

Selective Nasal Allergen Provocation Induces Substance P-Mediated Bronchial Hyperresponsiveness

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Although the concept of “global airway allergy” has become widely accepted during recent years, nasobronchial interaction and its mechanisms remain incompletely understood. The experimental study of the effect of nasal allergen deposition on lower airway pathology is hampered by the difficulty of avoiding lower airway penetration of the allergens. In ovalbumin-sensitized mice with experimental airway allergy, nasal allergen provocations were performed after complete anatomical separation of upper and lower airways by means of a tracheotomy. A canula was inserted in the trachea, and the trachea was ligated, thus inhibiting any passage of allergens from upper to lower airways. Mice showed bronchial hyperresponsiveness to methacholine as early as 4 hours after nasal allergen provocation in the absence of recruitment of inflammatory cells. An increased substance P (SP) concentration in the bronchial lumen was found, as well as an increased number of SP-positive pulmonary nerves. Treatment with a neurokinin (NK) 1 receptor antagonist abolished the allergen-induced bronchial hyperresponsiveness. Moreover, endobronchial administration of SP caused NK1 receptor-dependent bronchial hyperresponsiveness in mice with airway allergy. Nasal allergen provocation rapidly induces bronchial hyperresponsiveness via pulmonary up-regulation of SP and activation of NK1 receptors.

Keywords: asthma; rhinitis; nasobronchial interaction; substance P; bronchial hyperresponsiveness

The concept of “united airways allergy” has become progressively accepted during the last decade, and has been included in the state-of-the-art document “Allergic rhinitis and its impact on asthma” (1). The epidemiological association between the two disease entities is clear: up to 90% of individuals with asthma have been reported to have allergic rhinitis, and one-third of patients with allergic rhinitis suffer from asthma (2, 3). Similar immunological processes, such as IgE-dependent mast cell degranulation and T helper type 2 lymphocyte activation, lead to an inflammatory infiltrate of predominantly eosinophils in both allergic rhinitis and asthma (4, 5). The most convincing

CLINICAL RELEVANCE

We demonstrate, for the first time, a rapid development of bronchial hyperresponsiveness after a selective nasal allergen deposition, using a tracheotomy to separate upper and lower airways anatomically. We show that substance P was responsible for this reaction via binding to the neurokinin 1 receptor. This research improves our understanding of the concept of “United Airways.”

proof of the link between upper and lower airways comes from experimental studies in which allergen deposition in either nose or bronchi leads to inflammatory changes at a distant site of the airways (6, 7). The precise mechanisms underlying this bidirectional nasobronchial crosstalk remain speculative. Moreover, it cannot be excluded that the described bronchial effects of a nasal allergen provocation are caused by bronchial deposition of the allergens after inhalation. We used a novel approach to confirm the existence of nasobronchial interaction after a truly selective nasal allergen provocation. The selectivity of the nasal allergen deposition was ensured by means of a tracheotomy, which completely interrupts the anatomic connection between nose and bronchi. Pulmonary functional and inflammatory changes were addressed as early as 4 hours after allergen provocation.

MATERIALS AND METHODS

Mice

Male BALB/c mice (6 wk of age) were obtained from Harlan (Horst, The Netherlands) and kept under conventional pathogen-free conditions. Experimental procedures were approved by the local ethics committee of animal experiments.

Allergen Sensitization, Challenge, and Selective Nasal Provocation

Eosinophilic airway inflammation was induced with adjuvant-free ovalbumin (OVA; Sigma, St. Louis, MO), as previously described (8) (Figure 1). Briefly, mice were sensitized to OVA by seven intraperitoneal injections on alternate days (Days 1–14) and subsequently exposed to OVA by daily nebulizations (Days 33–40). After an allergen-free period of 10 days (Day 50), mice were anesthetized with 150 mg/kg ketamin and 10 mg/kg xylazine. Via a longitudinal incision, a tracheotomy was performed and an 18-GA polyethylene catheter was inserted in the trachea. Subsequently, a suture was tightly tied around the canula-containing trachea to avoid leakage of allergens from upper to lower airways. After tracheotomy, a selective nasal allergen provocation was performed by instillation of 1 mg of OVA in 10 μ l of saline (SAL) in the nose (OVA group). To control groups, either 10 μ l of SAL (SAL group) or 1 mg of BSA in 10 μ l of SAL (BSA group) (BSA

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grade V; MP Biomedicals, Illkirch, France) was instilled in the nose. All mice were killed for analyses by intraperitoneal injection of a lethal dose of pentobarbital 4 hours after provocation.

Measurement of Bronchial Hyperresponsiveness

Airway responses to increasing doses of inhaled methacholine (MCh) were measured using a forced oscillation technique (FlexiVent; SCIREQ, Montreal, PQ, Canada). For a detailed description, see the online supplement.

Bronchoalveolar Lavage and Histology

Lungs were lavaged as previously described (8), and differential cell counts were performed on May-Grünwald-Giemsa-stained cytospin preparations of the lavage fluid (see online supplement for details). Lung tissue was fixated in formaldehyde 6%, paraffin embedded, cut into 6- μ m sections, and stained with hematoxylin and eosin.

Substance P, Neurokinin A, and Neurokinin B ELISA

For the quantitative determination of substance P (SP), NKA, and NKB in bronchoalveolar lavage (BAL) fluid, we used commercially available ELISA kits from R&D Systems (Wiesbaden, Germany) and Bachem (Bubendorf, Switzerland) according to the manufacturer's instructions.

SP and NK1 Receptor Immunohistochemistry

Double immunofluorescence labeling of SP, the neuronal marker, protein-gene product (PGP) 9.5, or NK1 receptor was performed with the fluorescent Avidin kit (Vector Laboratories Inc., Burlingame, CA) according to the manufacturer's instructions and as previously described (9). For a detailed description of the technique, see the online supplement.

Endobronchial SP Application

OVA-sensitized mice with experimental airway allergy (Day 50) on the one hand, and naive mice on the other hand, were anesthetized with sodium pentobarbital (70 mg/kg intraperitoneal). An 18-gauge metal catheter was inserted in the trachea, and mice were connected to a computer-controlled small animal ventilator. Mice were challenged with an SP aerosol (SP acetate salt hydrate; Sigma), generated with an inline nebulizer and administered intratracheally directly through the ventilator for 5 seconds. One group of mice received an aerosol of 0.1 mg SP/ml SAL, and a second group received 0.5 mg SP/ml SAL. Control mice received SAL. Immediately after the endobronchial SP administration, bronchial hyperresponsiveness was measured as described earlier.

Treatment with L-732138

L-732138, a selective antagonist of the NK1 receptor, was purchased from Sigma and dissolved in DMSO 70% in SAL. L-732138 (500 μ g in 50 μ l DMSO 70%) or DMSO 70% alone (50 μ l) was administered intraperitoneally 1 hour before endobronchial SP application in OVA-sensitized mice with experimental airway allergy. Bronchial respon-

siveness to MCh was measured immediately after SP administration. In another experiment, L-732138 or its solvent was administered intraperitoneally 1 hour before tracheotomy and nasal deposition of SAL or OVA in OVA-sensitized mice with experimental airway allergy. Analyses were performed 4 hours after the provocation. The administered dose of L-732138 was based on the data of Zhang and colleagues (9).

Statistical Analyses

A Kruskal-Wallis test was performed to show differences between the groups. When significant differences between the groups were established, comparisons between two groups were performed by using the Mann-Whitney *U* test. With both methods, a difference was considered to be significant with a *P* value less than 0.05. Data are expressed as means ($\pm$ SEM).

RESULTS

Nasal Allergen Provocation Induces Bronchial Hyperresponsiveness

In OVA-sensitized mice with experimental airway allergy (Figure 1), a tracheotomy was performed on Day 50, followed by a nasal allergen provocation by means of instillation of OVA (1 mg in 10 μ l of SAL) in the nose. Control mice received the same volume of SAL (10 μ l). At 4 hours after the provocation, mice that had received nasal OVA showed significant bronchial hyperresponsiveness (BHR) to MCh as compared with control mice (Figure 2).

At 4 hours after nasal SAL, BSA, or OVA provocation, no significant differences in absolute eosinophil, neutrophil, lymphocyte, or monocyte cell counts in the BAL fluid were found between the groups (Table 1). On hematoxylin and eosin-stained histological lung sections, no differences were noted in peribronchial inflammatory cell infiltration (Figure 3).

Induction of Bronchial SP after Nasal Allergen Provocation

To explore which mechanisms are involved in the rapid induction of bronchial hyperresponsiveness after nasal allergen provocation, the concentration of the neuropeptides, SP, NKA, and NKB, was measured in BAL fluid 4 hours after tracheotomy and selective nasal provocation with SAL (10 μ l) or OVA (1 mg in 10 μ l). A significant induction of bronchial SP was found after nasal allergen provocation (*P* = 0.015; Figure 4A), whereas the concentrations of NKA and NKB were below detection limit (data not shown). Also in homogenized lung tissue of these mice, no NKA or NKB could be detected (data not shown). Double immunofluorescence labeling of SP and the neuronal marker, PGP 9.5, was performed on histological sections of lung tissue taken 4 hours after a selective nasal provocation with SAL (10 μ l) or OVA (1 mg in

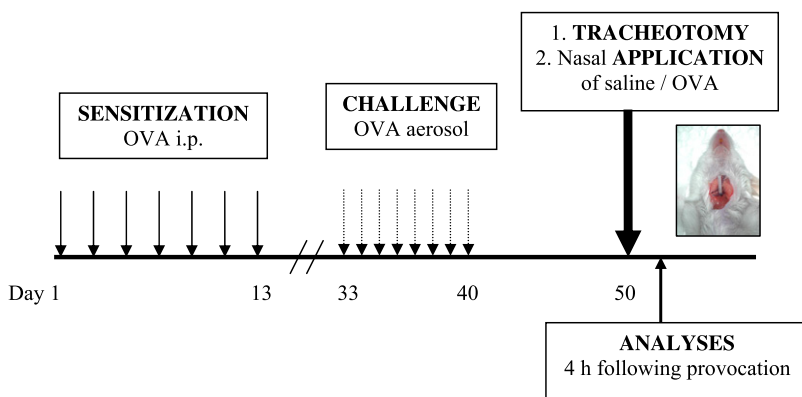


Figure 1. Protocol for sensitization and selective nasal allergen provocation in mice with experimental asthma. All mice were actively sensitized by seven intraperitoneal injections of 10 μ g ovalbumin (OVA) (grade V) in 0.5 ml of saline (SAL) on alternate days, from Day 1 to Day 13. From Day 33 to Day 40, mice were exposed daily for 5 minutes to nebulized OVA (10 mg/ml). On Day 50, a tracheotomy was performed, followed by selective nasal deposition of OVA in 10 μ l SAL. Control mice received 10 μ l SAL or BSA in 10 μ l SAL. Mice were killed 4 hours after provocation.

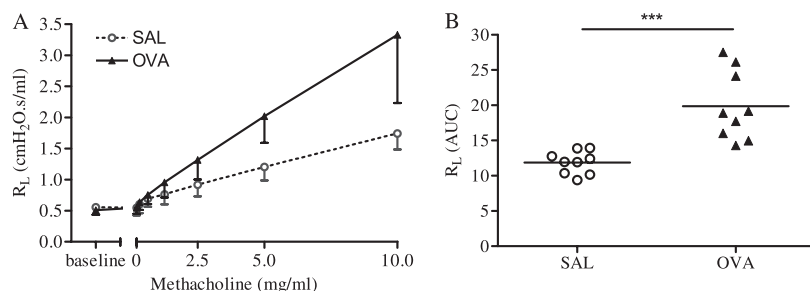


Figure 2. Bronchial hyperresponsiveness to methacholine (MCh) after selective nasal allergen provocation. SAL or OVA was applied in the nose of OVA-sensitized mice with experimental airway allergy after tracheotomy. At 4 hours after provocation, bronchial hyperresponsiveness to increasing doses of nebulized MCh was measured using a forced oscillation technique. The mean resistance was plotted against the MCh concentration (A) and the area under the curve (AUC) was calculated for each mouse (B). R_L , lung resistance. *** $P < 0.001$. Each group consisted of nine mice.

10 μ l) (Figures 4B and 4C). SP was clearly located in pulmonary nerves (Figure 4C), and increased in OVA compared with SAL mice ($P = 0.002$; Figure 4B). The total number of PGP 9.5-positive nerves was not significantly different between the OVA group and the SAL group (1.08 ± 0.17 [OVA] versus 0.66 ± 0.28 [SAL]; $P = 0.280$).

NK1 Receptor-Dependent Increase of Bronchial Hyperresponsiveness after Inhalation of SP in Mice with Experimental Airway Allergy

We next investigated whether endobronchial SP was capable of eliciting bronchial hyperresponsiveness without allergen stimulation. Therefore, different concentrations of SP (0, 0.1, and 0.5 mg/ml in SAL) were nebulized on Day 50 directly in the trachea via the tracheotomy, using the Flexivent artificial ventilation device. Bronchial responsiveness to MCh dose-dependently increased immediately after SP inhalation (Figure 5A). Pretreatment with the NK1 receptor antagonist, L-732138, inhibited the development of bronchial hyperresponsiveness (Figure 5B). Interestingly, no bronchial hyperresponsiveness could be elicited by endobronchial SP in naive mice (Figure 5C).

Up-Regulation of NK1 Receptor in Lungs of Mice with Allergic Airway Inflammation

Endobronchial SP administration only elicited bronchial hyperresponsiveness after previous airway allergen exposure, and was absent in naive mice. In search of a molecular substrate for this finding, we performed immunofluorescence labeling of the NK1 receptor on histological sections of lung tissue. The tissue was prelevated 4 hours after a selective nasal provocation with SAL (10 μ l) or OVA (1 mg in 10 μ l) after tracheotomy in previously OVA-sensitized and -challenged mice (Day 50). Sections of lung tissue from naive mice (Day 0) were also stained. Although no significant differences were found between the expression of NK1 receptor in lungs of mice with experimental airway allergy that were nasally provoked with SAL or OVA, there was an induction of NK1 receptor expression in both groups as compared with naive mice (Figures 6A and 6B) (SAL versus naive, $P = 0.009$; OVA versus naive, $P = 0.009$).

Pretreatment with the NK1 Receptor Antagonist L-732138 Inhibits BHR after Nasal Allergen Provocation

To investigate whether the rapid induction of SP in the lower airways was responsible for the bronchial hyperresponsiveness after nasal allergen provocation, we used the NK1 receptor antagonist, L-732138. L-732138 (500 μ g in 50 μ l 70% DMSO in SAL) or its solvent (50 μ l 70% DMSO in SAL) was injected intraperitoneally 1 hour before tracheotomy and selective nasal SAL (10 μ l) or OVA (1 mg in 10 μ l) application. In mice treated with L-732138 and challenged with OVA, the development of bronchial hyperresponsiveness 4 hours after nasal challenge was totally inhibited (Figure 7), and was comparable to the reaction of mice challenged with SAL.

DISCUSSION

Studying the mechanisms by which nasal allergen deposition influences lower airway pathology is hampered by the difficulty to perform truly selective nasal allergen provocations in allergic individuals. After nasal allergen deposition in patients, a proportion of the allergen may reach the lower airways via the airborne route and be responsible for the observed pulmonary effects. We took advantage of a mouse model of allergic airway inflammation to study the effects of a selective nasal allergen provocation on pulmonary functional and inflammatory responses. The selectivity of nasal allergen deposition was ensured here by means of a tracheotomy, interrupting the continuity of upper and lower airways, hence avoiding post-nasal drip and aspiration of allergens, inflammatory cells, and mediators.

Under the rigorous conditions of selective nasal allergen deposition, we observed a significant bronchial reaction. Nasal allergen deposition caused bronchial hyperresponsiveness after 4 hours, in the absence of an overt inflammatory influx in the bronchial lumen (Table 1). Simultaneously, we demonstrated SP release in the bronchial lumen (Figure 4A), whereas NKA and NKB were not detected. The tachykinins, SP, NKA, and NKB, are neurotransmitters belonging to the nonadrenergic, noncholinergic (NANC) nerve system. Although the NANC nerve system is basically a sensory nerve system, its nerve fibers can also be antidromically stimulated by action potentials that originate at different terminals of the same neuron, a phenomenon called axon reflex. The antidromic stimulation results in the so-called “neurogenic inflammation,” a local release of proinflammatory neuropeptides. In the airways, tachykinins have been related to antigen-induced bronchoconstriction, airway inflammation, and bronchial hyperresponsiveness in various animal models (10). Of the tachykinins, SP binds preferably to the NK1 receptor (11). SP was originally considered as a mere neurotransmitter, but it has become clear that a variety of nonneuronal cells are also capable of producing

TABLE 1. WHITE BLOOD CELL COUNTS IN BRONCHOALVEOLAR LAVAGE FLUID AFTER SELECTIVE NASAL ALLERGEN PROVOCATION

Cell Type	SAL (n = 6)	BSA (n = 8)	OVA (n = 8)
Eosinophils	2.0 ± 1.2	0.5 ± 0.2	1.5 ± 0.6
Lymphocytes	2.6 ± 0.8	1.3 ± 0.3	1.4 ± 0.3
Neutrophils	1.2 ± 0.3	1.3 ± 0.3	1.8 ± 0.7
Monocytes	7.7 ± 1.0	7.5 ± 1.1	6.9 ± 0.8

Definition of abbreviations: OVA, ovalbumin; SAL, saline.

White blood cells in bronchoalveolar lavage (BAL) fluid were counted 4 hours after nasal application of SAL (10 μ l), BSA (1 mg in 10 μ l), or OVA (1 mg in 10 μ l) in OVA-sensitized mice with experimental airway allergy after tracheotomy. Data represent absolute cell counts in BAL fluid $\times 10^{-4}$ /ml. Data are expressed as means ($\pm$ SEM).

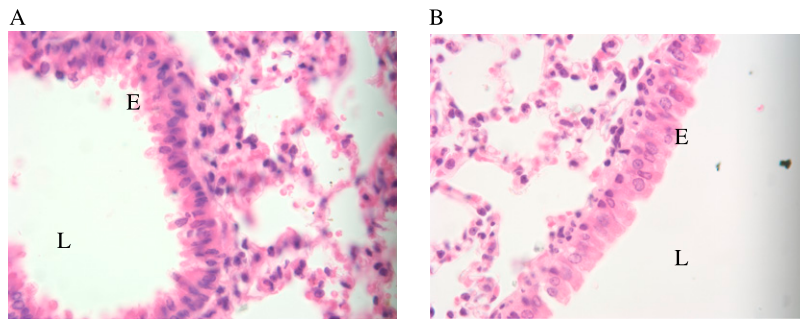


Figure 3. Absence of inflammatory cell recruitment to the lungs after selective nasal allergen provocation. SAL (A) or OVA (B) was applied in the nose of OVA-sensitized mice with experimental airway allergy after tracheotomy. The images show hematoxylin and eosin staining of lung tissue, prelevated 4 hours after the provocation. E, epithelium; L, lumen.

this molecule, especially in inflammatory conditions. Production of SP has been described in airway epithelial cells (12), airway smooth muscle cells (13), eosinophils (14), monocytes and macrophages (15), lymphocytes (16), and dendritic cells (17). To investigate the cellular source of SP in our experiments, immunofluorescence labeling was performed on histological sections of lung tissue after selective nasal deposition of OVA or SAL. Double staining for SP and PGP 9.5 showed that SP was located in pulmonary nerves (Figure 4C). The number of SP-positive nerves was increased in OVA-treated mice as compared with control animals (Figures 4B and 4C). The finding that SP is exclusively found in pulmonary nerves 4 hours after a nasal allergen provocation, whereas multiple cells are capable of SP production, can be explained by the absence of an overt influx of inflammatory cells in the lower airways at this time point.

We next proved that bronchial SP release is sufficient to cause rapid bronchial hyperresponsiveness in OVA-sensitized mice with experimental airway allergy. Endobronchial SP administration caused a dose-dependent increase of bronchial reactivity to MCh in these mice (Figure 5A). This rapid development of bronchial hyperresponsiveness by SP is dependent on the NK1 receptor, as shown by the complete inhibition of this effect by pretreatment with an NK1 receptor antagonist (Figure 5B). Interestingly, no effect of SP inhalation was seen in naive mice (Figure 5C). The residual allergic airway inflammation due to previous allergen exposures at the time of allergen provocation or SP administration in our model (i.e., 10 d after the last allergen exposure) probably results in a state of neural hyperresponsiveness in the airways, explaining the enhanced sensitivity to SP. Indeed, although an acute nasal allergen provocation did not cause an additional cellular influx

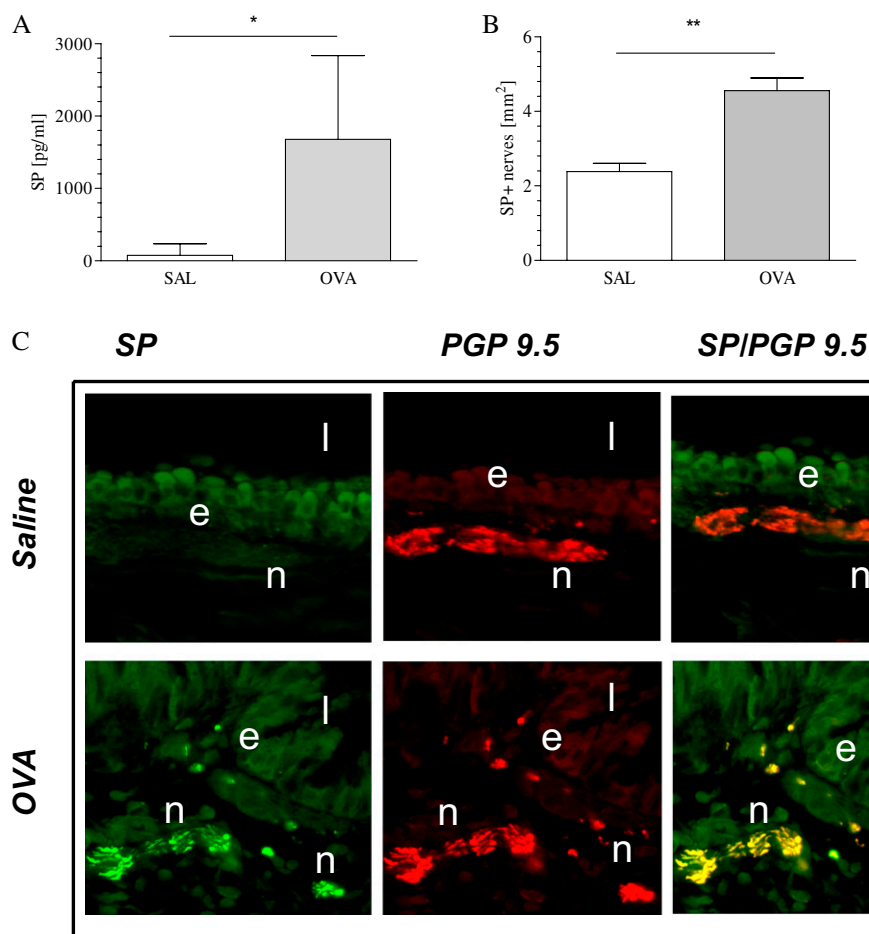


Figure 4. Bronchial induction of substance P (SP) after selective nasal allergen provocation. SAL or OVA was applied in the nose of OVA-sensitized mice with experimental airway allergy after tracheotomy. Mice were killed for analyses 4 hours after provocation. The concentration of SP was measured in bronchoalveolar lavage (BAL) fluid (A) (six mice per group). SP (green) and protein-gene product (PGP) 9.5 (Texas red) were labeled by immunofluorescence in lungs of mice treated with SAL or OVA. Depicted is one representative example for the immunofluorescence staining out of six (C). E, epithelium; L, airway lumen; N, nerves. The number of SP-positive nerves in each group was quantified and displayed as SP-positive nerves per square millimeter (B) (six mice per group). * $P < 0.05$.

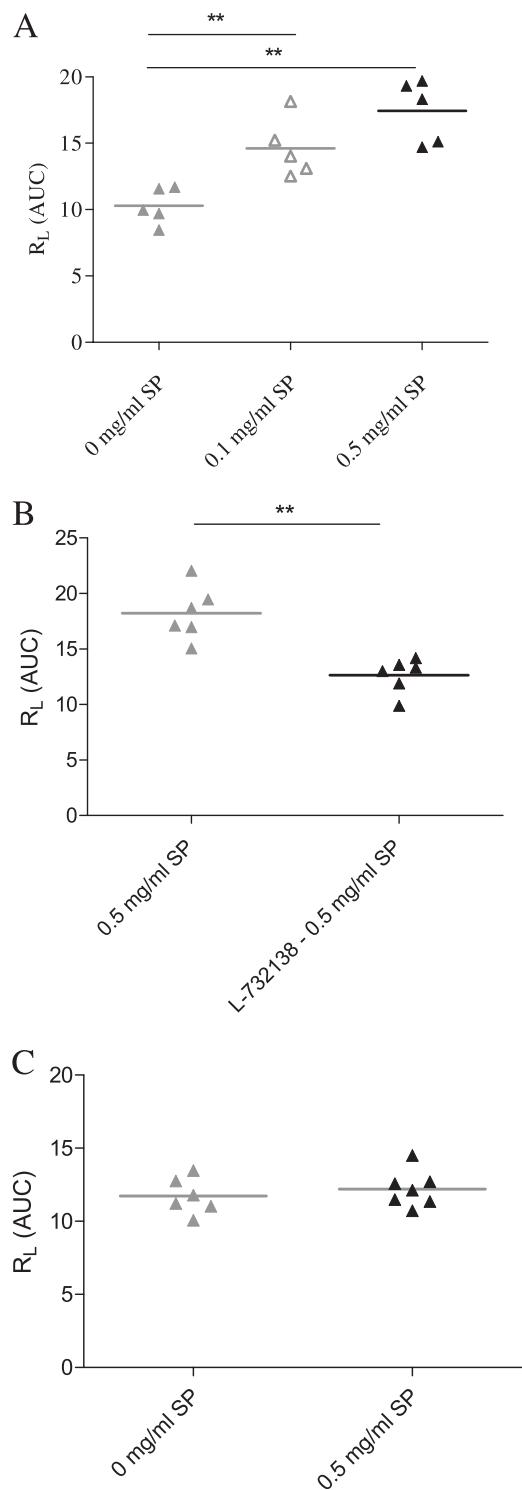


Figure 5. Bronchial responsiveness to MCh after endobronchial administration of SP. A tracheotomy was performed in OVA-sensitized mice with experimental airway allergy and SP (0, 0.1, 0.5 mg/ml in SAL) was nebulized directly in the trachea via the tracheotomy, using the Flexivent artificial ventilation device (A). In a second experiment, OVA-sensitized mice with experimental airway allergy were treated with the neurokinin (NK) 1 receptor antagonist L-732138 or its solvent (70% DMSO) 1 hour before tracheotomy and intratracheal nebulization of 0.5 mg/ml SP in SAL (B). In a third experiment, SP (0 or 0.5 mg/ml in SAL) was nebulized in the trachea of naive mice (C). In the three experiments, bronchial responsiveness to MCh was measured immediately after SP administration, using a forced oscillation technique. The mean resistance was plotted against the MCh concentration and the AUC was calculated for each mouse. Each group in (A) consists of five mice, in (B) of six mice, and in (C) of six (controls) or seven (SP 0.5 mg/ml) mice.

potential generation in airway sensory nerves (20), a lower activity of neutral endopeptidase, the inactivating enzyme of neuropeptides (21), or increased expression of tachykinin receptors in the airways (22, 23). We confirmed the latter in our model. We stained NK1 receptor on lung tissue. Although nasal provocation with allergen did not provoke a significant increase in NK1 receptor after 4 hours, as compared with mice provoked with SAL, both groups had a higher expression of the NK1 receptor as compared with naive mice (Figures 6A and 6B). This finding provides a molecular substrate for the neural hyperresponsiveness that we find in our model, although other mechanisms may be involved as well.

In the final experiments, mice were treated with a selective NK1 receptor antagonist before the nasal allergen challenge. This treatment completely inhibited the development of bronchial hyperresponsiveness, formally demonstrating that the rapid changes in bronchial responsiveness induced by nasal allergen challenge are mediated by the release of SP and its binding to the NK1 receptor (Figure 7).

It remains to be examined as to via which pathway nasal allergen contact leads to bronchial SP up-regulation. The fact that SP is located in pulmonary nerves suggests a neural reflex pathway between nose and bronchi. The so-called “nasobronchial reflex” was already described in the late 1960s. Nasal challenge with an irritant, such as aerosolized crystalline silica, caused an increase in pulmonary resistance, which could be blocked by pretreatment with subcutaneous atropine. This reflex was not present in subjects with an interruption of the second division of the trigeminal nerve (24, 25). Stimulation of the nose with cold, dry air also elicited increased lower airway resistance, which was abrogated by applying local anesthesia in the nose or inhalation of atropine (26). Of note, the nasobronchial reflex mechanisms remain controversial, because others were not able to reproduce these results (27), and due to the lack of data on the induction of bronchial symptoms after nasal allergen exposure via neural mechanisms. To explain our results, the efferent pathway of a nasobronchial reflex would not consist of parasympathetic activation, but of an efferent activation of NANC fibers within the vagal nerve. This contradicts the sensory nature of the NANC system.

Most evidence in literature points toward a systemic linkage of upper and lower airways. Increased blood eosinophilia and bone marrow eosinopoiesis have been reported to occur rapidly after allergen contact in both mice (28, 29) and humans (6, 30, 31). Induction of systemic IL-5 has repeatedly been found after allergen provocation and may represent the stimulus for bone marrow eosinopoiesis (8, 30, 32). The high and rapid absorption

at 4 hours after the provocation, we did find residual inflammatory cells in the BAL fluid of the sensitized mice nasally provoked with either SAL or OVA (Table 1). This bronchoalveolar cell content is clearly lower than inflammatory cell counts in mice at Day 41, immediately after allergen inhalation, where we find eosinophil counts averaging $25 \times 10^4/\text{ml}$ (8). Sensitization of vagal pulmonary C fibers by OVA has been described in a rat model of allergic asthma (18). Several mechanisms can explain neural hyperresponsiveness in allergic airway inflammation (19), such as a lower threshold for action

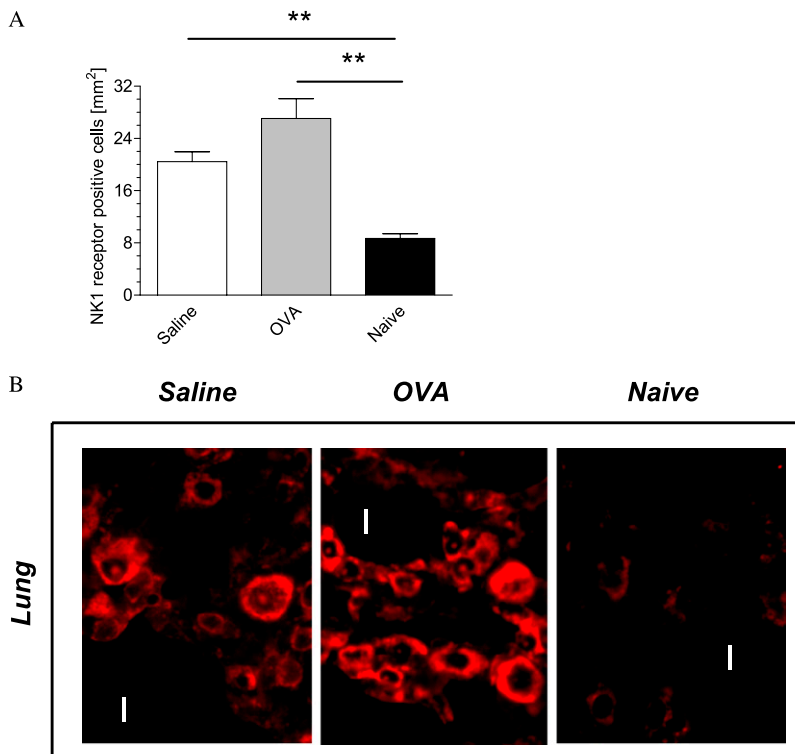


Figure 6. Induction of NK1 receptor in OVA-sensitized mice with experimental airway allergy. SAL or OVA was applied in the nose of OVA-sensitized mice with experimental airway allergy after tracheotomy. Mice were killed for analyses 4 hours after the provocation. NK1 receptor was labeled by immunofluorescence in lungs of SAL and OVA mice, and in lungs of naive mice. The number of NK1 receptor-positive cells in each group was quantified (six mice per group). $**P < 0.01$ (A). Depicted is one representative immunofluorescence for NK1 receptor staining out of six (B).

of allergens through the nasal mucosa that we previously reported (33) may be a more plausible explanation of the observed pulmonary effects. Systemically absorbed allergens may cause activation of resident pulmonary inflammatory cells and nerves after transportation of allergens to the pulmonary vessels.

In conclusion, we demonstrate a novel pathway of nasobronchial interaction in allergic asthma. Nasal allergen provocation rapidly induces BHR via SP release by pulmonary nerves.

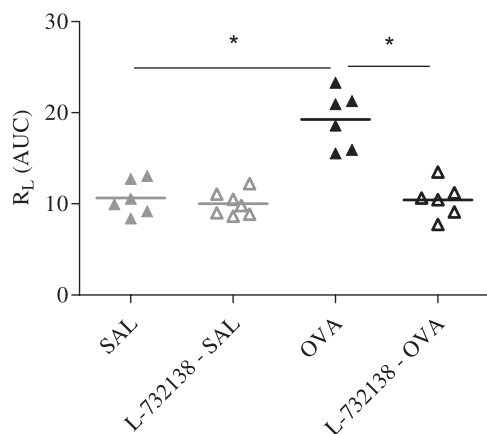


Figure 7. Pulmonary effects of a selective nasal allergen provocation after pretreatment with the NK1 receptor antagonist, L-732138. OVA-sensitized mice with experimental airway allergy were treated with the NK1 receptor antagonist, L-732138, or its solvent (70% DMSO) 1 hour before tracheotomy and nasal OVA or SAL application. At 4 hours after nasal allergen deposition, bronchial responsiveness to increasing doses of nebulized MCh was measured using a forced oscillation technique. The mean resistance was plotted against the MCh concentration, and the AUC was calculated for each mouse (A). $*P < 0.05$. Each group consists of six to seven mice.

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