JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2013. Vol. 7(Spl. Edn.), p. 201-207

Effects of Substance P on Eotaxin Expression in Rats Model of Allergic Rhinitis

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(Received: 03 March 2013; accepted: 14 April 2013)

Study the effection of substance P(SP) on Eotaxin in allergic rhinitis(AR) through SD rats. An AR model with SD rats was established by using ovalbumin then divided into four groups, one were continue sensitized by ovalbumin, and the second group were treated with treated with substance P (SP) as induction group, the third group were treated with substance P antagonist (SPA) as suppression group,and the others were treated with saline for control. The sneezing and nose rubbing and leukocyte infiltration of nasal lavage,IgE in blood serum,CO concentrations in plasma were recorded,then the expression of Eotaxin in nasal mucosa were determinated by real time RT-PCR and immunohistochemical, and Eotaxin level in serum and nasal lavage fluid were also detected by ELISA. The expression of Eotaxin and level of Eotaxin in serum and nasal lavage fluid of sensitized group were higher than those of control($P<0.01$), and all these items were show higher when treated with SP and lower as treated with SPA($P<0.05$). SP plays a significant role in the pathogenesis of AR through effection on Eotaxin, and the level of Eotaxin positive correlate with the level of SP.

Key words: Allergic Rhinitis; Expression; Eotaxin; Substance P; Antagonist.

Substance P (SP) was the first neuropeptide that was discovered and recognized as a sensory neurotransmitter by Lembeck[1]. Substance P (SP) is secreted by nerves and inflammatory cells, such as macrophages, eosinophils, lymphocytes, and dendritic cells. It acts by binding to the neurokinin-1 receptor (NK-1R)[2]. In airway tissues, it has been postulated that substance P is the transmitter of afferent sensory nerves which respond to various irritants, and is probably involved in allergic reactions occurring in airways[3-4]. SP has pro-inflammatory effect on epithelial cells and plays a key role in inflammatory diseases of the respiratory tract[5]. Capsaicin, the pungent component of hot pepperÿhas proved useful in the investigation of effects of neuronal stimulation. Intranasal capsaicin specifically stimulates afferent nerves consisting mostly of unmyelinated C fibers and some myelinated A-delta fibers. As a result it can trigger central and axonal reflexes, the latter being putatively mediated by the release of neuropeptides. Capsaicin as a blocking agent of neuropeptides, blocks the axon reflex and may exert a curative effect on allergic rhinitis[6].

Allergic rhinitis(AR) is a chronic inflammatory disease which is characterized by tissue eosinophillia. Eotaxin is a novel C-C chemokine with a potent and relatively specific chemoattractant activity towards eosinophils. It binds selectively to receptor CCR3, a receptor that is abundantly expressed on eosinophils[7]. It is not clear whether SP regulates Eotaxin expression. In

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this study, our aim is to elucidate the effect of substance P on Eotaxin expression in case of allergic rhinitis.

MATERIALS AND METHODS

Material and animal model

48 aged healthy male SD rats, weight 180–220g(shanghai laboratory animal center, Chinese Academy of Sciences), and animal models of allergic rhinitis were prepared according to the methods made by Al Suleimani M *et al.,*[2].48 guinea pigs were randomly divided into four groups, 36 guinea pigs were sensitized by ovalbumin (OVA,Sigma Inc.MD) then divide into three groups, one was continue nose inspired with OVA as AR group, the second group was treated with SP(SP ,Sigma Inc.MD) named as SP group, and the third was treated with S capsaicin (capsaicin, Sigma Inc.MD) as SPA group, and the control group were treated with saline for equal treatment. SP group was nose dripped SP according to the daily dose of 8μg/ml (50μl/ nostril, before nose inspired every day, and the SPA group was nose dripped capsaicin according to the daily dose of 2.5mg/ml (50μl/ nostril), and all groups continue to treat for one week.

Observation of sneezing and nose rubbing

Sneezing and nose rubbing were assessed as previously described by Al Suleimani M et al with modifications[2]. They were counted directly following nasal challenge, and for 30 min thereafter. A sneeze was characterized by an explosive expiration just after deep inspiration and a nose rub was characterized by an external perinasal scratch with the animal's forelimbs.

ELISA for Eotaxin in serum and Nasal lavage fluid (NLF)

NLF was collected from rats 1 h post-challenge as follows[5]: nasal cavities were washed with 2 ml of pre-warmed saline infused from the tracheal side. The recovered saline was collected from the anterior nares and centrifuged (2000r/min, 15 min).

Rats were anesthetized with pentobarbital (40mg.Kg-1 dose) intraperitoneal administration after last treatment, then were rapid decapitated and the serum and nasal mucosa were reserved for use,a part of nasal mucosa were put in liquid nitrogen rapidly and other mucosa embedded in paraffin for immunohistochemical, Eotaxin levels of serum and NLF were determined through ELISA methods(RB Inc.MD).

Immunohistochemical

Nasal mucosal biopsies of AR group and normal group were fixed in 10 % formalin solution, dehydrated in graded alcohols and embedded in paraffin. A transverse section was cut and stained with haematoxylin and eosin. Immunohistochemical staining was performed with rabbit polyelonal antibody against Eotaxin (1:1000 dilution,Santa Cruz Inc.MD)and an immunohistochemical assay kit (.Santa Cruz Inc.MD) and anti avidin-biotin-peroxidase (ABC) method were used. The histopathological assessment was performed by light microscopy and the presence of Eotaxin was indicated by the development of brown bands.

Total RNA extraction and cDNA synthesis

Samples of nasal mocusa were shipped and stored at -80 °C. They were then minced with a scalpel on dry ice and transferred immediately to 2 ml polypropylene tubes, homogenized and total RNA was extracted using TrizolTM reagent (Invitrogen Inc, MD) following the manufacturer's instructions. The concentration and purity of RNA were determined spectrophotometrically. Then the synthesis of cDNA were performed according to a cDNA cycle kit (,TaKaRa Inc,Japan.

Real time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)for HO-1 and HO-2 mRNA Expression

To determine the expression of the HO-1 and HO-2 gene in nasal mucosa, fluorescent quantitative real time RT-PCR assay was performed. The sequences of the primers (TaKaRa Inc, Japan) specific for Eotaxin were performed with sense (5'-CCC AGG TTC CAT CCC AAC T-3')and antisense (5'-TCA GCA CAG ATC TCT TTG CCA-3') primers, with an expected size of the amplified sequence of 153 bp. â-actin was used as control(sense: ATC ATG TTT GAG ACC TTC AAC A, antisense: CAT CTC TTG CTC GAA GTC,318bp). Then the incubation of cDNA and primer was performed at 94 °C for 5 min and the PCR reaction proceeded for 37 cycles: 95 °C for 30 s, 55 °C for 30 s, and 72 oC for 40 s in a programmable thermal cycler (Perkin Elmer Inc,U.S) using a thermostable Taq DNA polymerase (TaKaRa Inc,Japan) finalincubation at 72 oC for 7 minÿFluorescent product was measured by a single acquisition mode at 86 °C after each

cycle. After the completion of PCR amplification,a melting curve analysis was performed. For each sample,the amount of the target and of an endogenous control (β-actin, a housekeeping gene) were determined. The amount of the target molecule was then divided by the amount of the endogenous reference, to obtain a normalized target value. The PCR products were also electrophorased on a 1.5% agarose gel and visualized by ultraviolet light.

Statistical Analysis

A11 data were expressed as mean±S.D. Analysis of results was performed by using ANOVA for multiple comparison, and Pearson Correlation was used for the two-variable correlation analysis. P<0.05 was considered to be statistically significant.

RESULTS

Sneezing, nose rubbing induced by antigen

Sneezing frequency and number of nose rubbing in sensitized AR group were significantly increased (p<0.01) as compared with those in non-sensitized group, and increased further in SP treated group as compared with those in AR group(p<0.05), but significantly decreased in SPA treated group(p<0.05) (Fig. 1,2).

Concentration of Eotaxin in serum and NFL

The Eotaxin level in serum and NFL of AR group was higher than that of non-sensitized group by ELISA (p<0.05). Eotaxin level increased significantly after being treated with SP, and decreased after SPA administration as compared with AR group (p < 0.05) (Fig. 3A,B).

Expression of Eotaxin by real-time RT-PCR

The cumulative data for mRNA expression of Eotaxin is presented in Fig. 4,5. Eotaxin mRNA expression was upregulated in AR group as compared with control (p<0.05), and the expression was further increased after being stimulated with SP (p<0.05), whereas it was inhibited by SPA (p <0.05). Electrophoresis result showed that the order of Eotaxin mRNA expression

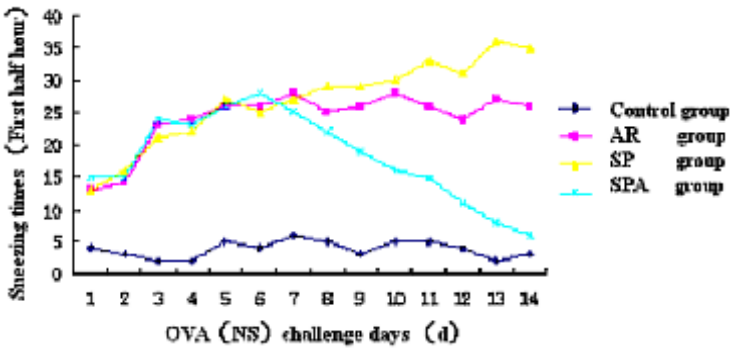


Fig. 1. The Sneezing of rats: SP induce an increase in the sneezes whereas SPA significantly inhibited the sneezes

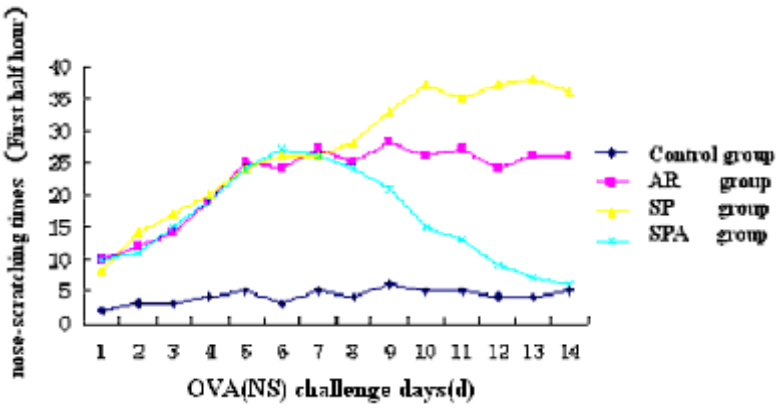
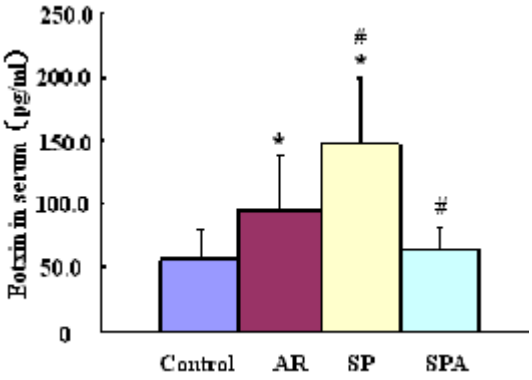
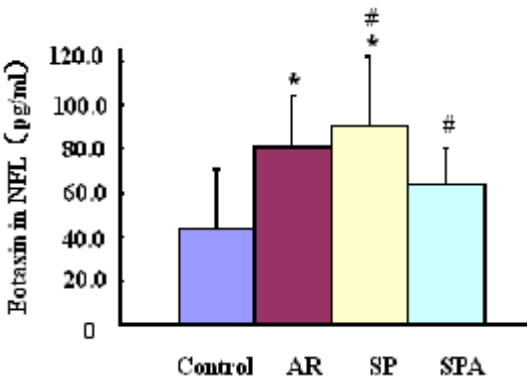


Fig. 2. The nasal scratching of rats: SP induce an increase in the nasal scratching times whereas SPA significantly inhibited the actions



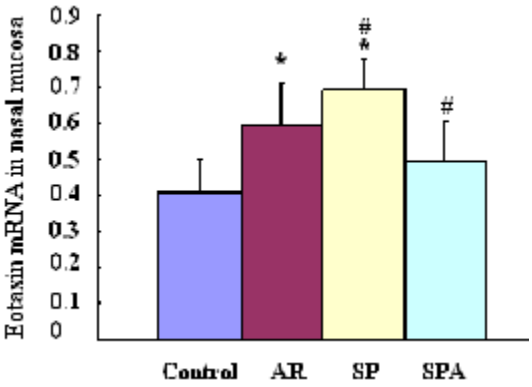
The serum Eotaxin level. Each column and vertical bar represents the mean ± S.D. *: Significantly different from the control group ($p < 0.05$). #:Significantly different from the AR group ($p < 0.05$)

Fig. 3(A). Eotaxin protein content in serum



The Eotaxin level of NFL. Each column and vertical bar represents the mean ± S.D. *: Significantly different from the control group ($p < 0.05$). #:Significantly different from the AR group ($p < 0.05$)

Fig. 3(B). Eotaxin protein content in NFL:



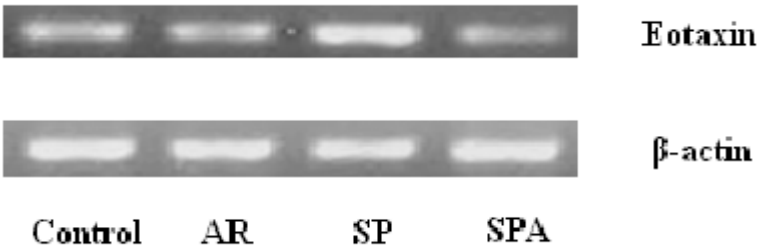
Each column and vertical bar represents the mean ± S.D. *: Significantly different from the control group ($p < 0.05$). #:Significantly different from the AR group ($p < 0.05$).

Fig. 4. Relative expression of Eotaxin mRNA in nasal mucosa of each group

levels from high to low was SP treated group, AR sensitized group, control group and SPA treated group in turn (Fig.5). It suggested that SP and SPA affect the expression of Eotaxin.

Immunohistochemical staining for Eotaxin

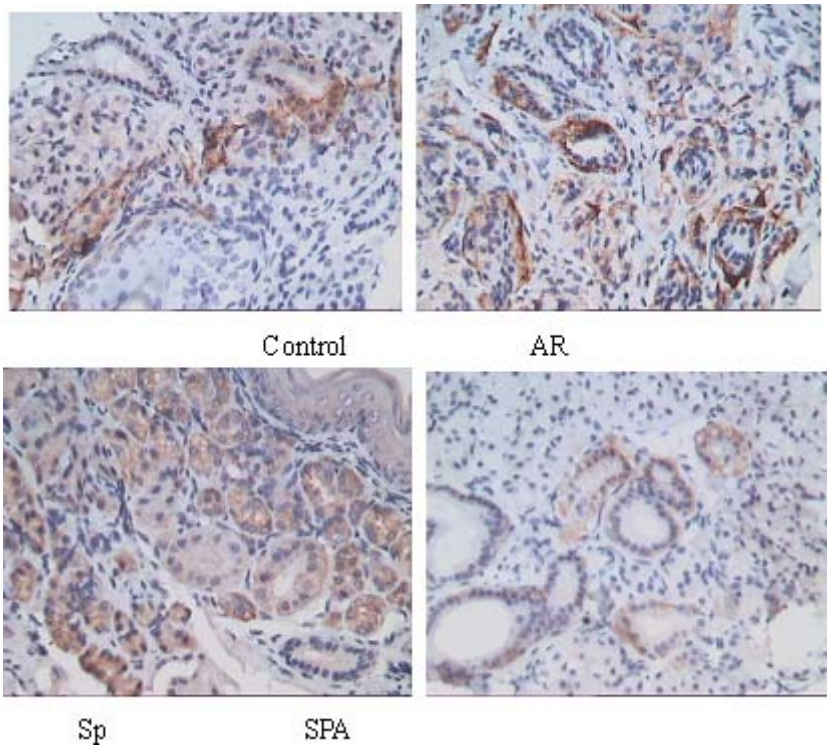
The yellow-brown cytoplasm represented positive signals of Eotaxin expression. Positive granules were not observed in the control group but found expressed in allergic nasal mucosa, and those were distributed mainly in the cytoplasm of seromucous glands, mesenchymal cells and inflammatory cells in lamina. Strongly positive staining was present in nasal mucosa of SP group and weak expression of Eotaxin was observed in lamina of mucosa of SPA group (Fig. 6). The total immunoreactivity for Eotaxin, in relation to the area



Size of PCR products are 370 bp(Eotaxin) and 240 bp(β-actin). Lanes left to right were group of normal, AR, SP,SPA. There was an increase in Eotaxin mRNA in AR group compared with control, and Eotaxin mRNA increased after SP treated and decreased after SPA treated, whereas there was no change in β-actin mRNA

Fig. 5. Image of gel of RT-PCR for Eotaxin (up) and β-actin (down) mRNA from nasal mucosa of guinea pigs.

of mucosal tissue, was 0.85 ± 0.34 in controls (n = 6), 2.23 ± 0.72 in rats after allergen challenge (n = 12), 4.70 ± 1.23 in sensitized guinea pigs after SP administration (n = 12), and 1.28 ± 0.75 in SPA treated group (n =12). There was an increase in Eotaxin immunoreactivity after allergen challenge ($p<0.05$), and an increase further in Eotaxin immunoreactivity after SP administration ($p<0.05$). However, there was a decrease in Eotaxin immuno reactivity after SPA treatment ($p<0.05$).



Immunoreactivity of Eotaxin in allergic nasal mucosa was found and cytoplasmic staining (brown) is seen in seromucous glands, mesenchymal cells and inflammatory cells of the lamina propria. A particularly intense staining was present in epithelium cells of SP group, and weak expression of Eotaxin was observed in SPA group. (original magnification × 40)

Fig. 6. Immunoreactivity of Eotaxin in nasal mucosa of groups

DISCUSSION

In the experimental results can be seen that SP can induce Eotaxin mRNA and protein expression accompany with the increased level of Eotaxin in sensitized rats serum and NLF, and it indicated that the increase of Eotaxin in AR. When SP was used as the induction agent of AR, Eotaxin expression in nasal mucosa and serum level were further increase, accompanied by AR symptoms aggravate. But when treated with capsaicin, the supresstion agent of SP[6]ÿthe AR rats show decrease of all items, also with the symptoms alleviate. It suggested that SP takes part in the pathogenic process of AR through effect on the level of Eotaxin.

Neuropeptides are contained in and released from a wide range of nerves. Chemically distinct, they exhibit characteristic patterns of localization within the peripheral and central nervous system and possess the ability to stimulate a range of diverse biological activities. SP was the first neuropeptide to be discovered and was recognized as a sensory neurotransmitter by Lembeck[1]. The sensory nerves that contain and release neuropeptides are primarily unmyelinated sensory C-fibres and myelinated A-fibres. SP is a vasodilator but is perhaps better known for its

ability to increase microvascular permeability leading to inflammatory oedema formation[8]. substance P binds to neurokinin 1 receptor (NK1R) on effector cells, increases microvascular permeability and promotes plasma extravasation from the intravascular to the extravascular space. Eosinophil activation by SP is reported to cause their degranulation, release of O_2^- , and thromboxane B2[2].

Eotaxin is a chemokine produced by a wide variety of cells. It is found in the airway lavage fluid of antigen-challenged animals and in humans with asthma and AR. Eotaxin signals exclusively via the CCR3 receptor, mediating eosinophil chemotaxis and activation[9,10]. Antigenic sensitization, even in the absence of antigen challenge, causes eosinophils to migrate to the airway nerves[11]. Chou, D.L et al illustrated that Eotxin is present in the airway nerves of guinea pigsÿhumans, and monkeys[12].In this study, it was also proved that eotaxin is present in smooth muscle and epithelium of nasal mucosa and highly expressed in AR rats.

Symptoms of AR aggravated by SP is possible for their degranulation through Eotaxin release. The stimulatory effect of SP on the degranulation of eosinophils is mediated via Eotaxin and is thought to be suppressed by SPA. Study of El-Shazly also proved that human eosinophil was release stimulated by SP[5]. Eosinophils from allergic and normal subjects differ in their chemotactic response to SP. SP induces degranulation and histamine and serotonin release from human and rat mast cells by a receptorindependent mechanism .Tachykinins, especially SP through the NK-1 receptor, induce a series of leukocyte responses to trigger and amplify the inflammatory processes, including upregulation of ICAM-1 expression on vascular endothelial cells and enhancement of neutrophil transendothelial migration, mediating leukocyte adhesion to the endothelial or epithelial cells in the nasal mucosa[2].

Both eosinophils and sensory C fiber neurons may be involved in the pathophysiology of AR. Nociceptive, unmyelinated C fiber nerve endings are present throughout the airways and contain neuropeptides, including SP. Several lines of evidence indicate that the products of C fibers can influence the function of eosinophils such that they promote eosinophil influx or bioactive mediator release[13-14]. The accumulation of eosinophils around airway nerves is critical to the physiological response to a variety of airway attacks[15].

The role of SP in AR has gradually been recognized for it affection of Eotaxin, and the SP may also serve a therapeutic role. SPA reduced both aeroallergen-induced inflammation and airway hyperresponsiveness in Rat through suppression of Eotaxin. So defining the regulation of Eotaxin in AR help us to found a therapeutic role of SP and it will remain an important avenue of investigation for the foreseeable future.

CONCLUSION

Findings of this study implicate a direct involvement of SP in the inflammatory process of AR and the effect of SP may attribute to the action of Eotain. SP acts as an important modulator of the inflammatory response in upper airway in AR. Understanding of these mechanisms is essential for future therapeutic strategies and the successful treatment of AR.

Competing interests

The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

This study was supported by National Science Foundation of Jiangshu province (No. BK2007545)).

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