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European Journal of Pharmacology 498 (2004) 287–294

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Involvement of enhanced neurokinin NK_3 receptor expression in the severe asthma guinea pig model

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Received 8 July 2004; accepted 13 July 2004
Available online 24 August 2004

Abstract

In this study, we investigated the involvement of neurokinin NK_3 receptors in a severe asthma model prepared by administering ovalbumin via inhalation three times to systemically sensitized guinea pigs. [^{3}H]senktide, a neurokinin NK_3 receptor ligand, showed significant specific binding to the lungs from the model animals, but not to those from negative control animals. The airway responsiveness to intravenous neurokinin B, a neurokinin NK_3 receptor agonist, was increased in the model, indicating an increase in functional NK_3 receptors. Furthermore, SB 223956 ((−)-3-methoxy-2-phenyl-*N*-[(1*S*)-phenylpropyl]quinoline-4-carboxamide), a selective neurokinin NK_3 receptor antagonist, significantly inhibited the ovalbumin-induced airway hyperresponsiveness to inhaled methacholine, but it did not show significant effects on the ovalbumin-induced airway narrowing and eosinophil accumulation. These results suggest that the expressed neurokinin NK_3 receptors in the severe asthma model are involved in the development of airway hyperresponsiveness.

Keywords: Airway hyperresponsiveness; Severe asthma model; (Guinea pig); Neurokinin NK_3 receptor

1. Introduction

Neurokinins, which include substance P, neurokinin A and neurokinin B, are a family of peptides, which share a common C-terminal sequence, Phe-X-Gly-Leu-Met-NH_2 (Casale, 1991; Maggi et al., 1995). Neurokinins are present in unmyelinated pulmonary C fibers in airways (Maggi et al., 1993; Otsuka and Yoshioka, 1993). Stimulation of the C-fibers evokes the release of the neurokinins, thereby eliciting a wide range of biological actions. These include vascular hyperpermeability, vasodilation, bronchoconstriction, airway hyperresponsiveness, cough, facilitation of cholinergic neurotransmission and mucus secretion (Rogers et al., 1989; Yasumitsu et al., 1996; Chapman et al., 1998).

The biological effects of neurokinins are mediated through three types of receptors, termed neurokinin NK_1, NK_2 and NK_3, which are members of the superfamily of G protein-coupled, seven-transmembrane-spanning receptors (Maggi et al., 1993). The three neurokinin receptors have been cloned and expressed. Substance P interacts preferentially with the neurokinin NK_1 receptor, neurokinin A with the neurokinin NK_2 receptor, and neurokinin B with the neurokinin NK_3 receptor (Joos et al., 1995). The neurokinin-receptor activation causes stimulation of phospholipase C leading to a phosphoinositide breakdown and elevation of intracellular calcium (Maggi et al., 1993). It has been recognized that the expression of neurokinin NK_3 receptor is limited mainly to the central and peripheral nervous system, while neurokinin NK_1 and NK_2 receptors are expressed both in the nervous system and in target

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doi:10.1016/j.ejphar.2004.07.071

organs, including airways (Maggi, 1993; Myers and Undem, 1993).

It has been reported that chronic treatment with a high dose of capsaicin, which depletes neurokinins from unmyelinated pulmonary C fibers, inhibits antigen-induced bronchoconstriction in sensitized guinea pigs (Warth et al., 1995), and inhibits antigen-induced pulmonary response and airway hyperresponsiveness to methacholine in repeatedly antigen-sensitized guinea pigs (Matsuse et al., 1991; Tiberio et al., 1997). In addition, involvement of neurokinins in an asthma guinea pig model has been shown using neurokinin NK_1, NK_2 and NK_1/NK_2 dual antagonists (Bertrand et al., 1993; Boichot et al., 1995; Kudlacz et al., 1996; Mizuguchi et al., 1996; Schuiling et al., 1999a,b). Thus, it is considered that neurokinins will play an important role in asthma. Indeed, it has been reported that neurokinin NK_1 and NK_2 receptor genes are expressed in asthmatics (Adcock et al., 1993; Bai et al., 1995), and that the concentration of substance P-like immunoreactivity in the lavage fluids of allergic asthma subjects is increased compared with that of normal subjects (Nieber et al., 1992). Recently, involvement of the neurokinin NK_3 receptor in the airways has been demonstrated in addition to that of neurokinin NK_1 and NK_2 receptors. Myers and Undem (1993) have shown that neurokinin-induced depolarization is mediated by the neurokinin NK_3 receptor in guinea pig bronchial parasympathetic ganglion neurons. In addition, it has been reported that neurokinin NK_3 antagonists inhibit citric acid-induced cough and airway hyperresponsiveness, and neurokinin-induced airway hyperresponsiveness in guinea pigs (Daoui et al., 1997, Daoui et al., 1998, Daoui et al., 2000; Emonds-Alt et al., 2002). However, the role of NK_3 receptor in asthma is still unclear, although Nenan et al. (2001) has shown that SR 142801 (osanetant, ((*R*)-(*N*)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl) piperidin-3-yl) propyl)-4-phenylpiperidin-4-yl-*N*-methylacetamide), a neurokinin NK_3 receptor antagonist, significantly decreases the recruitment of eosinophils in ovalbumin-sensitized mice.

In this study, in order to investigate the role of neurokinin NK_3 receptor in asthma, we developed a severe asthma model by ovalbumin inhalation three times (once a week) in systemically sensitized guinea pigs, and examined the binding affinity of [^{3}H]senktide, a neurokinin NK_3 receptor ligand, to the lung homogenate. Then, we examined the airway responsiveness to intravenous neurokinin B in naive and model animals to check whether the increased neurokinin NK_3 receptors function or not. Furthermore, we investigated the effects of SB 223956 ((−)-3-methoxy-2-phenyl-*N*-[(1*S*)-phenylpropyl]-quinoline-4-carboxamide), a selective neurokinin NK_3 receptor antagonist (Giardina and Raveglia, 1997), on antigen-induced airway narrowing, and airway hyperresponsiveness to inhaled methacholine and eosinophil accumulation in bronchoalveolar lavage fluid using the model.

2. Materials and methods

2.1. Animals

Male, 5-week-old Hartley guinea pigs were purchased from Japan SLC (Hamamatsu, Japan) and were used at the age of 6 weeks. The guinea pigs were kept in a room under a 12-h light/dark lighting cycle, and a room temperature and humidity set at 23±2 °C and 55±5%, respectively. The animals were provided food and tap water ad libitum. All animal experimental procedures were performed under the guideline for animal experiments in Sankyo (Tokyo, Japan).

2.2. Reagents

SB 223956 was synthesized in the Medicinal Chemistry Research Laboratories of Sankyo.

Ovalbumin, mepyramine maleate (mepyramine), sucrose, polyethyleneimine, TRIZMA® base (Tris), bovine serum albumin, chymostatin, leupeptin, phosphoramidon, gallamine triethiodide (gallamine) and methacholine chloride (methacholine) were obtained from Sigma (St. Louis, MO). Pentobarbital sodium (pentobarbital) was purchased from Abbott Laboratories (North Chicago, IL). Tetra-hydrothiophene 1,1-dioxide (sulfolane) was obtained from Kanto Chemical (Tokyo, Japan). Chloroform, manganese II chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) and bacitracin were from Wako (Osaka, Japan). Phosphate buffer (67 mM) was from Iatron Laboratories (Tokyo, Japan). Ethylenediamine tetraacetic acid (EDTA) was from Daiichi Pure Chemical (Tokyo, Japan). Neurokinin B were from Peptide Institute (Osaka, Japan). [Prolyl^{2,4}-3,4(n)-^{3}H]substance P ([^{3}H]substance P) and [^{3}H]SR 48968 (saredutant, ((*S*)-*N*-methyl-N[4-(4-acetylamino-4-phenyl piperidino)-2-(3,4-dichlorophenyl)butyl] benzamide) were from Amersham Pharmacia Biotech (Uppsala, Sweden). [Phenylalanyl-3, 4, 5-^{3}H]-senktide ([^{3}H]senktide) was from NEN™ Life Science Products (Boston, MA). Sodium dodecyl sulfate was from Bio-Rad Laboratories (Cambridge, MA).

2.3. Severe asthma model (Itoh et al., 1996)

The protocol is shown in Fig. 1. 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. (1978). Ovalbumin was suspended in physiological saline. A booster injection was given 1 week later in the same manner (Day 7). One week after the booster injection, 1% ovalbumin was inhaled for 3 min in the chamber using a nebulizer (NE-U11B, OMRON, Kyoto, Japan; Day 15). Thirty minutes before the inhalation, a histamine H_1 antagonist, mepyramine (10 mg/kg) was intraperitoneally injected in order to prevent acute fatal anaphylaxis and ensure that a high dose of antigen was inhaled. Mepyramine was dissolved in physiological saline. Then, a second (Day 22) and third (Day 29) inhalation was

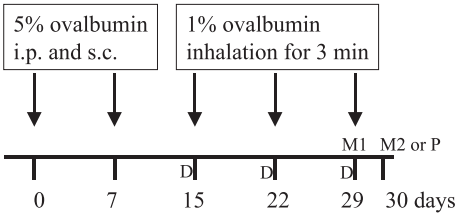

Fig. 1. Experimental protocol for the preparation of an asthma model. Guinea pigs were immunized weekly via intraperitoneal and subcutaneous administrations of ovalbumin on days 0 and 7, followed by inhalation of nebulized ovalbumin on days 15, 22 and 29. For the binding assay, lung samples were prepared on day 30 (P). In the experiments to investigate the effects of SB 223956 on asthma responses, SB 223956 (10 mg/kg) was administered intravenously 5 min before each antigen inhalation (D). The bronchial response to airway narrowing was measured on day 29 after ovalbumin inhalation (M1). On day 30, airway responsiveness to inhaled methacholine was measured. This was followed by the collection of bronchoalveolar lavage fluid (M2). In the experiments to investigate airway responsiveness to neurokinin B, the airway pressure was measured on day 30 (M2).

given in the same manner at 1-week intervals. The animals were fasted for 1 day before all three inhalations (Days 14, 21 and 28).

2.4. Receptor binding

2.4.1. Preparation of guinea pig lung membrane

The guinea pig lung membranes were prepared according to the method of Fujii et al. (1992) with a slight modification. Model animals administered antigen three times by inhalation (challenge (+)) and those administered antigen twice by inhalation (challenge (−)) as well as the negative control (naive) animals were exsanguinated under chloroform anesthesia and their lungs were removed rapidly. The isolated tissue was washed several times in ice-cold phosphate buffer (67 mM) and pooled (n=3–5). 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, Switzerland). After centrifuging at 1000×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. Then, the suspension was centrifuged at 12,000×g for 20 min at 4 °C. Next, the supernatant was centrifuged at 100,000×g for 60 min at 4 °C, and the pellets were suspended in Tris–HCl buffer (50 mM, pH 7.4). The suspension was frozen at −80 °C until use. Protein content was measured using Protein Assay Kit II (Bio-Rad Laboratories) before the binding assay.

2.4.2. [^{3}H]substance P binding

[^{3}H]substance P was used as a ligand for the neurokinin NK_1 receptor. Receptor binding was initiated by the addition of 250 μl of membrane (0.25 mg protein) to [^{3}H]substance P (final concentration 0.10–2.5 nM) and dimethyl sulfoxide (DMSO, final concentration 0.5%) in a final volume 500 μl of 50 mM Tris–HCl buffer (pH 7.4) containing 0.05% bovine serum albumin, 1.6 μg/ml chymostatin, 1.6 μg/ml leupeptin, 16 μg/ml bacitracin, 4 μg/ml phosphoramidon and 1.2 mM $MnCl_2$. The reaction mixtures were incubated at room temperature for 35 min. The incubation was stopped by rapid filtration with a harvester (Biomedical Research and Development Laboratories, Gaithersburg, MD) through Whatman GF/B glass filters (Biomedical Research and Development Laboratories) that were presoaked in 5 mM Tris–HCl (pH 7.4) buffer containing 0.1% polyethyleneimine and 0.04% bovine serum albumin. Each of the filters was washed three times with 5 ml of cooled (about 4 °C) 5 mM Tris–HCl buffer containing 0.04% bovine serum albumin and the radioactivity was measured with a liquid scintillation counter.

2.4.3. [^{3}H]SR 48968 binding

[^{3}H]SR 48968 (final concentration 1–10 nM) was used as a ligand for the neurokinin NK_2 receptor. The experimental conditions of use were the same as those mentioned above (Section 2.4.2).

2.4.4. [^{3}H]senktide binding

[^{3}H]senktide (final concentration 1–15 nM) was used as a ligand for the neurokinin NK_3 receptor. The conditions of use were the same as those mentioned above (Section 2.4.2) except for the following. The reaction mixtures were incubated at room temperature for 65 min in 5 mg/ml bovine serum albumin-coated plastic tubes, and the filter was washed three times with 5 ml of 5 mM Tris–HCl (pH 7.4) buffer containing 0.04% bovine serum albumin and 0.01% sodium dodecyl sulfate.

2.4.5. Scatchard plot analysis

The K_D and B_{max} values were calculated by Scatchard plot analysis (Rosenthal, 1967) using Kell Version 6 (Biosoft, Ferguson, MO).

2.5. Measurement of airway responsiveness to intravenous neurokinin B

The airway responsiveness was determined by examining neurokinin B-induced bronchoconstriction according to the method of Konzett and Rössler (1940). Negative control animals and asthma model animals 24 h after the last antigen inhalation were anesthetized with pentobarbital (30 mg/kg s.c.) and their trachea was cannulated. The cervical artery and vein were also cannulated to monitor the systemic blood pressure and heart rate, and to inject drugs, respectively. Gallamine (20 mg/kg i.v.) was administered and then, the animals were subjected to artificial ventilation/respiration with a constant-volume respiration pump (Type 7025, Ugo basile, Comerio-Varese, Italy) at a frequency of 60 airflows per min and a tidal volume of 8–10 ml/kg. Any change in airway pressure at a constant airflow was measured using a pressure transducer (TP-200T, Nihon Kohden, Tokyo, Japan)

connected to a side-arm of the tracheal cannula as airway pressure is proportional to tracheobronchial constriction. Neurokinin B (0.25–16 μg/kg at twofold-increasing doses) was injected intravenously every 5 min and the peak value of airway pressure was measured. Neurokinin B was dissolved in 50% sulfolane diluted with physiological saline. The increase in airway pressure was calculated by subtracting the value before neurokinin B injection (basal value) from the peak value of airway pressure.

2.6. Effects of SB 223956 on severe asthma model

SB 223956 at a dose of 10 mg/kg suspended in guinea pig serum that was diluted to 50% with physiological saline was administered intravenously 5 min before each antigen inhalation (Days 15, 22 and 29). Sensitized animals administered antigen three times by inhalation but without SB 223956 administration were used as positive controls.

2.6.1. Measurement of antigen-induced airway narrowing

As an index of airway narrowing, specific airway resistance (cm $H_2O \cdot s$) was determined by the method of Pennock et al. (1979) on a breath-by-breath basis in a double-chamber plethysmograph with a respiratory gas analyzer (Model P, System B-IV, Buxco Electronics, Sharon, CT). Specific airway resistance was measured before and at 0.25, 1, 2, 3, 4, 5 and 6 h after the last inhalation (on Day 29). The specific airway resistance value before the last antigen inhalation was determined before SB 223956 administration on Day 29.

The percent relative increase in specific airway resistance from the value before the antigen inhalation (basal value: 100%) was calculated as the degree of airway narrowing. The integrated areas (ΔAUC) under the time–response (percentage relative increase in specific airway resistance) curve were calculated from 0 to 2 h as the immediate bronchial response and from 2 to 6 h after antigen inhalation as the late bronchial response (Itoh et al., 1996).

2.6.2. Measurement of airway responsiveness to inhaled methacholine

Airway responsiveness was determined by measuring the concentration of methacholine required to increase airway resistance (by twofold), 24 h after the last antigen inhalation (Day 30). As an index of airway narrowing by methacholine, respiratory resistance (cm H_2O/ml/s) was automatically measured by a forced oscillation technique using Animalasto (TMC-2100, Chest MI, Tokyo, Japan) with a multinebulizer, based on the method of Mead (1960). In brief, guinea pigs were placed inside a body plethysmograph chamber, and a 30 Hz sine wave oscillation pressure was applied on the animal body surface. The respiratory flow rate through the mask and the box pressure was measured with a differential pressure transducer. Respiratory resistance was calculated according to the method of Hyatt et al. (1970). The mask flow, body pressure and resistance were recorded using a multichannel polygraph recorder.

Methacholine (32–4096 μg/ml at twofold-increasing doses) and physiological saline aerosols were generated using an ultrasonic nebulizer driven by compressed air. Physiological saline was inhaled for 1 min, and then, twofold-increasing concentrations of methacholine was inhaled for 1 min every minute. Next, the percentage relative increase in respiratory resistance from the value after physiological saline inhalation (base) was calculated. The minimum concentration of methacholine that increased the basal airway resistance by twofold in individual animals (PC_{200} in μg/ml) was calculated based on three methacholine concentrations and the percentage relative increase in respiratory resistance by nonlinear regression analysis.

2.6.3. Measurement of eosinophil infiltration

Bronchoalveolar lavage was performed, and the number of eosinophils in the bronchoalveolar lavage fluid was measured in the asthma model, 24 h after the last antigen inhalation (Day 30). After the guinea pigs were anesthetized with pentobarbital (30 mg/kg i.p.), their trachea was cannulated with a disposable intravenous catheter (3-Fr size, Atom Medical, 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 three equal volumes of physiological saline (10 ml/kg). The bronchoalveolar lavage fluid collected from each animal was immediately placed on an ice bath and centrifuged at 300×*g* for 10 min at 4 °C, and the precipitate was resuspended in 4 ml of physiological saline. Total cell counts were determined using a standard hemocytometer (Kayagaki Irika Kogyo, Tokyo, Japan). The proportion of eosinophils (%) was determined by preparing a smear stained with Diff-Quik solution (International Reagents, Kobe, Japan). A minimum of 500 cells per smear was counted under a light microscope (×400, BX-50-34-SP, Olympus Optical, 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.

2.7. Data analysis

The data from the in vivo studies were expressed as means±S.E.M. Comparisons between the negative control and model animals for neurokinin B-induced bronchoconstriction were made by the *t*-test using SAS® System Release 8.2 (SAS Institute, Cary, NC) and by the Bonferroni post hoc test. Comparisons between the positive control and SB 223956-treated animals for airway narrowing were made by the *t*-test, and those between the negative and positive controls, and positive control and SB 223956-treated animals for airway responsiveness and eosinophil accumulation, by the Mann–Whitney *U*-test, using SAS® System

Release 8.2. P values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Receptor binding to lungs

[^{3}H]substance P and [^{3}H]SR 48968 showed specific binding to the lungs of age-matched negative control (naive) animals, challenge (−) animals and challenge (+) animals in the severe asthma model. The K_D and B_{max} values for [^{3}H]substance P and [^{3}H]SR 48968 binding were almost same in the lungs of negative control animals, challenge (−) animals and challenge (+) animals (Table 1). [^{3}H]senktide did not show significant specific binding to the lungs of age-matched negative control animals or challenge (−) animals in the asthma model. On the other hand, in the challenge (+) animals, [^{3}H]senktide showed specific binding (Table 1).

3.2. Airway responsiveness to intravenous neurokinin B

We examined the airway responsiveness to intravenous neurokinin B in negative control (naive) animals and asthma model animals to verify whether the increased NK_3 receptors still function or not. An intravenous injection of neurokinin B induced bronchoconstriction in a dose-dependent manner in the negative control guinea pigs. In the asthma model animals, neurokinin B also induced bronchoconstriction in a dose-dependent manner. The neurokinin B dose–response curve of the model animals, however, shifted to the left, and the increasing airway pressure values at 0.5, 1 and 2 μg/kg of neurokinin B in the model animals were significantly greater than those in the negative control animals (Fig. 2).

Table 1
K_D and B_{max} values of [^{3}H]substance P, [^{3}H]SR 48968 and [^{3}H]senktide binding to membrane preparations of lung in naive and asthma model animals

		Ligand		
		[^{3}H]substance P	[^{3}H]SR 48968	[^{3}H]senktide
Negative control	K_D (nM)	0.093	4.2	ND
	B_{max} (fmol/mg protein)	33	290	ND
Challenge (−)	K_D (nM)	0.15	2.6	ND
	B_{max} (fmol/mg protein)	41	210	ND
Challenge (+)	K_D (nM)	0.15	2.5	5.1
	B_{max} (fmol/mg protein)	43	220	13

Each value represents the mean of the data performed in triplicate. In each group, pooled samples from three to five animals were used for the assay. Challenge (−): sensitized guinea pigs administered antigen twice by inhalation (without last antigen inhalation), challenge (+): sensitized guinea pigs administered antigen three times by inhalation, ND: not detected.

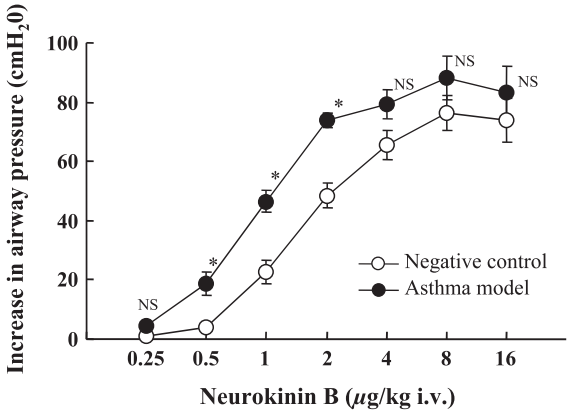

Fig. 2. Airway responsiveness to neurokinin B in negative control and asthma model animals. Neurokinin B (0.25–16 μg/kg at twofold-increasing doses) was injected intravenously every 5 min and the peak value of airway pressure was measured. Each point represents means±S.E.M. (n=8 for negative control and n=5 for asthma model group). Statistical analysis was carried out by the t-test and Bonferroni post hoc test, *P<0.05 and NS, not significant.

3.3. Effects of SB 223956 on severe asthma model

We investigated the effects of SB 223956, a neurokinin NK_3 receptor antagonist (Giardina and Raveglia, 1997), on antigen-induced airway narrowing, airway hyperresponsiveness to inhaled methacholine, and eosinophil accumulation in bronchoalveolar lavage fluid in the animal model. SB 223956 at a dose of 10 mg/kg was administered intravenously 5 min before each antigen inhalation (Days 15, 22 and 29).

3.3.1. Effect of SB 223956 on antigen-induced immediate and late bronchial responses

Fig. 3 shows the percentage change from the baseline of specific airway resistance after the last antigen inhalation in the asthma model (positive control). A biphasic bronchial

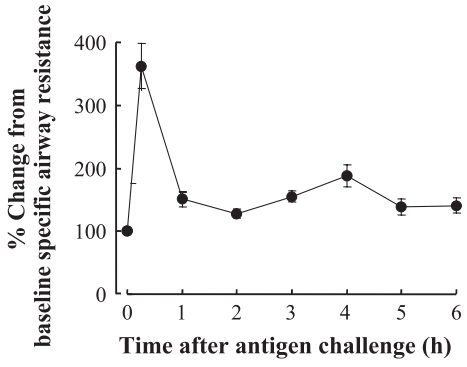

Fig. 3. Time–response (specific airway resistance) curve after antigen inhalation in model animals. A biphasic bronchial response, an immediate bronchial response that peaked at 15 min and late bronchial response that peaked at 4 h, was induced. Each point represents means±S.E.M. of 20 animals.

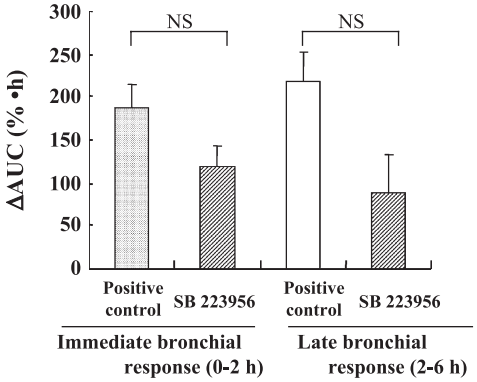

Fig. 4. Effects of SB 223956 on immediate and late bronchial responses in asthma model animals. We calculated ΔAUC, an integrated area under the time–response (percentage relative increase in specific airway resistance) curve to measure the intensity of airway narrowing. SB 223956 (10 mg/kg) was administered intravenously 5 min before each antigen inhalation, which was performed three times. Data represent means±S.E.M. (n=20 for positive control and n=5 for SB 223956-treated group). Statistical analysis was carried out by the t-test, NS, not significant.

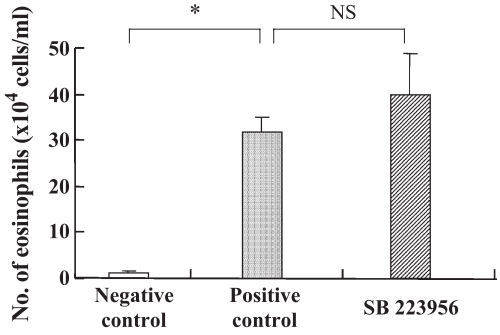

Fig. 6. Effect of SB 223956 on antigen-induced eosinophil accumulation in bronchoalveolar lavage fluid 24 h after the last antigen inhalation in asthma model animals. We counted the number of eosinophils in bronchoalveolar lavage fluid after the measurement of airway responsiveness to methacholine. SB 223956 (10 mg/kg) was administered intravenously 5 min before each antigen inhalation, which was performed three times. Data represent means±S.E.M. (n=10 for negative control, n=12 for positive control and n=5 for SB 223956-treated group). Statistical analysis was carried out by the Mann–Whitney U-test, *P<0.05 and NS, not significant.

response, an immediate bronchial response that peaked at 15 min and late bronchial response that peaked at 4 h, was induced. The naive animals did not show an increase in specific airway resistance. In Fig. 4, the integrated areas (ΔAUC) of immediate and late bronchial responses are shown. SB 223956 showed a tendency to decrease the ΔAUC of immediate and late bronchial responses, but these effects were not statistically significant.

3.3.2. Effect of SB 223956 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. The mean value of PC_{200} 24 h after the last antigen inhalation in the asthma model animals (positive control) was significantly decreased compared with that of the negative control (naive) animals (Fig. 5). SB 223956 significantly inhibited the airway hyperresponsiveness to methacholine (Fig. 5).

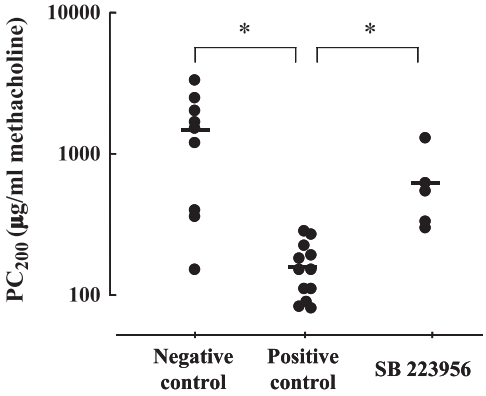

Fig. 5. Effect of SB 223956 on antigen-induced airway hyperresponsiveness to inhaled methacholine 24 h after the last antigen inhalation in asthma model animals. We used PC_{200}, the minimum concentration (μg/ml) of methacholine that increased the basal airway resistance by twofold in individual animals, as a measure of airway hyperresponsiveness. SB 223956 (10 mg/kg) was administered intravenously 5 min before each antigen inhalation, which was performed three times. Circles represent individual values of PC_{200}, and the horizontal bar, the mean value. Statistical analysis was carried out by the Mann–Whitney U-test, *P<0.05.

3.3.3. Effect of SB 223956 on antigen-induced eosinophil accumulation in bronchoalveolar lavage fluid

The number of eosinophils 24 h after the last antigen inhalation in the asthma model animals (positive control) was significantly increased compared with that of the negative control (naive) group (Fig. 6). SB 223956 did not affect the antigen-induced eosinophil accumulation in bronchoalveolar lavage fluid (Fig. 6).

4. Discussion

In this study, we have shown that the airway responsiveness was increased as a result of an increase in functional neurokinin NK_3 receptors, and the airway hyperresponsiveness to inhaled methacholine was inhibited after the administration of neurokinin NK_3 receptor antagonist, in a severe asthma guinea pig model.

[^{3}H]senktide showed specific binding in challenge (+) animals while the binding was not detected in the negative control and challenge (−) animals. We have previously evaluated [^{3}H]senktide binding to the naive guinea pig brain, which was reported to be rich in neurokinin NK_3 receptors (Guard et al., 1990), and the K_D and B_{max} values were 6.8 nM and 62 fmol/mg protein, respectively (unpublished data). Since the K_D value for [^{3}H]senktide binding to the lungs in the asthma model (5.1 nM: this study) was almost the same as that to the brain in the negative control animals, it is strongly suggested that the [^{3}H]senktide binding to lungs in the challenge (+) animals was to the neurokinin NK_3 receptor. In

addition, repeated antigen inhalation might be effective in increasing neurokinin NK_3 receptors because [^{3}H]senktide binding was observed in the challenge (+) animals but not in the challenge (−) animals.

The neurokinin B dose–response curve of the model animals shifted to the left compared with that of the negative control animals, indicating that the airway responsiveness to neurokinin B was increased in the model animals. Thus, it was confirmed that the increased neurokinin NK_3 receptors in the binding study were functional in vivo.

SB 223956, a selective neurokinin NK_3 receptor antagonist, significantly inhibited the airway hyperresponsiveness to inhaled methacholine in the model animals. This result suggests the involvement of neurokinin NK_3 receptors in the development of airway hyperresponsiveness to methacholine in the asthma model. This is in contrast with a previous result where SR 142801, a neurokinin NK_3 receptor antagonist, did not significantly modify the airway hyperresponsiveness to inhaled histamine in a rabbit asthma model (D'Agostino et al., 2002). It seems that this model was a mild asthma model in which systemically sensitized rabbits were made to inhale antigen once. When the systemically sensitized guinea pigs were made to inhale antigen only once, which corresponds to a mild asthma model, we were also able to observe that the neurokinin NK_3 receptor expression was not increased (unpublished data). On the other hand, in our severe asthma model, in which systemically sensitized guinea pigs were made to inhale antigen three times, neurokinin NK_3 receptor expression was enhanced. Therefore, the discrepancy in the results could be explained by the difference in neurokinin NK_3 receptor expression related to the asthma model (mild or severe) used.

Although it is difficult to provide an exact mechanism for the development of airway hyperresponsiveness at this time, the neurokinin NK_3 receptor is considered to be involved in the process. It has been reported that the inhalation of a neurokinin NK_3 agonist increased airway responsiveness to acetylcholine and the airway responsiveness was inhibited by a neurokinin NK_3 receptor antagonist in guinea pigs (Daoui et al., 2000). Thus, it is considered that endogenous neurokinins interact with the expressed neurokinin NK_3 receptor in model animals, which causes airway hyperresponsiveness. Further mechanism is still unclear, but one possibility is that the neurokinin NK_3 receptor stimulation then modifies the responsiveness of target cells via other substances and/or inflammatory cells. In relation to this, Braun et al. (1999) have demonstrated that co-cultured B- and T-lymphocytes could express neurokinin NK_3 receptor mRNA after activation by interleukin-5 and transforming growth factor β2. Another possibility is that the neurokinin NK_3 receptor stimulates target cells directly. This might induce an abnormality in the muscle itself, which could cause an increase in contractile activity in airway tissue as Ishida et al. (1990) reported in their in vitro experiments using tracheal preparations from guinea pigs that were repeatedly administered antigen. Another mechanism could be associated with an increase in cholinergic neural activity due to neurokinin NK_3 receptors since neurokinin NK_3 receptor stimulation has been demonstrated to facilitate cholinergic neurotransmission in guinea pig airways (Myers and Undem, 1993; Canning et al., 2002). Whatever the ensuing mechanism may be, we consider that the airway hyperresponsiveness was induced in the guinea pig asthma model as a result of an increase in neurokinin NK_3 receptors owing to stimulation by endogenous neurokinins.

SB 223956 did not show a significant effect on ovalbumin-induced immediate and late bronchial responses, and did not affect eosinophil accumulation 24 h after the last antigen inhalation in model animals. These results suggest that the neurokinin NK_3 receptor is mainly not involved in the development of bronchial response and eosinophil accumulation in model animals. On the other hand, Nenan et al. (2001) have reported that SR 142801, a neurokinin NK_3 receptor antagonist, induced a significant decrease in the recruitment of eosinophils in ovalbumin-sensitized mice. The reasons for the difference between our results and theirs are not clear, but it could be due to a difference in animal species (mice vs. guinea pigs), administration route of test compounds (inhalation vs. i.v.) and/or animal model used (mild vs. severe).

In conclusion, it was demonstrated that neurokinin NK_3 receptor expression was enhanced in the lungs of a severe asthma guinea pig, and that the expressed neurokinin NK_3 receptors were involved in the development of airway hyperresponsiveness.

Acknowledgement

The authors thank Dr. Kazuhiro Ito (National Heart and Lung Institute, Imperial College School of Medicine, London, UK) for his helpful suggestions and contribution in preparing this manuscript.

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