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Title:

Aclidinium inhibits cholinergic and tobacco smoke-induced MUC5AC in human airways.

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Running Head: Aclidinium reduces MUC5AC overexpression

Abstract: 200

Mucus hypersecretion and mucin MUC5AC overexpression are pathological features of chronic obstructive pulmonary disease (COPD). This study examines the inhibitory effect of aclidinium, a new long-acting muscarinic antagonist, on MUC5AC expression in human airway epithelial cells.

MUC5AC mRNA (RT-PCR) and protein expression (enzyme-linked immunosorbent assay and immunohistochemistry) were studied in human bronchial tissue and differentiated human airway epithelial cells activated with carbachol (100μM) or cigarette smoke extract in the absence or presence of aclidinium.

Carbachol increased MUC5AC mRNA and protein expression in human bronchus and cultured epithelial cells. Aclidinium inhibited the carbachol-induced MUC5AC mRNA and protein expression with potency (IC_{50}) ~1nM in human bronchus and cultured airway epithelial cells. AG1478, a selective inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase, inhibited carbachol-induced MUC5AC responses indicating EGFR transactivation. Aclidinium inhibited carbachol-induced phospho-EGFR and phospho-p44/42 MAPK expression. In cultured airway epithelial cells transfected with siRNA against muscarinic receptor subtypes, siRNA-M3 but not siRNA-M2 blocked carbachol-induced MUC5AC expression. Cigarette smoke-induced MUC5AC upregulation in cultured airway epithelial cells was suppressed by aclidinium.

In conclusion, aclidinium decreases carbachol and tobacco smoke-induced MUC5AC overexpression in human airway epithelial cells. This effect may contribute to the clinical efficacy of aclidinium in mucus hypersecretory diseases including COPD.

Keywords: Aclidinium; human airway epithelial cells; human isolated bronchus; mucin MUC5AC; muscarinic receptor subtypes, small interference RNA

Introduction: 330 count word

Mucus hypersecretion is an important feature of chronic inflammatory airway diseases such as chronic obstructive pulmonary disease (COPD) and asthma, and contributes to their morbidity and mortality [1]. MUC5AC is the predominant mucin gene expressed in healthy human airway epithelial cells, and its expression is augmented in smokers, COPD patients and asthmatics [2].

COPD and asthma are associated with increased pulmonary vagal activity [3]. Muscarinic antagonists are effective drugs for the treatment of COPD and certain forms of asthma because they exert an anticholinergic effect that results in relaxation of airway smooth muscle [4]. Furthermore, there is recent awareness of the existence of a non-neuronal cholinergic system in humans. Airway epithelial cells are endowed with this system that represents a previously unappreciated regulatory pathway in pulmonary inflammation and remodeling [5]. Dysfunction of the non-neuronal cholinergic system appears to be involved in the pathophysiology of asthma and COPD [6]. Therefore, these potential anti-inflammatory and anti-remodeling effects of the muscarinic antagonists shown in animal models [7] may be of added value to their established bronchodilation in the management of chronic respiratory diseases.

Aclidinium is a novel, long-acting, muscarinic antagonist that has reached Phase III clinical development for COPD treatment [8]. In preclinical studies, aclidinium demonstrated potent muscarinic antagonist activity comparable to ipratropium and tiotropium and long duration of action [9]. The aim of the present study was to characterize the effect of aclidinium on regulating carbachol-induced increase of MUC5AC expression in human isolated bronchus and well-differentiated human primary airway epithelial cells cultured in an air-liquid interface system. In addition, since tobacco smoke exposure is

associated with the pathogenesis of COPD and the steroid resistance in COPD and severe asthma [10], we examined the effects of aclidinium on cigarette smoke extract-induced increase of MUC5AC expression in human cultured airway epithelial cells. Aclidinium was found to effectively decrease carbachol and cigarette smoke-induced MUC5AC overexpression in human airway epithelial cells. Preliminary data of this study were presented at the American Thoracic Society [11].

Methods: 1233 count word

Human bronchial tissue experimental protocol

Human lung tissue was obtained from patients who had undergone surgery for lung carcinoma as previously outlined [12]. Experiments were approved by local ethics committee and informed consent was obtained. Tissue from a total of 12 patients was included in this study. All patients were smokers until the moment of lung carcinoma diagnosis (around 1 month before lung surgery). None of the patients included in this study had COPD and none of the patients were chronically treated with theophylline, β-adrenoceptor agonists, corticosteroids or anticholinergic drugs. Clinical details of different patients are showed in supplementary data 1.

Human bronchial tissues were pretreated with antagonists or their vehicles for 15 min prior to stimulation with carbachol and remained until termination of experiments. Carbachol was selected as a cholinergic agonist as it is widely used in the literature and is resistant to degradation by cholinesterases present in human bronchial epithelium [13]. In this study we used carbachol 100μM concentration since we observed that it was near maximal responses which is in keeping with other studies of goblet cell secretion and cell

culture models using cholinomimetics [14]. Therefore, this carbachol concentration was selected for all subsequent experiments.

Differentiated human bronchial epithelial cells cultured in air-liquid interface

Human bronchial epithelial cells were cultured and differentiated in Transwell inserts (Corning Costar, Buckinghamshire, UK) under air-liquid interface (ALI) conditions as previously described [15]. In brief, a multilayered bronchial epithelium was obtained by seeding cells ($8.25x10^4$ cells/insert) onto polyester inserts. Cells were submerged in differentiation media (50% DMEM in basal epithelial growth media (BEGM, Clonetics, UK) for the first 7 days. Cells were then cultured for an additional 21 days with the apical surface exposed to air. Based on results from pilot experiments in airway epithelial ALI cultures that studied the time-course of the expression of MUC5AC (1, 2, 6, 12 and 24 h), the carbachol response was obtained at 12 h, a time point similar to that studied in conventional culture models [12].

Mucin MUC5AC protein expression

MUC5AC protein was measured by enzyme-linked immunosorbent assay (ELISA) as outlined previously [12]. In brief, for MUC5AC ELISA, 100 μg total protein extracted from human bronchial tissues was incubated at 40ºC until dry. Plates were blocked with 2% BSA for 1 h at room temperature. After three washes, plates were incubated with 50 μl of mouse monoclonal antibody (mAb) to MUC5AC (clone 45M1, 1:100; Neomarkers, Fremont, CA, USA). After incubation with secondary horseradish peroxidase-goat anti-mouse (1:10,000) color reaction was read at 450 nm. To confirm ELISA results, Western

blot analysis of MUC5AC was carried out in human bronchial homogenates as previously reported [12].

For MUC5AC immunohistochemistry of human bronchus, specimens were fixed, cut into sections, stained with hematoxylin-eosin, and with Alcian blue and Periodic acid-Schiff (PAS) to visualize goblet cells, and incubated with mouse monoclonal antibody to MUC5AC (clone 45M1, 1:100; NeoMarkers, Fremont, CA) as previously reported [12].

Expression of phospho-EGFR and phospho-p44/42 MAPK

Protein expression of phospho-EGFR (Tyr845) and phospho-p44/42 MAPK (Thr202/Tyr204) was determined in cultured airway epithelial cells by PathScan® sandwich ELISA kit following the instructions of the manufacturer (Cell Signaling Technology, Beverly, MA, USA). A 15 min incubation time with carbachol was chosen because preliminary experiments indicated that there was little difference in phosphorylation between 5 and 15 min (data not shown). In some experiments, aclidinium was added 30 min before the addition of carbachol.

In additional experiments, the expression of phospho-p44/42 MAPK was determined by immunohistochemistry in human bronchial tissue using phospho-p44/42 MAPK rabbit mAb (Thr202/Tyr204, 1:100; Catalog nº: E7028, Sigma, USA) with appropriate rabbit and mouse IgG negative controls (Sigma, USA).

Real Time RT-PCR

Total RNA was isolated from human bronchial tissue or human epithelial cells differentiated in ALI as previously outlined [15]. cDNA was amplified with specific primers to MUC5AC, muscarinic receptors (M) 1-5 and GAPDH (as endogenous control)

using 7900HT Fast Real-Time PCR System (Applied Biosystem). The primers used to amplify cDNA were obtained from TaqMan Gene Expression Assays (Applied Biosystem) to MUC5AC (cat. nº: Hs01365616_m1), M1 (cat. nº: Hs00912795_m1), M2 (cat. nº: Hs00265208_m1), M3 (cat. nº: Hs00325478_m1), M4 (cat. nº: Hs00265219_s1), M5 (cat. nº: Hs00255278_s1) and GAPDH (cat. nº: 4352339E). Relative quantification of the different transcripts was determined with the $2^{-\Delta\Delta Ct}$ method using GAPDH as endogenous control. Results were normalized to respective time control relative expression as previously outlined [12].

Transfection of muscarinic receptor siRNA

Cultured human bronchial epithelial ALI cells were transfected with a commercial 50nM siRNA against M2 muscarinic receptor gene (Ambion, Austin TX, USA. PN 4392421) or with 50nM siRNA M3 muscarinic receptor gene (Ambion, Austin TX, USA. PN 4390815) or with 50nM siRNA control (Ambion, Huntingdon, Cambridge, UK), as outlined [16]. Briefly, ALI cultured bronchial epithelial cells were exposed to three successive solutions (4°C) containing (in mM): 1) 10 EGTA, 120 KCl, 5 ATP, 2 $MgCl_2$, 20 TES (pH 6.8; 20 min); 2) 120 KCl, 5 ATP, 2 $MgCl_2$, 20 TES and 50nM siRNA (pH 6.8; 3 h); and 3) 120 KCl, 5 ATP, 10 $MgCl_2$, 20 TES and 50nM siRNA (pH 6.8; 30 min). Subsequently, ALI epithelial cells were bathed in a fourth solution containing (in mM) 140 NaCl, 5 KCl, 10 $MgCl_2$, 5 glucose, and 2 MOPS (pH 7.1, 22°C) in which Ca^{2+} concentration was gradually increased from 0.01 to 0.1 to 1.8 mM every 15 min. Then, cells were cultured in differentiation media. The mRNA expression for M2 and M3 transcripts was determined by real time RT-PCR after 72 h post-silencing and compared with siRNA control at the respective time to determine silencing efficiency. Furthermore,

the M2 and M3 muscarinic receptor protein expression was measured by western blot after 72 h of silencing using a commercial antibodies against muscarinic receptors M2 (Sigma, USA, cat. nº M 9558) and M3 (Sigma, USA, cat. nº M0194) as previously described [15]. Protein expression was referred to β-actin (1:1000, Sigma, USA) expression as internal control. All experiments were performed in triplicate.

Cigarette smoke exposure of differentiated human airway epithelial cells

Differentiated bronchial epithelial cells were used to examine the MUC5AC responses after exposure to cigarette smoke extract (CSE) prepared as previously outlined [17]. Aqueous CSE was applied to the apical side of epithelial cells to mimic the *in vivo* pattern of cigarette smoke exposure. Briefly, smoke of research grade cigarettes 2R4F (Tobacco Health Research, University of Kentucky) was bubbled into a flask containing 25 ml of pre-warmed (37°C) DMEM medium obtaining a 100% CSE solution that was used within 30 min of CSE preparation. The CSE solutions were assessed by measuring absorbance at 320 nm. Stock solutions with absorbance values of 3.0±0.1 were used. To test for cytotoxicity from CSE exposure, cultured cells were treated with CSE concentrations of up to 30% for 24 h. No significant increase of supernatant lactate dehydrogenase content above control levels were observed (colorimetric assay Sigma; data not shown).

Statistics

Data are expressed as mean ± SEM of n experiments. In concentration-response experiments, the -log inhibitory concentration 50% (IC_{50}) was calculated by nonlinear regression to express compound potency (GraphPad Software Inc., San Diego, USA). Statistical analysis was

carried out by analysis of variance followed by appropriate *post hoc* tests including Bonferroni correction. Significance was accepted when $P<0.05$.

Materials

Drugs used in this study were from Sigma (St Louis, MO, USA) except aclidinium bromide that was synthesized at Almirall (Barcelona, Spain), and tyrphostin-AG1478 (Calbiochem; Nottingham, UK). All products were dissolved in distilled water mili-Q or in dimethyl sulfoxide (DMSO) at the concentration of 10^{-3}M except aclidinium which was dissolved in HCl 1N 1% (v/v) at the concentration of 10^{-3}M. Appropriate dilutions were obtained in freshly prepared medium. The DMSO final concentration in the assay solutions was ≤0.1% (v/v); this concentration was without significant effects on MUC5AC expression.

Results: 585 count word

Carbachol-induced MUC5AC overexpression in human bronchus is blocked by aclidinium

Carbachol (100 μM) augmented the MUC5AC mRNA and protein expression with peak values reached at 1 h and 3 h after carbachol exposure, respectively (Figure 1A). Furthermore, carbachol increased MUC5AC mRNA and protein expression in a dose-dependent manner reaching a peak value at 100 μM (Figure 1B). Thus, we selected this carbachol concentration for the rest of experiments.

The inhibitory potency values (-log IC_{50}) of aclidinium on carbachol-induced MUC5AC mRNA expression were ~1 nM (Figure 1C and 1D, and Table 1). Aclidinium (100nM) as well as atropine (1μM) and the selective inhibitor of EGFR tyrosine kinase, tyrphostin-

AG1478 (10 μM), abolished the carbachol-induced increase in MUC5AC mRNA and protein (Figure 1E). In contrast, the antagonist of nicotinic receptors hexamethonium (100μM) did not alter the MUC5AC response to carbachol (Figure 1E).

Immunohistochemistry experiments showed that MUC5AC immunoreactivity was localised in Alcian blue and PAS-stained goblet cells. The MUC5AC positive staining in airway epithelium was increased in carbachol-exposed preparations, and this augmentation was reduced in aclidinium-treated tissues (Figure 2). Additional immunohistochemical experiments addressed to assert the activation of downstream elements of the EGFR pathway (see below) showed that carbachol augmented the expression of phospho-p44/42 in human bronchial tissue and this effect was also blocked by aclidinium (Figure 2).

Carbachol-induced MUC5AC overexpression in cultured human ALI airway epithelial cells is blocked by aclidinium

In human bronchial epithelial ALI culture model, carbachol (100μM) increased significantly MUC5AC mRNA and protein after 12 h of incubation (Figure 3A). The carbachol-induced MUC5AC expression was suppressed by aclidinium and atropine in a concentration-dependent manner (Figure 3B and 3C, and Table 1). Similar findings were obtained with tyrphostin-AG1478 (10 μM), but not with hexamethonium (100μM) or with direct stimulation with nicotine (Figure 3D).

Furthermore, we observed that relative protein expression levels of phospho-EGFR and phospho-p44/42 were augmented at 15 min (2.02±0.21 and 2.29±0.17-fold increase, respectively; $n = 3$; $P<0.05$ vs. control at 15 min) and declined at 30 and 60 min following carbachol addition (Figure 3E). In this sense, aclidinium (100nM), atropine (1 μM) and

tyrphostin-AG1478 (10 μM) suppressed the carbachol (15 min incubation)-induced EGFR and p42/44 phosphorylation (Figure 3F, $P<0.05$).

Cigarette smoke-induced MUC5AC expression in cultured human ALI airway epithelial cells is blocked by aclidinium

CSE increased MUC5AC mRNA and protein expression in a time and concentration-dependent manner (Figure 4A and 4B). Based on these results, CSE (10%, 24 h) was selected for further experiments as producing consistent increases of MUC5AC mRNA and protein similar to those obtained with carbachol.

CSE (10%, 24 h)-induced increase of MUC5AC mRNA and protein expression was suppressed by aclidinium and atropine in a dose-dependent fashion (Figure 4C and 4D, and table 1). Furthermore, CSE-induced MUC5AC expression was inhibited by aclidinium (100nM) as well as atropine (1μM) and tyrphostin-AG1478 (10 μM), but remained unaltered in the presence of hexamethonium (100μM) (Figure 4E).

In other experiments performed in differentiated bronchial epithelial cells, CSE induced EGFR and p42/44 phosphorylation reaching a peak value at 30 min (Figure 5A). Moreover the CSE-induced EGFR and p42/44 phosphorylation was partially suppressed by aclidinium (100nM) as well as atropine (1μM) and tyrphostin-AG1478 (10 μM) (Figure 5B, $P<0.05$).

M3 muscarinic receptor subtype appears involved in MUC5AC response to carbachol and CSE in human cultured airway epithelial cells

Setting the expression level of M1 mRNA at 1, the amount of M2 mRNA expression in ALI airway epithelial cells was ~5.2 times higher and that of M3 was ~2.4-fold higher than

that of M1 (Figure 6A). Additional experiments demonstrated also the presence of M4 and M5 expression (data not shown). These data were corroborated at protein level, where M2 receptor was the most expressed followed by M3 (Figure 6B).

Furthermore, following CSE exposure, a significant increase in the mRNA and protein expression of M3 was observed without significant changes of the relative levels of M1 and M2 (Figure 6A and 6B).

Due to the low expression of M1, we studied the role of muscarinic M2 and M3 receptor subtypes in the MUC5AC secretion. To this respect, transfection of human ALI airway epithelial cells with siRNA-M2 or siRNA-M3 decreased the mRNA expression of M2 and M3 by ~62% and ~73% respectively, with the corresponding protein downregulation (Figure 7A). The siRNA-M2 did not affect the carbachol or CSE-induced MUC5AC mRNA expression whilst siRNA-M3 blocked the MUC5AC mRNA response to carbachol and CSE thus indicating a preferential role of M3 receptors in this response (Figure 7B and 7C). To further study of the implication of M1, M2 and M3 receptors in MUC5AC upregulation, we pre-incubated differentiated human ALI epithelial cells with pirenzepine 1μM (M1 antagonist), methoctramine 1μM (M2 antagonist) and p-fluoro-hexahydro siladifenidol 1μM (pFHHSid) (M3 antagonist). We detected that both, pirenzepine and methoctramine did not modify significantly the effect of carbachol and CSE on MUC5AC expression. In contrast, pFHHSid effectively attenuated carbachol and CSE-induced MUC5AC overexpression confirming data from siRNA experiments (Figure 7D and 7E)

Discussion:

In this study, we show that aclidinium potently inhibited the carbachol-induced MUC5AC overexpression in human bronchial tissue *in vitro* as well as the carbachol- and

cigarette smoke-induced augmentation of MUC5AC expression in differentiated human airway epithelial cells cultured in ALI. This is the first report showing a direct effect of a cholinergic agonist in up-regulating MUC5AC expression in human airway epithelial cells by activation of muscarinic receptors and transactivation of EGFR. Also, we showed that cigarette smoke-induced increase of MUC5AC expression *in vitro* can be effectively suppressed by the use of muscarinic antagonists. Since mucus hypersecretion is considered pathologically relevant in COPD and asthma, this inhibitory effect of aclidinium is of potentially added therapeutic value.

It is well established that goblet cell hypertrophy and hyperplasia occur in the large airways of habitual cigarette smokers with or without airway obstruction and result in epithelial mucin stores that are significantly higher than normal [18]. Goblet cells are located on the bronchial surface epithelium and MUC5AC is the best characterized mucin in this cell type, and its expression is directly correlated with airway obstruction [18-20]. Therefore, MUC5AC was selected for this study. While MUC5AC is increased directly by cigarette smoke in goblet cells [20], the role of other mucins such as MUC5B seems contradictory since MUC5B is not altered or indeed diminished in goblet cells from smokers and COPD patients [18, 19]. However, the major place of mucin production is located in submucosal glands in the central airways, where both MUC5AC and MUC5B are abundant in smokers and COPD patients [19, 21]. In this line, MUC5B is mainly produced in submucosal gland cells and is the most abundant in COPD sputum whilst MUC5AC is mainly produced in goblets cells and is the most abundant in smoker sputum without airway obstruction [21]. However seems that only MUC5AC may be induced by different stimulus while MUC5B appears more constitutively expressed and less sensitive to the inducible effect of irritants such as cigarette smoke [19]. This work is focused on the

MUC5AC production in goblet cells from surface epithelium due to the inducible characteristic of MUC5AC and its correlation with human airway obstruction found in COPD. However, since the major mucins are released predominantly from submucosal gland cells, it may be considered a limitation of the present study.

The human bronchial tissue *in vitro* is a preparation that has previously been shown to have a basal secretion of mucin MUC5AC produced principally by goblet cells [12].

The rapid time-course of MUC5AC secretion found in this work (Figure 1) was according to previous report for EGF [12] and for carbachol in goblet cells from rat conjunctiva [14].

In this *in vitro* model, aclidinium inhibited the augmented expression of MUC5AC induced by carbachol in a concentration-dependent fashion, with potency values ~1nM which is in the range of the potency of this muscarinic antagonist against cholinergic contraction in isolated trachea [22].

Immunohistochemical analysis of human bronchial tissues confirmed that carbachol exposure resulted in an augmented expression of MUC5AC positive stained cells in the airway epithelium that was paralleled by an increased presence of Alcian blue positive and PAS-stained goblet cells. Treatment with aclidinium effectively prevented this carbachol-induced overexpression of MUC5AC. In this point, it is interesting to note that the histochemical analysis of goblet cells not always is correlated with the immunohistochemical staining of MUC5AC as previously reported [19, 20], which may be take in account in the evaluation of this study.

The epidermal growth factor receptor and its tyrosine receptor kinase-based pathway mediate mucin production by airway epithelial cells in response to a variety of pathogenic and environmental insults [23]. The transactivation of EGFR following the stimulation of

muscarinic receptors has been described in different cell types including conjunctival goblet cells in relation to mucin secretion [24]. We confirmed and extended this finding by showing that carbachol-induced augmentation of MUC5AC mRNA and protein expression was mediated by the transactivation of EGFR and the subsequent phosphorylation of p44/42 MAPK. Thus, blockade of muscarinic receptors by aclidinium avoided transactivation of EGFR.

Because the human bronchial tissue contains a variety of different cell types, we also aimed to study the direct impact of carbachol on well-differentiated human primary airway epithelial cells with relation to MUC5AC expression. We found that carbachol-induced MUC5AC overexpression was abolished by aclidinium and atropine similarly to that found in tissue bronchium *in vitro* model, whilst hexamethonium did not reduce carbachol responses. Furthermore, nicotine (10μM) did not evoke a MUC5AC response which rules out nicotinic receptor activation in this mechanism. Consistent with the results observed in bronchial tissue, MUC5AC overexpression was consequence of the transactivation of the EGFR and the subsequent triggering of phosphorilated p44/42 MAPK signalling cascade as previously suggested in goblet cells from rat conjunctiva [24].

Next we investigated the role of muscarinic receptor subtypes on carbachol-induced MUC5AC overexpression. The presence of the five subtypes of muscarinic receptors was detected but we focused our study on M1, M2 and M3 receptors since only these subtypes exert well known physiological effects in the lungs [25]. We found a predominance of M2 and M3 muscarinic subtypes with low amounts of M1 receptors. A similar pattern of expression was described for cultured respiratory epithelial cells [5, 26]. In this sense, we found that siRNA specific for siRNA-M3 and the M3 antagonist pFHHSid attenuated the MUC5AC response to carbachol and CSE while siRNA-M2 and the M2 antagonist

methoctramine were without significant effects. These results suggest that the activation of muscarinic M3 receptor appears involved in the mucin MUC5AC response after cholinergic activation in differentiated human airway epithelium. The low expression of M1 receptors impeded the use of specific siRNA in the present study. However the use of the M1 antagonist pirenzepine was without effect which discards the M1 receptor in this process.

Cigarette smoke is widely used in *in vitro* studies due to its relevance in the pathogenesis of COPD [10]. The extent of the increases of MUC5AC expression obtained in the present study after cigarette smoke exposure, and the time point selected for this expression, are in keeping with findings from other studies [27]. In this work we observed that aclidinium with potency close to 1nM as well as atropine inhibited the CSE-induced MUC5AC. In contrast nicotinic receptors did not affect the MUC5AC upregulation. In this mechanism, the transactivation of EGFR after cigarette smoke exposure was also demonstrated by direct phosphorylation after CSE exposure and by the inhibition of MUC5AC expression obtained in the presence of tyrphostin-AG1478, a selective inhibitor of the EGFR tyrosine kinase. This result is consistent with the overproduction of mucin via EGFR found in response to cigarette smoke in the airway epithelial cell line NCI-H292 [28].

The activation of muscarinic receptors in response to cigarette smoke requires explanation. The epithelium of the airways possesses a non-neuronal cholinergic system and local release of acetylcholine may serve a variety of autocrine and paracrine functions [5]. Bronchial epithelial cells in culture express cholinesterases that inactivate acetylcholine [28]. Cigarette smoke is known to impose an important oxidative burden on exposed cells [10] that may degraded acetylcholinesterase and butyrylcholinesterase

molecules while cholineacetyltransferase is not affected thus resulting in tissue accumulation of acetylcholine [29]. On the other hand, we found an upregulation of muscarinic M3 receptors in airway epithelial cells exposed to CSE. This finding would be consistent with a recent study showing that human lung fibroblasts exposed to CSE dramatically increased cholineacetyltransferase and M3 muscarinic receptor expression [30] which may explain the effect of anti-muscarinics on tobacco smoke responses.

The results of this study indicate that aclidinium effectively decreased the MUC5AC overexpression elicited by cholinergic activation and cigarette smoke exposure in human bronchial epithelial cells. This direct inhibitory effect may have an additive effect in the clinical efficacy of aclidinium in mucus hypersecretory diseases such as COPD.

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Conflicts of Interest

EJM and JC received a research grant from Almirall. AG and MM are employees of Almirall.

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LEGEND TO FIGURES

Figure 1

Carbachol induces MUC5AC mRNA and protein expression in human isolated bronchus.

A) Time course of the relative expression of MUC5AC mRNA and protein in human isolated bronchus. The peak expression for MUC5AC mRNA was observed 1 h after stimulation with carbachol (CCh, 100μM), thus preceding the peak expression of MUC5AC protein at 3 h. B) CCh dose-dependently increased MUC5AC mRNA (1 h of stimulation) and protein (3 h of stimulation) expression. C, D) Concentration-dependent inhibition of the CCh 100μM-induced overexpression of MUC5AC mRNA and protein obtained in the presence of different concentrations of aclidinium and atropine under the experimental conditions described in Methods. E) Relative quantitation of MUC5AC mRNA and protein levels in human bronchus unstimulated (control) or stimulated with CCh (100μM) in the absence or presence of aclidinium (ACL; 100nM), atropine (ATR; 1μM), hexamethonium (HEX; 100μM) or tyrphostin AG1478 (10μM). Exposure time was 1 h for MUC5AC mRNA determination and 3 h for MUC5AC protein measurements. MUC5AC was determined using real time RT-PCR by the $2^{-\Delta\Delta Ct}$ method; points show the fold increase in expression of MUC5AC relative to GAPDH normalized to respective controls in time as mean±SEM of the $2^{-\Delta\Delta Ct}$ values. MUC5AC protein was determined in bronchial tissue by ELISA; points are mean±SEM. Data were obtained from three to five different patients. *P<0.05 vs. basal values. $^{\#}P$<0.05 vs. CCh.

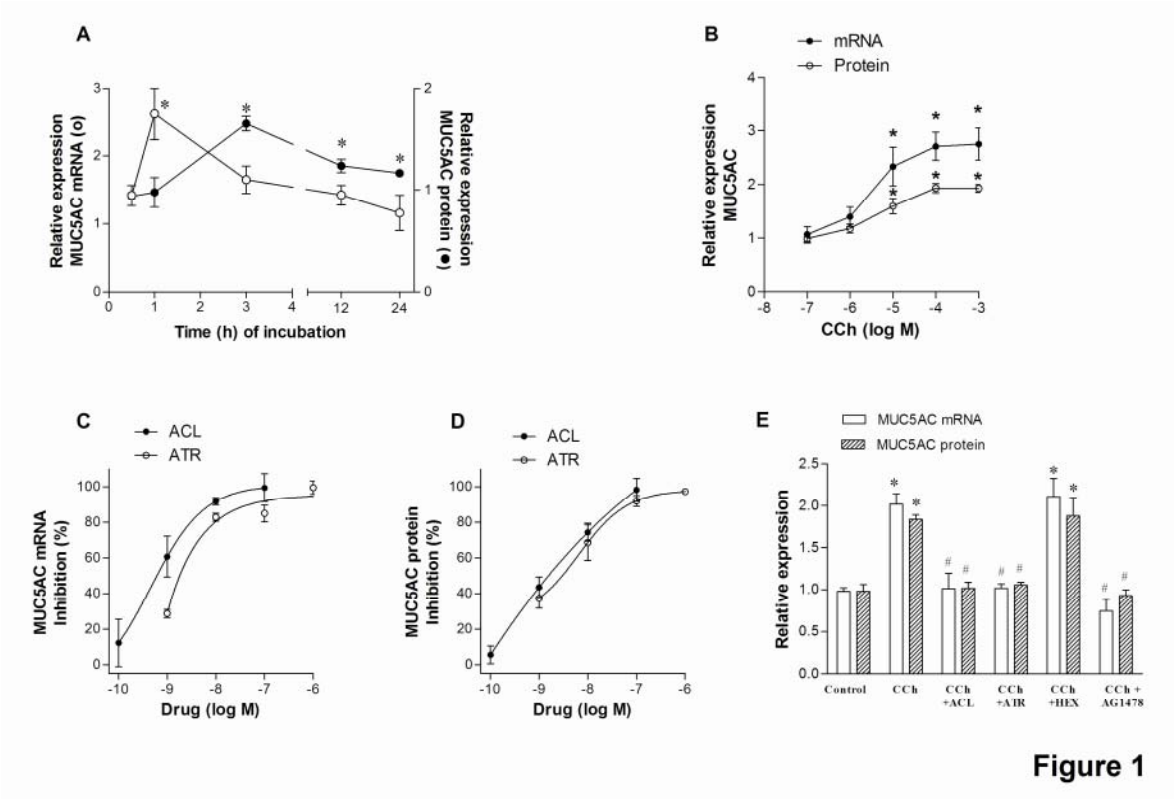

Figure 2

Photomicrographs of representative histological sections from human bronchial tissue unstimulated (control) or stimulated with carbachol for 3 h (CCh; 100µM) or 30 min (in case of phospho-p44/42 MAPK immunostaining) in the absence or presence of aclidinium (ACL; 100nM). Sections show staining with haematoxylin-eosin (H&E), Alcian blue, periodic acid-Schiff (PAS) or immunohistochemical staining of MUC5AC or phospho-p44/42 MAPK. Mucin stores in goblet cells appear as blue staining for alcian blue + cells and purple staining for PAS + cells. MUC5AC immunoreactivity was observed as brown staining in goblet cells. Ciliated cells showed no staining for MUC5AC. The sections demonstrate increased Alcian blue, PAS, MUC5AC and phospho-p44/42 staining in the tissues exposed to CCh which were prevented by the incubation with aclidinium for 15 min. Negative control is expressed as non-specific IgG. Scale bar: 25 µm.

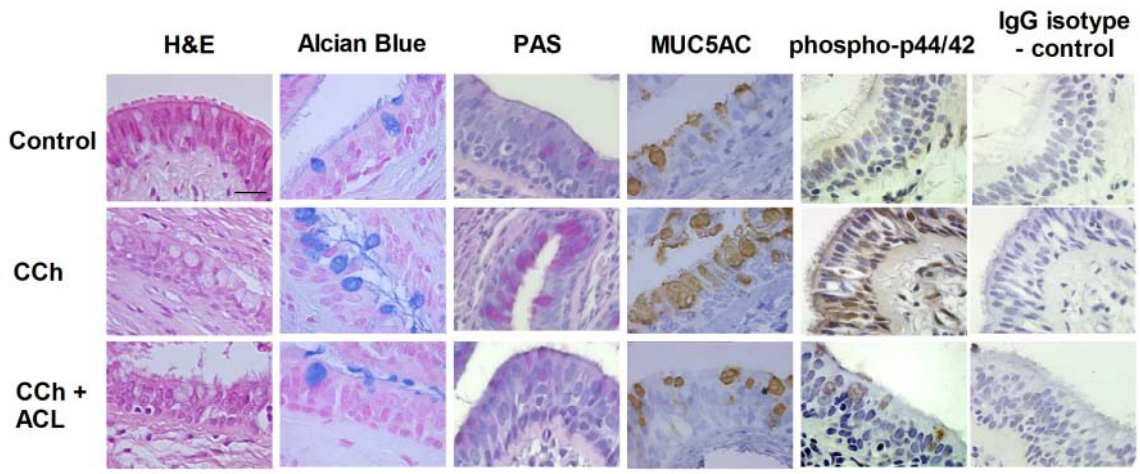

Figure 2

Figure 3

Carbachol induces MUC5AC mRNA and protein expression in differentiated human bronchial epithelial cells.

A) Time course of the relative expression of MUC5AC mRNA and protein in differentiated human bronchial epithelial cells in air-liquid interface system. The peak expression for MUC5AC mRNA and protein was observed 12 h after stimulation with carbachol (100μM). B, C) Concentration-dependent inhibition of the carbachol 100μM-induced overexpression of MUC5AC mRNA and protein obtained in the presence of different concentrations of aclidinium and atropine under the experimental conditions described in Methods. D) Relative quantitation of MUC5AC mRNA and protein in human bronchial epithelial cells cultured in air-liquid interface system, unstimulated (control) or stimulated with carbachol (CCh; 100μM) in the absence and presence of aclidinium (ACL; 100nM), atropine (ATR; 1μM), hexamethonium (HEX; 100μM) or AG1478 (10μM). Nicotine (NIC; 10μM) was without effect. Exposure time was 12 h for MUC5AC mRNA

and protein determination. Columns show the fold increase from control levels at 12 h as mean±SEM of 4 independent experiments. *P<0.05 from control; $^{\#}P$<0.05 vs. CCh. E) Relative time course of the EGFR and p44/42 phosphorylation in differentiated human bronchial epithelial cells in air-liquid interface system following CCh 100μM stimulation. Levels of EGFR and p44/42 phosphorylation are normalized to basal conditions. F) Relative expression of the EGFR and p44/42 phosphorylation in human bronchial epithelial cells cultured in air-liquid interface system, unstimulated (control) or stimulated with CCh 100μM in the absence and presence of ACL 100nM, ATR 1μM, HEX 100μM or AG1478 10μM. CCh exposure time was 15 min. Columns show the fold increase from control levels at 15 min as mean±SEM of 4 independent experiments. *P<0.05 from control; $^{\#}P$<0.05 vs. CCh.

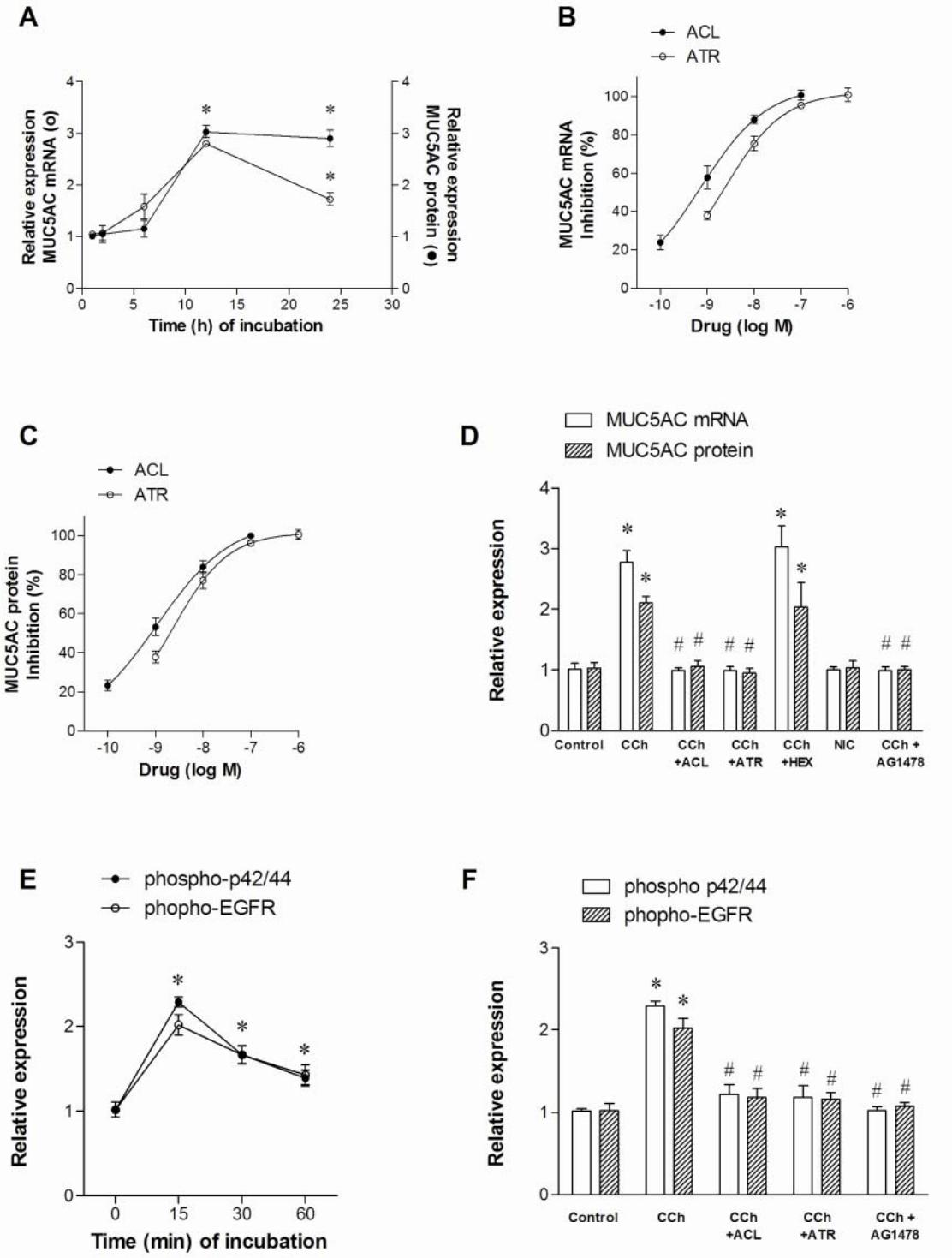
A
Relative expression MUC5AC mRNA (o)
Relative expression MUC5AC protein (●)
Time (h) of incubation
B
ACL
ATR
MUC5AC mRNA Inhibition (%)
Drug (log M)
C
ACL
ATR
MUC5AC protein Inhibition (%)
Drug (log M)
D
MUC5AC mRNA
MUC5AC protein
Relative expression
Control
CCh
CCh +ACL
CCh +ATR
CCh +HEX
NIC
CCh + AG1478
E
phospho-p42/44
phopho-EGFR
Relative expression
Time (min) of incubation
F
phospho p42/44
phopho-EGFR
Relative expression
Control
CCh
CCh +ACL
CCh +ATR
CCh + AG1478

Figure 3

Figure 4

CSE induces MUC5AC mRNA and protein expression in differentiated human bronchial epithelial cells.

A) Time course of the relative expression of MUC5AC mRNA and protein in differentiated human bronchial epithelial cells in air-liquid interface system. The peak expression for MUC5AC mRNA and protein expression was observed 24 h after stimulation with CSE 10%. B) CSE dose-dependently increased MUC5AC mRNA and protein expression. C, D) Concentration-dependent inhibition of the CSE 10%-induced overexpression of MUC5AC mRNA and protein obtained in the presence of different concentrations of aclidinium and atropine under the experimental conditions described in Methods. E) Relative quantitation of MUC5AC mRNA and protein in human bronchial epithelial cells cultured in air-liquid interface system, unstimulated (control) or stimulated with CSE 10% in the absence and presence of aclidinium (ACL; 100nM), atropine (ATR; 1μM), hexamethonium (HEX; 100μM) or AG1478 (10μM). Exposure time was 24 h for MUC5AC mRNA and protein determination. Columns show the fold increase from control levels at 24 h as mean±SEM of 4 independent experiments. **P*<0.05 from control; $^{\#}$*P*<0.05 vs. CSE.

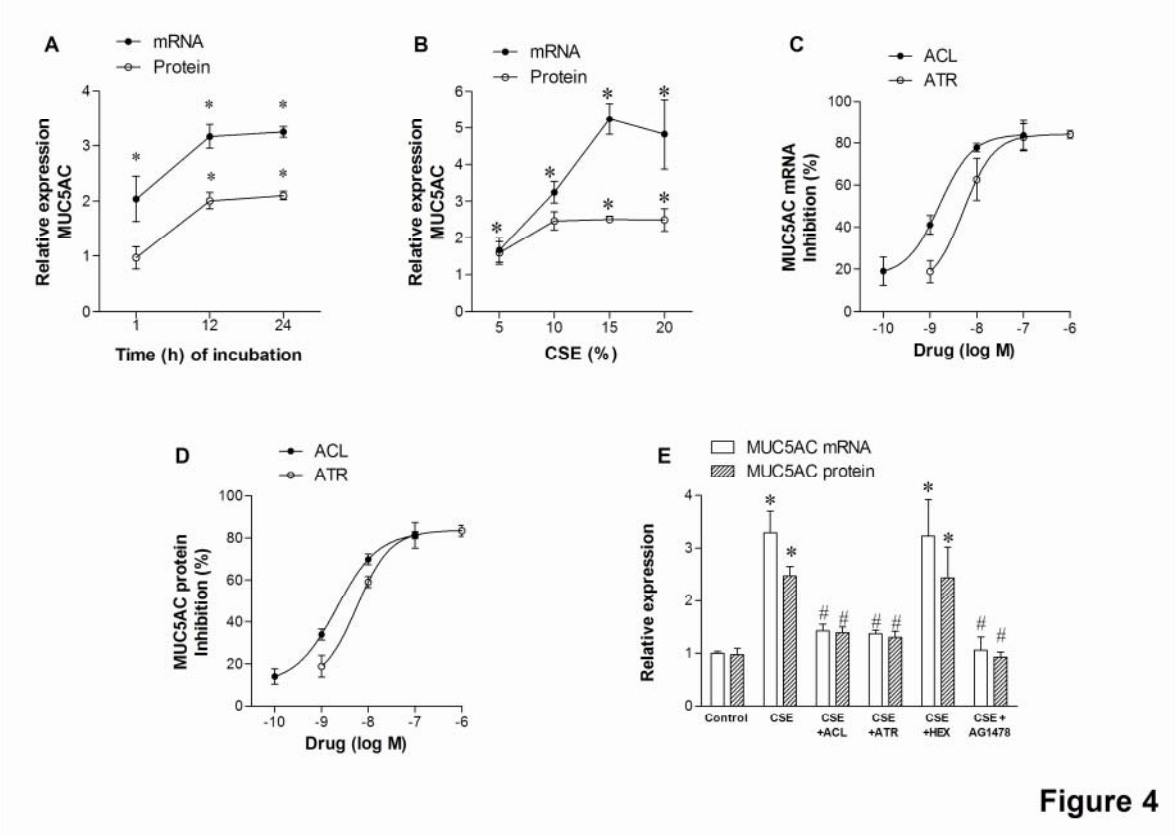

Figure 4

Figure 5

CSE induces EGFR and p44/42 phosphorylation is partially suppressed by antimuscarinics.

A) Relative time course of the EGFR and p44/42 phosphorylation in differentiated human bronchial epithelial cells in air-liquid interface system following CSE 10% stimulation. Levels of EGFR and p44/42 phosphorylation are normalized to basal conditions. B) Relative expression of the EGFR and p44/42 phosphorylation in human bronchial epithelial cells cultured in air-liquid interface system, unstimulated (control) or stimulated with CSE 10% in the absence and presence of aclidinium (ACL 100nM), atropine (ATR 1μM), or AG1478 10μM. CSE exposure time was 30 min. Columns show the fold increase

from control levels at 30 min as mean±SEM of 4 independent experiments. *P<0.05 from control; $^{\#}P$<0.05 vs. CSE.

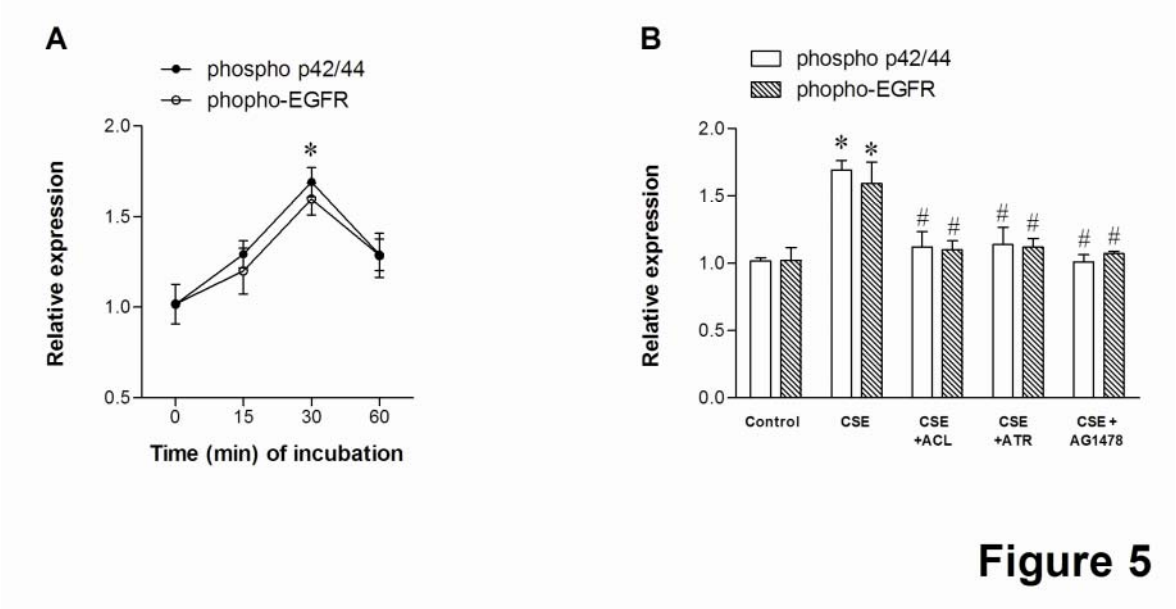

Figure 5

Figure 6

Relative expression of muscarinic receptor subtypes M1, M2 and M3 in human bronchial epithelial cells cultured as air-liquid interface in the absence (control) and presence of exposure to CSE. M receptor levels were measured by quantitative real-time PCR (panel A) and western blot (panel B). The level of the mRNA expression of M2 and M3 receptors in the controls is referred to the expression level of M1 receptors taken as unity. Columns are mean±SEM of 5 independent experiments. *P<0.05 from M1; #P<0.05 from the corresponding control at 24 h.

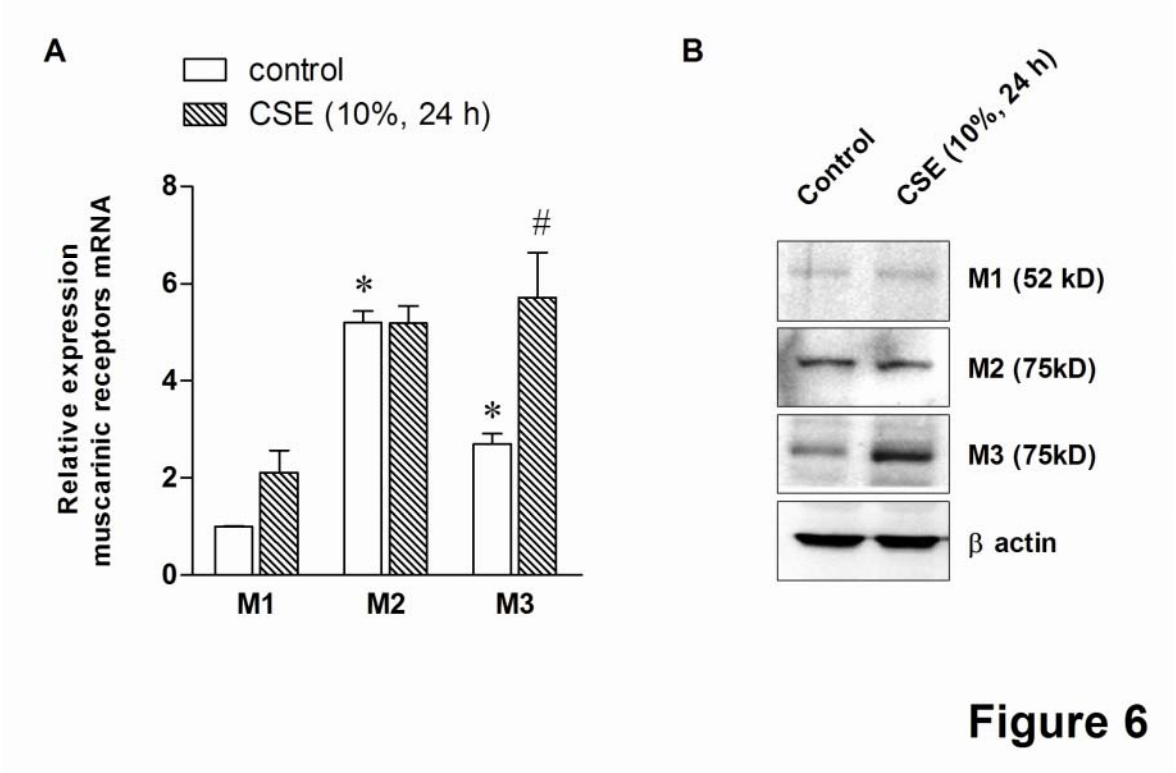

Figure 7

Effects of M2 and M3 targeted siRNA on the expression of their corresponding receptors (panel A) and on the carbachol and CSE-induced MUC5AC expression (panels B and C respectively) in differentiated human bronchial epithelial cells in air-liquid interface system. siRNA-M2 and siRNA-M3 resulted in a significant reduction in their respective muscarinic M2 and M3 mRNA and protein expression (panel A). Muscarinic receptor mRNA transcripts were measured by quantitative RT-PCR and protein expression by western blotting. The MUC5AC mRNA overexpression elicited by carbachol (CCh, 100μM) or CSE 10% was unaltered in the presence of siRNA-M2 but blocked by siRNA-M3 (panel B and C). Columns are mean±SEM of 3 independent experiments per condition. **P*<0.05 from corresponding controls; #*P*<0.05 from the response to CCh or CSE without siRNA-M3. D, E) In other experiments, differentiated human bronchial epithelial cells

were incubated in presence or absence of pirenzepine (M1 antagonist at 1μM), methoctramine (M2 antagonist at 1μM) or pFHHSid (M3 antagonist at 1μM) for 30 min before the stimulation with carbachol (CCh, 100μM, 12 h) or CSE (10%, 24 h). MUC5AC mRNA was then quantified by real time PCR. Columns are mean±SEM of 3 independent experiments. **P*<0.05 from basal conditions; #*P*<0.05 from CCh or CSE.

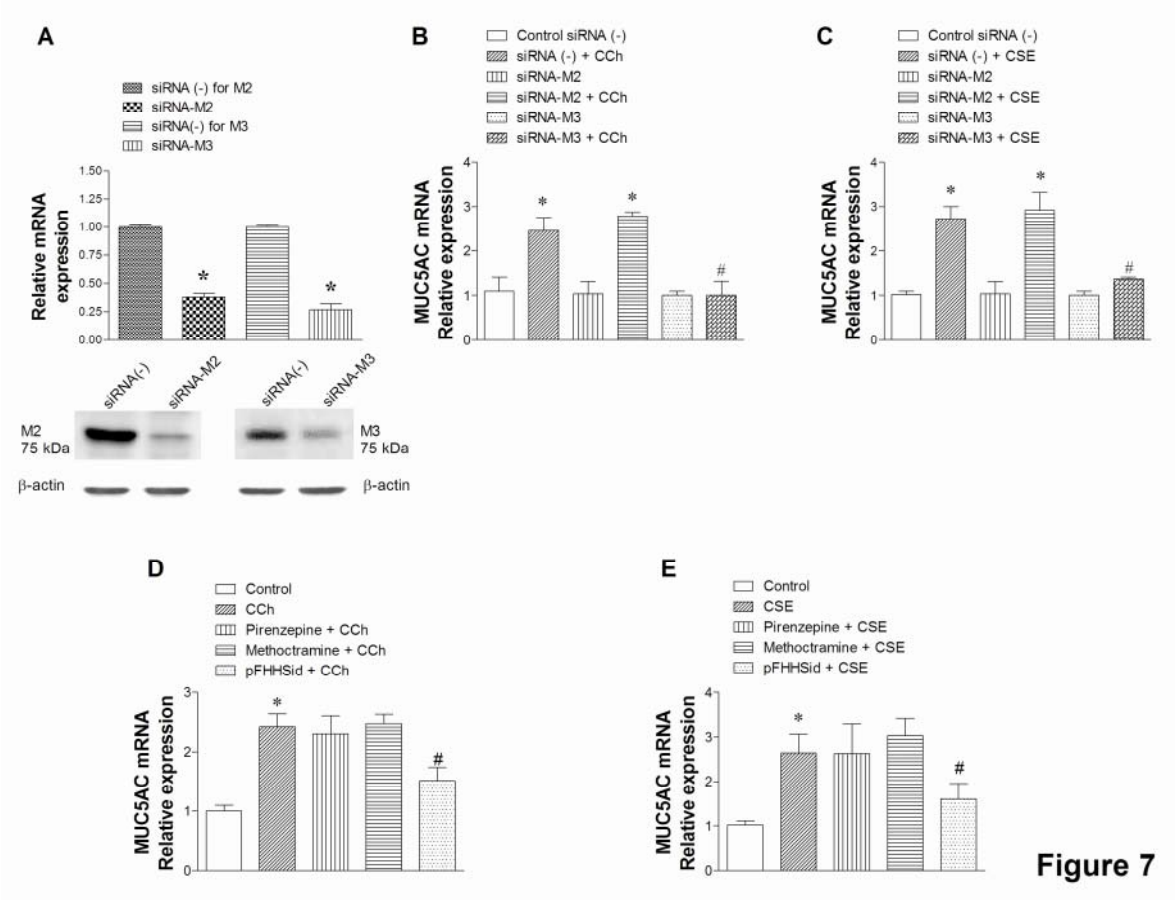

Figure 7

Table 1

Potency values of aclidinium as antagonist of carbachol- and cigarette smoke-induced augmentation of the expression of mucin MUC5AC mRNA and protein expression in human isolated bronchial tissue and human airway epithelial cells cultured in air-liquid interface system. The corresponding values for atropine are also given for comparison. Potency values are given as the –log IC_{50} values calculated from the concentration-dependent inhibition of the carbachol 100μM and cigarette smoke 10%-induced overexpression of MUC5AC mRNA and protein obtained in the presence of aclidinium (0.1, 1, 10 and 100 nM) and atropine (1, 10, 100 and 1000 nM) under the experimental conditions described in Methods. Data are mean±SEM of 3-5 independent experiments

	Potency values (-log IC_{50}) as inhibitor of	
	MUC5AC mRNA expression	MUC5AC protein expression
Carbachol in human isolated bronchial tissues		
Aclidinium	9.17±0.14	8.81±0.16
Atropine	8.61±0.08	8.78±0.09
Carbachol in human cultured airway epithelial cells		
Aclidinium	9.13±0.12	8.95±0.09
Atropine	8.69±0.05	8.60±0.06
Cigarette smoke in human cultured airway epithelial cells		
Aclidinium	8.80±0.12	8.67±0.11
Atropine	8.29±0.14	8.24±0.13