Muscarinic M₃ Receptor Stimulation Increases Cigarette Smoke-Induced IL-8 Secretion by Human Airway Smooth Muscle Cells

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Running head: M₃ receptors, cytokines and airway smooth muscle

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Abstract

Acetylcholine is the primary parasympathetic neurotransmitter in the airways and is known to cause bronchoconstriction and mucus secretion. Recent findings suggest that acetylcholine also regulates aspects of remodeling and inflammation through its action on muscarinic receptors. In the present study, we aimed to determine effects of muscarinic receptor stimulation on cytokine production by human airway smooth muscle cells (primary and immortalized cell lines). The muscarinic receptor agonists carbachol and methacholine both induced modest effects on basal IL-8 and IL-6 secretion, whereas the secretion of RANTES, eotaxin, VEGF-A and MCP-1 was not affected. Secretion of IL-8 and IL-6 was only observed in immortalized airway smooth muscle cells that express muscarinic M_3 receptors. In these cells, methacholine also significantly augmented IL-8 secretion in combination with cigarette smoke extract in a synergistic manner, whereas synergistic effects on IL-6 secretion were not significant. Muscarinic M_3 receptors were the primary subtype involved in augmenting cigarette smoke extract-induced IL-8 secretion, as only tiotropium bromide and muscarinic M_3 receptor subtype selective antagonists abrogated the effects of methacholine. Collectively, these results indicate that muscarinic M_3 receptor stimulation augments cigarette smoke extract induced cytokine production by airway smooth muscle. This interaction could be of importance in patients with COPD.

Introduction

The role of acetylcholine as a neurotransmitter that regulates airway smooth muscle contraction and mucus secretion in the airways is well established, and is mediated through muscarinic receptors [1]. Acetylcholine is an important regulator of airway smooth muscle tone, especially in patients that suffer from COPD, in whom cholinergic tone appears to be the primary reversible component of airflow obstruction. Therefore, anticholinergics are commonly used bronchodilators in COPD, and less frequently in asthma [2].

Recent findings show that the acetylcholine synthesizing enzyme choline acetyl transferase (ChAT) is also expressed by non-neuronal sources such as the bronchial epithelium and inflammatory cells including B and T lymphocytes, macrophages, mast cells, neutrophils and eosinophils [3-5]. These cells not only synthesize acetylcholine, but also express functional muscarinic receptors. A recent study showed that acetylcholine induces the release of neutrophil chemoattractants such as leukotriene B_4 from sputum cells of COPD patients [6]. These results are consistent with studies demonstrating that alveolar macrophages exhibit neutrophil, eosinophil and monocyte chemotactic activities in response to acetylcholine [7;8] and suggest that acetylcholine is a paracrine and/or autocrine mediator that regulates inflammatory processes.

Acetylcholine also triggers chemokine and cytokine release from structural cells that play a role in COPD pathogenesis. For example, bronchial epithelial cells exhibit neutrophil, eosinophil and monocyte chemotactic activity in response to acetylcholine [9;10]. Since the synthesis of non-neuronal acetylcholine is relatively high in bronchial epithelial cells [11], these results implicate a role for acetylcholine (autocrine or paracrine) in initiating inflammatory responses. This contention is supported by animal studies that demonstrate protective effects of anticholinergics on inflammation induced by inhaled allergen [12] or inhaled diesel particles

[13]. In addition, increased expression of ChAT has been reported in fibroblasts from active smokers and COPD patients [14]. Similar observations were reported in vitro in HFL-1 fibroblasts treated with cigarette smoke extract [14]. Whether acetylcholine regulates inflammatory responses in COPD is, however, largely unknown.

The airway smooth muscle cell is increasingly recognized as an important cellular source of pro-inflammatory cytokines and growth factors, including interleukin (IL)-6, IL-8, MCP-1, VEGF and others (see [15-17] for review) and it appears to play an important immunomodulatory role in airways diseases such as asthma and COPD. The immunomodulatory function of airway smooth muscle has mostly been studied in cell culture; these studies have revealed that G protein coupled receptor agonists, growth factors, cytokines and extracellular matrix proteins can induce the release of significant amounts of pro-inflammatory cytokines [15-17]. In addition, airway smooth muscle cells exhibit IL-8 release in response to cigarette smoke extract, which is strongly augmented by cytokines such as TNF-α [18]. The regulatory role of acetylcholine in these synthetic airway smooth muscle cell responses is largely unknown. A study using bovine tracheal smooth muscle strips showed that stimulation with carbachol resulted in increased expression of IL-8, cyclooxygenase (COX)-1 and -2 at the mRNA level [19]. This study together with the fact that airway smooth muscle expresses muscarinic M_2 and M_3 receptors in abundance suggest a role for these receptors in release of mediators. Therefore airway smooth muscle may represent a primary target of acetylcholine released by nerve and other cell types in the airways.

Based on the above mentioned observations, in the present study, we aimed to characterize the pro-inflammatory response of human airway smooth muscle cells induced by muscarinic receptor stimulation, and to investigate potential functional interactions with cigarette

smoke extract. In addition, we aimed to investigate the role of muscarinic M_2 and M_3 receptor subtype(s) involved in these responses.

Materials and Methods

Cell Culture

Human bronchial smooth muscle cell lines, immortalized by stable expression of human telomerase reverse transcriptase (hTERT), were used for the experiments. The primary cultured human bronchial smooth muscle cells used to generate each cell line were prepared as previously described [20] from macroscopically healthy segments of 2nd-to-4th generation main bronchus obtained after lung resection surgery from patients with a diagnosis of adenocarcinoma (Dr. H Unruh, Section of Thoracic Surgery, University of Manitoba, Canada). All procedures were approved by the Human Research Ethics Board of the University of Manitoba. As we have detailed previously [20], each cell line was thoroughly characterized to passage 10 and higher, and was shown to express a number of smooth muscle (sm) contractile phenotype marker proteins (eg. sm-myosin heavy chain (MHC), sm-α-actin, and desmin). For all experiments, passages 10-25 myocytes grown on uncoated plastic dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 U/ml streptomycin, 50 µg/ml penicillin and 10 % v/v FBS were used.

In selected experiments, primary human airway smooth muscle cells from two different commercial sources (Stratagene, La Jolla, CA and Clonetics, San Diego, CA) were used. These cells were cultured in DMEM/F12 nutrient mixture supplemented with 25 mM Hepes, 2.5 mM L-glutamine, 1% v/v non-essential amino acids, 10% v/v FBS (all from Invitrogen, Breda, The Netherlands) and antibiotics. Cells at passages 5-7 were used to perform experiments.

Treatments

Unless otherwise specified, cells were grown to confluence and serum-starved for 1 day in DMEM supplemented with antibiotics (50 U/ml streptomycin, 50 µg/ml penicillin, 1.5 µg/ml amphotericin B) and ITS (5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium) before each experiment. Cells were then subjected to different treatments for 24 h in DMEM supplemented with antibiotics. Muscarinic receptor antagonists (gallamine, 4-DAMP, DAU5884, tiotropium) were added 30 min prior to the addition of agonists. Cigarette smoke extract was prepared by combusting 2 University of Kentucky 2R4F research cigarettes (filters removed) using a peristaltic pump (Watson Marlow 323 E/D) and passing the smoke through 25 ml of FBS-free DMEM supplemented with antibiotics at a rate of 5 minutes / cigarette. The obtained solution was referred to as 100 % strength.

Multiplex cytokine assays

Undiluted supernatants of airway smooth muscle cell cultures were analyzed using the Bio-plex 200 and the Bio-plex human cytokine 27-plex assay (Bio-Rad, Veenendaal, The Netherlands) according to manufacturers instruction. The 27-plex assay kit contains beads conjugated with mAbs specific for interleukin-1β (IL-1β), IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, fibroblast growth factor (FGF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage-CSF (GM-CSF), interferon-γ (IFN-γ), interferon-inducible protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-α), MIP-1β, platelet-derived growth factor-BB (PDGF-BB), regulated on activation normal T-cell expressed and secreted (RANTES), tumor necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF). The detection limit

was ~10 pg/ml for all cytokines. Standard curves and the concentration of cytokines within samples were generated with the Bio-Plex Manager 4.1 software.

Cytokine ELISA

Cytokine levels were quantified in cell-free supernatants using enzyme-linked immunosorbent assays (ELISA), according to the manufacturer's instructions. The detection limit of the IL-8 ELISA was 1 pg/ml and 0.2 pg/ml for the IL-6 ELISA (both from Sanquin, Amsterdam, the Netherlands).

Polymerase Chain Reaction

RNA was isolated using the Qiagen RNeasy Minikit (Qiagen, Valencia, CA) in combination with the Qiagen RNase-Free DNase Set (Qiagen, Valencia, CA). The RNA concentration and purity were determined by measuring optical density measurements using the NanoDrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE). Reverse transcription of 500 ng of total RNA was performed using 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen, Breda, The Netherlands); 0.5 mM of dNTP's (Invitrogen, Breda, The Netherlands); 0.5 µg of oligo(dT)$_{15}$ primer (Promega, Madison, WI); RNasin RNase inhibitor 40 U (Promega, Madison, WI); 10 mM dithiothreitol (Invitrogen, Breda, The Netherlands); 50 mM Tris-HCl (pH 8.3); 75 mM KCl; and 3.0 mM MgCl$_2$ in a total volume of 20 µl. PCR was performed at 95°C for 4 minutes followed by 25 or 35 cycles 15 seconds denaturing, 15 seconds annealing and 30 seconds amplification using 0.5 pmol gene specific primers (table E1); nucleotide mix (Invitrogen, Breda, The Netherlands) in a final concentration of 0.08 mM each; 1 U of GoTaq Flexi DNA polymerase (Promega, Madison, WI); 5 µl of 5x

Green GoTaq Flexi reaction buffer (Promega) and different concentrations of $MgCl_2$ in a final volume of 25 µl. β-actin was used as a housekeeping gene. Products were visualised using a 1.5% agarose gel containing ethidium bromide.

Data analysis

Values reported for all data represent means ± SE, unless otherwise specified. The statistical significance of differences between means was determined by one-way Kruskal-Wallis ANOVA, followed by Dunn's multiple comparisons test. Differences were considered to be statistically significant when $p < 0.05$.

Results

Muscarinic receptors induce IL-8 and IL-6 secretion from airway smooth muscle cell lines that express muscarinic M_3 receptors

To evaluate the role of muscarinic receptors in the synthetic function of airway smooth muscle cells, we analyzed supernatants of airway smooth muscle cells treated with carbachol for an array of secreted proteins using a multiplex cytokine assay. We used a mixture of pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ) as a positive control for induction of cytokines, chemokines and growth factors and to normalize for heterogeneity in responses between cell lines. Using cells from one of the immortalized cell lines, we found that carbachol alone induced a modest increase in IL-8 and IL-6 release, compared to that induced by cytokine mixture, which induced release of several cytokines (e.g. IL-6), chemokines (including RANTES, eotaxin, MCP-1) and growth factors (e.g. VEGF-A) (Figure 1). A full list of mediators induced by the mixture of cytokines is listed in the online depository (Table E2). In the primary airway smooth muscle cells from the commercial sources little effect of carbachol exposure was seen, whereas the cytokine mixture induced the same pattern of cytokine release as we observed for the immortalized airway smooth muscle cells (data not shown).

We verified our findings on IL-6 and IL-8 release using a sandwich ELISA. Carbachol (0.01-100 μM) dose-dependently increased IL-6 and IL-8 release in the immortalized cell line, with a greater increase in IL-6 compared to IL-8 (Figure 2; donor 1). As in the Bio-plex assay, carbachol did not affect release of these factors in primary cultured cells (Figure 2; donors 2 and 3). As it has been shown that passage of primary cells in culture reduces the expression of muscarinic M_3 receptors, we assessed the mRNA expression of muscarinic receptors (M_1, M_2 and M_3 receptors). The immortalized cells expressed both M_2 and M_3 receptor mRNA in high

amounts, whereas the primary cells from the commercial sources only expressed mRNA for M_2 receptors (Figure 2). This difference between cell cultures suggested that M_3 receptors could be involved in the carbachol-induced IL-8 and IL-6 release. None of the airway smooth muscle cells we tested expressed the muscarinic M_1 receptor.

Muscarinic receptor stimulation augments IL-8 secretion induced by cigarette smoke extract

We next evaluated the effects of muscarinic receptor stimulation on cytokine secretion in combination with cigarette smoke extract. Cigarette smoking is the main risk factor for COPD, and an aqueous extract of cigarette smoke induces the expression of several pro-inflammatory cytokines by cells that play an immunomodulatory role in COPD, including airway smooth muscle cells [15;21]. In view of our results examining effects of muscarinic receptor ligation alone, and the differential expression pattern of muscarinic receptors between cell lines, we focused our next studies the secretion of IL-6 and IL-8 using only the immortalized cell lines (which retain muscarinic M_3 receptor expression – Figure 2). Moreover, our studies using these cells confirmed the functional expression of muscarinic M_3 receptors, as ascertained by the mobilization of intracellular Ca^{2+} in response to the muscarinic receptor agonists methacholine and acetylcholine, and by ligand binding assays [22].

Cigarette smoke extract induced a profound induction of IL-8 secretion that was concentration-dependent and maximal at the highest concentration tested (15 %)(Figure 3). The effects of higher concentrations were not evaluated as these induced significant cell death. Interestingly, cigarette smoke extract alone did not induce the secretion of IL-6, whereas methacholine alone did induce IL-6 (Figure 3), suggesting that its pro-inflammatory effects are quite specific. In addition to its ability to significantly increase IL-6 secretion, only a modest

effect of methacholine was observed for IL-8 secretion in some experiments. The most profound

induction of IL-8 secretion we observed occurred when cigarette smoke extract and

methacholine were combined. For instance, although methacholine alone did not induce

significant IL-8 secretion, it profoundly augmented the response induced by cigarette smoke

extract, particularly at lower concentrations of CSE (Figure 3). No synergistic effects of

methacholine and CSE on IL-6 secretion were observed. Further analysis of the interaction

between methacholine and cigarette smoke extract indicated that the effects of methacholine

were concentration dependent, with the most pronounced synergy observed at the highest

concentration methacholine tested (10 µM; Figure 4). Since the synergistic effect on IL-8

secretion was highest for 10 μM methacholine and 5 % cigarette smoke extract, we used these

conditions in further experiments to assess the muscarinic receptors involved.

Muscarinic M_3 receptors augment IL-8 secretion induced by cigarette smoke extract

Since our initial experiments suggested that the effects of muscarinic receptor stimulation on

cytokine secretion were observed only in cells that express muscarinic M_3 receptors, we next

characterized the role of muscarininc receptor subtypes further. To this aim, we selected subtype

selective muscarinic receptor antagonists DAU5884, 4-DAMP (M_3 selective) and gallamine (M_2

selective). The inhibitory effect of the clinically used muscarinic receptor antagonist tiotropium

was also evaluated. M_3 selective DAU5884 and 4-DAMP were applied in concentrations that

occupy the majority (>99%) of the muscarinic M_3 receptor population with relatively modest

(~30%) occupation of the muscarinic M_2 subtype, whereas the concentration of the M_2 selective

gallamine was chosen to ensure no significant effects on M3 receptors [23-26]. Interestingly,

both tiotropium and the M_3 selective 4-DAMP and DAU5884 completely inhibited the

synergistic effect of methacholine on cigarette smoke extract-induced IL-8 secretion, whereas the M_2 selective gallamine had no significant effect (Figure 5). Responses induced by cigarette smoke extract alone were not affected by the treatment with the antagonists. These results confirm that the primary receptor subtype involved in the synergism between methacholine and CSE is the muscarinic M_3 receptor.

Discussion

The results from our study indicate that muscarinic receptor stimulation induces a pro-inflammatory response of airway smooth muscle cells, and that the response induced by cigarette smoke extract is strongly enhanced. These results extend previous observations by others that have shown muscarinic receptor stimulation can induce IL-8 production by bronchial epithelial cells, and leukotriene B_4 production by monocytes, macrophages and bronchial epithelial cells [6;8-10;27]. Muscarinic receptor stimulation using carbachol was also previously shown to regulate pro-inflammatory gene transcription of IL-6, IL-8 and COX-2 induced by mechanical stimulation of airway smooth muscle strips [19]. Our current findings confirm these studies and provide novel insights as we demonstrate a strong synergistic interaction with the response induced by cigarette smoke extract. Collectively, these findings indicate that muscarinic receptors are coupled to pro-inflammatory gene expression in several cell types involved in COPD pathogenesis.

The results from our study indicate that methacholine synergy with CSE is primarily dependent on the muscarinic M_3 receptor subtype. Application of DAU 5884 or 4-DAMP in concentrations that are M_3 selective almost completely inhibited the synergy between methacholine and cigarette smoke extract, whereas the antagonist gallamine, applied in concentrations that are M_2 selective had no significant effect. It is clear therefore that the primary receptor subtype involved in synergy is the muscarinic M_3 receptor. This is further confirmed by our studies indicating that airway smooth muscle cells that lost expression of functional M_3 receptors in culture lack functional IL-6 and IL-8 responses to carbachol. The M_3-dependent nature of this effect is in line with findings reported for IL-8 production in epithelial cells [27], and complements our previous findings that indicate that the M_3 receptor subtype is the primary

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receptor subtype involved in airway smooth muscle contraction and in cell proliferation induced by methacholine [23-25;28]. Based on these findings, M_3 receptor subtype selectivity would appear an advantageous property of muscarinic receptor antagonists, especially since inhibition of M_2 autoreceptors on vagal nerve terminals is associated with enhanced acetylcholine release [3;29]. Indeed, the 'kinetic' muscarinic M_3 selective properties of tiotropium have been viewed as beneficial and new muscarinic M_3 receptor selective antagonists are currently under development [30-34]. Nonetheless, recent reports indicate that proliferative and pro-fibrotic responses of lung fibroblasts are mediated via muscarinic M_2 receptors and pertussis toxin sensitive $G_{i/o}$ proteins [35;36]. Therefore it is clear that to clarify the benefits and negative aspects of muscarinic receptor subtype selectivity requires future study.

Our study was performed using both primary airway smooth muscle cells and several immortalized airway smooth muscle cell lines. The immortalized smooth muscle cells retained expression of the muscarinic M_3 receptor and the capacity to regulate IL-8 secretion in concert with cigarette smoke, whereas the primary cells lost M_3 receptor expression in culture as well as their capacity to produce IL-8 in response to muscarinic receptor activation. The immortalized cells also retain the capacity to respond to contractile agonists (calcium signaling), the capacity for induction of contractile proteins, and the capacity to proliferate upon stimulation with growth factors [20;37]. These aspects make the immortalized cell lines a well suited model to study the role of muscarinic M_3 receptors in physiological responses of airway smooth muscle cells *in vitro*. However, translation of our results to intact organ systems, *in vivo* models and clinical studies is necessary to confirm the functional interaction of acetylcholine with cigarette smoke in patients.

Activation of the non-neuronal cholinergic system [38] in airway smooth muscle is likely not of major functional importance to cigarette smoke induced pro-inflammatory cytokine production, as we did not observe any effects of tiotropium or other muscarinic receptor antagonists on cells exposed to CSE alone. Nonetheless, in intact lungs stimulation of muscarinic receptors, for example by neuronal or inflammatory cell-derived acetylcholine, could augment the inflammatory response induced by smoking, suggesting CSE could promote an immunomodulatory role for muscarinic receptors on airway smooth muscle cells, thereby potentiating its pro-inflammatory effects. Although increased airway smooth muscle mass is found in patients with COPD [39], the role of airway smooth muscle cells in the inflammatory process observed in COPD has not yet been extensively studied. Airway smooth muscle bundles in COPD patients are infiltrated by neutrophils and $CD8^+$ T cells [40;41], suggesting that airway smooth muscle cells may actively recruit these cells. Also, *in vitro* studies have shown that airway smooth muscle cells produce IL-8 in response to many inflammatory mediators implicated in COPD, including TNF-α [42], IL-17 [43], neutrophil products such as LL-37 [44], bacteria [45] and cigarette smoke [18]. Combining cigarette smoke with TNF-α results in a synergistic increase in IL-8 release [46]. Our data also show that muscarinic receptor agonists can act in concert with cigarette smoke to induce the production of IL-8 by airway smooth muscle cells, suggesting that these cells, in addition to epithelial cells and inflammatory cells, could play an important role in the inflammatory process involved in neutrophil recruitment and exacerbations of COPD. In addition, our findings may have implications for our understanding of muscarinic receptor function on airway smooth muscle cells in smoking asthmatics.

Clinical studies have demonstrated that anticholinergic agents, in particular the long-acting anticholinergic drug tiotropium bromide, have beneficial therapeutic effects in COPD

patients that exceed their acute bronchodilatory effects. Several clinical studies suggest that tiotropium bromide may have beneficial effects on inflammation and/or remodeling in COPD, which is supported by several animal and cell culture studies that report effects of muscarinic receptors on cytokine production, cell proliferation, matrix protein production and airway remodeling (see [3-5] for review). However, prior to our present study, reports that have directly investigated the interaction of muscarinic receptors with cigarette smoke-induced responses had been lacking. Our results provide important new insights in this area; however, studies that investigate the effects of muscarinic receptor antagonists in animal models of COPD are clearly needed.

In conclusion, the results from our study indicate that activation of muscarinic M_3 receptors expressed by airway smooth muscle induces IL-6 and IL-8 production and augment the pro-inflammatory cytokine response induced by cigarette smoke extract. These results imply a role for the airway cholinergic system in modulating cigarette smoke-induced inflammation, which is of clear interest to COPD pathogenesis and treatment. Future studies are needed to investigate the role of this mechanism in COPD and to clarify the mechanisms involved.

Acknowledgements

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Figure legends

Figure 1 *Synthetic responses of airway smooth muscle cells induced by carbachol and a mixture of TNF-α, IL-1β, and IFN-γ.* Human airway smooth muscle cells (hTERT-immortalized) were grown to confluence and serum-deprived for 1 day in ITS supplemented media. Cells were subsequently treated with 100 μM of carbachol or a mixture of TNF-α, IL-1β, and IFN-γ (all at 20 ng/ml) for 24 hours in duplicate. Supernatants were harvested and analyzed using the human cytokine 27-plex assay. Data shown are means ± standard deviation of one experiment in duplicate.
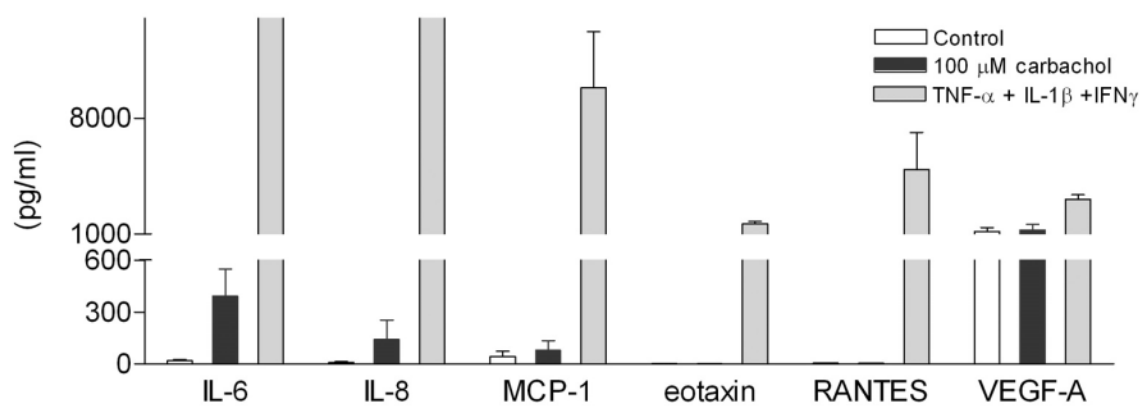
Figure 1

Figure 2 *Carbachol induces an increase in IL-6 and IL-8 release in cells expressing the muscarinic M₃ receptor only.* Human airway smooth muscle cells were grown to confluence and serum-deprived for 1 day in ITS supplemented media. Cells were subsequently treated in triplicate with increasing concentrations of carbachol as indicated. In addition, RNA of untreated cells was harvested. Supernatants were analyzed for IL-6 and IL-8 release. Expression of muscarinic receptors was assessed using reverse transcriptase PCR. Data shown for IL-6 and IL-8 release are means ± SE of triplicates per experiment for cells from three different

donors/sources (donor 1= immortalized ASM cells, donor 2= commercial source Strategene, donor 3= commercial source Clonetics).
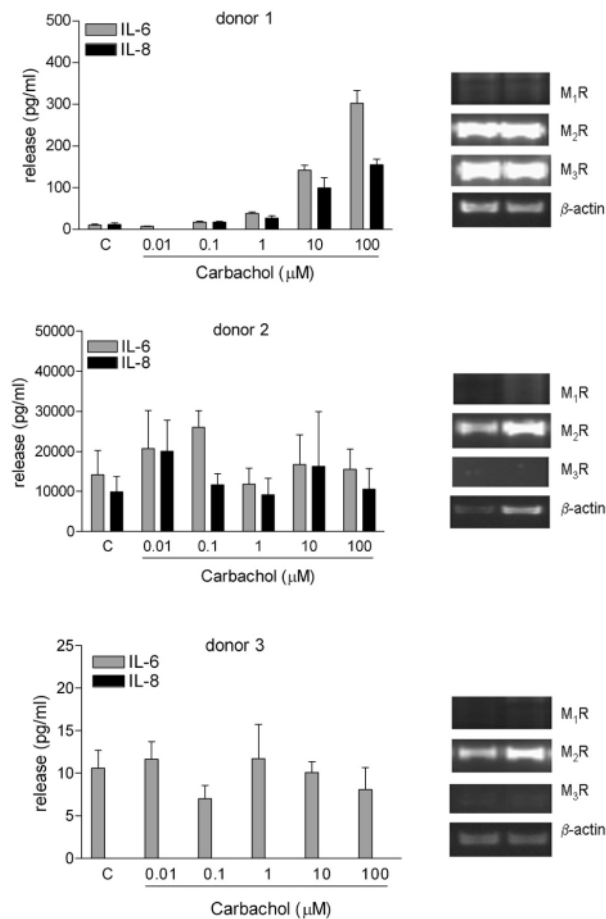

Figure 2

Figure 3 *Methacholine augments CSE-induced IL-8 secretion by airway smooth muscle.* Human airway smooth muscle cells were grown to confluence and subsequently serum-deprived for 1 day in ITS supplemented media. Cells were then treated with increasing concentrations of CSE, in the absence or presence of methacholine (MCh, 10 µM), as indicated. Supernatants were then analysed for the presence of IL-8 (panel A) or IL-6 (panel B). Data shown are the means ± SE of four to six experiments, each performed in duplicate. * $p<0.05$; ** $p<0.01$.
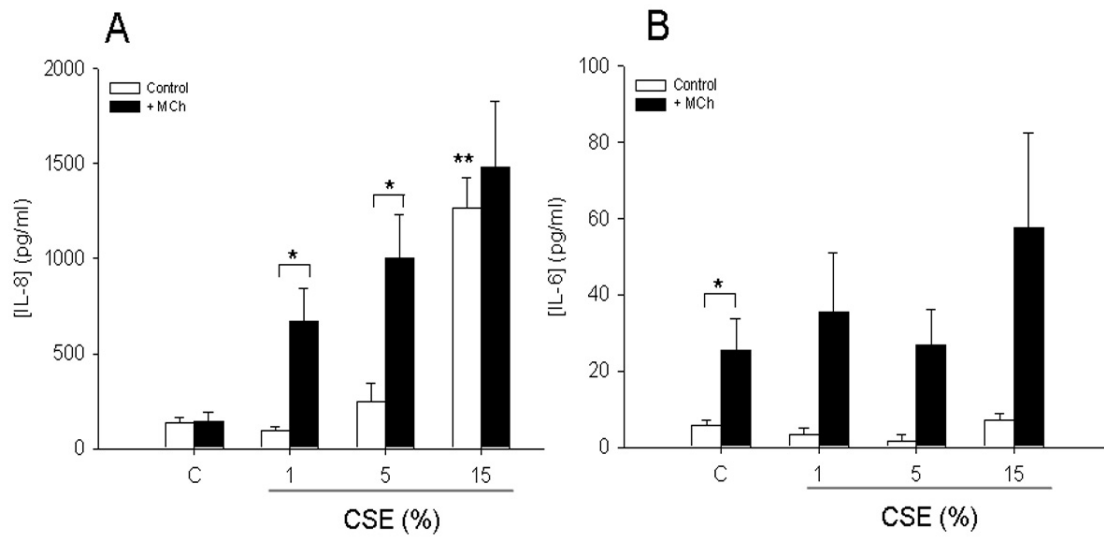
Figure 3

Figure 4 *Potentiating effects of methacholine on CSE-induced IL-8 secretion are concentration dependent*. Human airway smooth muscle cells were grown to confluence and subsequently serum-deprived for 1 day in ITS supplemented media. Cells were then treated with increasing concentrations of methacholine (MCh), in the absence or presence of 5 % CSE, as indicated. Supernatants were then analysed for the presence of IL-8. Data shown are the means ± SE of five experiments, each performed in duplicate. * $p<0.05$; *** $p<0.001$.
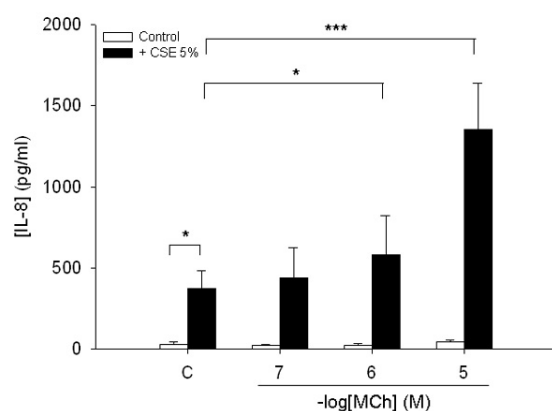
Figure 4

Figure 5 *Potentiating effects of methacholine on CSE-induced IL-8 secretion are muscarinic M_3 receptor dependent.* Human airway smooth muscle cells were grown to confluence and subsequently serum-deprived for 1 day in ITS supplemented media. Cells were then treated with methacholine (MCh; 10 μM), CSE (5 %) or its combination, in the absence or presence of muscarinic receptor antagonists (tiotropium, 4-DAMP, DAU5884, gallamine) as indicated. Supernatants were then analysed for the presence of IL-8. Data shown are the means ± SE of four to six experiments, each performed in duplicate. *** $p<0.001$ compared to control; # $p<0.05$ compared to CSE + MCh in the absence of antagonists.

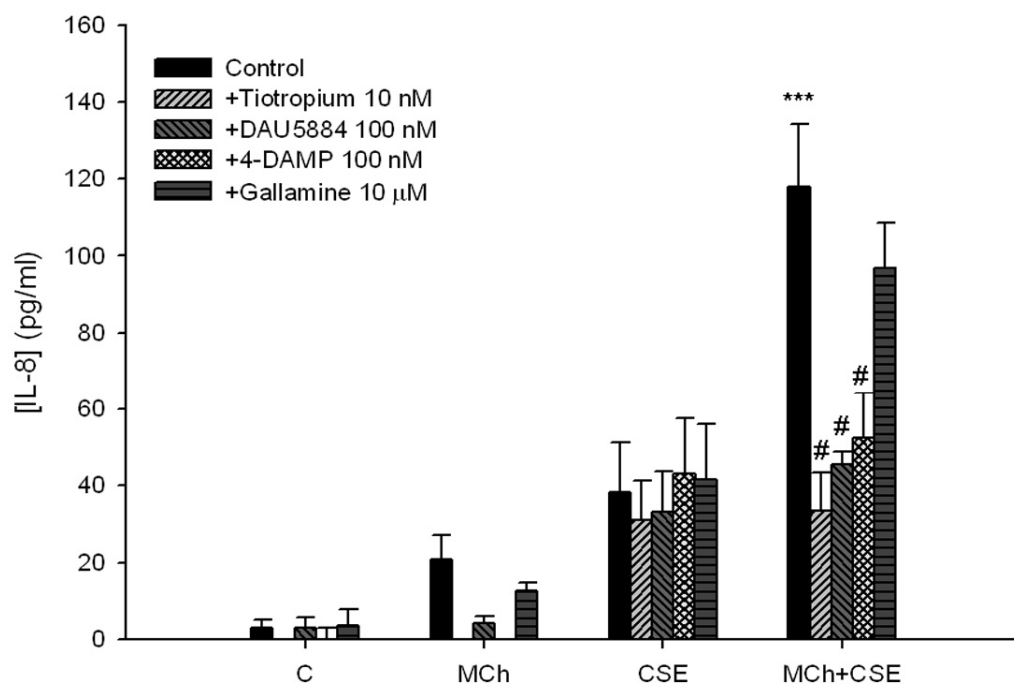Figure 5