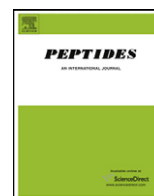




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Adrenomedullin level in the nasal discharge from allergic rhinitis cohort

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ARTICLE INFO

Article history:

Received 22 September 2010

Received in revised form 23 October 2010

Accepted 25 October 2010

Available online xxx

Keywords:

Nasal allergy

Mast cell

Nasal discharge

Neutral endopeptidase

ABSTRACT

Adrenomedullin (AM) is a potent hypotensive and vasodilatory peptide. AM may exert protective actions against the development of many diseases by modulating the blood circulation and body fluid balance. In addition to these functions, it has recently been reported to play important roles in the development of allergy and infections. The purpose of the present study was to demonstrate the existence of AM in the human nasal mucosa and to discuss whether AM might contribute to the pathogenesis of nasal congestion. We measured the total AM concentrations in the nasal discharge. The total AM concentration in the nasal discharge was significantly higher in the non-allergy group (72.1 ± 55.5 fmol/ml) than in the allergy group (37.1 ± 44.2 fmol/ml). By immunohistochemical examination, we identified AM-containing cells in the nasal mucosa from both subjects with and without nasal allergy, and also in nasal polyps. Moreover, those cells were positive for anti-tryptase antibody which recognizes mast cells. In nasal allergy, vasodilatation and increase in vascular permeability are characteristic features of the immediate phase response. Reduced AM levels in the nasal discharge may be associated with attenuation of both of these factors. On the other hand, immunohistochemical analysis demonstrated AM-immunoreactive cells in the chronic phase of rhinosinusitis. In the late and inflammatory phase, mast cells produce AM, which possibly acts as an inhibitor of inflammatory cell migration. In conclusion, AM may be actively secreted into the nasal discharge. AM in the nasal discharge may have protective and anti-inflammatory effects in the nasal mucosa.

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1. Introduction

Adrenomedullin is a potent hypotensive and vasodilatory peptide that was first isolated from a human pheochromocytoma [20]. It has been shown to be produced in several tissues, including adrenal glands, blood vessels, heart, kidneys and lungs [3,14]. Normal levels of immunoreactive AM have been determined in the human urine, plasma, cerebrospinal fluid, amniotic fluid, and saliva [9,18,26,31,39]. Elevated plasma AM levels have been identified in patients with a variety of diseases, including congestive heart failure, myocardial infarction, renal diseases, hypertensive disease, diabetes mellitus, stroke, and septic shock [5,7,10,11,15,16,21,24,33]. Under these pathological conditions, AM may exert a protective action against the adverse effects of the diseases by modulating the blood circulation and body fluid balance. In addition, the roles of AM in allergy and infection have

recently been reported [4,22,44,48]. Plasma AM levels have been reported to be increased during acute attacks of bronchial asthma [4]. AM has been shown to exhibit antimicrobial properties and been suggested to have a potential role in host defenses [1,2].

In the respiratory system, AM immunoreactivity has been reported to be observed in the bronchial mucosa, bronchial glands and type II pneumocytes of the lung [3,28]. However, little is known about the distribution of AM in the upper airways, including the nasal mucosa. The nasal cavity and turbinates are lined by pseudostratified columnar ciliated epithelium. The submucosa consists of nasal glands, nerves, extensive vasculature and cellular elements. Because of the highly vascular nature of nasal tissues, vascular dilatation can lead to significant nasal obstruction, which is one of the earliest and most consistent symptoms of allergic rhinitis [13,45]. Nasal mucosa also provides the first line of defense against potentially pathogenic microorganisms [6,41].

Mast cells have been reported as one of the AM-producing cells. Mast cells are also known to be a major contributor to the pathogenesis of nasal allergy. Recently, interactions between mast cells and AM have been identified. AM induces histamine release from the human mast cells through a receptor-independent pathway. Moreover, AM is chemotactic for human mast cells and stimulates MCP-1 mRNA expression [49]. MCP-1 is known to be a histamine-

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releasing factor and been shown to contribute to the prolonged allergic inflammatory cycle [8].

The purpose of the present study was to demonstrate the existence of AM in the human nasal mucosa and to discuss whether AM might contribute to nasal congestion. We assayed the total AM concentrations in nasal discharge samples by immunoradiometric assay. We hypothesized that mast cells are an important source of AM in the nasal mucosa. Therefore, immunohistochemistry was performed to identify the immunolocalization of AM in the nasal mucosa.

2. Materials and methods

2.1. Materials

Rabbit anti-human AM 1–52 polyclonal antibody was purchased from Peninsula Laboratories, Inc., (Belmont, CA). Murine anti-human mast cell tryptase monoclonal antibody was procured from Chemicon International, Inc., (Temecula, CA). The immunohistochemical staining system called Universal Immuno-Enzyme Polymer Method (Nichirei-Histofine™ Simple Stain MAX-PO-MULTI, Nichirei Co., Ltd., Tokyo) was used.

2.2. Study subjects

The subjects recruited for the nasal discharge assay in this study were 18 subjects, including 13 patients with seasonal Japanese cedar pollinosis and 5 patients with perennial allergic rhinitis associated with house dust mite allergy (7 females, 11 males, 32 ± 5 years old) and 12 healthy volunteers (5 females, 7 males, 36 ± 7 years old). For the immunohistochemical study, 6 patients with chronic rhinosinusitis (including 2 patients with perennial allergic rhinitis, 2 patients with nasal polyposis, 2 patients with non-allergic hypertrophic rhinitis) were included. All the patients with allergic rhinitis showed positive test results for mite nasal allergy or Japanese cedar pollinosis as detected by a clinical history of moderate to severe rhinosinusitis, rhinoscopic examination, nasal smear for eosinophilia, an intradermal test or determination of specific IgE antibodies by a capsulated hydrophobic carrier polymer-radioallergosorbent test (CAP-RAST). The inclusion criteria for allergic rhinitis are positive test results of CAP-RAST (score 2 or greater) or skin prick test (wheal > 5 mm) for either Japanese cedar, house dust or mite. The patients with non-allergic hypertrophic rhinitis are diagnosed who have hypertrophy of inferior turbinate under rhinoscopic examination, but negative for all other tests including nasal smear for eosinophilia and determination of specific IgE. The patients with nasal polyp were positive for eosinophiles in nasal smear. Healthy volunteers showed negative results in all tests. Patients with infectious rhinosinusitis were not included in this study. The protocol was approved by the ethics committee of Tokyo Woman's Medical University. Written informed consent was obtained from each of the participating patients before the start of the study.

2.3. Collection of nasal discharge samples

Nasal discharge samples were collected from both sides of the nasal cavity by active suction using a compressor of ENT therapy unit (Nagashima Medical Instruments Co., Ltd., Tokyo) and 12Fr tracheal suction catheter kit with sampling tube (Sumitomo Bakelite Co., Ltd., Tokyo). Suction catheter was cut with a length of 15 cm for nasal suction. Each sample, approximately 0.5 ml in volume, was quickly transferred to chilled tubes containing EDTA (1.5 g/L sample) and aprotinin (50,000 kIU/L sample), and then centrifuged at 4°C for 10 min at $400 \times g$. The supernatant was stored frozen at -80°C until later analysis, and diluted 1:5 or 1:10 with saline at

the time of the assay. After the assay, the concentration of AM was corrected for the ratio of dilution.

2.4. Detection of AM in the nasal discharge

The nasal discharge AM concentrations were measured by a specific immunoradiometric assay using a commercial kit (AM RIA Shionogi kits, Shionogi & Co., Ltd., Osaka, Japan). Total AM were measured by a one-step two-site IRMA. Briefly, two kinds of monoclonal antibodies that specifically determined AM 12–25 or AM(46–52)NH₂ peptide were used to detect human AM, as previously reported [36].

2.5. Immunohistochemistry

Nasal biopsy samples were collected from the inferior turbinates or nasal polyps during surgery for nasal obstruction. Specimens (approximately 5×10 mm) were immersed in 10% formalin and fixed for 2 days. Each specimen was embedded in paraffin and cut with a microtome into 4- μm thick sections. After deparaffinization, the sections were hydrated in 0.01 M phosphate-buffered saline (PBS) and incubated in 3% H₂O₂ in methanol for 15 min at room temperature to block endogenous peroxidase activity. Continuous sections were stained with anti-AM antibody or anti-human tryptase antibody.

According to a previously reported method [28], polyclonal antibody raised in rabbits against purified human AM 1–52 (Peninsula Laboratories, Inc., Belmont, CA) was applied as the primary antibody at a dilution of 1:100–1:300 for 1 h at 37°C . Control slides were similarly treated, using mouse or rabbit IgG1 in place of the primary antibody. Staining of mast cells was carried out using the same system, but using murine anti-human mast cell tryptase monoclonal antibody (Chemicon International, Inc., Temecula, CA), which recognizes mast cells, at a dilution of 1:100 as the primary antibody. Including secondary antibody, the immunohistochemical staining system called Universal Immuno-Enzyme Polymer Method (Nichirei-Histofine™ Simple Stain MAX-Peroxidase-MULTI, Nichirei Co., Ltd., Tokyo) was used. We counted the number of immunoreactive cells in 10 fields (chosen at random) of each slides (magnification, $200\times$) by double blind fashion.

2.6. Statistical analysis

Data were expressed as the mean $\pm$ standard deviation. Inter-group comparison was performed using the Mann–Whitney *U*-test. Differences with *p* values of less than 0.05 were considered to be significant.

3. Results

3.1. Nasal discharge AM concentrations

Measurable AM was present in all the nasal discharge samples collected from both the allergy and non-allergy subjects. The nasal discharge AM concentrations (Fig. 1) were significantly higher ($p < 0.05$) in the non-allergy group (72.1 ± 55.5 fmol/ml) than in the allergy group (37.1 ± 44.2 fmol/ml).

3.2. Immunohistochemistry

Adrenomedullin was detected in the nasal turbinate mucosa obtained from both patients with chronic allergic rhinitis and chronic non-allergic rhinitis (Fig. 2). The numbers of AM immunoreactive cells in nasal mucosa from each allergic rhinitis cohort were 31.2 ± 11.2 and 18.4 ± 6.5 . They were larger than those from two

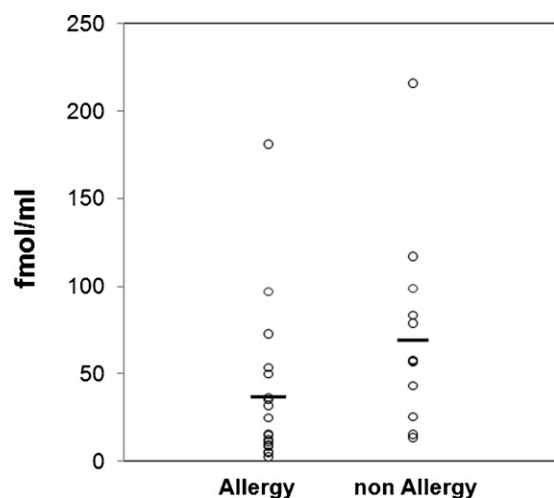


Fig. 1. The nasal discharge AM concentration. The nasal discharge AM concentration was significantly higher in the non-allergic group than in the allergic group.

non-allergic samples (6.6 ± 4.5 and 1.9 ± 1.4). Positive immunostaining for adrenomedullin was observed in the cells infiltrating the nasal mucosa. Most of the AM-immunoreactive cells also showed positive staining with anti-tryptase, which recognizes human mast cells (Fig. 3). Anti-AM and anti-tryptase double-positive cells were also detected in the nasal polyp tissue (Fig. 4). All the negative controls showed negative staining.

4. Discussion

The results of the present study demonstrate the presence of AM in human nasal discharge. The nasal discharge AM levels in healthy volunteers (72.1 ± 55.5 fmol/ml) were higher than the reported plasma AM levels in healthy volunteers (3.1 ± 1.0 fmol/ml) [21]. This finding suggests that the AM found in nasal discharge does not only represent a leakage from the blood circulation, but is actively secreted locally.

The immunostaining pattern in this study suggests that mast cells could be the source of the AM. To the best of our knowledge, this is the first report discussing the presence of AM in the upper airways.

In the nose, a system of capacitance vessels or sinuses plays an important role in regulating the width of the nasal passage. The sinuses have a thick layer of smooth muscle in their walls, and contraction and relaxation of this muscle causes changes in the blood capacity of these vessels. When the sinuses distend, they block the nasal lumen and cause nasal obstruction. Changes in the blood volume of these vessels are also thought to affect the filtering and air-conditioning functions of the nose [45]. These nasal blood vessels are innervated by both the sympathetic and parasympathetic nervous system. Moreover, in the presence of rhinitis, many inflammatory and neurogenic mediators are also released into the nasal mucosa that influence the severity of nasal congestion. The sensory nerve endings release neuropeptides, such as substance P, neurokinin A and calcitonin gene related peptide (CGRP). These substances stimulate mucus release from the submucosal glands and also cause vasodilatation and nasal congestion. In the presence of allergic rhinitis, leukotrienes and other chemical mediators such as histamine and thromboxane A_2 also contribute to the development of nasal congestion [13,30,45].

AM exhibits a 17% amino acid sequence homology to CGRP and also shares a part of the calcitonin receptor like receptor, however, receptor-associated protein-1 to 3 distinguish the specificity of the receptors [40]. CGRP-induced vasodilatation in the nasal mucosa is thought to play an important role in nasal congestion [27,37,43]. It is also possible that AM induces local vasodilatation, however, in the present study, we studied only ex vivo samples and could not examine the contribution of AM to vasodilatation and nasal congestion in the patients with allergic rhinitis.

From the results of the study conducted in AM-deficient mice, the defect in AM gene function did not affect the conventional mechanism of the allergic airway response, such as the production of specific IgE, Th1 and Th2 cytokines, leukotrienes, and tissue eosinophilia [47]. Although elevation of the plasma AM level

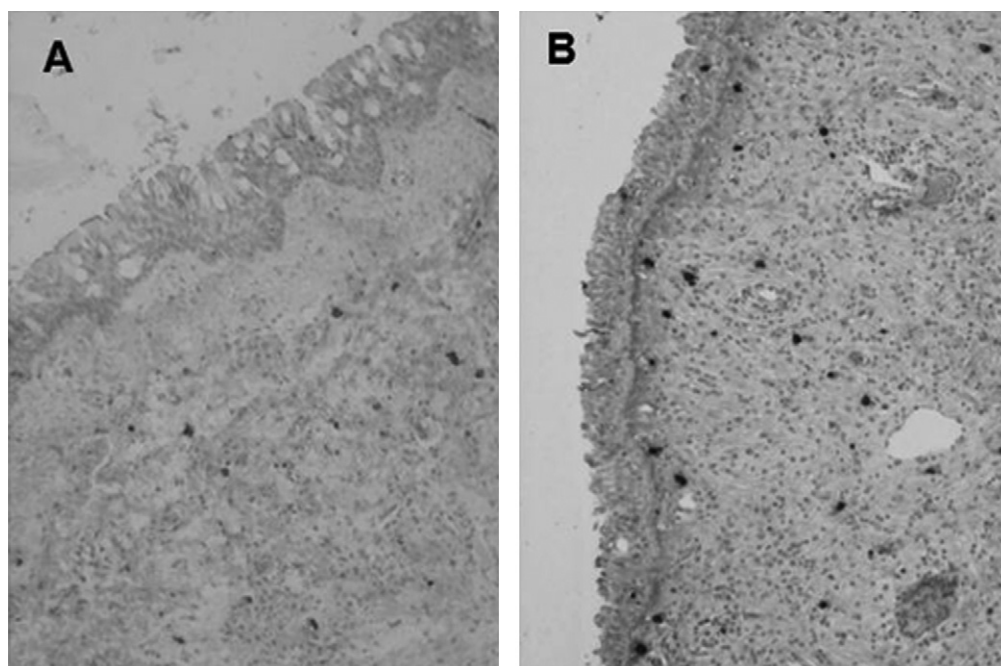


Fig. 2. Anti-adrenomedullin immunoreactive cells in the nasal mucosa. Anti-adrenomedullin immunoreactive cells were observed in the nasal mucosa, irrespective of the presence of allergic rhinitis. (A) Nasal mucosa without allergic rhinitis. (B) Nasal mucosa with allergic rhinitis.

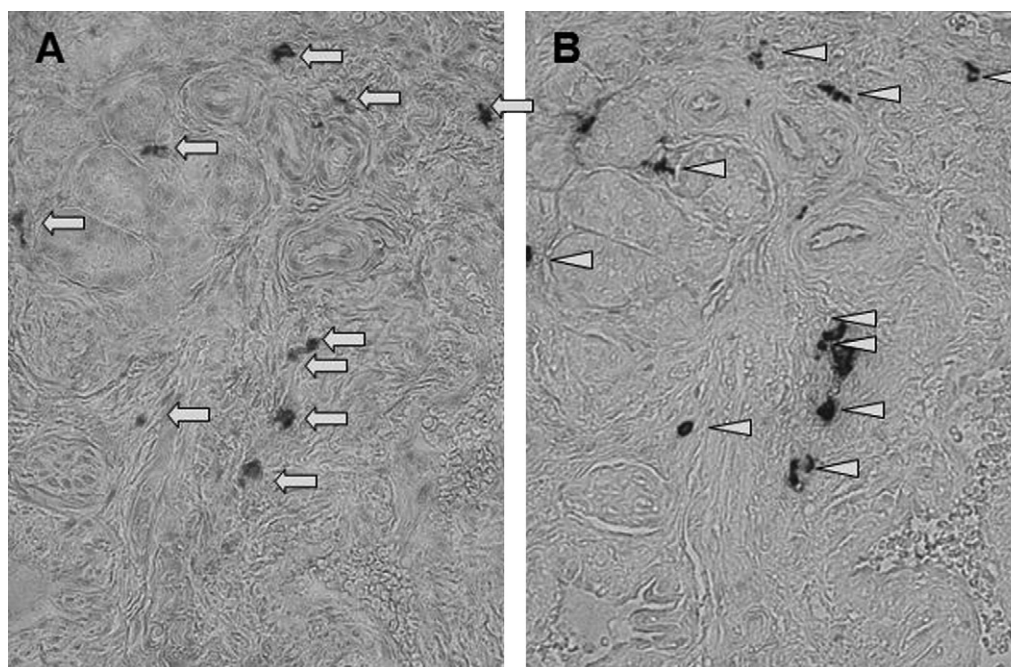


Fig. 3. Double-positive cells for anti-adrenomedullin antibody and anti-tryptase antibody. (A) Anti-adrenomedullin immunoreactive cells (arrow) in the nasal mucosa. (B) Anti-tryptase immunoreactive cells which recognize mast cells (arrow head) in a section contiguous with (A). These cells were double-positive for both antibodies.

has been reported during an asthma attack, this phenomenon is thought to represent a preventive mechanism against further bronchoconstriction [22]. Nasal discharge AM levels were found to be lower in patients with allergic rhinitis than in healthy volunteers. It is reported that AM acts as a chemoattractant to recruit mast cells and also increases the vascular permeability [49]. Considering these effects, it appears possible that the decrease of the AM levels in the nasal discharge in allergic rhinitis represents a protective mechanism. There are several factors such as LPS, inflammatory cytokines, hypoxia, and shear stress that increase AM expression in

vascular endothelial cells and other cells, but no negative regulators are reported [12,19,29,32,42].

Neutral endopeptidase (NEP) is known to degrade AM [25,46] and in the nasal mucosa, NEP-immunoreactive areas were present in the epithelium, serous cells of the submucosal glands, and endothelial cells of the small vessels [34]. Increase in the NEP activity in human nasal secretions by methacholine and histamine provocation has been reported. NEP in the nasal secretions has been thought to play a role in regulating the mucosal responses to luminal neuropeptides or other as yet uncharacterized NEP substrates

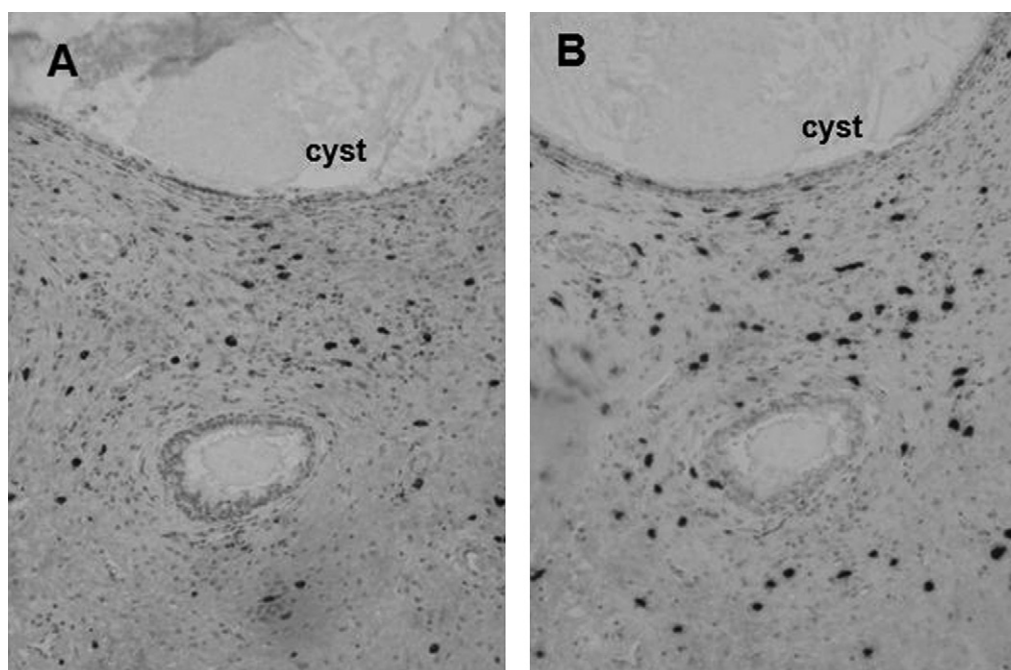


Fig. 4. Anti-adrenomedullin immunoreactive cells in a nasal polyp. (A) Anti-adrenomedullin immunoreactive cells in the cystic region of a nasal polyp. (B) Anti-tryptase immunoreactive cells in a contiguous section. Many of the cells were double-positive for both antibodies.

[35]. It is possible that NEP degrades AM, resulting in the reduced levels of AM in the nasal exudates of patients with allergic rhinitis.

On the other hand, AM acts as a histamine-releasing factor for mast cells and stimulates mast cells to produce MCP-1, which is also a histamine-releasing factor. Histamine is known to stimulate production of CC-chemokines including MCP-1, MCP-3, RANTES, and eotaxin [8]. Therefore, decrease of AM release also contributes to attenuation of the prolonged inflammatory cycle in allergic inflammation through regulating histamine release and CC-chemokine production.

In a study of pulmonary inflammation, it was shown that AM may play a role in the inhibitory regulation of inflammatory processes by several pathways, such as inhibition of binding of neutrophils to vascular endothelial cells and subsequent neutrophil emigration [17,38] and inhibition of cytokine secretion from macrophages, such as TNF- α , IL-6 and cytokine-induced neutrophil chemoattractant [17,23]. Although we have no direct evidence, the immunostaining pattern observed in the present study suggests that mast cells may compensate for the decreased content of AM. Compensated AM may contribute to inhibit migration of inflammatory cells to the site of allergic inflammation. AM can be a double-edged sword in the regulation of local allergic reactions and its regulation may differ from organ to organ.

In nasal allergy, the vascular tone and permeability is prominent in the immediate phase response. The reduced AM levels may protect against these responses. In the late and inflammatory phase, AM may act as an inhibitor of inflammatory cell migration. In the nasal discharge study, we recruited patients with allergic rhinitis in the acute phase and not chronic allergic rhinitis patients, therefore, it is possible that the actions of AM on the vascular tone and permeability are more prominent than the inflammatory responses induced by cytokines. Therefore, reduction in the levels of AM in the nasal discharge may be directed toward protection from allergic complications. On the other hand, an immunohistochemical study has demonstrated AM-immunoreactive cells in the chronic phase of rhinosinusitis. Further studies are required to clarify how modulation by AM differs between the upper and lower airways.

5. Conclusion

AM in nasal discharge is not an only leakage from blood circulation. AM is actively secreted into the nasal discharge. In the acute phase of allergic rhinitis, the nasal discharge AM concentrations are actually lower than those in healthy volunteers. AM has vasodilatory properties, as well as an inhibitory effect on the release of histamine and chemokines in the nasal mucosa. Considering these effects, the decrease in the nasal discharge AM concentrations in the acute phase of symptomatic allergic rhinitis may be protective. In chronic allergic inflammation, mast cells produce AM, that might act as an inhibitor of inflammatory cell emigration. Our findings suggest that the nasal discharge AM content might be regulated in a biphasic fashion by both degradation induced by NEP and production from mast cells.

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