough, which was carried out 24 h after an antigen challenge in sensitized guinea pigs. The same dose of phosphoramidon ($n = 8$) or vehicle ($n = 8$) was given 30 min before the capsaicin provocation in naive animals.

Effect of S-1452 on cough response

The TXA₂ receptor antagonist S-1452 in a dose of 0.01 or 0.1 mg/kg or vehicle (saline) was given intraperitoneally at 24 and 1 h before the measurement of cough response in sensitized or naive guinea pigs ($n = 6$ in each group).

Chemicals

The following chemicals were used: ovalbumin (Sigma, St Louis, MO, USA), aluminium hydroxide (Al(OH)₃) (Wako Pure Chemical Ind., Osaka, Japan), sodium pentobarbital (Abbott Laboratories, North Chicago, IL, USA), capsaicin (Sigma), dimethyl sulfoxide (DMSO) (Wako Pure Chemical Ind.), ethanol (Sigma), phosphoramidon (Sigma), atropine sulphate (Sigma), procaterol (Otsuka Pharmaceutical Co. Ltd, Osaka, Japan), physiological saline (Otsuka Pharmaceutical Co. Ltd) and S-1452 (Calcium 5(z)-1R, 2S, 3S, 4S-7-[3-phenylsulfonylamino]bicyclo [2.2.1] hept-2-yl]-5-heptenoate hydrate) (Shionogi Pharmaceutical Ind., Osaka, Japan).

Statistical analysis

All data were shown as mean $\pm$ standard error of the mean (SEM). Statistical differences were determined by the student's unpaired t-test and the student's paired t-test between groups and within group, respectively.

Relationship between eosinophil number in BAL fluid and number of coughs caused by inhaled capsaicin was analysed using simple regression analysis. Significance was based on a 95% confidence level ($P < 0.05$).

Results

The number of coughs induced by inhaled capsaicin 24 h after an antigen challenge in sensitized guinea pigs was significantly greater than that after the antigen or saline challenge in naive animals or after saline challenge in sensitized animals (Table 1), suggesting that airway allergy increases cough response. Total cells and eosinophils in BAL fluid were significantly increased 24 h after the antigen challenge in sensitized animals compared with the antigen or saline challenge in naive animals or saline challenge in sensitized animals (Table 1), showing the antigen-induced airway eosinophil accumulation. Total cell number and eosinophil percentage in the BAL fluid were significantly correlated to the number of coughs induced by each concentration of capsaicin (Table 2).

The time course of cough response to 10^{-4} M capsaicin after an antigen challenge in sensitized or naive guinea pigs is shown in Fig. 1. Although the number of capsaicin-induced coughs was significantly increased in sensitized guinea pigs compared with naive guinea pigs before the antigen challenge, the cough number was much greater 24 h after the antigen challenge. The antigen-induced increase in cough number decreased to the level of naive guinea pigs within 3 days.

Procaterol did not alter the number of coughs induced by inhaled capsaicin in naive guinea pigs or the increased cough number 24 h after the antigen challenge in sensitized animals (Table 3). Procaterol completely inhibited the increase in sRaw following the capsaicin provocation in sensitized and antigen-challenged guinea pigs. On the other hand, atropine significantly reduced the number of coughs induced by inhaled capsaicin in naive guinea pigs, but did not alter the increased cough number 24 h after the antigen challenge in sensitized animals (Table 3). Atropine completely protected the increase in sRaw after the capsaicin inhalation in antigen-exposed sensitized guinea pigs.

Capsaicin desensitization remarkably reduced the increased cough number induced by 10^{-6} and 10^{-4} M capsaicin 24 h after the antigen challenge in sensitized guinea pigs and significantly attenuated the number of 10^{-4} M capsaicin-induced coughs in naive guinea pigs (Fig. 2).

Phosphoramidon significantly increased the number of coughs elicited by 10^{-8} , 10^{-6} and 10^{-4} M capsaicin in naive guinea pigs. On the other hand, phosphoramidon did not affect the increased number of capsaicin-induced

Table 1. Number of coughs induced by inhaled capsaicin and cell differentials in bronchoalveolar lavage fluid obtained after completion of the capsaicin provocation performed 24 hours after antigen or saline challenge in actively sensitized or naive guinea pigs.

	Number of coughs			Bronchoalveolar lavage cell differentials				
	Concentration of inhaled capsaicin (M)			Total cells ($\times 10^5$ cells/ml)	Mac (%)	Neu (%)	Lym (%)	Eos (%)
	10^{-8}	10^{-6}	10^{-4}					
<i>Naive animals</i>								
Saline challenge ($n = 10$)	1.9 ± 0.3	2.3 ± 0.4	9.1 ± 0.8	18.2 ± 4.5	80.0 ± 1.9	7.1 ± 2.0	3.2 ± 1.0	9.4 ± 2.4
OA challenge ($n = 10$)	1.6 ± 0.3	3.6 ± 0.4	10.0 ± 1.4	15.2 ± 4.7	78.3 ± 3.5	7.3 ± 2.4	4.1 ± 2.6	10.2 ± 2.2
<i>Sensitized animals</i>								
Saline challenge ($n = 10$)	2.4 ± 0.3	2.6 ± 0.3	11.9 ± 0.9	30.1 ± 9.7	65.0 ± 4.4	14.9 ± 5.5	7.6 ± 3.3	12.6 ± 5.4
OA challenge ($n = 10$)	$4.2 \pm 0.8^*$	$6.1 \pm 1.1^*$	$33.1 \pm 3.0^\dagger$	$99.3 \pm 15.4^\dagger$	$50.4 \pm 4.1^*$	8.1 ± 4.4	4.7 ± 2.2	36.9 ± 5.8

OA, ovalbumin; Mac, macrophages; Eos, eosinophils; Neu, neutrophils; Lym, lymphocytes. * $P < 0.01$, $^\dagger P < 0.001$ vs saline challenge in naive animals, OA challenge in naive animals and saline challenge in sensitized animals.

coughs 24 h after the antigen challenge in sensitized guinea pigs (Fig. 3).

S-1452 significantly reduced the increased cough number elicited by each concentration of capsaicin solution 24 h after the antigen challenge in sensitized guinea pigs but not in naive animals (Fig. 4).

Discussion

We used capsaicin to elicit coughs in this study. Capsaicin is the active ingredient of red pepper and has been

presumed to produce cough mainly by stimulating C-fibre endings [13–15]. However, as capsaicin also stimulates some rapidly adapting receptors with myelinated fibers this view has been questioned [16,17]. We used capsaicin as a commonly used cough inducer to examine overall cough response in this study.

Xiang *et al.* [10] have shown that inhalation of 10^{-4} M capsaicin (nebulizer output of 0.25 mL/min) causes bronchoconstriction as well as coughing in guinea pigs. In our study, the nebulizer output was 0.037 mL/min, which was extremely small compared to that used by Xiang *et al.*

Table 2. Relationship between number of coughs induced by inhaled capsaicin and cell differentials in bronchoalveolar lavage fluid

	Number of coughs					
	Concentration of inhaled capsaicin (M)					
	10^{-8}		10^{-6}		10^{-4}	
	r	P value	r	P value	r	P value
Total cells ($\times 10^5$ cells/ml)	0.411	0.0196	0.456	0.0087	0.834	0.0001
Macrophages (%)	0.313	0.0814	0.490	0.0044	0.809	<0.0001
Lymphocytes (%)	0.274	0.1284	0.104	0.5706	0.030	0.8705
Neutrophils (%)	0.195	0.2836	0.048	0.8932	0.076	0.6787
Eosinophils (%)	0.460	0.0081	0.503	0.0033	0.878	<0.0001

Simple regression analysis was performed in four groups: sensitized and antigen-challenged, sensitized and saline-challenged, non-sensitized and antigen-challenged, and non-sensitized and saline-challenged guinea pigs. r = regression coefficient.

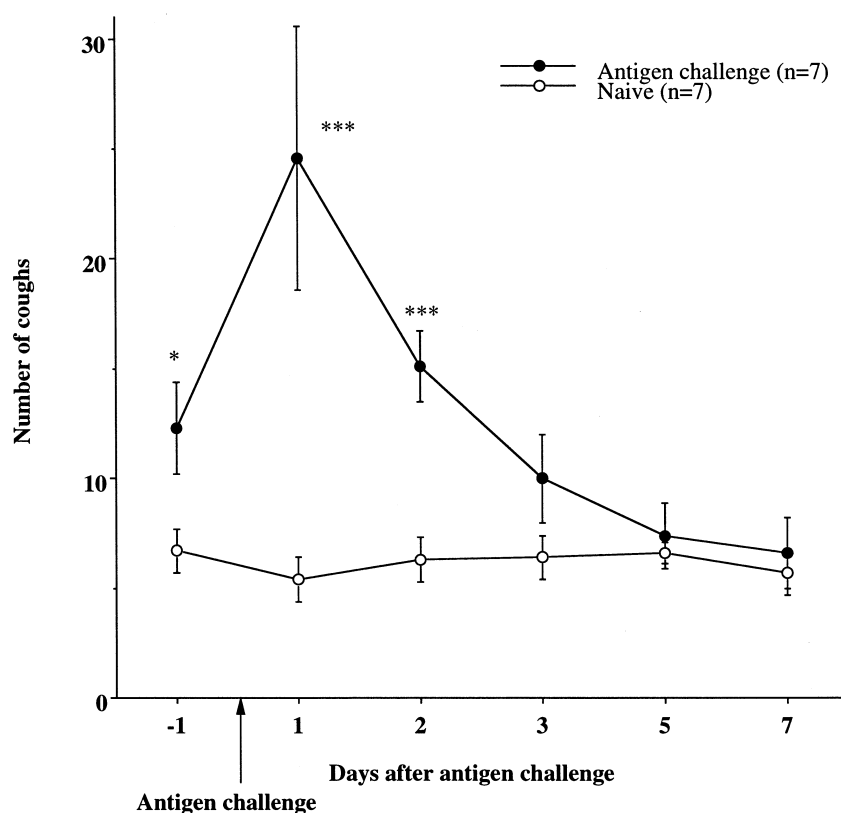


Fig. 1. Time course of 10^{-4} M capsaicin-induced coughs after an aerosolized antigen challenge in sensitized (closed circles) and naive guinea pigs (open circles). Capsaicin-induced coughs were measured 1 day before and 1, 2, 3, 5 and 7 days after the antigen challenge in sensitized or naive guinea pigs ($n = 7$ for each day and each group, a total of 84 animals). Different animals were used for each measurement to avoid the possibility of tachyphylaxis to repeated inhalation of capsaicin. * $P < 0.05$, *** $P < 0.001$ compared with naive guinea pigs.

Indeed, 10^{-4} M capsaicin elicited coughs but did not cause any significant increases in sRaw in naive guinea pigs, showing that 10^{-4} M capsaicin is appropriate for the measurement of cough response in our experimental system.

In the present study, the number of coughs induced by inhaled capsaicin was extremely increased 24 h after a single aerosolized antigen challenge in actively sensitized guinea pigs. Eosinophils were increased in BAL fluid after the antigen challenge in sensitized guinea pigs and the BAL eosinophil number was significantly correlated to the number of coughs caused by capsaicin, suggesting that airway allergy accompanied with airway eosinophilia may increase the cough response. On the other hand, the inhalation of 10^{-4} M capsaicin solution caused significant bronchoconstriction 24 h after the antigen challenge in sensitized guinea pigs, but not in naive animals. This finding is in agreement with many previous reports [18–20]. Thus, it is concluded that both cough response and bronchial responsiveness are increased 24 h after an antigen challenge in sensitized guinea pigs.

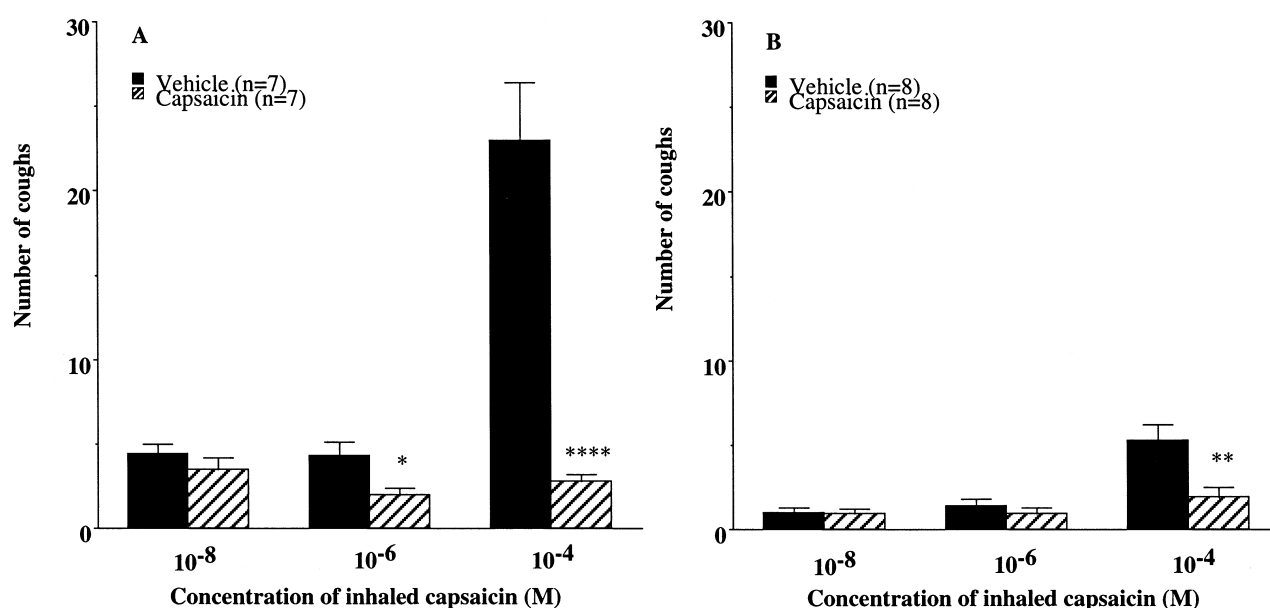
To clarify whether the antigen-induced increase in cough response is due to the increase in bronchial responsiveness or cough sensitivity we examined the effects of bronchodilators, procaterol and atropine, on the responses. Procaterol and atropine completely inhibited bronchoconstrictor response to inhaled capsaicin but did not influence the increased cough response. Accordingly, it is suggested that the antigen-induced increase in cough response results from the increased cough sensitivity, but is not mediated by bronchoconstrictor response or bronchial hyperresponsiveness. We previously reported that procaterol did not change cough sensitivity to inhaled capsaicin in normal subjects [21] and asthmatics [22] and the capsaicin cough sensitivity was not correlated with bronchial responsiveness to methacholine in asthmatics [23]. Thus, it is concluded that the increased cough response after an antigen challenge may be due to the increased cough receptor sensitivity in our animal model.

In contrast to the results in sensitized guinea pigs, atropine inhibited cough response to inhaled capsaicin in

Table 3. Effects of procaterol and atropine on number of coughs elicited by inhalation of increasing concentrations of capsaicin and specific airway resistance (sRaw) in naive guinea pigs and 24 hours after antigen challenge in sensitized guinea pigs (mean $\pm$ SEM).

	Number of coughs			Percent increase in sRaw	
	Concentration of inhaled capsaicin (M)			After treatment	After capsaicin provocation following treatment
	10 ⁻⁸	10 ⁻⁶	10 ⁻⁴		
<i>Procaterol</i>					
Naive animals					
Vehicle (saline) (n=10)	2.3 ± 0.4	2.5 ± 0.3	8.6 ± 0.6	-0.4 ± 5.2	0.4 ± 3.2
Procaterol 0.1 mg/kg (n=10)	2.3 ± 0.3	2.5 ± 0.2	7.3 ± 0.6	-3.6 ± 5.0	-0.1 ± 5.9
Sensitized and OA challenged animals					
Vehicle (saline) (n=10)	4.2 ± 0.8	6.1 ± 1.1	33.1 ± 3.0	2.7 ± 7.8	29.2 ± 10.0
Procaterol 0.1 mg/kg (n=10)	3.4 ± 0.6	4.7 ± 1.0	34.4 ± 1.5	-7.9 ± 5.0	-1.1 ± 7.2*
<i>Atropine</i>					
Naive animals					
Vehicle (saline) (n=5)	4.2 ± 0.5	3.0 ± 0.7	9.8 ± 1.1	1.7 ± 2.7	0.3 ± 4.6
Atropine 1 mg/kg (n=5)	3.2 ± 0.9	3.0 ± 0.6	6.8 ± 1.1*	-6.7 ± 4.1	-6.7 ± 7.5
Atropine 10 mg/kg (n=5)	2.4 ± 0.4	1.8 ± 0.7	5.6 ± 0.9**	-1.3 ± 9.8	-7.0 ± 7.7
Sensitized and OA challenged animals					
Vehicle (saline) (n=6)	4.2 ± 0.4	9.2 ± 0.9	20.0 ± 2.1	-3.3 ± 3.3	27.0 ± 14.1
Atropine 1 mg/kg (n=6)	4.2 ± 0.6	7.7 ± 0.7	18.3 ± 1.0	-12.1 ± 4.1	-5.2 ± 3.3**
Atropine 10 mg/kg (n=6)	3 ± 0.4	7.5 ± 0.6	17.5 ± 1.0	-11.4 ± 3.7	-11.4 ± 3.7**

* $P < 0.05$, ** $P < 0.01$ vs vehicle treatment.

**Fig. 2.** Effect of capsaisin desensitization on cough response to inhaled capsaisin 24 h after an antigen challenge in sensitized (A) and naive guinea pigs (B). Solid bars, vehicle; hatched bars, capsaisin desensitization. Values are means $\pm$ SEM; $n = 7$ and $n = 8$ in each group in A and B, respectively. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ compared with vehicle treatment.

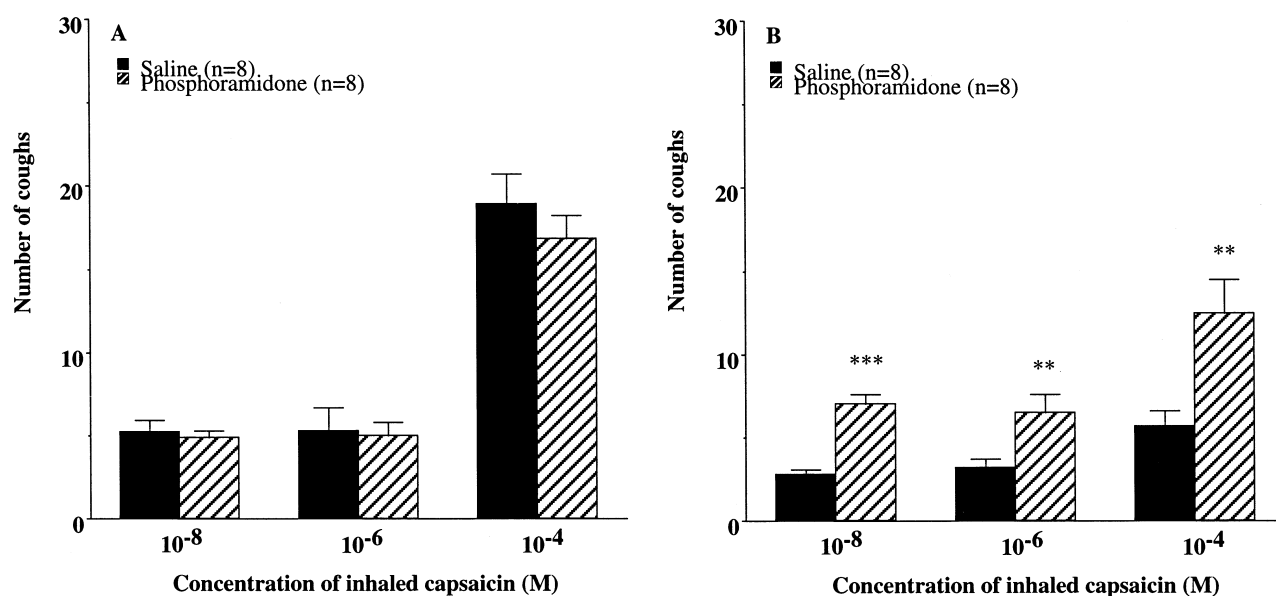


Fig. 3. Effect of the neutral endopeptidase inhibitor phosphoramidone on cough response to inhaled capsaicin 24 h after an antigen challenge in sensitized (A) and naive guinea pigs (B). Solid bars, saline; hatched bars, phosphoramidone. Values are means $\pm$ SEM; $n = 8$ in each group in A and B. ** $P < 0.01$, *** $P < 0.001$ compared with saline treatment.

naive animals. Anticholinergics have been shown to have an antitussive effect on capsaicin-induced cough in naive guinea pigs [24] which is in agreement with the present result. The antitussive effect of anticholinergics is controversial in human studies.

Lowry and coworkers [25] have shown that inhalation of 72 μ g ipratropium bromide decreases cough induced by distilled water in normal subjects and a small reduction in airway tone is associated with the reduced cough response. On the other hand, Fuller et al. [26], have shown that

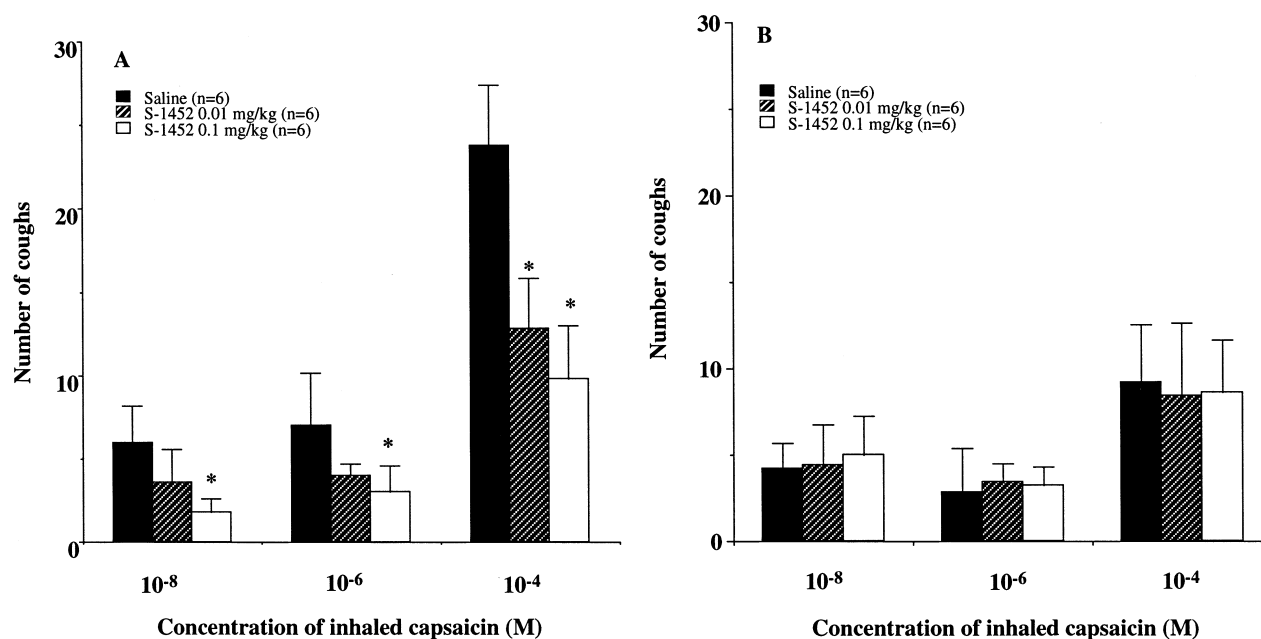


Fig. 4. Effect of the thromboxane A₂ receptor antagonist S-1452 on cough response to inhaled capsaicin 24 h after an antigen challenge in sensitized (A) and naive guinea pigs (B). Solid bars, saline; hatched bars, 0.01 mg/kg of S-1452; open bars, 0.1 mg/kg of S-1452. Values are means $\pm$ SEM; $n = 6$ in each group in A and B. * $P < 0.05$ compared with saline treatment.

250 µg of ipratropium bromide inhibits bronchoconstriction, but not cough, induced by inhalation of capsaicin in normal subjects. Pounsford and his colleagues [27] have reported that citric acid-induced cough is not influenced by 1000 µg of ipratropium bromide in normal subjects. We [28] also showed that 40 µg of ipratropium bromide did not change airway cough sensitivity to inhaled capsaicin in normal subjects. Thus, the involvement of cholinergic nerve activity in the cough response may be different between normal guinea pigs and humans. On the other hand, it has been controversial whether β_2 -adrenoceptor agonists have a direct effect on cough receptor sensitivity to tussive agents. Bolser and coworkers [24] reported that subcutaneous administration of salbutamol (0.003–3.0 mg/kg) reduced both antigen-induced cough probably mediated by bronchoconstriction (allergic cough) in sensitized guinea pigs and capsaicin-induced cough in naive guinea pigs. In contrast, Lalloo and his colleagues [29] showed that intraperitoneally administered terbutalin (0.05 mg/kg) did not affect the cough response to citric acid, capsaicin or hypertonic saline in naive guinea pigs and Girard et al. [30] reported that salbutamol given intraperitoneally in a dose of 0.3 mg/kg prevented bronchoconstriction, but not cough, caused by citric acid provocation in naive guinea pigs. The lack of direct effect of β_2 -adrenoceptor agonists on cough sensitivity is in agreement with our results that procaterol did not alter cough receptor sensitivity to capsaicin after an antigen challenge in sensitized guinea pigs or in naive animals while it prevented bronchoconstriction caused by capsaicin provocation in sensitized animals. These findings suggest that β_2 -adrenoceptor agonists have no direct effect on cough sensitivity in both guinea pigs and humans.

Subsequently, we studied the effects of capsaicin desensitization and phosphoramidon on the cough response after the antigen challenge in sensitized guinea pigs in comparison with the effects in naive guinea pigs to examine the role of sensory neuropeptides and neutral endopeptidase in the cough response. Capsaicin desensitization extremely reduced the number of coughs induced by capsaicin, especially by high concentration of capsaicin, both in sensitized and naive guinea pigs. These findings suggest that a high concentration of capsaicin elicits cough in a large part via release of sensory neuropeptides from C-fibre endings. The large parts of coughs induced by the lower concentration of capsaicin may be independent of neuropeptide release, probably via direct stimulation to irritant receptors. Phosphoramidon potentiated the capsaicin cough response in naive guinea pigs, but did not change the increased cough response after the antigen challenge in sensitized animals. The results suggest that inactivation of neutral endopeptidase by airway allergic reaction is responsible for the antigen-induced increase of cough sensitivity.

Regarding the role of sensory neuropeptides, Xiang *et al.* [10] have clearly shown that specific NK1 antagonists, FK-888 and SR-140333, do not influence capsaicin-induced cough in naive guinea pigs while the compounds reduce the increased cough response induced by multiple antigen challenge in sensitized guinea pigs. The authors [10] have also shown that the NK2 antagonist SR-48968 strongly inhibits capsaicin-induced cough both in naive and antigen-exposed sensitized guinea pigs. These findings suggest that NK2 receptors are involved in a common cough reflex pathway and NK1 receptors have a role in heightened cough sensitivity induced by airway allergic reaction.

Although the present study has shown antigen-induced inactivation of NEP which is a cause of the antigen-induced increase of cough sensitivity, the role of the NEP inactivation in asthma has been controversial. It has been shown that both antigen-induced protein plasma extravasation and bronchoconstriction are potentiated by NEP inhibition and reduced by tachykinin receptor antagonists in guinea pigs [31–34], and substance P is less efficiently degraded by NEP in inflamed airway of antigen exposed guinea pigs, suggesting antigen-induced NEP inactivation [35]. Indeed, we investigated the remarkable reduction of airway NEP activity 24 h after an antigen challenge in another guinea pig study (data not shown). In contrast, human studies have shown that inhalation of the NEP inhibitor thiorphan does not affect either the early or the late asthmatic bronchoconstriction to allergen inhalation in asthmatics [36], there is no difference in NEP activity in serum or BAL fluid between allergic asthmatics and healthy non-smokers [37], and there is no obvious reduction of NEP activity in mildly asthmatic subjects compared with control subjects [38]. Thus, it seems likely that airway NEP activity may not be important in the pathophysiology of human asthma and the influence of NEP inactivation on airway function may be different between guinea pigs and humans. Further studies are needed to clarify the role of antigen-induced inactivation of NEP in eosinophilic airway inflammation-associated diseases such as asthma, cough variant asthma, eosinophilic bronchitis without asthma and eosinophilic tracheobronchitis with cough hypersensitivity as described later.

Finally, we examined the effect of the specific TXA2 receptor antagonist S-1452 [39] on the antigen-induced increase in cough sensitivity to elucidate the involvement of TXA2, an inflammatory mediator. S-1452 inhibited the increased cough response after antigen challenge in sensitized guinea pigs while it did not influence the cough response of naive animals. It is thought that S-1452 has no non-specific or direct effect on the cough response and TXA2 is involved as an inflammatory mediator in the antigen-induced increase in cough response. Our earlier studies [40–42] have shown that bronchial responsiveness

to methacholine is reduced by TXA2 modifiers in stable asthmatic patients but not in healthy subjects. We [43] also reported that the cyclooxygenase inhibitor indomethacin decreased cough response to capsaicin in asthmatics but not in normal volunteers. These findings suggest that prostanoids including TXA2 are continuously released in inflamed airways. Accordingly, it is likely that intrinsic TXA2 released by airway allergic inflammation is an enhancer of cough sensitivity.

Eosinophilic airway inflammation is a fundamental feature of cough variant asthma (CVA) [44], eosinophilic bronchitis without asthma (EB) [45], as well as eosinophilic tracheobronchitis with cough hypersensitivity associated with atopy, abbreviated as atopic cough (AC) [4–6]. All the three eosinophilic airway disorders have been recognized to be causes of isolated chronic cough. Cough sensitivity is heightened in EB [46] and AC [4–6] whereas it is slightly increased or within normal limits in CVA [47]. Peripheral airway eosinophilia is a histological feature of CVA and EB as in bronchial asthma, but not of AC. Atopy is common in CVA and AC but the same as the general population in EB [47]. Bronchial responsiveness is increased and bronchodilators are effective in CVA, but not in EB or AC [47]. Thus, as increased cough sensitivity and eosinophilic airway inflammation, both of which are presented in our guinea pig model, are common features of AC and EB, it is unclear whether this animal model is relevant to AC, EB or both.

In conclusion, we clearly showed that airway allergic reaction induces a transient increase in cough sensitivity independent of bronchoconstriction or bronchial hyperresponsiveness, to which inactivation of neutral endopeptidase by airway allergy may contribute. This animal model may be useful in studying pathophysiology and treatment of pathological cough with increased cough sensitivity associated with allergy. However, there are many problems to be resolved, such as why cough sensitivity was heightened before the antigen challenge in sensitized guinea pigs, and why the antigen-induced increase in cough sensitivity promptly recovered to the level of naive guinea pigs.

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