

Greater Involvement of Neurokinins Found in Guinea Pig Models of Severe Asthma Compared with Mild Asthma

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Key Words

Airway hyperresponsiveness · Airway narrowing · Asthma model · Capsaicin · Eosinophil accumulation · Guinea pig · Neurokinin A · Neurokinins · Substance P

Abstract

Background: Involvement of neurokinins in asthma has been previously pointed out by several reports. However, the relationship between neurokinins and the severity of asthma has remained unclear. We developed a model of mild asthma (model I) and severe asthma (model II) in guinea pigs, and investigated the function of neurokinins in both models. **Methods:** In models I and II, systemically sensitized guinea pigs were made to inhale ovalbumin once and three times, respectively. Substance P (SP) and neurokinin A (NKA) concentrations in the bronchoalveolar lavage fluid (BALF) were measured in models I and II. Then, the effects of a capsaicin pretreatment, which depletes neurokinins, in both animal models on airway narrowing induced by the last ovalbumin inhalation, airway hyperresponsiveness to inhaled methacholine, and eosinophil accumulation in BALF, were investigated. **Results:** SP concentration tended to increase and the NKA concentration increased significantly in model II, but not in model I. Capsaicin pretreat-

ment significantly inhibited the late bronchial response that was observed 2–6 h after the last ovalbumin inhalation, airway hyperresponsiveness and eosinophil accumulation in model II. On the other hand, it had no effects on the responses in model I. **Conclusion:** It is suggested that the more severe the disease, the greater the involvement of neurokinins.

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Introduction

Neurokinins, which include substance P (SP), neurokinin (NK) A and NKB, are a family of peptides which share a common C-terminal sequence, Phe-X-Gly-Leu-Met-NH₂ [1, 2]. The biological effects of neurokinins are mediated through three types of receptors, termed NK₁, NK₂ and NK₃. Neurokinin receptors are members of the superfamily of G-protein-coupled, seven-transmembrane-spanning receptors, and neurokinin-receptor activation causes stimulation of phospholipase C, leading to a phosphoinositide breakdown and elevation of intracellular calcium [3]. The three neurokinin receptors have been cloned. SP interacts preferentially with the NK₁ receptor, NKA with the NK₂ receptor, and NKB with the NK₃ receptor [1, 2, 4].

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Neurokinins are synthesized and stored in neurons in the central nervous system and in the peripheral tissue [5]. In airways, neurokinins are present in unmyelinated pulmonary C fibers [3, 5]. The C fibers can be stimulated by several agents which elicit a wide range of biological actions in mammals. They include vascular hyperpermeability, bronchoconstriction, airway hyperresponsiveness, cough and mucus secretion [6–8]. Since these effects are characteristic of patients with pulmonary diseases such as asthma, it is considered that neurokinins play an important role in these diseases [6, 9–11]. Indeed, it has been reported that the NK₁ and NK₂ receptor genes are expressed in asthmatics [12, 13] and that the concentration of SP-like immunoreactivity in lavage fluids of allergic asthma subjects is high compared with that of normal subjects [14].

Capsaicin has been used as a research tool to elucidate the physiological effects of neurokinins [15–18]. Capsaicin pretreatment has been shown to inhibit antigen-induced bronchoconstriction in sensitized guinea pigs [19] and to inhibit antigen-induced pulmonary response and airway hyperresponsiveness to methacholine in guinea pigs repeatedly sensitized with antigen [20, 21]. In addition, Fischer et al. [22] have reported that neurokinin gene and peptide expression was induced after allergen exposure in nodose primary afferent neurons in ovalbumin-sensitized guinea pigs. These reports suggest that neurokinins play an important role in asthma models. However, the relationship between neurokinins and the severity of asthma has remained unclear.

In order to investigate at which stage neurokinins are involved, we developed a model of mild asthma by one ovalbumin inhalation (model I) and a model of severe asthma by three ovalbumin inhalations (model II) using systemically sensitized guinea pigs, and examined SP and NKA concentrations in bronchoalveolar lavage fluid (BALF) and neutral endopeptidase (NEP), a neurokinin-degrading enzyme, activity in the lungs. Furthermore, we examined the effects of capsaicin pretreatment on bronchial response to ovalbumin, airway hyperresponsiveness to inhaled methacholine, and eosinophil accumulation in BALF in both models.

Materials and Method

Animals

Male 5-week-old Hartley guinea pigs were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and were used at the age of 6 weeks. The guinea pigs were kept in a room with a 12-hour light/12-hour dark cycle, and a room temperature and humidity set at $23 \pm 2^\circ\text{C}$

and $55 \pm 5\%$, respectively. The animals were provided food and tap water ad libitum.

Materials

Capsaicin, salbutamol hemisulfate, ovalbumin, mepyramine maleate, methacholine chloride (methacholine), sucrose, Trizma® base (Tris), leucine aminopeptidase, 7-amino-4-methyl-coumarin (AMe-Cou), zinc acetate and phosphoramidon were obtained from Sigma Chemical Company (St. Louis, Mo., USA). Xylazine and theophylline were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Ketamine was obtained from Sankyo Co., Ltd. (Tokyo, Japan). Pentobarbital sodium was from Abbott Laboratories (North Chicago, Ill., USA), and chloroform, tragacanth gum powder (tragacanth) and calcium chloride (CaCl₂) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phosphate buffer was from Iatron Laboratories, Inc. (Tokyo, Japan), and ethylenediamine tetraacetic acid (EDTA) was from Daiichi Pure Chemical Co., Ltd. (Tokyo, Japan). Suc-Ala-Ala-Phe-AMeCou was from Bachem AG (Bubendorf, Switzerland), and N-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (Tes) was from Dojindo Laboratories (Kumamoto, Japan).

Capsaicin was suspended at a concentration of 50 mg/ml in 10% ethanol, 10% Tween 80 and 80% physiological saline. Ovalbumin was suspended in physiological saline. Salbutamol hemisulfate was dissolved in 5% tragacanth solution, mepyramine maleate, methacholine and theophylline was dissolved in physiological saline.

Asthma Models

Model of Mild Asthma (Model I). The animals were sensitized with 0.5 ml of 5% ovalbumin subcutaneously and 0.5 ml intraperitoneally according to the method described by Engineer et al. [23]. A booster injection was given 1 week later in the same manner. One week after the booster injection, 1% ovalbumin was inhaled for 3 min in the chamber using a nebulizer (NE-U11B; Omron Corp., Kyoto, Japan) (fig. 1). Thirty minutes before the inhalation, an H₁ antagonist, mepyramine maleate (10 mg/kg) was intraperitoneally injected in order to prevent acute fatal anaphylaxis and ensure that a high dose of antigen was inhaled. The animals were also fasted for 1 day before the inhalation.

Model of Severe Asthma (Model II) [24]. After 1% ovalbumin was administered as in model I, a second and third dose was administered by inhalation in the same manner at 1-week intervals (fig. 1). Mepyramine maleate (10 mg/kg) was intraperitoneally injected 30 min before each inhalation.

Measurement of Neurokinin Concentration

Preparation of Sample. Neurokinin concentrations in BALF from the following animals were measured: model I animals sensitized with antigen inhalation (challenge+) and those not sensitized with antigen inhalation (challenge–), and model II animals sensitized with antigen inhalation three times (challenge+) and those sensitized with antigen inhalation twice (challenge–) as well as naive (normal) animals. After the guinea pigs were anesthetized with pentobarbital sodium (30 mg/kg i.p.), their trachea was cannulated with a disposable intravenous catheter (3-Fr size; Atom Medical Corporation, Tokyo, Japan) connected to a 5-ml glass syringe filled with physiological saline fixed into the posterior lobe of the right lung, and the airway lumen was washed three times with 3 equal volumes of physiological saline (10 ml/kg). The BALF collected from each animal was immediately placed in an ice bath and centrifuged at 300 g for 10 min

at 4°C. The supernatant was used for the measurement of neurokinin concentration.

Enzyme-Linked Immunosorbent Assay

SP or NKA concentration in the BALF supernatant was measured using a competitive enzyme immunoassay kit (Peninsula Laboratories, Inc., San Carlos, Calif., USA). In brief, 50 µl of the standard (SP or NKA) or the supernatant was incubated with 25 µl of primary antibody to SP or NKA and 25 µl of biotinylated secondary antibody in 96-well immunoplates for 2 h at room temperature. After the plate was washed five times with 300 µl of assay buffer (10 mM of Tris buffer and 0.1% of Tween 20), 100 µl of streptavidin-horseradish peroxidase solution was added and incubated for 1 h at room temperature. After the plate was washed five times with 300 µl of assay buffer again, 100 µl of 3,3',5,5'-tetramethyl benzidine dihydrochloride solution was added and incubated for 10 min at room temperature. The reaction was terminated by adding 100 µl of 2 N HCl. The absorbance at 450 nm was measured with a micro-plate reader (Cytofluor Multi-Well Plate Reader; Applied Biosystems, Foster City, Calif., USA), and the SP and NKA concentration (pg/ml) was calculated from the standard curve obtained.

Capsaicin Pretreatment

Capsaicin treatment was conducted with the method of Ichinose and Barnes [25]. First, capsaicin (50 mg/kg) was subcutaneously injected 1 day after the booster injection in model I, and 1 day before the first antigen inhalation in model II, under anesthesia with ketamine (50 mg/kg i.m.) and xylazine (0.1 mg/kg i.m.). In order to prevent bronchoconstriction, salbutamol hemisulfate (2 mg/kg p.o.) and theophylline (50 mg/kg i.p.) were given 1 h and 30 min before capsaicin injection, respectively. This procedure was conducted only in the animals in which antigen-induced airway narrowing, airway hyperresponsiveness to inhaled methacholine and eosinophil infiltration were measured.

Measurement of Airway Narrowing

As an index of airway narrowing, specific airway resistance (sRaw: cm H₂O·s) was determined by the method of Pennock et al. [26] on a breath-by-breath basis in a double-chamber plethysmograph with a respiratory gas analyzer (Model P, System B-IV; Buxco Electronics, Inc., Sharon, Conn., USA). sRaw was measured before and 0.25, 1, 2, 3, 4, 5 and 6 h after the last antigen inhalation in model I and II.

The percent relative increase in sRaw from the value before antigen inhalation (basal value: 100%) was calculated as the degree of airway narrowing. Furthermore, the integrated areas (ΔAUC_{sRaw}) under the time-response (percent relative increase in sRaw) curve were calculated from 0 to 2 h after antigen inhalation as the immediate bronchial response (IBR, 0–2 h) and from 2 to 6 h as the late bronchial response (LBR, 2–6 h, model II only).

The inhibitory effect of capsaicin pretreatment was calculated as follows: percent inhibition = $(1 - B/A) \times 100$, where A is the mean value of ΔAUC_{sRaw} in non-capsaicin-treated animals of model I or II (control) and B is the mean value of ΔAUC_{sRaw} in capsaicin-pretreated animals of model I or II.

Measurement of Airway Responsiveness to Inhaled Methacholine

Airway responsiveness was determined by measuring the concentration of methacholine used to double Raw, 24 h after the last antigen inhalation. As an index of airway narrowing by methacholine,

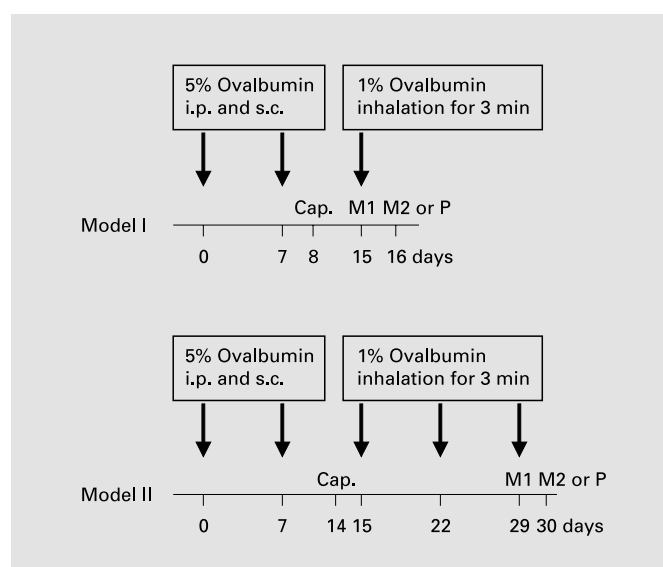


Fig. 1. Experimental protocol for the preparation of model I and II animals. Guinea pigs were immunized weekly via intraperitoneal and subcutaneous administration of ovalbumin on day 0 and 7, followed by inhalation of nebulized ovalbumin on day 15 (model I and II). In model II animals, additional doses of ovalbumin were administered on day 22 and 29. The bronchial response to airway narrowing was measured (M1) on day 15 (model I) and day 29 (model II) after ovalbumin inhalation. On day 16 (model I) and day 30 (model II), airway responsiveness to methacholine was measured (M2). This was followed by the collection of BALF and measurement of neurokinin concentration and NEP activity (P). For capsaicin pretreatment analyses, capsaicin (cap., 50 mg/kg) was injected subcutaneously on day 8 (model I) and day 14 (model II). Capsaicin pretreatment was conducted only in the animals in which antigen-induced airway narrowing, airway hyperresponsiveness to inhaled methacholine and eosinophil infiltration were measured.

respiratory resistance (Raw: cm H₂O/ml/s) was automatically measured by a forced oscillation technique using Animal-asto (TMC-2100; Chest MI Inc., Tokyo, Japan) with a multinebulizer, based on the method of Mead [27]. In brief, guinea pigs were placed inside a body plethysmograph chamber, and a 30-Hz sine wave oscillation pressure was applied on the animal's body surface. The respiratory flow rate through the mask and the box pressure was measured with a differential pressure transducer. The 30-Hz components of mask flow and box pressure were extracted with a lock-in amplifier. The resistance was calculated according to the method of Hyatt et al. [28]. The mask flow, body pressure and resistance were recorded using a multichannel polygraph recorder.

Methacholine (32–4,096 µg/ml at doubling doses) and physiological saline aerosols were generated using an ultrasonic nebulizer driven by compressed air. Physiological saline was inhaled for 1 min, and then, doubling concentrations of methacholine were inhaled for 1 min every minute. Next, the percent relative increase in Raw from the value after physiological saline inhalation was calculated. The minimum concentration of methacholine that doubled the basal Raw in individual animals (PC₂₀₀ in µg/ml) was calculated based on three

Table 1. Neurokinin concentration in BALF in model I and II animals

	Neurokinin concentration, pg/ml			
	n	model I	n	model II
SP				
Normal	5	51 ± 6	5	58 ± 7
Challenge–	4	40 ± 6	4	37 ± 5
Challenge+	4	47 ± 3	4	94 ± 24*
NKA				
Normal	5	7 ± 4	5	10 ± 2
Challenge–	4	19 ± 8	4	15 ± 6
Challenge+	4	16 ± 4	4	48 ± 15**

Results are mean ± SEM. Statistical analysis was carried out by Dunnett's test: * $p < 0.1$ vs. normal, ** $p < 0.05$ vs. normal; the other values are not significantly different from normal.

methacholine concentrations and the percent relative increase in Raw by nonlinear regression analysis.

The inhibitory effects of the capsaicin pretreatment were calculated as follows: percent inhibition = $[1 - (C - A)/(B - A)] \times 100$, where A is the mean value of PC₂₀₀ in naive animals (normal), B is the mean value of PC₂₀₀ 24 h after antigen inhalation in non-capsaicin-treated animals of model I or II (control) and C is the mean value of PC₂₀₀ 24 h after antigen inhalation in capsaicin-pretreated animals of model I or II.

Measurement of Eosinophil Infiltration

Bronchoalveolar lavage was performed as mentioned above, and the number of eosinophils in BALF was measured in models I and II. The BALF collected from each animal was immediately placed in an ice bath and centrifuged at 300 g for 10 min at 4°C, and the precipitates were resuspended in 4 ml of physiological saline. Total cell counts were determined using a standard hemocytometer (Kayagaki Irika Kogyo Co., Ltd., Tokyo, Japan). Differential cell counts were measured with smears stained with Diff-Quik solution (International Reagents Corporation, Kobe, Japan). A minimum of 500 cells per smear was counted under a light microscope (×400, BX-50-34-SP; Olympus Optical Company Ltd., Tokyo, Japan) and the proportion of eosinophils (%) was measured. Then, the number of eosinophils was calculated by multiplying the proportion of eosinophils and the total cell count.

The inhibitory effects of the capsaicin pretreatment were calculated as follows: percent inhibition = $[1 - (C - A)/(B - A)] \times 100$, where A is the mean value of eosinophils in BALF from naive animals (normal), B is the mean value of eosinophils in BALF from non-capsaicin-treated animals of models I and II 24 h after antigen inhalation (control) and C is the mean value of eosinophils in BALF from capsaicin-pretreated animals of models I and II 24 h after antigen inhalation.

NEP Activity Assay

Preparation of Sample. The challenge+, challenge– and normal guinea pigs of model I and II were exsanguinated under chloroform

anesthesia and their lungs were removed rapidly. The isolated tissue was washed several times in ice-cold phosphate buffer (1/15 M) and pooled. Then, the tissue was homogenized in ice-cold Tris-HCl buffer (50 mM, pH 7.4) containing 120 mM NaCl, 5 mM KCl, and 0.32 M sucrose using a Polytron® homogenizer (Kinematica AG, Switzerland). The homogenate was centrifuged at 1,000 g for 10 min at 4°C. The supernatant was resuspended in Tris-HCl buffer (50 mM, pH 7.4) containing 10 mM EDTA and 300 mM KCl. The suspension was centrifuged at 12,000 g for 20 min at 4°C. The supernatant was centrifuged at 100,000 g for 60 min at 4°C and the supernatant was used for the assay of NEP activity.

Measurement of NEP Activity. The assay was conducted according to the method of Zimmerman et al. [29] with a slight modification. Each sample was incubated with 0.2 mM substrate (Suc-Ala-Ala-Phe-AMeCou) in 50 mM Tes buffer containing 5% DMSO, 5 µg/ml leucine aminopeptidase, 50 µM zinc acetate and 10 mM CaCl₂ for 30 min at room temperature. The amount of AMeCou was determined by measuring the fluorescence with a spectrofluorometer (F2000; Hitachi Koki Co., Ltd., Tokyo, Japan) at 0 and 30 min after the addition of substrate. Activation and emission wavelengths were 383 and 455 nm, respectively. The assay was also performed in the presence of phosphoramidon (10 µM), an NEP inhibitor.

NEP activity was calculated as follows: NEP activity (pmol AMeCou/min/mg protein) = $[(A - B) - (C - D)]/(30 \times E)$, where A is the amount of AMeCou at 30 min (pmol) without phosphoramidon, B is the amount of AMeCou at 30 min (pmol) with phosphoramidon, C is the amount of AMeCou at 0 min (pmol) without phosphoramidon, D is the amount of AMeCou at 0 min (pmol) with phosphoramidon and E is the protein content (mg). Protein content was measured using a protein assay kit II (Bio-Rad Laboratories, Cambridge, Mass., USA).

Data Analysis

The data were expressed as means ± standard error of the mean (SEM). Comparisons for airway narrowing were made by Welch's test, those for airway responsiveness and eosinophil accumulation by the Mann-Whitney U test, and those for neurokinin concentration by Dunnett's test, using SAS® System Release 8.2 (SAS Institute Inc., Cary, N.C., USA). p values <0.05 were considered to be statistically significant, and those <0.1 to show a slight effect.

Results

Neurokinin Concentration in BALF

The SP concentration of age-matched normal control (naive) animals for model I was 51 ± 6 pg/ml, and that for model II was 58 ± 7 pg/ml. In model I, the SP concentrations in challenge– animals (40 ± 6 pg/ml) and challenge+ animals (47 ± 3 pg/ml) were not significantly different from those in normal animals. In model II, the SP concentration in challenge+ animals increased to 94 ± 24 pg/ml compared with that in normal animals whereas challenge– animals showed no significant change (37 ± 5 pg/ml) (table 1).

The NKA concentration in age-matched normal control (naive) animals for model I was 7 ± 4 pg/ml, and that

for model II was 10 ± 2 pg/ml. In model I, the NKA concentrations in challenge– animals (19 ± 8 pg/ml) and challenge+ animals (16 ± 4 pg/ml) were not significantly different from those in normal animals. On the other hand, in model II, the NKA concentration in challenge+ animals (48 ± 15 pg/ml) was significantly increased compared with that in normal animals (table 1), but the challenge– animals showed no significant change (15 ± 6 pg/ml) (table 1).

Effects of Capsaicin Pretreatment on Antigen-Induced IBR and LBR in Models I and II

Figure 2 shows the percent change from the baseline of specific airway resistance (sRaw) after the last antigen inhalation. In model I animals, only IBR, which peaked at 15 min, was induced. The maximal increase in percent change from the baseline of sRaw was $290 \pm 37\%$, and the increase in ΔAUC_{sRaw} for IBR was $176 \pm 21\% \cdot h$ (fig. 3). In model II animals, a biphasic bronchial response, IBR and LBR, which peaked at 4 h, was induced. The increases in percent change from the baseline of sRaw at 15 min and 4 h were $361 \pm 37\%$ and $188 \pm 18\%$, and the increases in ΔAUC_{sRaw} for IBR and LBR were $189 \pm 26\% \cdot h$ and $217 \pm 35\% \cdot h$, respectively (fig. 3). The effects of capsaicin pretreatment on the increase in ΔAUC_{sRaw} for IBR and LBR in model I and model II animals are shown in figure 3. Capsaicin pretreatment had no effect on IBR in model I ($160 \pm 34\% \cdot h$) and in model II ($151 \pm 44\% \cdot h$) animals, but significantly inhibited LBR to a value of $79 \pm 40\% \cdot h$ (64% inhibition).

Effects of Capsaicin Pretreatment on Antigen-Induced Airway Hyperresponsiveness to Methacholine

The airway responsiveness to inhaled methacholine was determined by measuring PC_{200} 24 h after the last antigen inhalation (fig. 4). The mean value of PC_{200} of normal (naive) animals was $1,500 \pm 350$ $\mu g/ml$. The

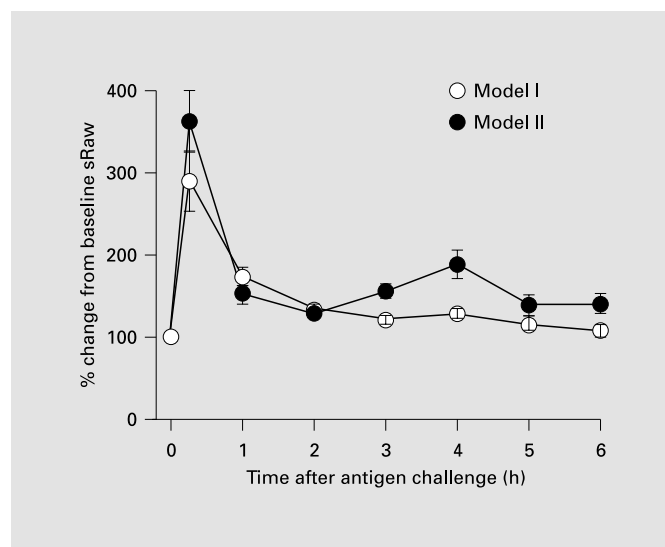


Fig. 2. Time-response (sRaw) curve after antigen challenge in model I and II animals. In model I animals, only IBR that peaked at 15 min was induced. In model II, a biphasic bronchial response, IBR and LBR that peaked at 4 h, was induced. Each point represents means $\pm$ SEM ($n = 4$ for model I and $n = 20$ for model II).

Fig. 3. Effects of capsaicin pretreatment on IBR and LBR in model I and II animals. We calculated ΔAUC_{sRaw} , an integrated area under the time-response (percent relative increase in sRaw) curves to measure the intensity of airway narrowing. Capsaicin (50 mg/kg) was injected subcutaneously 1 day after a booster injection in model I animals, and 1 day before the first antigen inhalation in model II animals. Data represent means $\pm$ SEM ($n = 4$ for model I and II capsaicin-treated group, $n = 4$ for model I control, and $n = 20$ for model II control). Statistical analysis was carried out by Welch's test, $**p < 0.05$; NS = not significant.

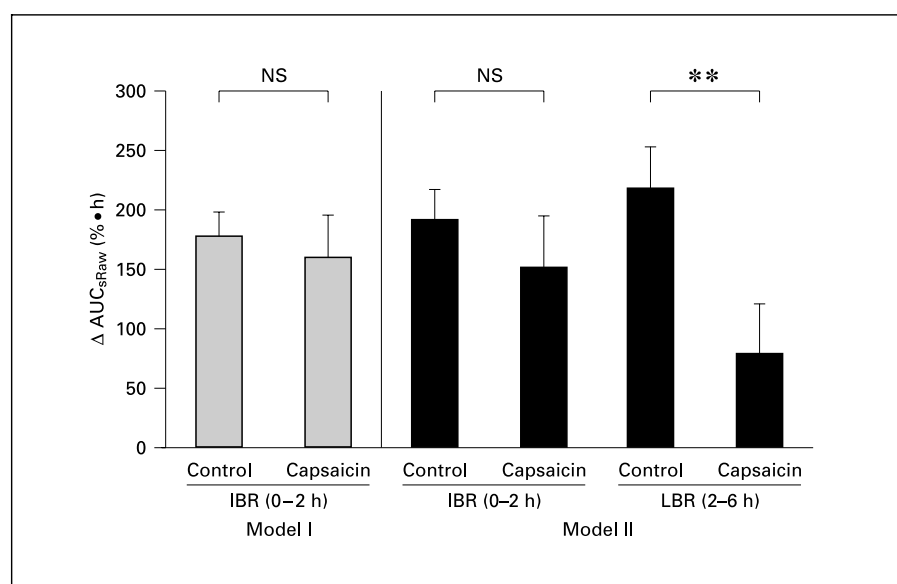
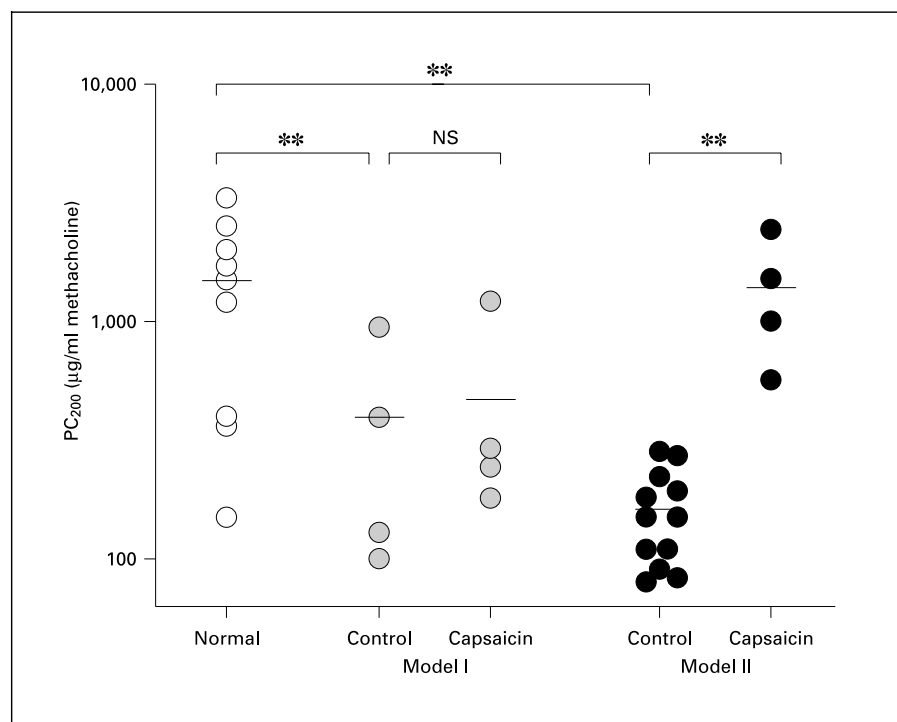


Fig. 4. Effects of a capsaicin pretreatment on antigen-induced airway hyperresponsiveness to inhaled methacholine 24 h after the last antigen inhalation in model I and II animals. We used PC₂₀₀, the minimum provocative concentration (μg/ml) of methacholine that doubled the basal Raw in individual animals, as a measure of airway hyperresponsiveness. Capsaicin (50 mg/kg) was injected subcutaneously 1 day after a booster injection in model I animals, and 1 day before the first antigen inhalation in model II animals. Circles represent the individual values of PC₂₀₀, and the horizontal bar, the mean. Statistical analysis by the Mann Whitney U test, ** p < 0.05, and NS, not significant.



mean value of PC₂₀₀ 24 h after the antigen inhalation in model I animals significantly decreased to 400 ± 200 μg/ml compared with normal animals. In model II animals, a significant decrease in PC₂₀₀ (160 ± 20 μg/ml) was also observed. Capsaicin pretreatment had no improving effect on airway hyperresponsiveness to methacholine 24 h after antigen inhalation in model I animals (480 ± 240 μg/ml). On the other hand, in model II animals, capsaicin pretreatment significantly inhibited the airway hyperresponsiveness to methacholine to a value of $1,400 \pm 390$ μg/ml (93% inhibition) (fig. 4).

Effects of Capsaicin Pretreatment on Antigen-Induced Eosinophil Accumulation in BALF

In normal (naïve) animals, BALF contained $1.4 \pm 0.3 \times 10^4$ eosinophils/ml. In model I, the number of eosinophils 24 h after the antigen inhalation was significantly increased to $27.5 \pm 4.4 \times 10^4$ cells/ml compared with the normal group. In model II, a significant increase ($31.6 \pm 3.2 \times 10^4$ cells/ml) was also observed (fig. 5). Capsaicin pretreatment did not inhibit the eosinophil accumulation in model I animals ($26.4 \pm 1.6 \times 10^4$ cells/ml). However, in model II animals, capsaicin pretreatment significantly inhibited eosinophil accumulation, down to a value of $15.6 \pm 5.5 \times 10^4$ cells/ml (53% inhibition) (fig. 5).

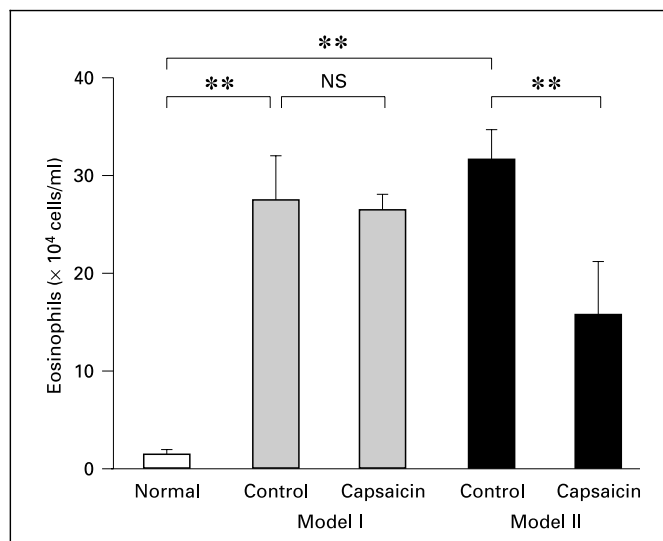


Fig. 5. Effects of capsaicin pretreatment on antigen-induced eosinophil accumulation in BALF 24 h after the last antigen inhalation in model I and II animals. We counted the number of eosinophils in BALF after the measurement of airway responsiveness to methacholine. Capsaicin (50 mg/kg) was injected subcutaneously 1 day after a booster injection in model I animals, and 1 day before the first antigen inhalation in model II animals. Data represent means $\pm$ SEM (n = 10 for normal, n = 4 for model I and II capsaicin-treated group, n = 4 for model I control, and n = 12 for model II control). Statistical analysis was carried out by the Mann-Whitney U test, ** p < 0.05; NS = not significant.

Table 2. NEP activities in the lungs of model I and II animals

	NEP activity, pmol AMeCou/min/mg	
	model I	model II
Normal	63.3	59.6
Challenge–	65.8	47.2
Challenge+	37.4	34.8

The NEP activity was monitored by measuring the fluorescence of AMeCou released with an excitation wavelength of 383 nm and emission wavelength of 455 nm. Each value was obtained from a pooled sample of 3–5 animals.

NEP Activity in Lung

The NEP activity in age-matched normal (naive) control animals of model I was 63.3 pmol AMeCou/min/mg. The value of challenge– animals was 65.8 pmol AMeCou/min/mg, which was almost equal to the value of normal animals (table 2). The NEP activity of challenge+ animals was decreased to 37.4 pmol AMeCou/min/mg (table 2).

The NEP activity in age-matched normal (naive) control animals of model II was 59.6 pmol AMeCou/min/mg (table 2). The NEP activity of challenge– animals decreased to 47.2 pmol AMeCou/min/mg and that of challenge+ animals was decreased to 34.8 pmol AMeCou/min/mg (table 2).

Discussion

Antigen inhalation by sensitized guinea pigs produced IBR, decreased PC₂₀₀ which represents the intensity of airway hyperresponsiveness, and increased the number of eosinophils in BALF in model I animals. In model II animals, a biphasic bronchial response (IBR and LBR), a lower PC₂₀₀ value and a greater number of eosinophils were observed.

The SP and NKA concentrations in BALF did not change in normal, challenge–, and challenge+ animals of model I. However, in model II animals, the SP concentration showed an increasing trend, and NKA concentration was significantly increased in the challenge+ animals compared with the normal animals. These data showed that the involvement of neurokinins in model II animals is greater than that in model I animals, suggesting that a depletion of neurokinins would inhibit the asthma response in model II animals. Repeated antigen inhalation might play a key role in the increase of SP and NKA in

BALF because the SP and NKA concentration in BALF in model II animals did not change in the challenge– animals but increased in the challenge+ animals compared with normal animals.

A chronic treatment with a high dose of capsaicin has been shown to deplete the tissue content of neurokinins [17]. We have previously confirmed that a subcutaneous administration of 50 mg/kg capsaicin to guinea pigs, 7 days before measurement, blocked the vagal nerve-stimulated nonadrenergic noncholinergic bronchoconstriction, which demonstrated that the bronchoconstriction was completely inhibited by neurokinin receptor antagonists (unpublished data). In this study, capsaicin pretreatment did not show significant effects on IBR, airway hyperresponsiveness, or eosinophil accumulation in model I animals. On the other hand, in model II animals, capsaicin pretreatment significantly inhibited LBR, airway hyperresponsiveness and eosinophil accumulation. These results also demonstrate that the involvement of neurokinins in model II animals is greater than that in model I animals.

In this study, LBR was significantly inhibited by capsaicin pretreatment, and the concentration of NKA and SP in BALF was increased in challenge+ animals of model II. As the main biological action of NKA is bronchoconstriction via NK₂ receptors [6], NKA might be involved in the LBR of model II animals. Although it has been reported that capsaicin pretreatment depletes not only neurokinins but other neuropeptides such as vasoactive intestinal polypeptide and calcitonin gene-related peptide (CGRP) [15], the main mediators involved would be neurokinins because vasoactive intestinal polypeptide and CGRP do not have bronchoconstricting activity in guinea pigs [30, 31]. On the other hand, IBR was not significantly inhibited by capsaicin pretreatment in model I and model II animals, indicating that neurokinins were not highly involved in the ovalbumin-induced IBR. These results are consistent with the previous observations that capsaicin pretreatment did not prevent the allergen-induced immediate bronchoconstriction in guinea pigs [32, 33], and that intravenous administration of neurokinin receptor antagonist had no effects on the immediate ovalbumin-induced bronchoconstriction [34]. On the other hand, other reports have shown that capsaicin pretreatment inhibited respiratory system resistance and elastance after antigen inhalation in guinea pigs repeatedly sensitized with ovalbumin aerosol [20], and capsaicin pretreatment resulted in a decreased immediate pulmonary mechanical response to antigen inhalation in sensitized guinea pigs [19]. These differences between their studies and ours might be

due to differences in the antigen sensitization method used, the parameters evaluated (respiratory system resistance vs. sRaw), and the testing conditions of the animal (anesthetized vs. conscious). Furthermore, we have previously shown that ovalbumin-induced bronchoconstriction was inhibited by histamine H₁ receptor antagonist, thromboxane synthase inhibitor and leukotriene receptor antagonist in the guinea pig model of mild asthma [35]. Thus, the main mediators for the development of IBR would be products from mast cells, although we were not able to exclude the involvement of neurokinins because SP activates airway mast cells [10].

Airway hyperresponsiveness to inhaled methacholine was completely inhibited by capsaicin pretreatment in model II animals, suggesting that neurokinins play an important role in the development of airway responsiveness in the model of severe asthma. This is consistent with studies in which capsaicin pretreatment inhibited the airway hyperresponsiveness to acetylcholine after antigen inhalation in guinea pigs repeatedly sensitized with antigen [21]. It has been reported that SP or NKA inhalation increased airway responsiveness to acetylcholine in rats [36] and in guinea pigs [37], and that the neurokinin-induced airway responsiveness was inhibited by neurokinin receptor antagonists [36, 37]. Therefore, the increased SP and NKA in airways could be considered to cause the airway hyperresponsiveness. Although the further mechanism is unclear, one possibility is that the increased SP and NKA changed the responsiveness of the target cells. Consequently, smooth muscle hyperreactivity was observed as mentioned in the report of Ishida et al. [38], which describes enhanced contractile activity in the tracheal preparations of guinea pigs after repeated administration of the antigen. Another mechanism could be associated with an increase in cholinergic neural activity by increased SP and NKA since neurokinins have been demonstrated to facilitate cholinergic neurotransmission in guinea pig airways [39, 40]. There is also the possibility that the increased epithelial permeability allows a higher concentration of methacholine to reach the smooth muscle producing hyperresponsiveness since endogenous neurokinins induce mucosal edema in rats [41]. It has also been reported that antigen-induced airway hyperresponsiveness is attenuated in sensitized CGRP-deficient mice [42] compared with sensitized wild-type mice. Therefore, we did not entirely exclude the involvement of other neuropeptides such as CGRP in the development of airway hyperresponsiveness. On the other hand, capsaicin pretreatment had no effect on airway hyperresponsiveness to inhaled methacholine in model I animals. We have previously

reported that ovalbumin-induced airway hyperresponsiveness was inhibited by thromboxane synthase inhibitor in mild asthma guinea pig model [35]. These observations suggest that the major mediator of the development of airway hyperresponsiveness in the model of mild asthma is a substance unrelated to neurokinins, e.g. thromboxane.

Eosinophil accumulation in BALF was significantly inhibited by capsaicin pretreatment in model II, suggesting that neurokinins contribute to the development of eosinophil accumulation. Numao and Agrawal [43] reported that neurokinins significantly enhanced a chemotactic response to platelet-activating factor and leukotriene B₄ in human eosinophils, and Kudlacz and Knippenberg [44] reported that SP aerosols elicited an eosinophil influx of BALF in guinea pig. A nebulized exposure of CGRP has also been shown to cause an increase in eosinophil numbers in the BALF of rats [45]. Therefore, the inhibitory effect of capsaicin pretreatment on eosinophil accumulation in model II animals might result from a depletion of neurokinins as well as effects by other neuropeptides such as CGRP. In contrast to our results, Tiberio et al. [20] reported that capsaicin treatment tended to attenuate the eosinophil accumulation in BALF or in the airway wall in guinea pigs repeatedly sensitized with ovalbumin aerosol, and Matsuse et al. [21] demonstrated that capsaicin treatment did not alter eosinophil infiltration into the epithelium of airways in guinea pigs repeatedly sensitized with antigen [21]. These differences might be due to differences in the eosinophil accumulation assay method used because eosinophil accumulation was evaluated based on a histological examination, whereas Tiberio et al. performed a bronchoalveolar lavage in addition to the histological examination. Another possible reason for the discrepancy could be due to the difference in the evaluation timing of eosinophil accumulation (72 h after antigen inhalation, Matsuse et al. [21]; immediately after antigen inhalation, Tiberio et al. [20]; 24 h after antigen inhalation, this study). On the other hand, capsaicin pretreatment had no effect on eosinophil accumulation in model I animals. This is consistent with the previous observation in which capsaicin pretreatment did not affect increase in BALF cell counts after antigen inhalation in sensitized guinea pigs [32]. We have previously observed that ovalbumin-induced eosinophil accumulation is inhibited by thromboxane synthase inhibitor and platelet-activating factor antagonist in a guinea pig model of mild asthma [46]. These observations suggest that the main mediator of the development of eosinophil accumulation in animal models of mild asthma is a substance unrelated to neurokinins, e.g. thromboxane or platelet-activating factor.

NEP activity in challenge+ animals was decreased compared with normal animals of model I. In addition, the NEP activity in challenge- animals tended to decrease and that in challenge+ animals showed a further decrease compared with that in normal animals of model II. However, SP and NKA concentrations in BALF increased only after antigen inhalation in model II animals. These results suggest that the decrease in pulmonary NEP activity does not trigger an increase in SP and NKA concentrations. Antigen inhalation caused SP and NKA production in model II, and then, the decreased NEP activity may have enhanced the SP and NKA levels.

Reversible airway constriction and prolonged nonspecific airway hyperresponsiveness are characteristic features of human asthma. Asthma is recognized by signs of eosinophilic airway inflammation, and mild or moderate asthma is well-controlled by inhaling steroids. However, in severe asthma, administration of high doses of steroids

will often not control the asthma [47]. In this study, capsaicin pretreatment inhibited asthma responses, and SP and NKA concentrations increased in model II animals, but not in model I animals. Thus, it is speculated that neurokinins contribute more against severe asthma than mild to moderate asthma.

In conclusion, our study demonstrated that the involvement of neurokinins in experimental severe asthma is greater than in experimental mild asthma. This observation might be related to the increased concentrations of SP and NKA in BALF.

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