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Role of nicotinic receptors and acetylcholine in mucous cell metaplasia, hyperplasia and airway mucus formation *in vitro* and *in vivo*

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Abstract

Background—Airway mucus hypersecretion is a key pathophysiological feature in number of lung diseases. Cigarette smoke/nicotine and allergens are strong stimulators of airway mucus; however, the mechanism of mucus modulation is unclear.

Objectives—Characterize the pathway by which cigarette smoke/nicotine regulates airway mucus and identify agents that decrease airway mucus.

Methods—IL-13 and gamma-aminobutyric acid receptors (GABA_ARs) are implicated in airway mucus. We examined the role of IL-13 and GABA_ARs in nicotine-induced mucus formation in normal human bronchial epithelial (NHBE) and A549 cells, and secondhand cigarette smoke and/or ovalbumin-induced mucus formation *in vivo*.

Results—Nicotine promotes mucus formation in NHBE cells; however, the nicotine-induced mucus formation is independent of IL-13 but sensitive to the GABA_AR antagonist picrotoxin (PIC). Airway epithelial cells express $\alpha 7/\alpha 9/\alpha 10$ nicotinic acetylcholine receptors (nAChRs) and specific inhibition or knockdown of $\alpha 7$ - but not $\alpha 9/\alpha 10$ -nAChRs abrogates mucus formation in response to nicotine and IL-13. Moreover, addition of acetylcholine or inhibition of its degradation increases mucus in NHBE cells. Nicotinic but not muscarinic receptor antagonists block allergen or nicotine/cigarette smoke-induced airway mucus formation in NHBE cells and/or in mouse airways.

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Conclusions—Nicotine-induced airway mucus formation is independent of IL-13 and $\alpha 7$ -nAChRs are critical in airway mucous cell metaplasia/hyperplasia and mucus production in response to various pro-mucoid agents, including IL-13. In the absence of nicotine, acetylcholine may be the biological ligand for $\alpha 7$ -nAChRs to trigger airway mucus formation. $\alpha 7$ -nAChRs are downstream of IL-13 but upstream of GABA_AR $\alpha 2$ in the MUC5AC pathway. Acetylcholine and $\alpha 7$ -nAChRs may serve as therapeutic targets to control airway mucus.

Keywords

cigarette smoke; nicotine; nicotinic acetylcholine receptors; gamma-aminobutyric acid receptors; acetylcholine; airway mucus

Introduction

Normal mammalian airway epithelium produces and is coated by mucins such as MUC5B and MUC5AC and, following stimulation by an allergen/infection, MUC5AC is the predominant mucin produced in human airways. These mucins assist in clearing inhaled particulate matter from the airways¹. However, excessive mucous cell metaplasia and mucus hypersecretion contribute to the pathology of many respiratory diseases such as chronic obstructive pulmonary disease (COPD), asthma, and cystic fibrosis². In addition, excessive mucus production prolongs lung infections and decreases lung function³. Cigarette smoke is a strong inducer of airway mucus production and a major risk factor for asthma, bronchitis, and COPD^{4, 5}. Moreover, recent studies suggest that nicotine promotes airway mucus formation^{6, 7}; however, the mechanism by which cigarette smoke/nicotine promotes mucus formation is not well-established. Studies have demonstrated that Th2-cytokines, particularly IL-13, are key mediators of mucous cell metaplasia/hyperplasia and mucus production^{8–10}; however, in a rat allergic asthma model, chronic nicotine treatment strongly downregulated IL-4 and IL-13 production, but increased mucous cell metaplasia and mucus production in the lung¹¹. Thus in this model of allergic asthma, nicotine might stimulate mucus formation independent or semi-independent of IL-13.

A number of non-neuronal cells, including T cells, macrophages, and lung epithelial cells, express nicotinic acetylcholine receptors (nAChRs) and may synthesize acetylcholine^{12, 13}. Mucus-producing lung epithelial cells from rats, mice, and humans also express several different GABA_A receptor (GABA_AR) subunits¹⁴, which have been implicated in airway mucus formation¹⁴. Nicotine is a major constituent of cigarette smoke and in the central nervous system nicotine activates GABA_AR in some neurons. Moreover, the $\alpha 7/\alpha 9/\alpha 10$ -nAChR antagonist methyllycaconitine (MLA) moderates mucus formation in the monkey lungs⁶. We hypothesized that in airway epithelial cells nicotine activated GABA_ARs, through nAChRs, thereby promoting mucus formation. In this communication, we show that while normal human bronchial epithelial (NHBE) cells express $\alpha 7$, $\alpha 9$, and $\alpha 10$ -nAChR subunits, $\alpha 7$ -nAChRs play a critical role in mucous cell metaplasia and mucus formation in NHBE cells. Moreover, (a) nicotine promotes mucus formation independent of IL-13, (b) the normal biological ligand for the nAChRs in the bronchial epithelial cells for mucus formation may be acetylcholine, and (c) antagonists of nAChRs but not muscarinic receptors suppress mucus formation *in vivo* and *in vitro*.

METHODS SUMMARY

NHBE and A549 cells were cultured using standard procedures¹⁵. DO11.10 OVA-TCR transgenic on a BALB/c background were exposed to air, secondhand smoke (SS) or Nicotine (1.5 mg total particulate material/m³), heat-aggregated OVA aerosol (5 mg/m³), or OVA plus SS or nicotine for 2 weeks (6 h/day, 5 days/week). In some experiments, normal

(wild-type) BALB/c mice were sensitized to *Aspergillus fumigatus* extract as described previously¹⁶. Where indicated mice were subcutaneously implanted with mecamylamine (MM) containing mini-osmotic pumps (2 mg/kg body weight/day) 3 weeks prior to exposure¹⁷. In another group of BALB/c mice, animals were first exposed subcutaneously to saline- (control) or MM-containing Alzet pumps for 2 wk and then sensitized with the allergen *Aspergillus fumigatus* extracts as in the Methods section of this article's Online Repository. To determine the effects of nicotine, IL-13, or acetylcholine on NHBE or A549 cells, cells were treated with nicotine base (100 nM), recombinant human IL-13 (10–50 ng/ml), or indicated concentrations of neostigmine bromide (NB) respectively, and the cultures were harvested approximately 48 h later. GABA_AR, nAChR, or muscarinic receptor inhibitors were added at the indicated concentrations 2 h prior to the addition of IL-13, nicotine, or NB. NHBE cells (5-μm-thick sections) were stained using alcian blue-periodic acid-Schiff's (AB-PAS) staining for mucus^{18, 19}, MUC5AC, or GABA_ARα2 using appropriate reagents, and the cell number and mucus volume were determined by microscopy^{11, 20}. Mouse lung sections were stained for Muc5ac- and GABA_ARα2 using Immunohistochemistry (IHC). Total RNA was isolated from lung tissues, NHBE cells, and A549 cells using TRI-Reagent. GABA_ARα2-specific mRNA was assayed by using SuperScriptTM III One-Step RT-PCR with Platinum[®] Taq. RT-PCR primers for GABA_ARα2 were: forward 5'-AGGCTTCCGTTATGATACAG, reverse 5'-AGGACTGACCCCTAATACAG and GAPDH: forward 5'-CCCATCACCATCTTCCAGGAG and reverse 5'-TTCACCACCTTCTTCTTGATGTCAT. qPCR was performed using a TaqMan One-Step RT-PCR kit containing AmpliTaq Gold[®] DNA polymerase with ABI primers and probes. Fold differences were determined by the 2^(-ΔΔCT) method²¹. nAChR subtypes were knocked down by specific siRNAs; changes in specific mRNAs were determined 48 h after siRNA treatment. Protein levels of GABA_ARα2 were determined by Western blot analysis¹⁷. GraphPad Prism Software 5.03 was used to determine statistical significance by two-way ANOVA. Detailed methods are given in the Methods section of this article's Online Repository.

RESULTS

Nicotinic receptors are critical in mucus formation

We examined the effects of nicotine (100 nM) on mucus formation in NHBE cells grown at Air Liquid Interface (ALI). This is a realistic concentration of nicotine and is severalfold lower than the EC₅₀ concentrations (10–100 μM) of nicotine/nicotine agonists required to activate the ligand-gated cationic channel in neurons²². As seen by AB-PAS mucus staining (Fig. 1A), control (Cont) NHBE cells have a low baseline level of mucus; however, when the cells were treated with nicotine, IL-13, or IL-13 + nicotine for 48 h (predetermined optimal time), the mucus content in these cells increased strongly. Furthermore, the α7/α9/α10-nAChR-specific antagonist MLA (Fig. 1A) as well as the non-selective nAChR antagonist MM (Fig. 1B) suppressed the nicotine + IL-13-induced mucus formation in NHBE cells. MLA also blocked the increase in mucus formation in NHBE cells in response to either nicotine or IL-13 (see Fig E1). To determine whether nicotine and/or IL-13 affected mucous cell hyperplasia and metaplasia, we measured the number of mucous cells/mm basal lamina and the volume of mucus-containing cells (mucus volume/mm³ basement membrane), respectively. Nicotine and IL-13 significantly increased both mucous cell numbers (Fig. 1C, left panel) and volume (Fig. 1C, right panel), and these effects were blocked by MLA. These results suggest that both nicotine and IL-13 affect mucous cell physiology and require the activation of nAChRs (α7 and/or α9/α10). Although in some experiments a combined treatment with nicotine and IL-13 appeared to increase cell volume over the individual treatment with IL-13 or nicotine, these differences varied from experiment to experiment and were not statistically significant (data not shown). Thus

nicotine and IL-13 may affect the same downstream pathway(s) for mucous cell hyperplasia/metaplasia and mucus production.

Nicotine and IL-13 increase MUC5AC

Mucin glycoproteins are the major constituents of airway mucus. MUC5AC is the dominant mucin gene expressed in airway goblet cells²³, and IL-13 is known to increase MUC5AC expression in these cells²⁴. To ascertain whether nicotine and/or IL-13 also induced the expression of MUC5AC, NHBE cells were treated with nicotine and/or IL-13, and MUC5AC expression was examined by qPCR and IHC staining using the MUC5AC-specific antibody. Nicotine as well as IL-13 significantly increased the mRNA expression of MUC5AC; however, combined treatment with nicotine and IL-13 did not cause significantly higher MUC5AC expression than nicotine or IL-13 alone, and pretreatment with MLA blocked the increase in MUC5AC by nicotine + IL-13 (Fig. 1D). Similar results were observed by scoring for MUC5AC protein by IHC staining (Fig. 1E).

Nicotine-induced MUC5AC is independent of IL-13

IL-13 is the critical cytokine in mucus formation and MUC5AC expression^{8–10, 25}. Although unlikely, it was possible that nicotine promoted MUC5AC/mucus formation by inducing IL-13 in NHBE cells. To ascertain this possibility, we determined IL-13 mRNA by qPCR in NHBE cells before and after nicotine treatment. Unlike human Jurkat cells (positive control), qPCR analysis of NHBE cells (up to 40 cycles) did not show any detectable expression of IL-13 mRNA in the presence or absence of nicotine (Fig. E2). Thus, while both nicotine and IL-13 induce mucus formation and MUC5AC expression in NHBE cells, the nicotine-induced MUC5AC expression and mucus formation do not necessarily require IL-13.

$\alpha 7$ -nAChRs are required for mucus formation

Neuronal nAChRs are pentameric structures and, in mammals, nAChRs are derived from eight α subunit ($\alpha 2$ – $\alpha 7$, $\alpha 9$, and $\alpha 10$) and three β subunit ($\beta 2$ – $\beta 4$) genes; however, $\alpha 7$ and $\alpha 9$ form functional homomeric receptors²⁶. Moreover, $\alpha 10$ subunits are functional only in the presence of $\alpha 9$ subunit²⁷; $\alpha 7$ and $\alpha 10$ co-localize in rat sympathetic neurons²⁸. Many non-neuronal cells, including T cells²⁹, mast cells³⁰, and macrophages express nAChRs; mast cells express full-length $\alpha 7$, $\alpha 9$, and $\alpha 10$ nAChRs that respond interdependently to low concentrations of nicotine³⁰. To ascertain whether a specific nAChR subtype mediated the effects of nicotine on mucus formation in NHBE cells, we determined the expression of nAChR subunits ($\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\alpha 9$, $\alpha 10$, and $\beta 4$) in these cells. Examining the expression of $\alpha 3$, $\alpha 4$, and $\alpha 7$ nAChR subunits was warranted because they are expressed in the lung tracheal tissue³¹, and genome-wide association studies show single nucleotide polymorphisms in the gene cluster encoding $\alpha 3/\alpha 5/\beta 4$ nAChR subunits in lung cancer³². Our qPCR analysis suggested that $\alpha 3$, $\alpha 4$, $\alpha 5$, and $\beta 4$ were essentially undetectable in NHBE cells (not shown); however, the cells expressed $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -nAChR subunits (Fig. E3). After normalizing with GAPDH, the mRNA expression of $\alpha 7$ was much higher than $\alpha 9$ and $\alpha 10$ (i.e., $\alpha 7$ was detectable around 27 while $\alpha 9/\alpha 10$ around 35 cycle of qPCR analysis). Thus, NHBE cells express much higher levels of $\alpha 7$ - than $\alpha 9/\alpha 10$ - mRNA.

NHBE cells are generally refractory to various transfection/transduction approaches and we were unable to knock down the expression of $\alpha 7$ -, $\alpha 9$ -, or $\alpha 10$ - nAChR subunits in these cells by siRNA (not shown). Therefore, to evaluate the role of $\alpha 7$ - and/or $\alpha 9/\alpha 10$ -nAChR subunits in mucus production, we used receptor subtype-specific conotoxin peptides to inhibit $\alpha 7$ - and $\alpha 9/\alpha 10$ -nAChRs in NHBE cells. At lower concentrations, the conotoxin peptides ArIB[V11L, V16D] and RgIA preferentially inhibit $\alpha 7$ - and $\alpha 9/\alpha 10$ -nAChRs, respectively^{33, 34}. Using these peptides at concentrations that showed minimum cross

inhibition, only ArIB[V11L, V16D] significantly reduced both nicotine- (Fig. 2A) and IL-13-induced (Fig. 2B) mucus production. Moreover, ArIB[V11L, V16D] (Fig. 2C), but not RgIA (not shown) also suppressed nicotine- and/or IL-13-induced expression of MUC5AC mRNA by qPCR. Thus, $\alpha 7$ -nAChRs are critical in the induction of airway mucus by nicotine and IL-13.

GABA_ARs are downstream of $\alpha 7$ -nAChRs

IL-13 has been shown to induce GABA_ARs in the human bronchial epithelial cell line A549¹⁴. Although A549 cells do not produce mucus, they contain mRNAs for $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ - (Fig. E4) as well as $\alpha 3$ -, $\alpha 4$ -, and $\beta 2$ -nAChRs (not shown). Inhibiting GABA_ARs with picrotoxin (PIC) also blocked mucus production in NHBE cells¹⁴. We used three approaches to determine whether the effects of nicotine on mucus formation involved GABA_ARs. First, we showed that the non-selective GABA_AR antagonist PIC inhibited the IL-13 + nicotine-induced mucus (AB-PAS staining, Fig. 3A) and MUC5AC proteins (IHC staining, Fig. 3B) in NHBE cells or by IL-13 and nicotine individually (see Fig E1). Similarly, PIC also blocked the IL-13 + nicotine-induced mucous cell hyperplasia (Fig. E5A), metaplasia (Fig. E5B), and MUC5AC mRNA expression (Fig. E5C) in NHBE cells. Second, in NHBE cells, nicotine and/or IL-13 induced the expression of GABA_AR $\alpha 2$ as detected by RT-PCR (Fig. 3C) and qPCR (Fig. 3D); the nicotine-induced expression of GABA_AR $\alpha 2$ mRNA seen by RT-PCR was blocked by the $\alpha 7, \alpha 9, \alpha 10$ -nAChR-specific antagonist MLA (Fig. 3E). Moreover, as assayed by IHC, MLA also blocked the nicotine-induced expression of GABA_AR $\alpha 2$ in NHBE cells (Fig. 3F). Third, A549 and NHBE cells express a number of GABA_AR subtypes. RT-PCR analysis suggested that both nicotine and IL-13 upregulated the expression of GABA_AR $\alpha 2$ in A549 cells (Fig. 3G). Indeed, among several GABA_AR subtypes (GABA_AR $\alpha 2$, GABA_AR $\beta 2$, GABA_AR γ , and GABA_AR π) only the expression of GABA_AR $\alpha 2$ was consistently upregulated by IL-13 and nicotine in these cells (not shown). Moreover, MLA suppressed the GABA_AR $\alpha 2$ expression in A549 cells (Fig. E6). Thus, $\alpha 7$ - and/or $\alpha 9/\alpha 10$ -nAChRs are involved in the increased expression of GABA_AR $\alpha 2$ by nicotine in A549 cells. To identify the type of nAChR subtype among $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ that regulates GABA_AR $\alpha 2$ expression and mucus formation, we used siRNA approach to individually knockdown $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -nAChRs in A549 cells. Specific siRNA treatment selectively decreased the mRNA expression of $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -nAChRs by approximately 65%, 80%, and 70%, respectively (Fig. E7). As seen by Western blot (Fig. 3H), the knockdown of $\alpha 7$, but not $\alpha 9$ and $\alpha 10$ significantly decreased nicotine-induced expression of GABA_AR $\alpha 2$ in A549 cells. In these cells, the nicotine-induced expression of GABA_AR $\alpha 2$ mRNA was also blocked by the $\alpha 7$ -specific ArIB[V11L, V16D] but not $\alpha 9/\alpha 10$ -specific RgIA conotoxin peptide (Fig. 3I). Because MLA blocked both nicotine- and IL-13-induced expression of GABA_AR $\alpha 2$, activation of nAChRs must precede the activation of GABA_AR $\alpha 2$ during mucus formation.

Mecamylamine blocks GABA_AR $\alpha 2$ and mucus production in mice

Cigarette smoke promotes goblet cell hyperplasia and mucus formation³⁵. Similarly, sensitization with allergens such as OVA, ragweed, and Aspergillus extracts promotes mucus formation in airways^{11, 16}. As seen by immunostaining, a 2-week inhalation exposure of OVA-TCR transgenic mice on the BALB/c background to OVA and/or SS strongly upregulated GABA_AR $\alpha 2$ expression (Fig. 4A) and mucus formation (Fig. 4B) in the airways. However, when the animals were pretreated with the nAChR antagonist MM prior to exposure to SS + OVA, the amplifying effects of SS + OVA on GABA_AR $\alpha 2$ expression (Fig. 4A) and airway mucus formation (Fig. 4B) were lost. Moreover, Western blot analysis of the lung extracts also indicated that MM blocked the OVA- and SS-induced expression of GABA_AR $\alpha 2$ (Fig. 4C). MM by itself had no detectable effect on normal bronchial epithelium, such as gross histopathology or nuclear condensation (not shown). Similarly,

qPCR analysis indicated that MM also blocked the SS- and/or OVA-induced Muc5ac mRNA expression in the lung (Fig. 4D). In a separate experiment, lungs from BALB/c mice, exposed to air (control) or nicotine inhalation (1.5 mg/m³, 6 h/day) for 2 weeks, exhibited increased expression of GABA_ARα2 protein by Western blot analysis (Fig. 4E) and Muc5ac expression by qPCR (Fig. 4F). Although OVA T cell receptor transgenic mouse model has been used extensively for delineating the mechanism of allergic asthma, a weakness of this model is that more than 90% of the peripheral T cells in these mice are directed to OVA and does not necessarily require sensitization with an allergen³⁶. Therefore, it is possible that the results may not be applicable to normal antigenic/allergic responses. To address this possibility, we used *Aspergillus fumigatus* extracts containing the allergen in allergic bronchopulmonary aspergillosis³⁷ as the sensitizing allergen in normal BALB/c mice¹⁶. Results presented in Fig. 5 suggest that, as in the OVA transgenic system, MM suppressed the mucus formation in response to *Aspergillus* in normal BALB/c mice, including the increase in expression of Muc5ac (Fig. 5A), airway mucus formation by AB-PAS (Fig. 5B), and GABA_ARα2 IHC staining (Fig. 5C). In addition, MM inhibited the inflammatory response and *Aspergillus*-induced rise in eosinophils and lymphocytes by BAL cell differential count (Fig. E8A), the expression of the proinflammatory cytokines IL-13 (Fig. E8B) and IFN-γ (Fig. E8C) in the lung. Together these results suggest that nicotine promotes GABA_ARα2 and mucus expression similar to that of cigarette smoke, and nAChRs play a critical role in both allergen- and cigarette smoke/nicotine-induced airway mucus formation *in vivo*.

Role of acetylcholine in mucus formation in NHBE cells

If the activation of nAChRs on airway epithelial cells were to play a decisive role in mucus formation even in the absence of nicotine/cigarette smoke (e.g., nonsmokers), it is important to understand how mucus-promoting molecules such as allergens would activate nAChRs *in vivo*. Nicotine is not normally found in mammals; rather nicotinic cholinergic transmission is mediated by the neurotransmitter acetylcholine³⁸. There is increasing evidence that airway epithelial cells have the enzymes to synthesize, degrade, and transport acetylcholine^{12, 39}. qPCR analysis indicated that NHBE cells express primarily M3 and lower levels of M1 and M2 muscarinic receptors (Fig. E9A). Acetylcholine is a highly labile molecule and difficult to assay. Therefore, to ascertain that acetylcholine promotes mucus formation in airway epithelial cells, we determined: (a) whether inhibiting the degradation of acetylcholine by the acetylcholinesterase inhibitor neostigmine bromide (NB) promoted mucus formation in NHBE cells. NHBE cells were treated with indicated concentrations of NB in the absence of IL-13 or nicotine. As little as 5 μM NB (Fig. 6A) significantly upregulated the mucus formation in NHBE cells. NB also increased MUC5AC mRNA to less impressive but highly statistically significant level (Fig. 6B). (b) Acetylcholine (100 μM) was added to NHBE cells and 48 h later the cells were analyzed for MUC5AC and GABA_ARα2 mRNA expression by qPCR. Compared to control cells, addition of acetylcholine significantly increased the expression of both MUC5AC and GABA_ARα2 by approximately 2 fold (Fig. E9B and E9C). These results suggest that acetylcholine is a trigger for mucus formation in airway epithelial cells. However, it should be noted that we were unable to detect acetylcholine in NHBE cells in the presence of NB and IL-13. It is likely that the cellular level of acetylcholine in these cells is lower than the sensitivity of our HPLC assay.

Acetylcholine is the biological ligand for both nicotinic and muscarinic receptors. To ascertain that acetylcholine mediates its pro-mucoid effects through nAChRs, NHBE cells were treated with the non-selective muscarinic receptor antagonist atropine prior to NB or IL-13. Fig. 6C shows that atropine had no significant effect on the MUC5AC mRNA expression induced by NB, IL-13, or NB + IL-13. Similarly, atropine did not affect mucus formation (AB-PAS staining) in NHBE cells in response to NB or IL-13 (Fig. 6D).

Moreover, unlike MLA, the increased level of GABA_Aα2 in NHBE cells in response to nicotine or IL-13 + nicotine was not affected by atropine treatment (Fig. 6E). These results suggest that, in the absence of nicotine or nicotine-containing substances, acetylcholine may be the critical molecule in the activation of nAChRs for mucus production in NHBE cells.

DISCUSSION

Nicotinic acetylcholine receptors are seen in tissues from both vertebrates and invertebrates such as nematodes and insects⁴⁰. In the central nervous system nAChRs are ligand-gated ion channels that mediate fast synaptic cholinergic transmission and these properties are strongly conserved across species. nAChRs are present in neuronal³² and many non-neuronal cell types^{13, 29, 30}. At least in some non-neuronal cell type nAChRs are not ligand-gated ion channels but signal through second messengers²⁹. So far, the function nAChRs in non-neuronal cells is described primarily as regulatory⁴¹; however, the near ubiquitous presence of nAChRs in various cell types and their evolutionary conservation suggest that these receptors may have some critical functions outside the central nervous system⁴². Results presented herein indicate that nAChRs are essential for airway mucus production as well as mucous cell hyperplasia and metaplasia in response to nicotine and allergen *in vivo* and/or *in vitro*, and it is likely that nAChRs are critical in other cellular functions.

Nicotine is immunosuppressive and anti-inflammatory⁴¹ and our previous *in vivo* experiments indicated that chronic low-dose nicotine exposure significantly inhibited some parameters of allergic asthma, including a dramatic reduction in Th2 cytokines/chemokines such as IL-13, IL-4, IL-5, eotaxin, and atopy, yet the nicotine treated rats exhibited increased mucous cell metaplasia and mucus formation in the airways¹¹. Possible explanations for these paradoxical results are that nicotine either decreased the amount of IL-13 required or substituted for IL-13 in mucus production. Our results clearly indicate that nicotine acts independent of IL-13 in promoting mucus formation and mucous cell metaplasia/hyperplasia. The ability of nAChR inhibitors to block nicotine- and IL-13-induced mucus production suggest that both IL-13 and nicotine activate nAChRs to trigger mucus formation, and IL-13 effects are upstream of nAChRs.

Previous studies have shown that IL-13 affects mucus by increasing GABA_AR expression in NHBE cells¹⁴. We showed that GABA_AR activation is downstream of nAChR activation in mucus formation and MUC5AC expression, and of the known GABA_AR subtypes expressed in NHBE cells, GABA_Aα2 was the only one that was significantly upregulated by IL-13 and nicotine in NHBE cells. GABA_Aα2 was also the only GABA_AR subtype whose expression was increased by OVA and/or secondhand cigarette smoke in OVA-TCR transgenic BALB/c mice. The interaction between nAChRs and GABA_AR has been shown in the central nervous system⁴³ and, in *C. elegans*, cholinergic motor neurons activate GABAergic neurons⁴⁴. Moreover, rhesus monkeys exposed prenatally to nicotine show increased GABA signaling in the lungs⁶; however, the significance of this observation is not clear because prenatal nicotine exposure also affects development of several organs including the lung⁴⁵. Thus, while the mechanism by which nicotine promotes GABAergic response has not been fully delineated, it is clear that GABA_Aα2 are critical in nicotine and IL-13 mediated mucus formation.

To ascertain the role of nAChRs in the regulation of airway mucus *in vivo*, we used two models of allergic asthma to trigger mucus formation. OVA TCR transgenic BALB/c mice (a frequently used model for the lung allergic responses) were exposed to OVA and/or SS. These treatments promoted airway inflammation (leukocytic infiltration in the lung), airway mucus formation, and increased expression of Muc5ac and GABA_Aα2 in the lung. However, when the animals were treated with the non-selective nAChR antagonist MM, the

inflammatory and mucoid responses to OVA and/or SS were significantly reduced, suggesting that nAChRs are intimately involved in the regulation of allergen-induced inflammation and mucus formation. The major question about the suitability of using OVA TCR transgenic animals to study the regulation of allergic asthma is that while normal naïve (unimmunized) animals have extremely low frequency of antigen-specific T cells⁴⁶, the majority of the peripheral T cells in the OVA transgenic animals are directed to OVA and do not require immunization to detect OVA-induced T cell proliferation³⁶, although in the absence pre-sensitization by OVA, the cells may not differentiate into Th2 type cells. Therefore, we used normal (wild-type) BALB/c mice and the allergen - *Aspergillus fumigatus* extracts that requires sensitization to induce airway responses¹⁶ and causes allergic bronchopulmonary aspergillosis in humans⁴⁷. In these animals, MM was able to ameliorate *Aspergillus*-induced airway inflammation and various indices of airway mucoid response. Thus, activation of nAChRs is critical in allergen-induced mucous cell metaplasia and airway mucus formation.

Recently, MLA was shown to suppress mucus formation in monkey lungs⁶. Although MLA was believed to preferentially block $\alpha 7$ -nAChRs, recent reports suggest that MLA also reacts with $\alpha 9/\alpha 10$ -nAChRs⁴⁸. NHBE cells express $\alpha 7$ -, $\alpha 9$ - and $\alpha 10$ - nAChR, and $\alpha 10$ are functional only in the association with $\alpha 9$ subunits²⁷; moreover, $\alpha 7$ and $\alpha 9$ colocalize in rat sympathetic neurons²⁸. In rat mast cells, the suppressive effect of nicotine on leukotriene production is mediated by an interdependent action of $\alpha 7$ -, $\alpha 9$ - and $\alpha 10$ - nAChR³⁰. Therefore, to identify the nAChR subtype(s) that regulated mucus formation, we used specific conotoxin peptides to inhibit $\alpha 9/\alpha 10$ - and $\alpha 7$ -nAChRs and observed that only the $\alpha 7$ -specific conotoxin peptide ArIB[V11L,V16D]^{33, 34} blocked the nicotine- and IL-13-induced mucus production in NHBE cells. This inference was further confirmed by the demonstration that knockdown of $\alpha 7$ - but not $\alpha 9/\alpha 10$ -specific mRNA blocked the nicotine- and IL-13-induced expression of GABA_AR $\alpha 2$ in A549 cells.

While these results clearly implicate $\alpha 7$ -nAChRs in mucous cell physiology and mucus production, it was important to understand how the activation of these receptors would be regulated in nonsmokers (i.e., in the absence of nicotine). *In vivo* cholinergic transmission involves both nicotinic and muscarinic receptors and is mediated by acetylcholine. There is increasing evidence that many non-neuronal cells, including airway epithelial cells express enzymes to synthesize, degrade, and transport acetylcholine^{12, 39}. Indeed, blocking the degradation of acetylcholine by the cholinesterase inhibitor NB promoted mucus formation and increased MUC5AC expression in NHBE cells in the complete absence of IL-13 or nicotine. Acetylcholine is the biological ligand for both nAChRs and muscarinic receptors, and the bronchial epithelial cells have functional muscarinic receptors⁴⁹. Results with MLA and the atropine suggest that muscarinic receptors are not involved in the IL-13 or NB (acetylcholine)-induced mucus formation in bronchial epithelial cells. Although with the use of nAChR inhibitors we were able to show that the effects of IL-13 on mucus formation in NHBE cells is regulated by nAChRs, we were unable to show that IL-13 induces detectable levels of acetylcholine in these cells. Nonetheless, it is likely that in the absence of nicotine, acetylcholine is important in airway mucus formation and mucous cell hyperplasia/metaplasia. Together, our results suggest that $\alpha 7$ -nAChRs, GABA_AR $\alpha 2$, and the acetylcholine metabolic pathway(s) may serve as potential targets to control airway mucus formation. A tentative scheme by which nAChRs may regulate airway mucus is presented in Fig. 7.

Acknowledgments

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List of Abbreviations

AB-PAS	Alcian blue-periodic acid-Schiff
ALI	Air liquid interface
COPD	Chronic obstructive pulmonary disease
GABA_AR	γ -aminobutyric acid type A receptor
IHC	Immunohistochemistry
MLA	Methyllycaconitine
MM	Mecamylamine
nAChRs	Nicotinic acetylcholine receptors
NHBE	Normal human bronchial epithelial
NB	Neostigmine bromide
PIC	Picrotoxin

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Key Message

This study shows that nicotine and acetylcholine promote mucus formation independent of IL-13, and totally dependent on the activation of $\alpha 7$ -nAChRs. Moreover, nicotinic receptor antagonists block mucus formation.

Figure 1. Nicotine and IL-13 promote mucus formation in NHBE cells through nicotinic and GABA_A receptors

In NHBE cells IL-13/nicotine (Ni)-induced mucus is suppressed by: 1 μ M MLA (**A**) and 1 μ M MM (**B**). MLA blocks mucous containing cells (**C, left panel**), mucus cell volume (**C, right panel**), MUC5AC mRNA (**D**), and MUC5AC-positive cells (**E**). Experiments were repeated at least five times and bars represent $\pm$ SEM.

Figure 2. $\alpha 7$ -nAChR-specific conotoxin peptides suppress nicotine/IL-13-induced mucus in NHBE cells
Effects of RgIA (500 nM) and ArIB[V11L, V16D] (500 nM) on Ni-induced (**A**) and IL-13-induced (**B**) mucous cell responses, and on Ni/IL-13-induced MUC5AC mRNA by qPCR (**C**). Each experiment was repeated at least three times, and bars on MUC5AC are mean $\pm$ SEM from three inserts.

Figure 3. GABA_ARs and α7-nAChRs are critical in nicotine/IL-13-induced mucus formation
 In NHBE cells, effects of 50 μM picotoxin (PIC) on mucus- (A) and MUC5AC-positive cells (B); Ni/IL-13-induced GABA_Aα2 expression is detected by RT-PCR (C), qPCR (D), and IHC (E), and blocked by 1 μM MLA (F). In A-549, Ni/IL-13-induced GABA_Aα2 (G) is blocked by Si-RNA knockdown of α7-nAChRs (H) and α7-nAChR-specific conotoxin peptide ArIB[V11L-V16D] (I).

Figure 4. MM blocks OVA/SS/Ni-induced airway mucus, Muc5ac, and GABA_ARα2 expression in OVA-transgenic mice

Mice received indicated treatments and lungs were tested for: GABA_ARα2 by IHC (A), mucus by AB-PAS (B), GABA_ARα2 Western blots of lung extracts (C), lung Muc5ac qPCR (D), GABA_ARα2 Western blots of lung extracts (E), and lung Muc5ac qPCR (F). The results represent two independent experiments (3–6 mice/group).

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Figure 5. Aspergillus induces mecamlamine-sensitive GABA_ARα2 expression and mucus formation in the lung

BALB/c mice were sensitized with *Aspergillus fumigatus* extracts, and where indicated received MM. Lungs were tested for: Muc5ac by qPCR (A), mucus by AB-PAS staining (B), and GABA_ARα2 by IHC (C). The results represent two independent experiments with 3–6 mice/group.

Figure 6. Neostigmine bromide (NB) promotes mucus and formation in NHBE cells
 NHBE cells were treated with indicated concentrations of NB or 1 μ M atropine (Atr) and scored for: NB-induced mucus (A) and MUC5AC (B). Atropine effects on NB/IL-13-induced MUC5AC (C), mucus (D) and GABAAR α 2 protein expression (E). The experiment in A/B/E was repeated twice. Bars represent mean $\pm$ SEM from 3 separate inserts.

Figure 7. Potential relationship between nAChRs and mucus formation in NHBE cells

Allergens or IL-13 directly or indirectly increase acetylcholine in airway epithelial cells. Acetylcholine activates $\alpha 7$ -nAChRs that increases $\text{GABA}_A \text{R}\alpha_2$ expression that is blocked by nAChR antagonists (MM, MLA, ArIB[V11L-V16D]). Increased $\text{GABA}_A \text{R}\alpha_2$ stimulates mucus formation that is blocked by the $\text{GABA}_A \text{R}$ antagonist PIC. Dashed lines represent: not formally proven interactions.