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mRNA expression of tachykinins and tachykinin receptors in different human tissues

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Abstract

The tachykinins substance P, neurokinin A and neurokinin B are involved in many pathophysiological processes. A reverse transcription–polymerase chain reaction (RT-PCR) assay was used to analyse the expression of *TAC1* and *TAC3*, the genes that encode substance P/neurokinin A and neurokinin B, respectively, and the genes encoding the tachykinin NK_1, NK_2 and NK_3 receptors in different human tissues. The data show that tachykinins and their receptors mRNAs are broadly distributed in different human tissues being present in neuronal and non-neuronal types of cells. The presence of *TAC3* and the tachykinin NK_3 receptor (*TACR3*) in a wide variety of peripheral tissues argue for a still unexplored role of this ligand–receptor pair in mediating visceral effects of tachykinins. We found, for the first time, that *TAC3* and *TACR3* mRNAs are expressed in human airways and pulmonary arteries and veins, providing further evidence for the involvement of this system in lung physiopathology.

Keywords: Tachykinins; Tachykinin receptors; mRNA expression; Peripheral airways; (Human)

1. Introduction

The tachykinins are a family of evolutionary conserved peptides, which have been implicated in the regulation of many physiological and pathological processes. In mammals, the best known members of the family are substance P, neurokinin A and neurokinin B. The gene that encodes substance P and neurokinin A in humans is termed *TAC1*. Transcription of this gene generates a pre-mRNA that could be spliced into four different mRNA isoforms, α, β, γ and δ (Nawa et al., 1984; Harmar et al., 1986; Krause et al., 1987). Substance P is encoded by all four isoforms, whereas the neurokinin A sequence is present in β and γ *TAC1* mRNAs (Krause et al., 1987; Harmar et al., 1986, 1990). Neurokinin B is the only tachykinin encoded by the *TAC3* gene (Page et al., 2000).

The tachykinin peptides are mainly expressed in neuronal tissues (Otsuka and Yoshioka, 1993; Severini et al., 2002). Substance P and neurokinin A are present in the central nervous system (CNS) and also in primary afferent neurons innervating peripheral tissues. They are released from nerve endings at both the spinal cord and the peripheral level playing a role as excitatory neurotransmitters (Otsuka and Yoshioka, 1993; Patak et al., 2000; Lecci and Maggi, 2003). Neurokinin B has been detected almost exclusively at the level of the central nervous system and its physiological role remains mainly unknown (Moussaoui et al., 1992; Patacchini et al., 2000). However, it has been shown that substance P and neurokinin A are also expressed in non-neuronal cells, such as human endothelial cells and different types of inflammatory and immune cells from human, rat and mouse (Linnik and Moskowitz, 1989; Ho et al., 1997; Germonpre et al., 1999; Lecci and Maggi, 2003). Recent reports have also indicated the presence of neurokinin B precursor mRNA in the human and rat placenta (Page et al., 2000) and uterus (Cintado et al., 2001; Patak et al., 2003). These data argue for

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a broader distribution for these peptides that may not only act as neurotransmitters but also have an endocrine function.

Tachykinins bind to three distinct G protein-coupled receptors denoted NK_1, NK_2 and NK_3 (Takeda et al., 1991; Gerard et al., 1990; Takahashi et al., 1992). The genes that encode the tachykinin receptors have been named tachykinin receptor 1 (*TACR1*), tachykinin receptor 2 (*TACR2*) and tachykinin receptor 3 (*TACR3*) (human gene nomenclature approved by the Human Genome Organization). The three tachykinin receptors are heterogeneously distributed within each species (Regoli et al., 1994; Lecci and Maggi, 2003). In addition, there are marked species-related differences in their pattern of expression (Lecci and Maggi, 2001; Patak et al., 2002, 2003), suggesting that data obtained in one species cannot be extrapolated to other ones. An analysis of the distribution of tachykinins and their receptors in human tissues is therefore essential to assess their functional role at central and peripheral levels. In the present study, we have analyzed the mRNA expression of *TAC1*, *TAC3* and the tachykinin receptors in different human tissues. Due to the great number of studies suggesting a role of tachykinins in lung physiopathology (Lecci and Maggi, 2003; Joos et al., 2003), we paid special attention to their expression at lung tissues.

2. Materials and methods

2.1. Tissue preparation

The project was approved by the Ethic Committee of Consejo Superior de Investigaciones Científicas (CSIC, Spain) and conforms to the principles outlined in the Declaration of Helsinki. Samples of human lung were obtained from four males (age range 60–64) and one female (age 56) patients who had undergone lobectomy or pneumectomy for lung carcinoma. Large bronchi (internal diameter: 3–6 mm), pulmonary veins and arteries (2–5 mm internal diameter) were carefully removed from the macroscopically normal regions of the diseased lung and dissected free from adjoining connective tissue and lung parenchyma. Tissue pieces were immediately submerged in RNAlater (Ambion, Huntingdon, UK) within 1.5–4 h after surgery and then stored at −20 °C until use.

A human total RNA master panel (BD Biosciences Clontech, Palo Alto, CA) was also used to assess the mRNA expression of tachykinins and their receptors in 20 additional human tissues. The expression of all the genes was analysed in two different human panels, in which each mRNA is a pool from different individuals.

2.2. RNA isolation and reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was extracted from human bronchi, pulmonary artery or pulmonary vein by using the method of Chomczynski and Sacchi (1987). Residual genomic DNA was removed by incubating the RNA samples with RNase-free, fast protein liquid chromatography pure DNase I (Amersham Biosciences, Essex, UK) and RNasin (Promega, Madison, USA). First strand cDNA was synthesized from each RNA (2 μg) of the above indicated tissues and from the human total RNA master panel using Moloney murine leukemia virus reverse transcriptase and random hexamers according to the manufacturer's instructions (first-strand cDNA synthesis kit, Amersham Biosciences). The resulting cDNA samples were amplified by PCR using a DNA thermal cycler (MJ Research, Watertown, USA) and specific oligonucleotide primers designed with the analysis software Primer 3 (Rozen and Skaletsky, 2000). The structure of the primers used is shown in Table 1. The primer set used to amplify *TAC1* was designed against a sequence common to all mRNA isoforms. Two different isoforms have been described for tachykinin NK_1 and NK_2 receptors (Fong et al., 1992; Candenas et al., 2002). The primer pair designed to analyse the expression of *TACR1* allows the amplification of both the short and long isoforms. In addition, we used a second set of primers to amplify specifically the long *TACR1* isoform (Table 1). Two different primer pairs were also designed to analyse the expression of the two known splice variants of the tachykinin NK_2 receptor. The first set

Table 1
Sequences of forward (F) and reverse (R) primers of indicated target genes and the size expected for each PCR-amplified product

Gene	Forward primer	Reverse primer	Amplicon size (bp)	Reference
TACR1 (long + short)	5′-ATGCCCAGCAGAGTCGTGT-3′	5′-TCGTGGTAGCGGTCAGAGG-3′	194	Takeda et al. (1991)
TACR1 (long)	5′-GACCGCTACCACGAGCAAGT-3′	5′-CGTGAAGAGCAGTTGGAGGTC-3′	415	Takeda et al. (1991)
TACR2 (α)	5′-CAGCCACAACATCTGGTACTTTG-3′	5′-GACGGTGGAGTAGAAGCACTGA-3′	235	Gerard et al. (1990)
TACR2 (α + β)	5′-CAGCCATGTTYGTCAGCATC-3′	5′-TSCCCAGGATGAAGTAGAGGT-3′	477 ($NK_2R\alpha$) 282 ($NK_2R\beta$)	Gerard et al. (1990)
TACR3	5′-TTGCGGTGGACAGGTATATGG-3′	5′-GGCCATTGCACAAAGCAGAG-3′	178	Takahashi et al. (1992)
TAC1	5′-ACTGTCCGTCGCAAAATCC-3′	5′-ACTGCTGAGGCTTGGGTCTC-3′	212	Harmar et al. (1986)
TAC3	5′-CCCCCGAGAGCAGAATAGGT-3′	5′-CCAGGGTCAGGTAGAAAAAGATGG-3′	171	Page et al. (2000)
β-Actin	5′-TCCCTGGAGAAGAGCTACGA-3′	5′-ATCTGCTGGAAGGTGGACAG-3′	362	Nakajima-Iijima et al. (1985)

Primers for β-actin, used as an internal control, are also shown.

allows the amplification of a sequence only present in the long *TACR2* α isoform, while the second one permits the simultaneous visualization of both the α and the truncated *TACR2* β isoforms (see Table 1). Amplification of the β-actin gene transcript was used to normalize and control the efficiency of RT-PCR reactions among the samples. All cDNAs were serially diluted and the expression of β-actin was quantified in each dilution by real-time quantitative PCR using the iCycler iQ real-time detection system (Bio-Rad Laboratories, CA, USA) and SYBR green (Molecular Probes, Leiden, The Netherlands) as previously described (Patak et al., 2003). Equal amounts of templates were then amplified with specific primer pairs for tachykinins, their receptors and β-actin. All primers were synthesized and purified by Amersham Biosciences.

PCR mixes contained 0.2 μmol primers, 1.5 U of Taq polymerase (Amersham Biosciences), the buffer supplied, 2.5 mmol $MgCl_2$, 200 μmol dNTP's and cDNA (5 ng) in 25 μl. After a hot start (2 min at 94 °C), the parameters used for PCR amplification were 10 s at 94 °C, 20 s at 60 °C and 30 s at 72 °C. Cycle numbers were 35 for tachykinins and tachykinin receptors and 24 for β-actin. The PCR products were separated by gel electrophoresis, stained with ethidium bromide and visualized and photographed under UV transiluminator (Spectronics, New York, USA). Each PCR experiment, with the cDNA from each tissue, was carried out in triplicate and controls containing no reverse transcriptase and no template were included. Amplicon sizes were verified by comparison with a DNA mass ladder and the identity of each PCR product was established by DNA sequence analysis as previously described (Pinto et al., 1999).

3. Results

By using RT-PCR, we observed the presence of single transcripts corresponding to the size predicted for *TAC1* (212 bp) and *TAC3* (171 bp) (Figs. 1 and 2). *TAC1* mRNA was mainly expressed in the whole brain, heart, spleen, salivary, thyroid and adrenal glands. A minor expression was observed in the cerebellum, kidney, prostate, skeletal muscle, testis, trachea, uterus and bone marrow. The *TAC1* transcript was undetectable in the thymus, fetal and adult liver, the lung and the placenta (Fig. 1). *TAC3* was strongly expressed in the cerebrum, placenta, testis and kidney. Compared to its mRNA expression in the whole brain, *TAC3* was expressed in significant, but lower levels in the prostate, bone marrow, salivary gland, skeletal muscle, thymus and adrenal gland (Fig. 1). Of the assayed tissues, the only ones where *TAC3* expression was undetectable, even after amplification of higher amounts of cDNA, were the cerebellum, the liver and the spleen.

The tachykinin NK_1 receptor mRNA (194 base pairs) was detected in all tissues assayed and was strongly expressed in many of them. The highest expression was detected in the whole brain and the lowest expression was found in the cerebellum, liver, spleen and testis (Fig. 1). Using the second set of primers to detect specifically the long *TACR1* variant, we observed that it was expressed in all tissues analysed (data not shown). It was therefore impossible to determine the sites of expression of the short *TACR1* isoform.

The long tachykinin NK_2 receptor mRNA (α isoform) was expressed in high levels in the prostate, uterus, trachea and lung and in moderate levels in testis, skeletal muscle and fetal brain. A low mRNA level was detected in the placenta, heart, kidney, liver, salivary and adrenal gland and cerebellum (Fig. 1). By using a second primer pair that allowed the simultaneous visualization of both *TACR2* isoforms, we observed that α *TACR2* was the predominant splice variant. β *TACR2* was only present, at variable levels, in those tissues that also express the α isoform (data not shown).

The highest levels of the tachykinin NK_3 receptor mRNA were found in the cerebrum, kidney, lung, placenta and

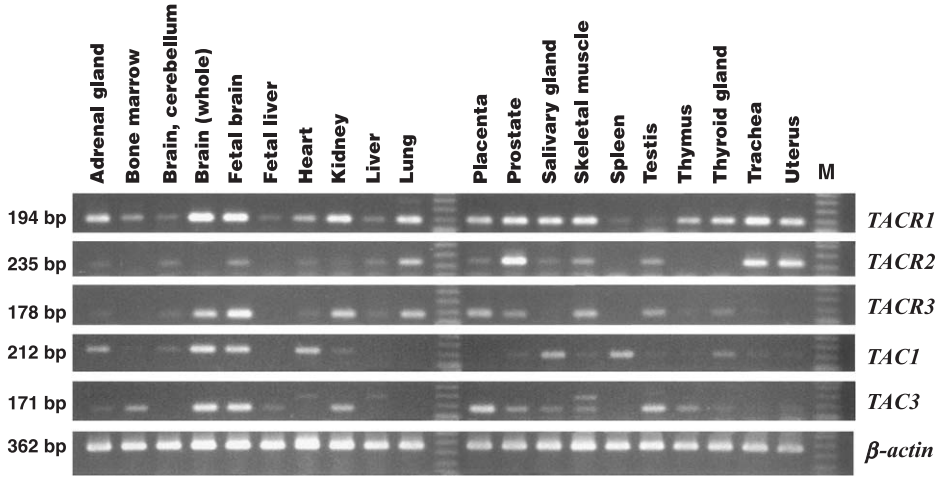

Fig. 1. Agarose gel showing expression of *TACR1*, *TACR2*, *TACR3*, *TAC1* and *TAC3* mRNAs in different human tissues. Equal aliquots of each cDNA were amplified by PCR for 24 (β-actin) or 35 cycles (target genes) with specific primer pairs. M, molecular weight standards.

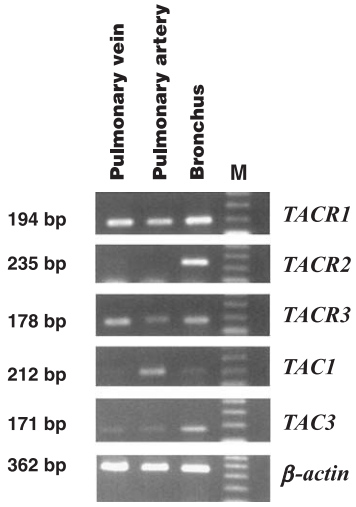

Fig. 2. Agarose gel showing expression of *TACR1*, *TACR2*, *TACR3*, *TAC1* and *TAC3* mRNAs in human bronchi, pulmonary arteries and veins. Equal aliquots of each cDNA were amplified by PCR for 24 (β-actin) or 35 cycles (target genes) with specific primer pairs. M, molecular weight standards.

skeletal muscle. *TACR3* was also expressed in significant amounts in the prostate and testis and a lower expression was observed in the cerebellum, thyroid, salivary and adrenal glands, thymus, heart, liver and trachea.

The results above showed that *TAC1*, *TAC3* and the three tachykinin receptors were expressed, in different levels, in the human peripheral airways. Therefore, we analysed further the expression of these genes in lung tissues. Human bronchi, pulmonary arteries and pulmonary veins were obtained from five different patients. Single transcripts corresponding to *TAC3*, *TACR1* and *TACR3* were detected in the different tissues from all patients (Fig. 2). *TAC1* was found in all five human bronchi and pulmonary arteries assayed, but only in pulmonary veins from two patients. The highest *TAC1* expression was observed in pulmonary arteries (Fig. 2). *TACR2* was expressed in high amounts in all bronchial cDNAs. A low mRNA expression of this tachykinin receptor was detected in two of the five pulmonary arteries and in two of the five pulmonary veins.

4. Discussion

This study shows that mRNAs of tachykinins and their receptors are broadly distributed in different human tissues. In this context, the data show for the first time that *TAC3* and *TACR3* mRNAs are expressed, at different levels, in the human peripheral airways.

Substance P- and neurokinin A-immunoreactivity has been detected in sensory nerves supplying a wide number of mammalian peripheral tissues, including the respiratory, gastrointestinal and genito-urinary tracts (Regoli et al., 1994; Patak et al., 2000; Lecci and Maggi, 2003; Joos et al., 2003). However, in the present study, *TAC1* mRNA was mainly found in the whole brain, heart, spleen, salivary, adrenal and thyroid glands, but its expression was very faint or undetectable in the lung, trachea, uterus, testis and prostate. In nerves, tachykinins are produced as inactive preprotachykinins that are translated and processed into the active neuropeptides mainly in the cell soma (Krause et al., 1987; Page et al., 2003). The tachykinin peptides are therefore present in nerve terminals and can be detected by immunochemistry. Conversely, their precursor mRNAs are absent or present in very small amounts (Wang et al., 1997) in nerve terminals and cannot be easily detected by RT-PCR. This and the present results lead to the following conclusions: (a) *TAC1* mRNAs presently found in human peripheral tissues are mainly located in cells of non-neuronal origin and (b) sensory neurons are probably the most important source of substance P/neurokinin A in peripheral tissues.

Immunohistochemical studies failed to identify neurokinin B in peripheral sensory nerves from different mammalian species (Moussaoui et al., 1992; Patacchini et al., 2000). For this reason, it was considered during many years that neurokinin B was present almost exclusively in the CNS. However, recent data have shown that neurokinin B is present in the human and rat placenta (Page et al., 2000) and uterus (Cintado et al., 2001; Patak et al., 2003). In fact, in our study, we found that the distribution of *TAC3* mRNA in human tissues was as wide as or even wider than that of *TAC1* mRNA. *TAC3* was strongly expressed in the brain but was also abundant in different peripheral tissues, including placenta, testis, prostate, kidney, bone marrow and salivary glands. This may suggest that, as occurs with the recently discovered tachykinins hemokinin-1 and endokinins (Zhang et al., 2000; Page et al., 2003), *TAC3* is primarily expressed in non-neuronal cells in the human periphery. Nevertheless, the possibility that *TAC3* mRNA might be present, at least in part, in peripheral ganglia or nerve terminals (Baluk and Gabella, 1989; Myers et al., 1996; Wang et al., 1997) cannot be excluded.

The tachykinin NK_1 receptor mRNA was ubiquitously expressed and appeared in all tissues assayed. This is in agreement with previous data in different mammalian species, including humans (Tsuchida et al., 1990; Ho et al., 1997; Lecci and Maggi, 2003; Page et al., 2003). The fact that *TACR1* is strongly expressed in many of the assayed tissues supports the notion that this receptor plays an essential role in mediating tachykinin effects in human tissues. Two different *TACR1* isoforms with length differences in its C-terminal tails have been described (Fong et al., 1992). In humans, there are conflicting reports regarding the expression of the short splice variant (Page and Bell, 2002; Caberlotto et al., 2003). Since we found an expression of the long isoform in all tissues assayed, we were unable to determine whether the short isoform was present or not in human tissues.

The tachykinin NK_2 receptor is primarily expressed in peripheral tissues and its expression in the CNS appears to be restricted to specific brain nuclei (Tsuchida et al., 1990;

Saffroy et al., 2003). We observed a significant expression of *TACR2* mRNA in the cerebellum. Interestingly, in spite of the high expression of *TAC1*, *TAC3*, *TACR1* and *TACR3* in the whole brain, the expression of all these genes in the cerebellum was low or absent. Further studies are needed to determine the meaning of these results.

A truncated splice variant of *TACR2*, named β *TACR2*, has recently been identified (Candenas et al., 2002). Our data show that the long *TACR2* α isoform was predominant in human tissues and that β *TACR2* was only visualized in tissues expressing α *TACR2*. The functional significance of β *TACR2* as well as whether this variant may be translated into an active protein is still unknown. However, its wide distribution in human tissues suggests that the truncated isoform may, at least, act as a regulator of tachykinin NK_2 receptor protein levels.

The tachykinin NK_3 receptor is mainly expressed in the CNS and has only been detected in certain peripheral tissues, such as the human uterus and skeletal muscle, the rat portal and mesenteric vein, and certain enteric neurons from the gut of different species (Tsuchida et al., 1990; Regoli et al., 1994; Johnson et al., 1998; Mileusnic et al., 1999; Page and Bell, 2002; Patak et al., 2003). Studies using selective agonists or antagonists point to a central role of the tachykinin NK_3 receptor, which might be involved in depression and anxiety (Massi et al., 2000). On the contrary, the role of this tachykinin receptor type at the peripheral level remains elusive. The present results show that *TACR3* mRNA receptor is abundant in the human whole brain, but is also widely distributed in different peripheral tissues, supporting a role for this receptor in mediating some of the peripheral effects of tachykinins. In this same context, our findings show that tachykinins and their receptors are broadly represented in several tissues such as the kidney, the prostate, the testis or the skeletal muscle, where their effects have been poorly studied. This suggests that the functional significance of the tachykinin system and, particularly, of neurokinin B and the tachykinin NK_3 receptor at the human peripheral level merits further investigation.

A great body of evidence indicate that tachykinins may play an important role in lung physiopathology with respect to bronchial hyperresponsiveness, airway constriction, and inflammation and cough (Joos et al., 2003; Lecci and Maggi, 2003). The effects on airway smooth muscle constriction are mainly mediated by the tachykinin NK_2 receptor while the tachykinin NK_1 receptor is mainly involved in microvascular leakage with the subsequent oedema formation (Naline et al., 1989; McDonald et al., 1996). The presence of the tachykinin NK_1 and NK_2 receptor proteins and/or their mRNAs have been demonstrated by distinct experimental approaches in the airways of different mammalian species including humans (Black et al., 1992; Adcock et al., 1993; Bai et al., 1995; Mapp et al., 2000; Pinto et al., 2002; Maghni et al., 2003). On the contrary, the tachykinin NK_3 receptor protein and/or its mRNA have not been detected in the mammalian peripheral airways (Black et al., 1992; Bai et al., 1995; Pinto et al., 2002). However, there is increasing evidence from functional studies that the tachykinin NK_3 receptor plays a role in controlling pulmonary function and may increase neuronal activity and responsiveness of target cells (Myers and Undem, 1993; Myers et al., 1996). In vivo studies have shown that aerosol administration of neurokinin B or of selective agonists for the tachykinin NK_3 receptor in guinea pigs elicited airway hyperresponsiveness (Daoui et al., 2000) or potentiation of histamine-induced airway microvascular leakage (Daoui et al., 2001). These effects were abolished by selective tachykinin NK_3 receptor antagonists (Daoui et al., 2000, 2001). In addition, SR 142801 ((*R*)-(*N*)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl)-4-phenylpiperidin-4-yl)-*N*-methylacetamide) or SB 235375 ((−)-(*S*)-*N*-(alpha-ethylbenzyl)-3-(carboxymethoxy)-2-phenylquinoline-4-carboxamide) inhibited substance P-, citric acid- or fenoterol-induced airway hyperreactivity to acetylcholine (Daoui et al., 1997; Hay et al., 2002; Pinto et al., 2002). A peripheral effect of SB 235375 was suggested by Hay et al. (2002) since this drug is characterized by a low CNS penetration.

A role for the tachykinin NK_3 receptor in controlling pulmonary function has also been demonstrated in human isolated tissues. Indeed, in a model of airway hyperresponsiveness on human isolated bronchi, interleukin-1β or nerve growth factor were able to induce a potentiation of the contraction provoked by the specific tachykinin NK_1 receptor agonist [Sar^9,$MetO_2^{11}$]SP and the hyperresponsiveness was abolished by SR 142801 (Barchasz et al., 1999; Vincent et al., 1999). The results presented in this paper show that *TAC3* and *TACR3* mRNAs are constitutively expressed in human airways and support previous results showing that tachykinin receptor agonists or antagonists may exert their effects through either stimulation or inhibition of the tachykinin NK_3 receptor in the human lung. The additional observation that *TAC1*, *TACR1* and *TACR2* are broadly and differentially expressed throughout the human peripheral airways further supports the involvement of this system in the development of airway diseases such as asthma. The differential expression of tachykinin and tachykinin receptor mRNAs in pulmonary arteries and veins also suggests a possible role in the regulation of the pulmonary vasculature.

In conclusion, the present study shows that mRNAs of tachykinins and tachykinin receptors are widely expressed in neuronal and non-neuronal human tissues. The presence of *TAC3* and *TACR3* mRNAs in a wide variety of peripheral tissues argue for a role of this ligand–receptor pair in mediating visceral effects of tachykinins. The broad distribution of the tachykinin system, and particularly, the expression of neurokinin B and the tachykinin NK_3 receptor in human peripheral airways provides further evidence for the involvement of these peptides in the development of airway hyperreactivity.

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