

Tachykinin NK₃-receptor deficiency does not inhibit pulmonary eosinophilia in allergic mice

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Accepted 5 July 2004

Abstract

Tachykinins are important in the development of pulmonary inflammation in mice but the tachykinin receptor subtype mediating this response has not been defined. To elucidate the role of tachykinin NK₃-receptors on allergen-induced pulmonary inflammation, studies were performed on ovalbumin (OVA) sensitized and challenged mice with genetic disruption of the tachykinin NK₃-receptor (NK₃^{-/-}). Aerosol OVA (0.5%) challenge produced eosinophil influx into the bronchoalveolar lavage fluid and lung tissue, goblet cell hyperplasia and damage to the airway epithelium of both NK₃^{-/-} mice and in wild type control mice (NK₃^{+/+}). There was no difference in the magnitude of these allergic inflammatory pulmonary responses between NK₃^{-/-} and NK₃^{+/+} mice. These results find no role for tachykinin NK₃-receptors on the pulmonary eosinophilia and lung damage after antigen challenge in mice.

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Keywords: Tachykinin NK₃-receptor; Allergen; Eosinophil; Mice

1. Introduction

Tachykinins represent a family of peptide neurotransmitters that include substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). Tachykinins bind to three pharmacologically distinct receptor subtypes termed tachykinin NK₁-, NK₂- and NK₃-receptors with SP binding preferentially to the tachykinin NK₁-receptor, NKA binding preferentially to the tachykinin NK₂-receptor and NKB binding preferentially to the tachykinin NK₃-receptor [1–3]. Tachykinins have a number of important effects in the lungs including airway smooth muscle contraction, airway blood vessel dilation, increased microvascular permeability, increased mucus gland secretion and augmentation of neurotransmission in airway cholinergic nerves [2,4]. Tachykinins also recruit and activate inflammatory cells in the airway

[5,6] and in a recent study in allergic mice, inhalation of aerosolized SR 142801, a tachykinin NK₃-receptor antagonist, inhibited the influx of inflammatory cells, including eosinophils into the bronchoalveolar lavage fluid after antigen challenge [7]. These results suggest an involvement of tachykinin NK₃-receptors in allergen-induced inflammatory cell recruitment into the lungs. However, this has not been validated with other tachykinin NK₃-receptor antagonists. Furthermore, locally high concentrations of SR 142801, which may be present after its inhalation, may have pharmacological effects that are totally unrelated to inhibition of the tachykinin NK₃-receptor as in vitro studies demonstrate inhibitory effects of SR 142801 on tachykinin NK₁- and NK₂-receptors and this compound also possess weak binding activity to opiod receptors and Na⁺ and Ca⁺⁺ channels [8–10].

In the present study, a tachykinin NK₃-receptor deficient mouse (NK₃^{-/-}) was used to define the role of tachykinin NK₃-receptors on pulmonary eosinophilic inflammation, ep-

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ithelial damage, and increase in mucus-secreting cells in the airway epithelium after antigen challenge.

2. Materials and methods

2.1. Animals

The NK₃ deficient mice were generated in 129sv X C57Bl/6J strains in the following manner. Two BAC clones encompassing the murine NK₃ genomic locus were identified from a mouse 129sv-derived embryonic stem (ES) cell library using a probe corresponding to the first 300 bp of the cDNA sequence (Genbank Accession [AF23341](#)). Fragments of these BACs containing murine NK₃ coding sequence were identified by hybridization with the full-length NK₃ cDNA.

A targeting vector was constructed in the pOSDupDel vector (O. Smithies, University of North Carolina, Chapel Hill, NC, USA). The targeting vector included approximately 8.5 kb of genomic DNA starting from the promoter region. In the targeting vector exon I was replaced with the neomycin resistance (NEO) gene. This resulted in the removal of sequence from the start codon through the third transmembrane domain.

This targeting vector was linearized by *Not I* restriction digestion and electroporated into ES cells. Neomycin-resistant ES cell clones were screened for homologous recombination by PCR and Southern blot analysis.

Five NK₃ targeted clones were injected into C57Bl/6J blastocysts (Jackson Labs, Bar Harbor, ME). Chimeras from two clones were mated with C57Bl/6J females and transmitted the targeted NK₃ allele to their offspring. Heterozygous mice were bred and live offspring of the expected +/+, +/- and -/- NK₃ genotypes were identified using the primers HGf968 (5'-CTGTCATGTTGCTTTCTACTCC-3'), HGf1824 (5'-CACCCTAAGCCATAGGC-3') and Osdupr1583 (5'-TTCGCCAATGACAAAGACGCTGG-3') which amplify a 856 bp fragment of the wt allele and a 600 bp fragment of the targeted allele. The null phenotype was confirmed by RT-PCR analysis of NK₃ RNA expression from mice. Total RNA was isolated from the brains of either wt or NK₃ deficient mice (RNEasy Isolation Kit, Qiagen, Valencia, CA). One-step RT-PCR (Invitrogen, Bethesda, MD) was carried out using the following oligonucleotide primer pairs. C0046 (5'-GGTAAACCTGGCTTTCTCCGAC-3') and C0047 (5'-GGGATGAAACCTGGTGGTCTTG-3') amplify an 810 bp product only from wt mice. OSDupFA (5'-GCCTTCTATCGCCTTCTTGACGAG-3') and R764 (5'-AATACCATCGTTGAAAGGGTCC-3') amplify an 850 bp product only from NK₃-deficient mice comprising the junction of the inserted neomycin resistance gene and intron I of the NK₃ gene. RNA loading was monitored by amplification of β -actin RNA (primers from BD Biosciences Clontech, Palo Alto, CA). Note that expression analysis was used to confirm the null phenotype since attempts to

detect NK₃ expression by radioligand binding failed to do so in wild-type mice (likely due to low expression level). All mice were maintained under specific pathogen-free conditions.

2.2. Sensitization, antigen challenge and collection of tissues and fluids

Mice were sensitized by intraperitoneal (i.p.) injection of 15 μ g ovalbumin (OVA, Sigma, St. Louis, MO) absorbed onto 2 mg of aluminum gel (alum; Reheis, Berkeley Heights, NJ, USA). Non-sensitized mice received i.p. injection of alum only. On day 5, the mice were given a booster injection of the same antigen or alum, i.p. Twelve days after sensitization, the mice were challenged with aerosolized OVA (0.5%) that was generated by an ultrasonic nebulizer (DeVilbiss, Somerset, PA, USA; model Ultra-Neb 99). The aerosol was circulated through a large plexiglass chamber and mice were placed into the chamber for 1 h in the morning and for the same period of time in the afternoon (6 h apart) of a single day. Forty-eight hours after the OVA challenge, the mice were sacrificed by CO₂ inhalation, the trachea catheterized and two separate 0.3 ml aliquots of isotonic saline were injected and withdrawn from the tracheal cannula. The total cell counts of this bronchoalveolar (BAL) sample were performed by hemocytometry. Slides were prepared on a Shandon Cytospin 3 (Shandon, Pittsburgh, PA, USA) at 250 rpm for 10 min, fixed with methanol and stained with Hema 3 (Fisher Scientific, Pittsburgh, PA, USA). Differential counts on at least 200 cells were done to enumerate the eosinophils in each sample.

For the histopathological evaluation of lung tissues the lungs were perfused in situ by flushing the pulmonary vasculature with buffered saline and fixed in 10% phosphate-buffered formalin. Paraffin sections of the lung tissues were then stained with either hematoxylin-eosin for eosinophil identification or periodic acid-Schiff/alcin blue stain (PAS/AB) for goblet cell identification [11]. Using five random areas, the number of eosinophils in peribronchial and alveolar regions were counted at 400 $\times$ magnification. The number of PAS-staining goblet cells was enumerated in a unit length (250 μ m) of airway mucosal layer. Damage to the airway epithelium and presence of mucus in the airway lumen were semi-quantitatively graded with numerical scores from 0 to 3 (0: none, 1: slight, 2: moderate, 3: severe).

2.3. Statistical analysis

Data are presented as the mean of group $\pm$ SEM. Statistically significant effects between the different treatment groups were determined by ANOVA/*t*-test procedures of StatView Program (Abacus Concepts Inc., Berkeley, CA, USA). A *P*-value less than 0.05 was accepted as statistically significant.

2.4. Animal care and use

This study was conducted with prior approval from the Animal Care and Use Committee of Schering-Plough Research Institute, which is a facility accredited by the American Association of Animal and Laboratory Animal Care (AAALAC).

3. Results

3.1. Identification of NK_3 KO mice

Brains were removed from mice after the completion of experiments and the phenotype was confirmed by monitoring the expression of tachykinin NK_3 receptor RNA as described in Section 2. Representative results are shown in Fig. 1. Total RNA isolated from each mouse was subjected to one-step RT-PCR analysis using primer pairs that detected either the wild-type NK_3 (C0046/C0047), or the disrupted locus (OS-DupFA/R764) or β -actin. Shown in Fig. 1, the wild-type NK_3

product was detected in three of the mice while the expected disrupted product was detected in the other three mice. Amplification of β -actin indicated that the RNA loaded in each reaction was similar. RT-PCR analysis also indicated that there was equivalent expression of tachykinin NK_1 and NK_2 receptor RNA in both $NK_3^{+/+}$ and $NK_3^{-/-}$ mice (data not shown).

3.2. Studies involving NK_3 KO mice

OVA challenge to sensitized, wild type ($NK_3^{+/+}$) mice increased the number of total cells and eosinophils appearing in the BAL fluid compared to values seen in non-sensitized $NK_3^{+/+}$ mice (Fig. 2). The magnitude of the OVA-induced increase in total cells and eosinophils in $NK_3^{-/-}$ mice was similar to that seen in wild-type controls (Fig. 2). OVA challenge to sensitized $NK_3^{+/+}$ mice increased the number of eosinophils in the peribronchial and peribronchiolar regions of the lung, produced slight damage to the airway epithelium and increased the number of PAS⁺/AB⁺ cells in the bronchial epithelium (Table 1). There were no differences between wild type ($NK_3^{+/+}$) and NK_3 -deficient mice ($NK_3^{-/-}$) in the magnitude of the tissue eosinophilia, damage to the airway epithelium or on the number of PAS positive staining cells after OVA challenge to sensitized animals (Table 1).

Baseline respiratory system resistance measured by a forced oscillation technique [11] in non-sensitized $NK_3^{+/+}$ mice (0.81 ± 0.05 cmH₂O ml⁻¹ s⁻¹) was not different from resistance values in non-sensitized $NK_3^{-/-}$ mice (0.89 ± 0.01 cmH₂O ml⁻¹ s⁻¹). Furthermore, bronchoconstrictor reactivity in these mice, measured as the dose of i.v. methacholine increasing respiratory system resistance by 100%

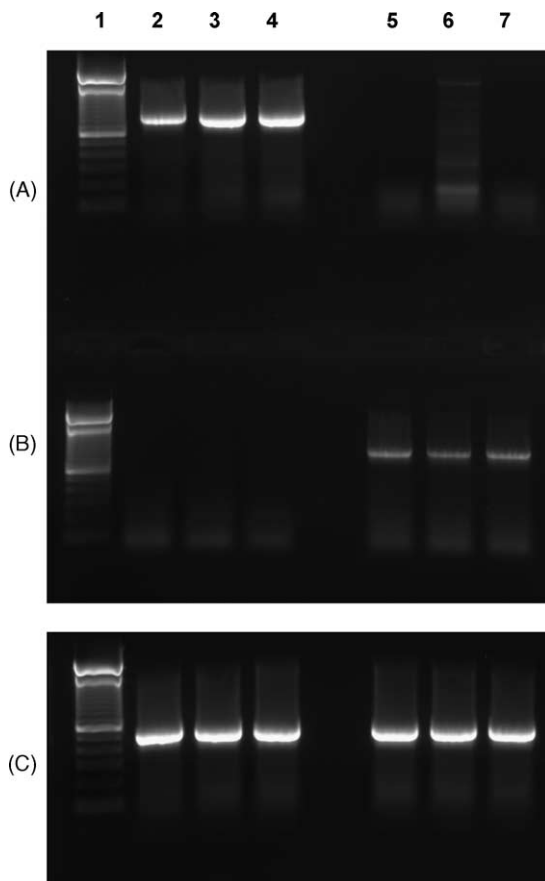


Fig. 1. Identification of phenotype by NK_3 RNA expression analysis. Total RNA isolated from mouse brains was subjected to RT-PCR (40 PCR cycles) as described in Section 2. Three $NK_3^{+/+}$ (lanes 2, 3 and 4) and three $NK_3^{-/-}$ (lanes 5, 6 and 7) mice were analyzed in this experiment. Primers identifying the wild-type locus were used in part A, those identifying the knock-out locus were used in part B and primers for β -actin were used in part C. Lane 1 contains DNA size marker.

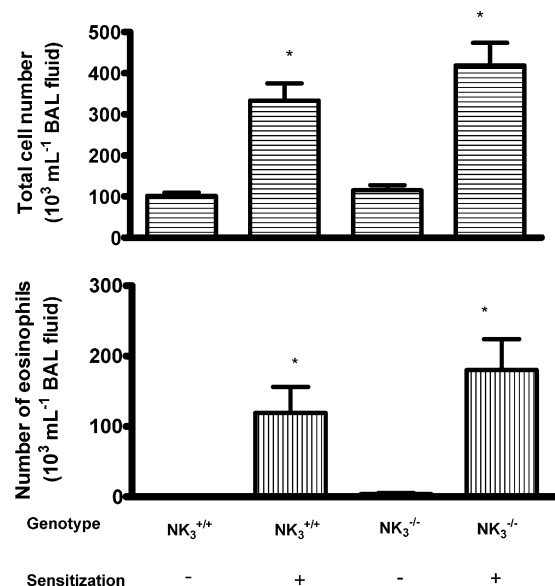


Fig. 2. Effect of OVA aerosol challenge on total cells and eosinophils in the BAL fluid of sensitized and non-sensitized $NK_3^{+/+}$ and $NK_3^{-/-}$ mice. Values represent mean $\pm$ SEM ($n = 9$ –12 per group). *, $P < 0.05$ compared to non-sensitized mice.

Table 1

Effect of OVA challenge on epithelial damage, tissue eosinophils and number of PAS⁺ mucus secreting cells in the airways of non-sensitized and sensitized NK₃^{+/+} and NK₃^{-/-} mice.

Genotype	Sensitization	Epithelial damage ^a	Number of eosinophil/field ^b		Number of PAS ⁺ -cells ^c
			Peribronchial	Peribronchiolar	
NK ₃ ^{+/+}	Non-sensitized	0.04 ± 0.04*	0.0 ± 0.0*	0.0 ± 0.0*	0.47 ± 0.22*
	Sensitized	0.60 ± 0.10	23.3 ± 3.9	7.2 ± 0.7	8.85 ± 1.58
NK ₃ ^{-/-}	Non-sensitized	0.10 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.37 ± 0.17*
	Sensitized	0.65 ± 0.1	28.0 ± 5.1	10.6 ± 2.4	7.43 ± 1.79

^a Epithelial damage was graded numerically (0: none, 1: slight, 2: moderate, 3: severe).

^b Number of eosinophils per high power field (400×).

^c Number of PAS⁺ cells per 250 μm epithelium. Values represent mean ± SEM (*n* = 10 per group).

* *P* < 0.05 compared to sensitized, NK₃^{+/+} mice.

over baseline (PD₁₀₀), was not different between NK₃^{+/+} (PD₁₀₀ = 0.24 ± 0.03 mg kg⁻¹) and NK₃^{-/-} (PD₁₀₀ = 0.25 ± 0.02 mg kg⁻¹) mice.

4. Discussion

The results in this study find no difference in the magnitude of the pulmonary eosinophilia, damage to the airway epithelium and increase in mucus secreting cells in the airways in sensitized, ovalbumin-challenged NK₃^{-/-} mice compared to that seen in the wild type control mice. These results do not support the hypothesis that tachykinin NK₃-receptors are important for the development of lung inflammation and inflammatory cell recruitment into the airways of antigen-challenged mice as was suggested in a study involving the tachykinin NK₃-receptor antagonist, SR 142801 [7].

Genetically targeted transgenic and knock-out mice are often used to evaluate the role of inflammatory mediators in disease processes [12,13] and, in general, represent an excellent method to upregulate or disrupt the function of the mediator in question. For example, gene-targeted disruption of the tachykinin NK₁-receptor in mice has been used as a means of defining an important role for the tachykinin NK₁-receptor on immune complex-induced inflammation in the lungs [13], inflammatory cell influx caused by injection of IL-1β [12] and on contractile responses of murine airway smooth muscle to antigen [14] and electrical field stimulation [15]. In this study, targeted depletion of the tachykinin NK₃-receptor was performed in mice and disruption of the gene expressing the tachykinin NK₃-receptor was confirmed by the fact that no mRNA for this tachykinin receptor was found in the NK₃^{-/-} mice but was found in the wild-type controls. There was, however, mRNA for tachykinin NK₁- and NK₂-receptors detected in the brains of the NK₃^{-/-} mice indicating that the targeting vector was selective for the tachykinin NK₃-receptor subtype. In addition, no detectable difference was observed in the level of NK₁ and NK₂ mRNA between NK₃^{+/+} and NK₃^{-/-} mice. These mice appear healthy, are fertile and show no difference in baseline pulmonary function

and bronchoconstrictor reactivity to methacholine compared to that seen in the wild type control mice.

In a previous study in mice that were sensitized and challenged with aerosolized ovalbumin, Nénan et al [7] found that treatment of these mice with inhaled SR 142801, a tachykinin NK₃-receptor antagonist, inhibited the pulmonary eosinophilia due to antigen challenge. However, the interpretation of these results is not entirely clear, as there are questions as to the selectivity of this compound for the tachykinin NK₃-receptor subtype. SR 142801 also possesses antagonist activity at both tachykinin NK₁- and NK₂-receptors and it also binds to μ-opioid receptors and Na⁺ and Ca⁺⁺ channels [8–10]. Therefore, it is uncertain if locally high concentrations of the drug in the lung, which is a condition that might exist after inhalation of the drug, will produce its pharmacological effect through mechanisms unrelated to inhibition of the tachykinin NK₃-receptor. Furthermore, the expression of tachykinin NK₃-receptor mRNA has not been demonstrated in peripheral tissues, including the lung [16] and the tachykinin NK₃-receptor subtype has only been demonstrated on neural tissue [1–3]. To investigate the role of the tachykinin NK₃-receptor on allergen-induced inflammatory responses in murine lungs in more detail, we used the genetically engineered tachykinin NK₃-receptor knock-out mice to measure the magnitude of the pulmonary eosinophilia in the BAL fluid and lung tissue, the damage to the airway epithelium and the number of PAS positive mucus secreting cells in the airway of sensitized and antigen-challenged mice. Our results show that quantitatively there were no differences in the magnitude of each of these parameters between the knock-out mice and the wild-type controls.

Tachykinins are the primary mediators producing the neurogenic inflammatory response [1–6] with the tachykinin NK₁-receptor as the dominant receptor subtype involved with this response [12,13,17–20]. Neurogenic inflammation occurs in murine airways after antigen challenge and this response involves the tachykinin NK₁-receptor subtype [17]. Indeed, pretreatment of these allergic mice with a tachykinin NK₁-receptor antagonist inhibited the influx of leukocytes into the BAL fluid and lungs following antigen challenge [17]. In addition, it was shown in this study that

NK₁-receptors were indeed upregulated by induction of an inflammatory response. It is possible that after sensitization and challenge, the tachykinin NK₁-receptor expression may be even more upregulated in the absence of tachykinin NK₃-receptors which may offset an effect of NK₃-receptor deficiency. However, our findings would suggest that even if this were the case the overall contribution of the tachykinin NK₃-receptor to immune inflammation in murine lungs is minimal at best.

Acknowledgement

The authors thank Ms. Maureen Frydlewicz for the preparation of this manuscript.

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