ACLDINIUM INHIBITS CIGARETTE SMOKE-INDUCED LUNG FIBROBLAST TO MYOFIBROBLAST TRANSITION

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Running title: Aclidinium bromide mitigates myofibroblast markers
ABSTRACT

Cigarette smoking contributes to lung remodeling in chronic obstructive pulmonary disease (COPD). As part of remodeling, peribronchiolar fibrosis is observed in small airways of COPD patients and contributes to airway obstruction. Fibroblast to myofibroblast transition is a key step of the peribronchiolar fibrosis formation. This in vitro study examines the effect of cigarette smoke on bronchial fibroblast to myofibroblast transition, and whether aclidinium bromide inhibits this process. Human bronchial fibroblasts were incubated with aclidinium bromide (10^{-9}M-10^{-7}M) and exposed to cigarette smoke extract. Collagen type I and alpha-smooth muscle actin expression were measured by real-time PCR and Western blotting as myofibroblast markers. Intracellular reactive oxygen species, cAMP, ERK 1/2 and choline acetyltransferase were measured as intracellular signaling mediators. Cigarette-smoke-induced collagen type I and alpha-smooth muscle actin was mediated by the production of reactive oxygen species, the depletion of intracellular cAMP and the increase of ERK1/2 phosphorylation and choline acetyltransferase. These effects could be reversed by treatment with the anticholinergic aclidinium bromide, by silencing mRNA at muscarinic receptors M1, M2 or M3, or by the depletion of extracellular acetylcholine by treatment with acetylcholinesterase. Non-neuronal cholinergic system is implicated in cigarette smoke-induced bronchial fibroblast to myofibroblast transition which is inhibited by aclidinium bromide.

Keywords: Anticholinergic, cigarette smoke, COPD, Non-neuronal cholinergic system.
INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by airflow limitation that is progressive and not fully reversible. Cigarette smoking is the main risk factor for COPD and contributes to structural changes in airways during COPD progression [1]. Structural changes in COPD patients are characterized by loss of alveolar wall (emphysema), vascular remodeling with pulmonary hypertension, mucus hypersecretion or peribronchiolar fibrosis [1]. As part of fibrotic alterations in COPD, structural changes are seen primarily in small airways. The severity of the disease appears to correlate with thickening of the wall of small airways caused by fibrosis and infiltration of inflammatory cells, which contributes to airflow obstruction [2]. Accumulation and persistence of myofibroblasts is believed to contribute to development of small airway fibrosis. In this respect, under chronic inflammatory conditions, resident lung fibroblasts are activated and transformed into a more contractile, proliferative and secretory-active myofibroblast phenotype, characterized by increased expression of extracellular matrix components and alpha-smooth muscle actin (α-SMA), which contribute to the increase of lung remodeling progression and airway bronchoconstrictor responsiveness [3].

Parasympathetic activity is increased in the airways of COPD patients and is the basis for the use of anticholinergic therapy [4]. Anticholinergics constitute a particularly important bronchodilator therapy in COPD and certain forms of asthma [5]. Furthermore, anticholinergics in animal models have shown potential anti-inflammatory and anti-remodeling effects [6], which may be of additional value to their classical bronchodilator effects.

Recently, it has been proposed that acetylcholine in the airways may be released by non-neuronal cell types, such as airway epithelial cells and lung fibroblasts. Therefore, a
dysfunction of a non-neuronal cholinergic system may contribute to the pathophysiology of asthma and COPD [7]. It has been shown that anticholinergic treatment inhibits cigarette smoke-induced mucin hypersecretion in human bronchial epithelial cells [8], as well as cigarette smoke-induced lung fibroblast proliferation [9]. Furthermore, choline acetyltransferase (ChAT), the intracellular enzyme responsible for acetylcholine production, is upregulated in both lung fibroblasts from COPD patients and fibroblasts stimulated with cigarette smoke [9]. However, no data exist concerning the role of cigarette smoke on fibroblast to myofibroblast transition, as well as the potential inhibitory effect of anticholinergics.

Aclidinium bromide is a novel, inhaled long-acting muscarinic antagonist compound with low systemic activity that has completed phase III clinical development for COPD treatment [10]. In preclinical studies, aclidinium bromide demonstrated potent muscarinic-antagonist activity, comparable to ipratropium bromide and tiotropium bromide, and long duration of action [11]. In clinical trials, aclidinium bromide has provided sustained bronchodilation, similar to that observed with tiotropium bromide, a good safety profile and low incidence of anticholinergic adverse events [10].

In this study, we investigated the role of aclidinium bromide on the increase of myofibroblast markers collagen type I and $\alpha$-SMA elicited by chronic cigarette smoke exposure in human bronchial fibroblast, as well as the intracellular pathways involved in this process.

**METHODS**

See the online supplement for further details of the methods used.
Isolation and cultivation of human fibroblasts

Human bronchial fibroblasts were obtained from patients undergoing surgery for lung carcinoma who gave informed consent, as previously described [12]. Cultivation and characterization of fibroblasts were performed as described elsewhere [12]. Protocol for obtaining human tissue was approved by the local ethical review board for human studies (General Hospital of Valencia, Spain). See online supplement for details.

Preparation of cigarette-smoke extract and incubations

Cigarette-smoke extract (CSE) solutions were prepared as previously described [13]. Briefly, the smoke of a research cigarette (2R4F, Tobacco Health Research, University of Kentucky) was bubbled into a flask containing 25 ml of pre-warmed (37°C) Dulbecco’s Modified Eagle Medium. The resulting solution was defined as CSE at 100%. CSE at 10% reportedly corresponds to exposure associated with smoking approximately 1–2 packs/d [14]. Before stimulation, subconfluent cell monolayers were deprived of serum for 24 h. Human bronchial fibroblasts were stimulated with CSE (0–10%) for different periods of time (0–72 h) replacing culture medium and stimulus every 24 h. Different drug modulators were added 30 minutes before stimulus. See online supplement for details.

Real-time RT-PCR

Total RNA was isolated from cultured human bronchial fibroblasts by using TriPure® (Roche, Indianapolis, IN) and reversely transcribed and amplified with specific primers. Relative quantification of these different transcripts was determined with the $2^{-\Delta\Delta Ct}$ method
using glyceraldehyde phosphate dehydrogenase as endogenous control (Applied Biosystems) and normalized to control group. See online supplement for details.

Transfection of siRNAs
Small interfering RNA (siRNA) for M1, M2 and M3 receptors and the scrambled siRNA control were purchased from Ambion (Huntingdon, Cambridge, UK). The transfection reagent used was lipofectamine-2000 (Invitrogen, Paisley, UK) at a final concentration of 2 µl ml⁻¹. See online supplement for details.

Western blotting
Western blot analysis was used to detect changes in collagen type I (138 kD), α-SMA, p-ERK1/2 (42–44 kD), M1 (52 kD), M2 (70 kD), M3 (75 kD), p67phox (67 kD), NOX4 (67 kD) and ChAT (65 kD) from bronchial fibroblast lysates. See online data supplement for details of Western blot analyses and antibodies used.

DCF fluorescence measurement of reactive oxygen species
2′, 7′-dichlorodihydrofluorescein diacetate (H₂DCF-DA, Molecular Probes, Nottingham UK) was used to monitor the intracellular reactive oxygen species (ROS) in bronchial fibroblasts. See online supplement for details.

cAMP assay
Human lung fibroblasts were cultured in 96-well plates until ~95% of confluence. Following different treatments, cells were lysed and intracellular cAMP content was
determined with cAMP Biotrak enzyme immunoassay according to manufacturer’s instructions (Amersham, UK). Results were expressed as fmol/well.

**Analysis of results**

Data presented as mean ± standard error of mean (SEM) of n experiments. Statistical analysis of data was performed by analysis of variance followed by Bonferroni test (GraphPad Software Inc., San Diego, CA). Significance was accepted when $P < 0.05$.

**RESULTS**

**Aclidinium bromide attenuates CSE-induced myofibroblast markers in human bronchial fibroblast cultures**

CSE, at 2.5% concentration, upregulated collagen type I and $\alpha$-SMA in a time-dependent manner, reaching peak values following 48 h of CSE 2.5% exposure (Figures 1A, 1B). Furthermore, CSE dose-dependently increased collagen type I and $\alpha$-SMA mRNA and protein expression reaching statistical significance at 2.5% concentration following 48 h of exposure (Figure 1C-F). Thus, we selected this CSE concentration to evaluate myofibroblast markers expression in future experiments.

In other experiments, aclidinium bromide was added 30 minutes before CSE 2.5% and further incubated for 48 hours. Aclidinium bromide dose-dependently reduced the CSE-induced collagen type I and $\alpha$-SMA mRNA and protein expression, reaching maximal inhibitory value at $10^{-7}$M (Figure 2A-C). Similar results were observed for atropine, reaching significant inhibition of both myofibroblast markers at 1µM, suggesting the participation of cholinergic pathway in this process (Figure 2D-F). Both, aclidinium or
atropine by themselves did not show any effect on myofibroblast markers (Figure E1 and E2, online supplement).

**Aclidinium bromide reduces intracellular ROS elevated by CSE in human bronchial fibroblasts**

In our experiments on human primary lung fibroblasts, CSE (2.5 to 10%) dose-dependently increased intracellular ROS generation, reaching a significant value after 2 h of stimulation that was sustained for 24 h (Figure 3A). Pretreatment of bronchial fibroblasts with aclidinium bromide dose-dependently reduced the CSE-induced ROS by nearly 50% at 10^{-7} M after 24 h of CSE 2.5% stimulation (Figures 3B, 3C). In parallel experiments, quenching of ROS by apocynin (100 µM) or NAC (1 mM), as well as increasing cAMP with its analog dbcAMP suppressed CSE 2.5%-triggered intracellular ROS production (Figure 3C).

NADPH oxidase complex is constituted by several cytosolic and plasmatic membrane subunits [15]. Among the NADPH oxidase subunits analyzed, we found that the cytosolic p67phox and the plasmatic membrane unit NOX4 were the most expressed in bronchial fibroblasts under basal conditions (Figure 4A). Following 24-hour exposure, CSE 2.5% upregulated both mRNA transcripts and protein expression of p67phox and NOX4, which were dose-dependently reverted to near control values by exposure to aclidinium bromide 10^{-7} M (Figure 4B–4D).
CSE Activation of Non-Neuronal Cholinergic System is Inhibited by Aclidinium Bromide

Previous reports indicated that CSE activates a non-neuronal cholinergic system in lung fibroblast, but the mechanism remains unclear. Since ChAT is the intracellular enzyme responsible for acetylcholine synthesis, we next explored the effect of CSE on ChAT expression. In this regard, CSE 2.5% increased ChAT expression, which was dose-dependently inhibited by aclidinium bromide and by the antioxidants apocynin 100 µM and NAC 1 mM (Figure 5A).

Intracellular levels of cAMP and phosphorylation of ERK1/2 have been related to the activation of human lung fibroblast [12, 16]. In this work we observed that CSE 2.5% reduced intracellular cAMP levels and increased the ERK1/2 phosphorylation following 24 h of exposure (Figure 5B-5D). Aclidinium bromide pretreatment dose-dependently reduced the cAMP downregulation, as well as the increase of ERK1/2 phosphorylation (Figure 5B, 5C). Furthermore, the antioxidant treatment with apocynin 100 µM or NAC 1 mM also reversed the CSE-induced cAMP downregulation and ERK1/2 phosphorylation (Figure 5B, 5D). Since CSE may activate a non-neuronal cholinergic system, we added the enzyme AChE (10 U/ml) to remove any extracellular acetylcholine during the 24-h period of CSE 2.5% stimulation. AChE partially reversed cAMP downregulation and ERK1/2 phosphorylation induced by CSE (Figure 5B, 5D).

The antioxidants N-acetyl-L-cysteine (NAC) 1 mM and apocynin 100 µM, as well as the cAMP analog dbcAMP and the ERK1/2 inhibitor PD98059 10 µM partially suppressed the CSE-induced collagen type I and α-SMA mRNA and protein expression (Figure 6A–6B). Moreover, both aclidinium bromide 10⁻⁷M and AChE 10 U/ml were also able to suppress
the CSE-induced collagen type I and α-SMA mRNA and protein expression in human bronchial fibroblasts, which implicates a non-neuronal cholinergic system in the upregulation of myofibroblast markers (Figure 6A–6B). To further study the role of non-neuronal cholinergic system in the myofibroblast transformation, lung fibroblast were incubated with the inhibitor of choline uptake transporter hemicholinium-3 at 50µM, or with the acetylcholinesterase inhibitor neostigmine 10µM before CSE 2.5% exposure. Hemicholinium-3 significantly inhibited the CSE-induced collagen type I and α-SMA mRNA and protein expression, while neostigmine significantly increased the CSE-induced collagen type I and α-SMA mRNA and protein expression (Figure 6C-6D). In absence of CSE, the different drugs assayed did not show any effect on mRNA or protein expression of collagen type I and α-SMA (Figure E3 and E4, online supplementary data).

**CSE Upregulation of Myofibroblast Markers is Partially Mediated by M1, M2 and M3 Receptors**

Human bronchial fibroblasts transfected with siRNA for M1, M2 or M3 selectively suppressed specific M receptors without affecting other M receptors (Figure 7A-7B). In this line, siRNA for all three M receptors showed suppression of CSE-induced myofibroblast markers collagen type I and α-SMA (Figure 7C), indicating that all three M receptors may be involved in mediating the induction of the myofibroblast markers.

**DISCUSSION**

The main and novel results of this study are that (1) CSE increased the myofibroblast markers collagen type I and α-SMA in human bronchial fibroblast through a mechanism mediated by increase of intracellular ROS, depletion of cAMP, and phosphorylation of...
ERK1/2; (2) CSE activated a non-neuronal cholinergic system by means of over-expression of ChAT, mediating increase of myofibroblast markers; (3) the anticholinergic aclidinium bromide was able to attenuate the CSE-induced myofibroblast markers through inhibition of ROS generation, cAMP depletion, ERK1/2 phosphorylation, and ChAT over-expression induced by CSE. These new findings provide in vitro evidence of the anti-remodeling effect of aclidinium bromide on human bronchial fibroblasts in those cigarette smoke exposure situations that may contribute to the amelioration of the peribronchiolar fibrosis observed in smokers with COPD.

It is known that airflow limitation in COPD patients occurs in distal airways considered as non-cartilaginous-conducting airways with an internal diameter <2 mm. In this work we carefully dissected small bronchi of ~2 mm to obtain bronchial fibroblasts, which may represent the place where peribronchiolar fibrosis occurs [17]. Bronchiolar fibroblast transformation into myofibroblast is considered a key step in the process of increased thickness of the small airways, reducing airways radius and enhancing airflow limitation. Myofibroblasts share phenotypic characteristics with fibroblasts and airway smooth muscle cells. In this regard, myofibroblasts are characterized by secretion of extracellular matrix components, such as collagen type I, a characteristic that is shared with fibroblasts but not with smooth muscle cells, and by formation of contractile apparatus such as α-SMA, a characteristic that is shared with airway smooth muscle cells but not with fibroblasts [3]. Therefore, we selected both of these molecular markers to analyze the myofibroblast-like phenotype. Several growth factors and proinflammatory mediators such as TGFβ1, IL-13 or CTGF have been described as inducers of myofibroblast transition [18]; however, no data were available on cigarette smoke, the main risk factor for COPD. In this work, we prepared CSE as we and others have described previously [13, 14], which approximately
corresponds to exposures associated with smoking slightly fewer than 0.5 packs/d to slightly fewer than 2 packs/d of cigarettes for CSE 2.5% and 10%, respectively. Currently, several in vitro studies have been focused on effect of CSE on lung fibroblasts; however, no data are available on effect of CSE on bronchial fibroblast to myofibroblast transition. In this work, we observed that CSE promoted myofibroblast markers over-expression, which is compatible with the myofibroblast-like phenotype [3]. Furthermore, the CSE-induced expression of myofibroblast markers was mediated by intracellular ROS production, which was significantly increased after 2 h of exposure and remained at least until 24 h. The ROS increase preceded the collagen type I and α-SMA over-expression (after 48 h), suggesting a role of intracellular ROS as second messenger. Similar time-response of ROS production has previously been reported in human primary lung fibroblasts [19]. Intracellular ROS production in response to CSE is mediated by direct activation of the NADPH complex [20]. The NADPH complex is constituted by several cytosolic and plasmatic membrane units, which vary depending on the cell type. Thus, in human lung fibroblasts, the NADPH oxidase components p47phox, p67phox, p22phox and NOX4 have been observed [21]. In this work, we found that the NADPH units p67phox and NOX4 were the most expressed in bronchial fibroblasts. The cytosolic p67phox unit is mobilized under certain conditions to activate the membrane plasmatic units NOX1, NOX2 or NOX3 to produce O’ and/or H2O2. In contrast, the plasmatic membrane unit NOX4 does not require interaction and activation by cytosolic regulatory subunits, so its activation is directly related with its expression [15]. In this regard, we found that CSE induced the upregulation of both p67phox and NOX4 NADPH oxidase units after 24 h of stimulation, which was in accordance with the increase of intracellular ROS.
Recent reports have related expression of NOX4 as a key factor of fibroblast progression and fibroblast to myofibroblast transition [22]. Therefore, a treatment diminishing NOX4, and in turn, intracellular ROS, may prevent myofibroblast transition. We found that 24-h exposure to either the antioxidant NAC or the NADPH oxidase inhibitor apocynin inhibited the CSE-induced p67phox and NOX4 upregulation. These antioxidant modulators were also able to reduce myofibroblast markers induced by CSE, establishing a link between oxidative stress and myofibroblast transition. The anticholinergic aclidinium bromide attenuated myofibroblast markers induced by CSE and that this action was mediated in part by the inhibition of the p67phox and NOX4 expression, as well as by the consequent reduction of intracellular ROS generated by CSE. These results suggest that CSE activates a non-neuronal cholinergic system. Previous reports have suggested that cigarette smoke may activate a non-neuronal cholinergic system in different cell types, including airways and human lung fibroblasts. For example, we have recently shown that CSE promotes synthesis and release of the mucin MUC5AC in differentiated bronchial epithelial cells by a mechanism mediated by release of acetylcholine, and can be inhibited by aclidinium bromide [8]. Furthermore, human lung fibroblasts from COPD patients have shown an increase of M receptors, as well as ChAT expression, an effect that was mimicked in healthy lung fibroblasts after CSE exposure [9]. In our experiments, AChE prevented the CSE-induced expression of myofibroblast markers, which is consistent with the presence of a non-neuronal cholinergic system. Other evidence to support the activation of a non-neuronal cholinergic system by cigarette smoke includes the upregulation of ChAT, the intracellular enzyme responsible of acetylcholine synthesis. We observed that CSE upregulated ChAT expression, which was prevented by the antioxidants NAC and apocynin, as well as by aclidinium bromide, suggesting the participation of intracellular
ROS. Further evidence of the participation of non-neuronal cholinergic system was supported by the inhibition of myofibroblast transition blocking choline uptake with hemicholinium, and increasing myofibloblast markers inhibiting acetylcholinesterase with neostigmine.

Nowadays it is believed that cigarette smoke contains more than 6000 compounds and possibly this list will growth in line with the new analytical techniques available [23]. Taken in account this assertion, we know that cigarette smoke extract (CSE), have the advantage of containing all of the compounds inhaled by smokers. However, due to the very complexity of CSE, it is difficult to identify the specific agent mediating precise effect because differences found for particular concentrations and durations of exposure of a given agent of CSE. To that end, one can perform a dose–response and time course and make some crude calculations to suggest that exposure mimics what might happen in vivo. Thus for example, low concentrations of CSE (under 5%) have shown proliferative effects on lung fibroblasts [24-26], while higher (more than 10%) concentrations showed inhibitory effects on lung fibroblast proliferation [27], thus indicating that between the ~6,000 substance of cigarette smoke, there are some proliferative and other anti-proliferative compounds that may act depending of their final balance.

In the present work we have used 2.5% of CSE, which is in agreement with the low doses of CSE demonstrating proliferative effects in previous studies [24-26] which support fibroblast activation in the process of myofibroblast transition [28].

Preliminary data observed in our laboratory indicated that CSE at 2.5% concentration had a slight increase of fibroblast proliferation after 48 h of exposure (based on BrDU incorporation assay; data not shown). However, this increase was not enough to perform inhibitory experiments with aclidinium bromide. This was the main reason to discard these
experiments, since the well established myofibroblast markers collagen type I and α-SMA are more reliable in mechanistic studies [3]. However, even if one could calculate exposure, experiments performed with CSE cannot mimic all of the components of the microenvironment that exist in living systems (as cell-cell interactions). Thus, despite limitations, results from these studies allow to determine the capacity of CSE to influence cellular functions while eliminating other variables.

Intracellular cAMP is a second messenger that mediates a high number of anti-inflammatory processes. In addition to its anti-inflammatory actions, cAMP also controls the inhibition of fibroblast activation, as well as the myofibroblast transition [29]. Thus, a decrease of the levels of cAMP could promote fibroblast to myofibroblast transition. This is evidenced by TGFβ1, which increases the expression and activity of phosphodiesterases, the enzymes that degraded cAMP, promoting myofibroblast transition [30]. In the case of cigarette smoke, it has been shown that hydrogen peroxide, a component of tobacco smoke, swiftly elevates the activity of phosphodiesterase 4D3 attributed to PI3K and ERK-dependent phosphorylations [31]. In this respect, we have previously observed that CSE reduces intracellular cAMP levels in human differentiated bronchial epithelial cells by means of phosphodiesterase 4B upregulation (data not shown). In this work, we observed that CSE decreases intracellular levels of cAMP and that this effect was partially reversed by the antioxidant NAC and aclidinium bromide, suggesting a role of intracellular ROS. Furthermore, AChE also attenuated the CSE-reduced cAMP, which indicates the involvement of the non-neuronal cholinergic system. This may be explained by the fact that the main M receptor expressed on lung fibroblasts is the M2 [32], which is coupled to Gi protein, so stimulation of M2 by means of a non-neuronal cholinergic system could also
promote the inhibition of adenylate cyclase (AC)/cAMP pathway, and therefore, myofibroblast transition.

In line with our results, recently, it has been found that PGE2, as a potent cAMP enhancer, may inhibit the activation of fibroblast and the transformation into myofibroblast [33, 34]. Thus, we cannot discard that one of the mechanisms of aclidinium bromide mediating the increase of cAMP and consequently the inhibition of myofibroblast transition could be the increase of PGE2 release.

Another pathway we investigated was ERK1/2 signaling. Previous studies have shown that ERK1/2 participates in fibroblast to myofibroblast transition [35] and that CSE directly phosphorylates ERK1/2 [9]. Thus, CSE-induced myofibroblast transition could be mediated in part by ERK1/2. In this work, we observed that CSE induced the phosphorylation of ERK1/2, which was reduced by aclidinium bromide and by the antioxidant treatment. Moreover, AChE also attenuates the CSE-induced ERK1/2 phosphorylation, suggesting that the activation of M receptors is also implicated. Previous results support the notion that M receptor activation increases collagen expression in human lung fibroblasts by means of ERK1/2 activation [12]. Therefore, a non-neuronal cholinergic system may also be participating in the process of CSE-induced ERK1/2 phosphorylation. Thus, the role of ERK1/2 in the CSE-induced myofibroblast markers was confirmed by the inhibitory effect of the ERK1/2 antagonist PD98059.

Based on these results, we may conclude that CSE activates M receptors in human bronchial fibroblasts by a non-neuronal cholinergic system, and that this mechanism is involved in the upregulation of the myofibroblasts markers. However, the answer to which isoform of M receptors plays a significant role in this mechanism is unclear. It has been shown that the inhibition of the Gi protein with pertussis toxin reduces the fibroblast
proliferation and collagen expression observed in response to muscarinic agonists [12], suggesting a key role for the M2 receptor. However, antagonists for M1, M2 and M3 were all effective at inhibiting the fibroblast proliferation induced by acetylcholine [9], although the specific M antagonists available are not fully selective [36]. To address this issue, we selectively silenced M1, M2 and M3 genes using siRNA. Our results suggest that all M1, M2 and M3 receptors participate in CSE-induced expression of myofibroblast markers.

In summary, we have demonstrated that cigarette smoke, which is the main risk factor of COPD and participates in lung remodeling, may increase myofibroblast markers collagen type I and α-SMA through the activation of a non-neuronal cholinergic system, which is attenuated by the anticholinergic aclidinium bromide. Therefore, results observed in this work support that aclidinium bromide may play a role in regulating peribronchiolar fibrotic remodeling of COPD in addition to the classical bronchodilator activity.
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Competing interests

AG and MM are employees of Almirall.
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Figure legends

Figure 1. CSE time- and dose-dependently increases collagen type I (Col Type I) and alpha-smooth muscle actin (α-SMA) in human bronchial fibroblasts. Human lung fibroblasts were stimulated with cigarette smoke extract (CSE) at the indicated times (A, B) or for 48 h at the indicated concentrations (C to F). (A to D) After incubation, the RNA was extracted and subjected to RT-PCR with Col type I and α-SMA-specific primers and probes. (E, F) After incubation, total protein was extracted and western blots were performed with specific antibodies for Col Type I and α-SMA. Bars from graphs E and F represent densitometries of three different western blots. Each bar represents the mean ± SEM of 4 (A to D) or 3 (E and F) independent experiments. One-way repeated measures analysis of variance (ANOVA): \( P < 0.001 \) (A to F). Post hoc Bonferroni test: \(*P < 0.05\) compared with solvent controls.
Figure 2. Aclidinium bromide dose-dependently reduces the CSE-induced collagen type I (Col Type I) and alpha-smooth muscle actin (α-SMA) in human bronchial fibroblasts. Human lung fibroblasts were stimulated with cigarette smoke extract (CSE) for 48 hours. Aclidinium bromide (ACL) or atropine (ATR) were added to the medium at the indicated concentrations 30 minutes before CSE. Total RNA (A, B and D, E) and protein (C and F) were extracted after the incubation period. A, B and D, E graphs show RT-PCR for Col Type I (A and D) or α-SMA (B and E). Graphs C and F represent values of densitometry of Col Type I or α-SMA protein expression relative to β-actin and normalized to solvent controls. Representative western blots of Col Type I and α-SMA are shown. Each graph represents the mean ± SEM of three experiments for western blots and four independent experiments for RNA experiments. One-way repeated measures analysis of variance
(ANOVA): $P < 0.001$ (A–C). Post hoc Bonferroni test: $^*P < 0.05$ compared with solvent controls; $^#P < 0.05$ compared with stimulus.

**Figure 3.** Aclidinium bromide mitigates CSE-induced intracellular ROS generation in human bronchial fibroblasts. (A) Human bronchial fibroblasts were loaded with H$_2$DCF-DA 30 minutes before cigarette smoke extract (CSE) stimulation. CSE dose and time-dependently increased intracellular ROS in human bronchial fibroblasts. (B and C) Human bronchial fibroblasts were loaded with H$_2$DCF-DA in the presence or absence of aclidinium bromide (ACL; 10$^{-10}$M-10$^{-7}$M), apocynin (APO; 100 µM), N-acetyl-l-cysteine (NAC; 1 mM), dbcAMP (1 mM) or vehicle for 30 minutes. Excess H$_2$DCF-DA was removed by
washing with PBS. ACL, APO, NAC, dbcAMP or vehicle were appropriately replenished before cells were exposed to CSE. (B) Representative DCF fluorescence images following 24 h of basal or CSE 2.5% stimulation in presence or absence of ACL. (C) ACL dose-dependently attenuated the CSE 2.5%-induced intracellular ROS after 24 h of stimulation. Similar results were found for APO, NAC and dbcAMP. Results represent the means ± SEM of three independent experiments. One-way repeated measures analysis of variance (ANOVA): $P < 0.001$ (A and C). Post hoc Bonferroni test: *$P < 0.05$ compared with solvent controls; #$P < 0.05$ compared with stimulus.

**Figure 3**

**Figure 4.** Aclidinium bromide attenuates the CSE-induced p67phox and NOX4 upregulation in human bronchial fibroblasts. (A) RNA was obtained from primary human bronchial fibroblasts and mRNA transcripts for NOX1, p67phox, p47phox and NOX4 were quantified and normalized setting p67phox as 100%. (B–D) Aclidinium bromide (ACL)
dose-dependently attenuated the cigarette smoke extract (CSE) 2.5%-induced p67phox and NOX4 mRNA (B and C) and protein (D) upregulation following 24 h of stimulation. Results represent the means ± SEM of 3 independent experiments. One-way repeated measures analysis of variance (ANOVA): $P < 0.001$ (A–C). Post hoc Bonferroni test: 

* $P < 0.05$ compared with solvent controls; 
# $P < 0.05$ compared with stimulus.

Figure 5. CSE activates non-neuronal cholinergic system, decreases cAMP and phosphorylates ERK1/2. Human bronchial fibroblasts were pre-incubated for 30 minutes
with (A-C) aclidinium bromide (ACL: $10^{-9}$M-$10^{-7}$M), (A, B and D) apocynin (APO: 100 µM), N-acetyl-L-cysteine (NAC: 1 mM) or (B, D) acetyl cholinesterase (AChE: 10 U/mL) followed by the stimulation with CSE at 2.5% over 24 h. (A) total protein was extracted and western blots for choline acetyltransferase (ChAT) and β-actin, as internal control, were performed. Representative western blots are shown from three different experiments. (B) Cells were lysed and intracellular cAMP was quantified using cAMP Biotrak enzyme immunoassay (EIA) system. (C, D) Total protein was extracted and phosphorylated ERK1/2 and non-phosphorylated ERK1/2 (as internal control) protein expression were determined by western blots. Bars from graphs A, C and D represent densitometries of three different western blots. Results represent the means ± SEM of 3-4 independent experiments. One-way repeated measures analysis of variance (ANOVA): $P < 0.01$. *Post hoc* Bonferroni test: *$P < 0.05$ compared with solvent controls; #$P < 0.05$ compared with stimulus.
Figure 6. The CSE-induced collagen type I (Col Type I) and alpha-smooth muscle actin (α-SMA) are partially mediated by a non-neuronal cholinergic pathway. Human bronchial fibroblasts were pre-incubated for 30 minutes with aclidinium bromide (ACL: 10^{-7} M), apocynin (APO: 100 µM), N-acetyl-L-cysteine (NAC: 1 mM), PD98059 (10 µM), dbcAMP (1 mM), acetyl cholinesterase (AChE: 10 U/ml), hemicholinium-3 (HC-3: 50 µM) or neostigmine at 10 µM followed by the stimulation with cigarette smoke extract (CSE) at 2.5% over 24 hours. Total RNA (A and C) or protein (B and D) were extracted to quantify mRNA transcripts and protein expression for Col Type I and α-SMA using appropriated primers and antibodies. Panel A and C represent values of Col Type 1 and α-SMA mRNA expression...
expression normalized to control group using GAPDH as internal control. Panel B and D represent values of densitometry of Col Type I or α-SMA protein expression relative to β-actin and normalized to solvent controls. Representative western blots of Col Type I and α-SMA are shown. Each graph represents the mean ± SEM of 3-4 independent experiments for western blots and for RNA experiments. One-way repeated measures analysis of variance (ANOVA): $P < 0.001$ (A and B). Post hoc Bonferroni test: *$P < 0.05$ compared with solvent controls; #$P < 0.05$ compared with stimulus.

Figure 6
**Figure 7.** CSE increases myofibroblast markers by a non-neuronal cholinergic signaling mediated by M1, M2 and M3 receptors. Human bronchial fibroblasts were selectively transfected with silencing mRNA (siRNA: 50 nM) for M1, M2 and M3 or with the negative control siRNA(-) for 48 hours using lipofectamin at final concentration of 2 µg/ml. (A and B) All siRNA targeting M1, M2 and M3 genes were able to reduce mRNA and protein expression for M1, M2 and M3 significantly without effecting other M receptors. β-actin was assayed as positive control. Cells transfected with siRNA for M1, M2 and M3 genes attenuated the cigarette smoke (CSE) 2.5%- induced Col Type I and α-SMA protein upregulation. Results are the mean ± SEM of three independent experiments, and representative of three independent experiments per condition for western blots. One-way repeated measure analysis of variance (ANOVA): \( P < 0.001 \) (A). *Post hoc* Bonferroni test: \* \( P < 0.05 \) compared with siRNA (-) controls.