Airway hyperresponsiveness induced by chronic exposure to cigarette smoke in guinea pigs: role of tachykinins

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Wu, Zhong-Xin, and Lu-Yuan Lee. Airway hyperresponsiveness induced by chronic exposure to cigarette smoke in guinea pigs: role of tachykinins. J. Appl. Physiol. 87(5): 1621–1628, 1999.—This study was carried out to determine whether tachykinins released from lung C-fiber afferents play a part in the bronchial hyperreactivity induced in guinea pigs by chronic exposure to cigarette smoke (CS). Two matching groups of young guinea pigs were exposed to either mainstream CS (CS group) or air (control group) for 20 min twice daily for 14–17 days. There was no difference in the baseline total pulmonary resistance (RL) between the two groups, but the baseline dynamic lung compliance was reduced (~19%) in CS animals. The responses of RL to intravenous injections of ACh, neuropeptide (NK) A, and capsaicin were all markedly increased in CS animals; for example, ACh at the same dose of 5.06 µg/kg increased RL by 207% in the control group and by 697% (n = 8; P < 0.001) in the CS group. The increased responsiveness was accompanied by significant increases in the numbers of neutrophils, eosinophils, and macrophages in the bronchoalveolar lavage fluid in CS animals. Pretreatment with SR-48968 and CP-99994, antagonists of NK1 and NK2 receptors, respectively, did not alter the response of RL to ACh in control animals, but it abolished the elevated bronchoconstrictive response in the CS animals. Furthermore, the immunoreactivities of substance P and calcitonin gene-related peptide in the bronchoalveolar lavage fluid collected after capsaicin challenge were significantly increased in CS animals. These results show that chronic exposure to CS induced airway mucosal inflammation accompanied by bronchial hyperreactivity in guinea pigs and that the tachykinergic mechanism plays an important role in this augmented responsiveness.

Airway inflammation; bronchoconstriction; capsaicin; neurokinin receptors; bronchopulmonary C fibers

It is known that a high incidence of bronchial hyperreactivity exists in human smokers (3, 9, 27). Experimental evidence of bronchial hyperreactivity induced by prolonged exposure of airways to cigarette smoke (CS) has also been reported in animal models (5, 7, 13, 17, 25), but the underlying mechanism is not fully understood. Airway epithelial injury and mucosal inflammation caused by chronic exposure to CS has been suggested to play a critical role. The fact that bronchial hyperreactivity occurs in animals and humans after airway mucosal inflammation has been induced by a variety of other experimental methods (6, 28, 31) seems to support this hypothesis.

Lung and airways are extensively innervated by nonmyelinated C-fiber afferents. Stimulation of these sensory endings is known to elicit reflex bronchoconstriction via the cholinergic reflex pathway (4) and also to trigger the release of tachykinins, which can induce potent bronchoconstrictive effects (2, 24, 29). Hence, one possible mechanism by which bronchial hyperreactivity is induced by chronic exposure to CS may involve bronchopulmonary C fibers because airway mucosal inflammation has been suggested to increase the sensitivity of theseafferent endings (2, 15). Thus a given level of inhalation challenge by a bronchoactive substance may evoke a greater discharge of these afferents and consequently a more severe bronchoconstriction mediated through both the cholinergic reflex pathway and tachykinin release; the latter may cause a more dominant effect in guinea pigs because tachykinins have potent bronchoconstrictive effects in this species. The purpose of this study was to characterize a possible role of tachykinins in the airway hyperresponsiveness induced by chronic exposure of the airways of guinea pigs to CS.

METHODS

The procedures described below were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health, and were also approved by the University of Kentucky Institutional Animal Care and Use Committee.

Materials

Pancuronium bromide (Elkins-Sinn Pharmaceuticals), ACh chloride (Sigma Chemical), neuropeptide (NK) A (Peninsula Laboratories), and CP-99994 (Pfizer) were each diluted in saline. Stock solution of capsaicin (Cap; 400 µg/ml; Sigma Chemical) was prepared in a vehicle of 10% Tween 80, 10% ethanol, and 80% isotonic saline. SR-48968 (Sanofi Recherche) was first dissolved in polyethylene glycol (average mol wt: 200; Sigma Chemical) and then diluted in saline at a 1:1 ratio to a final concentration of 0.67 mg/ml.

Chronic Exposure of Airways to CS

Young male Hartley guinea pigs of similar age and weight (initial wt 250–300 g) were randomly divided into two groups to be exposed to either mainstream CS (CS group) or air (control group). The chronic smoke exposure was carried out by the University of Kentucky Tobacco and Health Research Institute staff according to a standard protocol (10). In brief, each awake guinea pig was held in place by a wire-mesh restrainer with only the nose and mouth exposed to an exposure chamber. A puff of smoke (35 ml, 50% concentration) generated from a University of Kentucky Reference Cigarette 2R1 was drawn into the chamber each minute. Guinea pigs inhaled smoke during the first 20 s; the residual smoke was then vacuumed away and replaced by fresh air during the remainder of the 1-min exposure. Ten puffs of smoke can be generated from each 2R1 cigarette (85-mm overall length and
Tidal volume (VT) was adjusted according to the body weight to maintain abolition of the corneal withdrawal reflexes. The trachea was cannulated just below the larynx through a tracheotomy. The animals were placed in a heated cage. A heating pad was placed under the animal to maintain a constant body temperature.

Within 2 h after the last smoke or sham (air) exposure period, guinea pigs were anesthetized with α-chloralose (100 mg/kg ip) and urethane (500 mg/kg ip), and supplemental doses of the same anesthetics were administered whenever necessary to maintain abolition of the corneal and withdrawal reflexes. The trachea was cannulated just below the larynx through a tracheotomy. The animals were placed in a supine position and were ventilated with a respirator (model 683, Harvard) at a constant rate of 44 breaths/min. Tidal volume (Vt) was adjusted according to the body weight of each animal (8 ml/kg). The right jugular vein and carotid artery were cannulated for intravenous (iv) injections and for arterial blood pressure (ABP) measurement with a pressure transducer (model P23AA, Statham). A catheter for measuring intrapleural pressure (Pip) was inserted into the right intrapleural cavity through a surgical incision between the fifth and sixth ribs; this incision was subsequently sutured and further sealed airtight with silicone jelly. The pneumothorax was then corrected by briefly opening the intrapleural catheter to ambient air during a held hyperinflation (3 × Vt).

The animals were paralyzed with pancuronium bromide (30 µg/kg iv) during the experiment to prevent spontaneous breathing after the smoke-inhalation challenge. Additional doses of pancuronium bromide were given only after the effect of the original dose had worn off and the depth of anesthesia had been checked. Additional doses of anesthetics were administered, whenever necessary, to abolish the reflex changes in ABP and heart rate in response to pain induced by toe pinch. A heating pad was placed under the animal to maintain the body temperature at ~36°C during the experiment.

Inflammatory Cell Analysis in Bronchoalveolar Lavage (BAL) Fluid

BAL fluid was obtained by injecting 10 ml sterile saline (5 ml, twice) via the tracheal cannula; the solution was immediately withdrawn and reinjected. The collected BAL fluid (~7 ml) was centrifuged at 1,500 rpm for 10 min, and the pelleted cells were treated with Tris-buffered ammonium chloride solution (pH 7.2) for lysis of red blood cells. The remaining cells were washed once with phosphate-buffered saline supplemented with 1% fetal calf serum and 0.1% antibiotics (GIBCO, Long Island, NY). Total cell counts were determined by using a hemocytometer. Differential leukocyte counts were performed on cytospin slides that were prepared by centrifuging BAL cells on a glass slide using a cytospin centrifuge and staining them with a Diff-Quick staining kit (Jorgensen Laboratories, Loveland, CO). A minimum of 500 leukocytes were counted by using standard morphological criteria (30).

RIA of Neuropeptides

In both control and CS groups, BAL fluid was obtained within 1 min after a bolus injection of Cap (3.5 µg/kg), and phosphoramidon was added to give a final concentration of 3 × 10⁻⁶ M to inhibit the enzyme activity of neutral endopeptidase. Substance P-like immunoreactivity (SP-LI) and calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) were measured by RIA; the release of CGRP was measured in this experiment because it is known to be colocalized and coreleased with tachykinins from C-fiber endings and is more stable than tachykinins after release (24). Supernatant fractions of the collected BAL fluid were partially purified with Sep-Pak C₁₈ columns. After the columns were washed with 0.1% trifluoroacetic acid (TFA; 45 ml), CGRP and SP were eluted from columns with 60% acetonitrile (HPLC grade) in 0.1% TFA. The samples were lyophilized and reconstituted in RIA buffer, containing 0.1 M phosphate buffer (pH 7.4), 0.01% NaN₃, 50 mM NaCl, and 0.1% Triton X-100. For the measurement of CGRP, 200 µl of sample were incubated at 4°C for 24 h with 100 µl of anti-CGRP antibody (anti-human CGRP II antibody, Peninsula Laboratories), which cross-reacts with both CGRP I and CGRP II from both humans and rats. Standard curves were established with synthetic CGRP (rat sequence), ranging from 2.5 to 1,000 pg/assay tube. Then, 100 µl of 125I-CGRP (human sequence, Amersham) were added to each tube and allowed to incubate for an additional 24 h at 4°C. Finally, 100 µl of goat anti-rabbit IgG (second antibody; Peninsula Laboratories) and 100 µl of normal rabbit serum (Peninsula Laboratories) were added and incubated for 2 h at room temperature, after which 0.5 ml of RIA buffer was added and centrifuged (1,700 g, 4°C) for 20 min. After supernatant fractions had been decanted, the gamma radioactivity in the remaining pellet was counted. The level of SP-LI was measured in a manner similar to that for CGRP described above.

Experimental Protocol

Three series of experiments were carried out.

Study series 1: Dose responses to bronchoconstrictor challenges. The dose-response curves of RL and Cdyn to bolus injections (0.2-ml volume) of ACh (1.0–5.06 µg/kg), NKA (0.2–0.67 µg/kg), and Cap (0.5–1.68 µg/kg) were determined in each animal by successively increasing the concentration of the injectates by 50% at intervals of 7–10 min; at least 30 min elapsed between the tests for two dose-response curves. These dose responses were then compared between control (n = 8) and CS (n = 8) animals to verify airway hyperreactivity. The lungs were hyperinflated (3 × Vt) periodically and also at 2–3 min before each injection to maintain a constant volume history (26). The tests of the responses to these bronchoconstrictor challenges were initiated within 3 h after the last smoke or sham (air) exposure in each animal.

Study series 2: Role of tachykinins. The dose-response curves of RL to ACh and to Cap were determined before and after pretreatment with CP-99994 (0.3 mg/kg iv), the selective antagonist of NK₁ receptor, and SR-48968 (0.3 mg/kg iv), the selective antagonist of NK₂ receptor, in both control (n =
6) and CS (n = 10) animals. In two additional groups of guinea pigs (control group, n = 6; CS group, n = 6), the dose-response curves of RL to Cap (0.5–1.68 µg/kg) were determined both before and after bilateral cervical vagotomy and then were compared to evaluate a possible contribution of vagally mediated reflex to the CS-induced bronchial hyperresponsiveness.

Study series 3: Assessments of airway inflammation and tachykinin release. BAL fluid was obtained, and its differential leukocyte counts were determined in both control (n = 13) and CS (n = 13) guinea pigs; eight guinea pigs in each group were also used in study series 1. In separate groups of guinea pigs, BAL fluid was obtained within 1 min after a bolus injection of Cap (3.5 µg/kg), and SP-IL and CGRP-IL were measured by RIA in both control (n = 5) and CS (n = 5) animals.

Statistical Analysis

To pool the data from all the animals for statistical analysis, we chose the six breaths immediately before NKA and Cap challenges as the baselines and the six consecutive breaths with peak increases in RL within 30 breaths after the NKA and Cap injections as peak responses. The response to ACh was analyzed in the same manner, except that only three breaths were averaged for the peak responses because of the much shorter duration of the bronchoconstrictive effect of ACh. A two-way ANOVA was used for the statistical analysis; one factor was the treatment effect of chronic exposure to smoke, and the other factor was the effect induced by injections of bronchoactive agents or NK-receptor antagonists. When the two-way ANOVA showed a significant interaction, pairwise comparisons were made with a post hoc analysis (Fisher’s least significant difference). Data are reported as means ± SE. A P value < 0.05 was considered significant.

RESULTS

Dose Responses to Bronchoconstrictor Challenges

The average dose of TPM delivered during the chronic smoke exposure was 17.3 ± 0.6 mg·kg⁻¹·day⁻¹, and the average blood HbCO level measured in the CS animals at the end of the smoke exposure was 10.2 ± 1.0%. After exposure to CS for 14–17 consecutive days, the average baseline Cdyn of CS animals was 0.79 ± 0.09 ml/cmH₂O, which was ~19% smaller than that (0.98 ± 0.07 ml/cmH₂O; P < 0.05) of the matching control group exposed to room air. There was no difference between the control and CS animals in the baseline RL (control: 122 ± 8 cmH₂O·ml⁻¹·s; CS: 138 ± 11 cmH₂O·ml⁻¹·s; P > 0.05). However, the bronchomotor responses to iv injections of ACh, NKA, and Cap were all increased significantly in CS animals; for example, ACh at the same dose of 5.06 µg/kg increased RL by 207% in the control group and by 697% (n = 8; P < 0.001) in the CS group (Figs. 1 and 2). In addition, the bronchoconstriction induced by the same dose of ACh lasted substantially longer in CS animals (Figs. 1 and 2A). Injection of NKA induced a bronchoconstriction that was slower in onset but longer lasting than that produced by ACh; similarly, the bronchoconstrictive response to NKA was clearly augmented in CS animals (Fig. 2B). The bronchoconstrictive response to Cap injection was also markedly elevated in CS animals in a pattern similar to that induced by ACh (Fig. 2C). The degree of the increased responsiveness in CS animals is dose dependent: the bronchoconstrictive response is augmented to a greater degree at higher doses of the bronchoconstrictor challenges (Fig. 3).

Role of Tachykins

Pretreatment with CP-99994 and SR-48968 did not change the baseline RL and Cdyn in either control or CS animals. Furthermore, this pretreatment did not alter the dose response of RL to ACh in control animals (Fig. 4A). However, administration of these two NK-receptor antagonists in combination significantly attenuated the enhanced bronchomotor response to ACh caused by chronic exposure to CS (Fig. 4C); for example, RL caused by injection of ACh at the dose of 5.06 µg/kg was 0.67 ± 0.09 cmH₂O·ml⁻¹·s in CS animals (compared with 0.22 ± 0.05 cmH₂O·ml⁻¹·s in control animals; P < 0.001), and this exaggerated response of ΔRL to the same dose of ACh was reduced to 0.36 ± 0.05 cmH₂O·ml⁻¹·s after pretreatment with CP-99994 and SR-48968 (n = 10; P < 0.001) (Fig. 4C). Thus, after

![Fig. 1. Experimental record illustrating the responses of transpulmonary pressure (Ptp), respiratory flow (V), and arterial blood pressure (ABP) to ACh injections in a control (A; wt: 397 g) and a cigarette smoking (CS) guinea pig (B; wt: 410 g). Arrows were added to depict time when ACh (5.06 µg/kg) was injected into venous catheter as a bolus.](http://jap.physiology.org/)
pretreatment with these antagonists, there was no significant difference (\(P > 0.05\)) between control and CS groups in the peak RL in response to the same doses of ACh (Fig. 4, A and C). Furthermore, pretreatment with these NK1- and NK2-receptor antagonists completely abolished the bronchomotor response to Cap in both control and CS animals (Fig. 4, B and D).

In separate groups of guinea pigs, chronic exposure to CS augmented the bronchomotor response to Cap, but the responses of RL and Cdyn to Cap were not...
significantly affected by bilateral cervical vagotomy in either control (n = 6) or CS (n = 6) animals (Fig. 5).

Assessments of Airway Inflammation and Tachykinin Release

The total number of leukocytes in the BAL fluid collected from CS animals was ~300% of that in the control animals; there were marked increases in the numbers of neutrophils, eosinophils, and macrophages in the BAL fluid of CS animals (Table 1), indicating that airway inflammation was induced by chronic exposure to CS.

In the BAL fluid obtained from CS animals (n = 5) after a bolus injection of Cap (3.5 µg/kg), the level of SP-LI was 13.43 ± 1.71 fmol/ml and that of CGRP-LI was 6.04 ± 0.69 fmol/ml; these levels were significantly higher than those obtained from control animals (n = 5) after injection of the same dose of Cap (SP-LI: 7.03 ± 1.03 fmol/ml, P < 0.05; CGRP-LI: 4.02 ± 0.47 fmol/ml, P < 0.05; Fig. 6).
Results obtained from this study show that chronic exposure of guinea pigs to cigarette smoke induces airway inflammation, as exemplified by the marked increases in total number of neutrophils, eosinophils, and macrophages in the BAL fluid. Indeed, it is well documented that airway inflammation and mucosal injury occur commonly in human smokers (3, 9, 27). Accompanying this airway inflammation is bronchial hyperresponsiveness to the challenges of a variety of bronchoactive substances, including ACh, NKA, and Cap (Figs. 2 and 3). This part of the results should not be a surprise, because airway mucosal inflammation is a prominent feature of bronchial hyperreactivity (2, 6, 28) and CS-induced bronchial hyperreactivity to a variety of bronchoactive agents (e.g., acetylcholine, histamine, substance P, etc.) has been reported previously in guinea pigs (5, 7, 13, 17, 25). More importantly, our results further demonstrate that, after pretreatment with antagonists to NK₁ and NK₂ receptors, the bronchial hyperresponsiveness found in the chronically smoking guinea pigs was completely abolished, indicating the critical involvement of tachykinins in these augmented responses.

Immunocytochemical evidence clearly demonstrates that tachykinins, synthesized in the cell bodies of C neurons, are localized in the peripheral endings of the afferents innervating the lungs and airways of various species, including humans (23, 24, 29). Activation of these sensory endings by chemical irritants not only elicits reflex bronchoconstriction via the cholinergic pathway (4) but also triggers the local release of tachykinins from the afferent endings; these tachykinins can produce potent bronchoconstrictive effects on airway smooth muscle (2, 24, 29). The tachykinergic response is characterized by intense and sustained bronchoconstriction that is resistant to treatment with atropine, persists even after vagotomy, and can be prevented either by depletion of tachykinins from these sensory endings or by pretreatment with tachykinin receptor antagonists (11, 20, 24, 29). This tachykinergic component of the bronchoconstrictive responses to chemical irritants appears to vary among different species; it is particularly pronounced in the guinea pig. Indeed, bilateral cervical vagotomy did not significantly attenuate the augmented bronchoconstrictive response to Cap, further indicating the dominant role of tachykinergic mechanism in the CS-induced bronchial hyperresponsiveness in this study (Fig. 5).

The three bronchoconstrictive substances chosen for this study are known to induce bronchoconstriction through different mechanisms. ACh is the primary neurotransmitter mediating the cholinergic contraction of airway smooth muscle by activating M₃ receptors on the airway smooth muscle. NKA produces bronchoconstriction by activating NK₂ receptors and is a potent bronchoconstrictor, particularly in guinea pigs (24). The bronchoconstrictive response to Cap is primarily produced by its potent and selective stimulatory effect on bronchopulmonary C-fiber afferents and the resulting tachykinin release (nondicholinergic mechanism) and the cholinergic reflex mechanism; the latter has a relatively weak or negligible bronchconstrictive effect in guinea pigs (11, 20, 24). Despite these differences, the bronchoconstrictive responses to all three bronchoactive substances were markedly augmented in guinea pigs chronically exposed to cigarette smoke. Moreover, in a limited number of experiments (n = 4 in both control and CS groups) carried out in a similar manner as reported in this study, we have also found a significant increase in the bronchomotor response to iv injection of a high dose of histamine (6.74 µg/kg) in the CS-exposed guinea pigs (R. F. Morton and L. Y. Lee, unpublished observations). Altogether, these data have confirmed that prolonged exposure to cigarette smoke...
induces bronchial hyperreactivity in guinea pigs (5, 8, 13, 17, 25).

It has been suggested that airway mucosal injury and inflammation can "sensitize" these bronchopulmonary C-fiber afferents (2, 15). Indeed, inflammatory mediators such as prostaglandins, thromboxanes, and histamine that are locally produced in the lung have been shown to enhance the excitability of these afferents (21, 22). Hence, one possible explanation for our observation in this study is that airway inflammation induced by chronic exposure to CS may have enhanced the sensitivity of these afferent endings in the lung; a given level of stimulus such as Cap may, therefore, trigger a greater intensity of discharge of these afferents, thus releasing a larger amount of tachykinins. This hypothesis is supported by our data obtained from direct measurements showing that greater amounts of NKA and CGRP were released in the lungs of chronic-smoking animals than in the lungs of control animals in response to the same level of Cap challenge.

It has been shown that airway inflammation inhibits the enzyme activity of neutral endopeptidase (NEP) that is present on the membranes of various cell types (including epithelium and nerve fibers) in the airways and can cleave tachykinins immediately after their release (8, 29). In addition, various components of cigarette smoke are also known to depress the activity of NEP (7). Hence, another plausible explanation for chronic-smoking-induced bronchial hyperreactivity is that the attenuated NEP activity may have contributed to the augmented bronchoconstrictive effect of endogenous tachykinins. An increased release of tachykinins, a decreased degradation, or both are responsible for bronchial hyperresponsiveness; this finding is consistent with our data showing that the enhanced response was completely prevented by pretreatment with tachykinin-receptor antagonists (Fig. 4).

Our observation that the bronchoconstrictive response to the challenges of exogenous tachykinin is also augmented in the chronic-smoking animals (Figs. 2B and 3B) further suggests that some additional factors are probably also involved. Previous investigators have reported that the tachykinin-receptor mRNA expression is higher in the airways of human smokers than in those of nonsmokers (1); these investigators have suggested that chronic airway inflammation may have induced such increases in tachykinin-receptor gene expression. An increase in the densities of tachykinin receptors in the airway smooth muscle may then lead to an augmented response to a given level of endogenously released or exogenously administered tachykinins.

Pretreatment with tachykinin-receptor antagonists did not cause any change in the dose response of Rl to ACh in control animals (Fig. 4A). However, the same pretreatment substantially attenuated the elevated response to ACh in chronic-smoking animals (Fig. 4C). These results suggest that endogenous tachykinins are not involved in the bronchomotor response to ACh in control (healthy) guinea pigs, whereas tachykinins play an important role in the augmented bronchomotor response to ACh in guinea pigs with airway inflammation generated by chronic exposure to cigarette smoke.

There are several possible sites where this potentiating effect of tachykinins may have taken place in response to ACh. Electrophysiological studies have offered strong evidence suggesting that ACh nicotinic receptors are present on the membranes of pulmonary C-fiber endings in dogs (19). Presumably, when the excitability of these afferent endings is enhanced by chronic smoking-induced airway inflammation, they can be activated by exogenous ACh and can subsequently release tachykinins. An excitatory effect of tachykinins on the NK2 receptors on postganglionic prejunctional cholinergic nerve terminals has also been described (12, 14). In addition, others have reported that tachykinins can facilitate cholinergic ganglionic transmission (32). Although an increase in the bronchomotor response to endogenous or exogenous ACh has also been reported previously in guinea pigs with airway inflammation induced by other means (16, 31, 33), the underlying mechanism cannot be determined in the present study.

The average baseline Cdyn of guinea pigs chronically exposed to cigarette smoke was significantly smaller than that of the matching control animals (Figs. 2 and 3), and the cause of this difference is not fully understood. The smaller Cdyn was not caused by a difference in body weight: CS animals, 426 ± 7 g; control animals, 409 ± 8 g (P > 0.05). Pretreatment with NK1- and NK2-receptor antagonists in combination did not significantly alter the baseline Cdyn in CS animals, suggesting that tachykinins are not primarily responsible for the reduced Cdyn caused by the chronic exposure to smoke. Our data have clearly shown that chronic smoking induces airway inflammation in the CS animals; it is possible that the effect of endogenously released inflammatory mediators on lung periphery may be involved, at least in part, in the reduced lung compliance in these animals.

In conclusion, chronic exposure to CS induced marked increases in the numbers of neutrophils, eosinophils, and macrophages in the BAL fluid, accompanied by distinct airway hyperresponsiveness to intravenous injections of ACh, NKA, and Cap in guinea pigs; the increased responsiveness was abolished by pretreatment with NK1- and NK2-receptor antagonists. On the basis of these results, we suggest that the airway hyperreactivity induced by chronic smoking may result from an increased release of tachykinins from bronchopulmonary C-fiber endings. Other factors such as an inhibited enzymatic activity of the neutral endopeptidase and/or increased densities of NK receptors in the airway smooth muscle may be also involved.

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