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## Original article

## Secretoneurin is released into human airways by topical histamine but not capsaicin

**Background:** The neuropeptide secretoneurin, with potential relevance to leukocyte trafficking, is present in nerves of the nasal mucosa in allergic rhinitis and may be released in response to allergen and histamine exposure. There is no information on the occurrence and mechanisms of release of secretoneurin in healthy human airways.

**Methods:** The presence of secretoneurin in nasal biopsies and its release in response to nasal capsaicin and histamine challenges were examined. Symptoms and lavage fluid levels of fucose were recorded as markers of effects in part produced by neural activity. Bronchial histamine challenges followed by sputum induction and analysis of secretoneurin were also carried out.

**Results:** Nerves displaying secretoneurin immunoreactivity abounded in the nasal mucosa. Nasal capsaicin challenge produced local pain ( $P < 0.05$ ) and increased the levels of fucose ( $P < 0.05$ ), but failed to affect the levels of secretoneurin. Nasal histamine challenge produced symptoms ( $P < 0.05$ ) and increased the mucosal output of secretoneurin ( $P < 0.05$ ) and fucose ( $P < 0.05$ ). Bronchial histamine challenge increased the sputum levels of secretoneurin ( $P < 0.05$ ).

**Conclusions:** We conclude that secretoneurin is present in healthy human airways and that histamine evokes its release in both nasal and bronchial mucosae. The present observations support the possibility that secretoneurin is involved in histamine-dependent responses of the human airway mucosa.

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Secretoneurin is a neuropeptide derived by proteolysis from its precursor secretogranin II (1). We recently demonstrated extensive secretoneurin immunoreactivity in the nerves of the nasal mucosa in allergic rhinitis (2). In these patients, increased nasal lavage fluid levels of secretoneurin were observed during seasonal allergen exposure (2). Moreover, in a preliminary study, topical exposure to histamine intriguingly increased the output of secretoneurin (2). Our observations, together with previous *in vitro* data on potential roles of secretoneurin in leukocyte trafficking (3–5), suggest that this neuropeptide may be involved in the pathophysiology of airway inflammation. However, the mechanisms involved in airway release of secretoneurin are unknown.

In the present study involving healthy subjects, we examined the nasal mucosal expression of secretoneurin. Furthermore, we explored the effects of topical challenges, on nasal secretoneurin output, known to produce increased neural activity, i.e. capsaicin, the archetypical sensory nerve-challenge agent, and histamine

(6, 7). To provide a positive control for effects likely mediated by increased neural activity, we analyzed lavage fluid levels of fucose, a global marker of secretion (8). We also carried out bronchial histamine challenges followed by sputum induction and secretoneurin analysis.

## Methods

### Subjects

The subjects had no history of allergy (negative skin-prick test), no history of recent nasal or bronchial disease (normal spirometry), and no history of recent drug treatment. The study was approved by the regional Ethics Committees and informed consent was obtained.

### Nasal challenges

In the first group ( $n = 10$ , 22–28 years), concomitant nasal challenges/lavages were performed using a pool-device (9). The pool-fluid

volume was 15 ml and the dwell time 10 min. At separate visits, capsaicin (0–300 ng/ml) and histamine solutions (0–400 µg/ml) were administered at 10-min intervals. Ten minutes after each challenge/lavage any smart/pain, sneeze, secretion, or blockage was scored on a scale from 0 to 3. The lavage fluids were centrifuged and the supernatants frozen (–20°C). Two 30-s saline lavages were carried out before each challenge/lavage series to create baseline conditions.

#### Bronchial challenges and sputum induction

In the second group ( $n = 15$ , 24–31 years), histamine challenges were carried out using a dosimetric delivery system based on a jet nebulizer (Spira Electro 2; Respiratory Care Center, Hämeenlinna, Finland) (10). Challenges were continued until a cumulative dose of 3160 µg had been given or until a 20% decrease in forced expiratory volume in 1 s (FEV1) from baseline was achieved (Vitalograph-Compact II, Vitalograph, Buckingham, UK). Thereafter, aerosolized hypertonic saline (4.5%) was inhaled at resting ventilation rate for 40 min using an ultrasonic nebulizer (Aerosonic; DeVilbiss Health Care, Somerset, PA, USA) (10). The subject was then instructed to rinse the mouth three times with 20 ml water and to cough the sputum into a container. The sample was frozen (–20°C) for later analysis.

#### Nasal biopsies

In the third group ( $n = 6$ , 21–50 years), nasal biopsies were taken from the inferior turbinate under local anesthesia using a cutting forceps with a drilled-out punch (2). The specimen were immersed for 4–6 h in paraformaldehyde (4.0%, pH 7.2), rinsed in phosphate-buffered saline (PBS) with 10% sucrose, frozen in mounting medium, and stored at –80°C.

#### Analyses of secretoneurin and fucose

Secretoneurin levels were measured by a radioimmunoassay as described previously (1). Fucose levels were measured using a parallel ligand-exchange chromatography in combination with fluorescence detection as described previously (8).

#### Immunohistochemistry

Cryostat sections (10 µm) were processed for demonstration of secretoneurin, protein-gene product 9.5 (PGP), vesicular acetylcholine transporter (VAChT), tyrosine hydroxylase (TH), vasoactive intestinal peptide (VIP), and calcitonin gene-related peptide (CGRP), using indirect immunofluorescence as described previously (2). The site of the antigen-antibody reaction was revealed by application of secondary antibodies labelled with fluorescein isothiocyanate or Texas red as described previously (2). No immunoreactivity could be detected in sections incubated in the absence of primary antisera. To reveal co-existence of antigens, simultaneous double or sequential immunostaining was performed as described previously (2).

## Results

#### Nasal capsaicin challenge

The capsaicin challenges were associated with a marked nasal smart/pain response. The scores of smart/pain were  $0.1 \pm 0.1$  after isotonic saline,  $0.6 \pm 0.2$  after capsaicin

30 ng/ml ( $P < 0.05$ ), and  $2.1 \pm 0.3$  after capsaicin 300 ng/ml ( $P < 0.05$ ). Capsaicin also increased the nasal output of fucose (Table 1). In contrast, capsaicin failed to increase the nasal lavage fluid levels of secretoneurin (Table 1).

#### Nasal and bronchial histamine challenge

The nasal histamine challenges produced significant sneezing, secretion, and blockage (not shown) as well as increased nasal output of fucose (Table 1). Furthermore, histamine produced a significant increase in luminal entry of secretoneurin (Table 1). Inhalational histamine increased the sputum levels of secretoneurin (Fig. 1). In addition, this challenge produced a 26% reduction in FEV1 ( $P < 0.05$ ).

Table 1. Levels of secretoneurin and fucose in nasal lavages obtained at baseline and at combined challenge and lavage with histamine and capsaicin solutions (mean  $\pm$  SEM)

	Secretoneurin (pM)	Fucose (µM)
Histamine (0 µg/ml)	0.20 $\pm$ 0.07	32.59 $\pm$ 12.64
Histamine (40 µg/ml)	0.93 $\pm$ 0.60	43.23 $\pm$ 12.83
Histamine (400 µg/ml)	0.61 $\pm$ 0.09*	110.18 $\pm$ 36.49*
Capsaicin (0 ng/ml)	0.48 $\pm$ 0.08	41.23 $\pm$ 14.20
Capsaicin (30 ng/ml)	0.43 $\pm$ 0.07	56.75 $\pm$ 40.32
Capsaicin (300 ng/ml)	0.38 $\pm$ 0.06	135.33 $\pm$ 51.00*

Data were examined using the Friedman's test and the Wilcoxon signed rank test. \* $P$ -values  $< 0.05$  were considered significant.

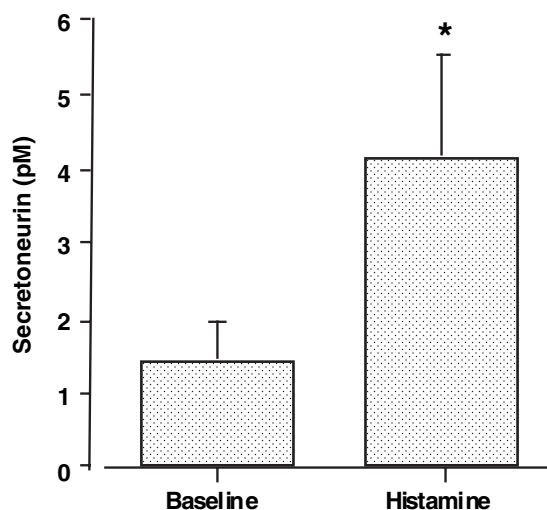
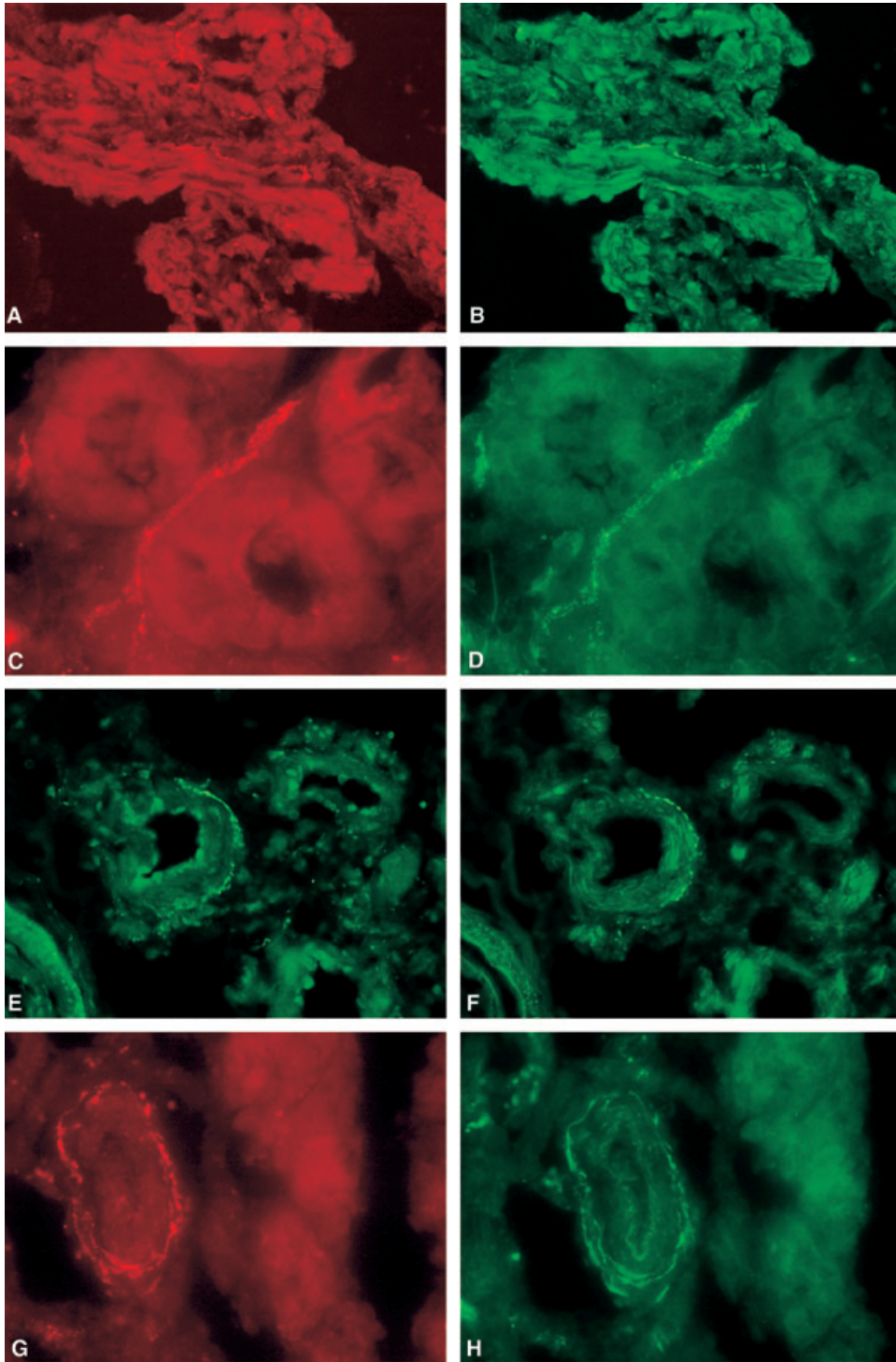


Figure 1. Sputum levels of secretoneurin at baseline and following inhalational histamine challenge (mean  $\pm$  SEM). The levels of secretoneurin were elevated by the histamine exposure. Data were examined using the Wilcoxon signed rank test. \* $P$ -values  $< 0.05$  were considered significant.



*Figure 2.* Secretoneurin-immunoreactive nerves in nasal biopsies. Double immunostaining for secretoneurin (A) and calcitonin gene-related peptide (CGRP) (B), secretoneurin (C) and vasoactive intestinal peptide (VIP) (D), demonstrated that the majority of CGRP and VIP immunoreactive nerve fibers also contained secretoneurin. On the other hand, many secretoneurin positive fibers lacked CGRP and VIP. Double or sequential immunostaining showed secretoneurin immunoreactivity (E, G) in a population of vesicular acetylcholine transporter (VChAT)-containing (F) and in a population of tyrosine hydroxylase (TH)-containing (H) fibers. The weaker specific staining and more intense autofluorescence in (E) is explained by the washing procedure that was performed between the two immunostainings.

## Nasal distribution of secretoneurin and co-localization experiments

The antiserum raised against secretoneurin recognizes both the free peptide and its precursors (1). Nerves displaying secretoneurin were frequent in all biopsies examined (Fig. 2). These nerves were distributed mainly around the seromucous glands and blood vessels in the subepithelial tissue. Secretoneurin immunoreactive fibers were also seen in nerve bundles deep in the mucosa, whereas their presence just beneath and within the epithelium was scarce. Secretoneurin was detected exclusively in nerve fibers.

The staining pattern of secretoneurin was compared with the distribution of CGRP, a neuropeptide mainly localized to sensory nerves (11). A majority of CGRP-positive nerves also expressed secretoneurin (Fig. 2A, B). However, most secretoneurin-immunoreactive fibers lacked CGRP. VIP is a neuropeptide mainly localized in the parasympathetic nerves (11, 12). A vast majority of VIP-immunoreactive nerve fibers also expressed secretoneurin (Fig. 2C, D), whereas many secretoneurin-positive fibers lacked VIP. As markers of adrenergic and cholinergic nerve fibers, we employed TH and VAcHT, respectively (13). Secretoneurin immunoreactivity was found in a population of VAcHT-containing nerves (Fig. 2E, F) as well as in a population of TH-containing nerves (Fig. 2G, H).

## Discussion

The present study demonstrates a rich occurrence and a widespread distribution of secretoneurin-immunoreactive nerves in the healthy nasal mucosa. Furthermore, it demonstrates that topical histamine increases the levels of secretoneurin in nasal as well as bronchial airway secretions. In contrast, capsaicin fails to increase the mucosal output of secretoneurin although, like histamine, it stimulates airway nerves and evokes reflexes. It may be speculated that effects of a prominent autacoid such as histamine in part are mediated by released secretoneurin.

In our previous study, the occurrence of secretoneurin in patients with allergic rhinitis was demonstrated (2). In the present study, a similar occurrence and distribution were observed in healthy subjects. Accordingly, secretoneurin was found mainly around blood vessels and glands in the subepithelial tissue. Although no quantitative analysis was made, there was no apparent evidence that the occurrence of secretoneurin-containing nerves was different from that observed in patients with allergic rhinitis.

Topical histamine increased the nasal as well as bronchial output of secretoneurin (this study). The mechanism by which histamine exerts this acute effect

remains to be clarified. *In vitro* studies have shown that histamine may induce production of secretogranin II messenger RNA, i.e. the precursor of secretoneurin (14). However, the present acute response to histamine may not involve *de novo* protein synthesis. Instead, based on the present neural distribution of secretoneurin and the effects evoked by histamine, i.e. reflex-mediated secretion (7) and sensory symptoms, increased neural activity leading to secretoneurin release seems possible. It may be speculated that activation of histamine receptors on nerves such as the slow-conducting nerves that mediate itch may induce secretoneurin release either directly or through a reflex action. The present differential effects of capsaicin and histamine may support the involvement of this group of afferent nerves rather than small-diameter C-fibers that mediate capsaicin-induced smart and pain. The nerves that are the actual source of secretoneurin released at histamine challenge remain to be examined.

It has been suggested that secretoneurin may induce or modify inflammatory processes (3–5). For example, this neuropeptide has been demonstrated to induce chemotaxis of immature dendritic cells and to arrest mature dendritic cells, suggesting that these cells may be recruited to and are maintained at sites of inflammation by a mechanism involving secretoneurin activity (5). Furthermore, secretoneurin may act as a chemoattractant for monocytes and eosinophils (3, 4). These *in vitro* observations, together with our demonstrations of secretoneurin immunoreactivity in the nasal mucosa of healthy subjects (this study) and of patients with allergic rhinitis (2), suggest that secretoneurin may be a mediator of importance in the recruitment of inflammatory cells and, possibly, neuroimmune interactions.

We conclude that the healthy nasal mucosa features secretoneurin and that topical challenge with histamine but not capsaicin produces an increased mucosal output of this neuropeptide. These observations, together with our previous findings in allergic rhinitis (2) and *in vitro* findings on the potential roles of secretoneurin in leukocyte trafficking (3–5), suggest a role for secretoneurin in histamine-dependent responses of the human airway mucosa and in conditions characterized by airway inflammation such as allergic rhinitis and asthma.

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