

Original article

Muscarinic receptors, leukotriene B₄ production and neutrophilic inflammation in COPD patients

Background: Acetylcholine (ACh) plays an important role in smooth muscle contraction and in the development of airway narrowing; preliminary evidences led us to hypothesize that ACh might also play a role in the development of airways inflammation in chronic obstructive pulmonary disease (COPD).

Methods: We evaluated the concentrations of leukotriene B₄ (LTB₄) in induced sputum, and the expression of ACh M1, M2, and M3 receptors in sputum cells (SC) obtained from 16 patients with COPD, 11 smokers, and 14 control subjects. The SC were also treated with ACh and the production of LTB₄ assessed in the presence or absence of a muscarinic antagonist (oxitropium). In blood monocytes, we evaluated LTB₄ release and activation of the extracellular signal-regulated kinases (ERK) pathway after treatment with ACh.

Results: The LTB₄ concentrations were higher in COPD than in controls ($P < 0.01$) and correlated with the number of neutrophil ($P < 0.01$). The M3 receptors expression was increased in COPD subjects when compared to smokers and control ($P < 0.05$ and 0.0001 , respectively), while M2 expression resulted decreased ($P < 0.05$ and 0.01). The ACh-induced LTB₄ production was observed in peripheral blood monocytes, and was sensitive to ERK inhibition. Similarly, ACh significantly increased neutrophil chemotactic activity and LTB₄ released from SC of COPD patients only, and these effects were blocked by pretreatment with the inhibitor of ERK pathway PD98059.

Conclusions: The results obtained show that muscarinic receptors may be involved in airway inflammation in COPD subjects through ACh-induced, ERK1/2-dependent LTB₄ release. Muscarinic antagonism may contribute to reduce neutrophil infiltration and activation in COPD.

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†During the course of this study Prof. Maurizio Vignola untimely died after suffering a malignant leukemia. We wish to dedicate this work to his memory.

Key words: chronic obstructive pulmonary disease; ERK1/2; induced sputum; leukotriene B₄; muscarinic receptors.

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Inflammation in chronic obstructive pulmonary disease (COPD) is characterized by an increased infiltration of neutrophils, lymphocytes and macrophages into the airways (1). Among these cells, neutrophils play an important role in the pathogenesis of airway inflammation in COPD due to their ability to release many mediators, such as elastase, metalloproteases and oxygen-derived radicals which promote tissue inflammation and damage (2). It is likely that neutrophil accumulation in the airways of COPD subjects is driven by an increased release of mediators exerting a chemotactic effect on these cells (3). Although the precise mechanism by which neutrophils traffic from blood to the lung tissue remains unsettled, leukotriene B₄ (LTB₄), whose levels are

increased in induced sputum of COPD subjects (4), may represent a mediator contributing to neutrophils influx into the airways and, possibly, to disease progression.

Acetylcholine (ACh) contributes to the control of airways tone and bronchial secretion and it is an important factor contributing to airway obstruction in airway diseases (5). Morphologic evidence of a neuronal supply of ACh to the airways was reported in the last century and cholinergic innervation has been demonstrated in the airways of various species (6). It is possible that in asthma and COPD, released ACh may diffuse to the alveolar spaces and bronchial lumens exerting inflammatory effects, but the involvement of nonneuronal sources of ACh (7) in inflammatory disease have also recently

drawn considerable attention, as patients with atopic dermatitis were shown to have a substantial increase in their skin ACh content (8). Recent studies in an animal model showed that ACh is involved in the regulation of inflammatory processes through the release of eosinophil and neutrophil chemotactic activity from bronchial epithelial cells (9) and alveolar macrophages (10), and that these ACh pro-inflammatory activities could be related to an increased release of LTB₄ by M3 ACh stimulation (10).

The activity of ACh is mediated by five different mammalian muscarinic receptors (M1–M5) of which only M1, M2, and M3 are known to exert physiological effects in the lungs (11). The cholinergic activation of M3 receptors causes smooth muscle contraction in the airways while M2 receptors act as autoreceptors, located prejunctionally on postganglionic nerves, limiting the release of ACh and reducing smooth muscle contraction (12). M1 or M3 receptor stimulation promotes the activation of protein kinase C (PKC) activating intracellular signals (13) such as the mytogen-activated protein kinase (MAPK) pathway (14), while M2 cholinergic stimulation inhibits the adenylate cyclase activity and consequently results in a decrease of intracellular cyclic AMP (cAMP) levels (13).

In the present study, we assessed the potential involvement of muscarinic receptors expressed on inflammatory cells in airway inflammation of COPD patients. The LTB₄ concentrations were measured in sputum supernatants (SSs), and the expression of muscarinic M1, M2, and M3 receptors was assessed on inflammatory cells recovered from induced sputum of control, smokers without airflow obstruction and COPD subjects. Furthermore we tested the involvement of M receptors and the role of ERK pathway activation in ACh-stimulated release of chemotactic activity and LTB₄ in SC as well as in peripheral blood monocytes.

Methods

Patients

The study was performed on three groups of subjects: 16 patients with COPD (70% of which active smokers), 11 active smokers without COPD (smokers without airflow obstruction) and 14 non-smoker control subjects (Table 1).

COPD was defined and classified according to the criteria reported by GOLD guidelines (15). All subjects were smokers or ex-smokers (38, 24–52 packs/year) and were classified as stage ≥ 1 according to GOLD. All COPD patients having routine chest X-ray and computed tomographic scan that showed obvious emphysema were excluded. Reversibility test to bronchodilator was performed to exclude an asthmatic component, and the increase in FEV₁ was lower than 12% and 200 ml, when compared to basal values, in all COPD subjects.

Smokers without airflow obstruction had a pulmonary function within the normal range.

COPD and smokers without airflow obstruction groups included subjects with chronic bronchitis as described by the presence of cough

Table 1. Demographic characteristic of Subjects

	Control	Smoker	COPD
Number	14	11	16
Sex (male/female)	9/5	6/5	10/6
Age (years)	60 (36–73)	63 (32–72)	67 (61–72)
Current smokers	0/14	11/11	11/16
Packs/year	0	50 (28–56)	38 (24–52)
FEV ₁ (%)	109 (101–115)	93 (91–100)	57 (44–84)*
Chronic bronchitis	0	3/11	7/16

Results are expressed as median (25–75%) COPD, chronic obstructive pulmonary Disease. Statistical analysis was carried out by nonparametric Bonferroni Dunn's *post hoc* test.

* $P < 0.0001$ vs control; $P < 0.0001$ vs smoker.

and sputum production for at least 3 months/year over the previous 2 years (Table 1).

Normal subjects were also studied as a control group. None of these subjects had ever suffered from asthma, chronic bronchitis or from any bronchial or respiratory tract infection during the month preceding the study. All subjects were lifelong non-smokers, and their pulmonary function was within the normal range.

Subjects were excluded if they had had a bronchial infection during the month preceding the study. No subject had received corticosteroids, anticholinergics, or theophylline in any form during the 2 months prior to the study.

The local ethic committee approved the study, and participating subjects gave their informed consent.

Sputum induction and processing

Sputum was induced by the nebulization of sterile hypertonic saline solution as previously described (16).

Sputum processing was performed according to the methods of Hargreave et al. (17). Cytospins were used for differential cell counts and for immunocytochemistry as previously described (18), and air-dried slides were stored at -80°C until use for immunocytochemical staining.

Measurement of LTB₄

Concentrations of LTB₄ in SS and in supernatants of SC or monocytes stimulated with ACh, were assessed by HPLC separation followed by specific enzyme immunoassay (EIA) using a commercially available kit (Amersham International plc Little Chalfont, Buckinghamshire, UK), as previously described (16).

Immunocytochemistry of muscarinic receptors

The immunostaining of M1, M2, and M3 muscarinic receptors on SC was examined in 14 control, nine smokers and 16 COPD subjects, with the use of: a rabbit polyclonal antibody directed against the human mAChR M1 (H-120 Santa Cruz Biotechnology Inc., Santa Cruz CA, recognizes human, mouse and rat mAChR M1); a goat polyclonal antibody directed against the human mAChR M2 (C-18 Santa Cruz Biotechnology, recognizes human, mouse and rat mAChR M2); a rabbit polyclonal antibody directed against the human mAChR M3 (H-210 Santa Cruz Biotechnology, recognizes human, mouse and rat mAChR M3). These antibodies were used at the dilutions of 1 : 10, 1 : 10, and 1 : 20, respectively, for overnight

incubation. The AChR antibodies were shown to be specific for the muscarinic ACh receptor subtype against, which they were raised as they do not cross-react with other subtypes or with nicotinic receptors (19). The slides were stained by the immuno-alkaline-phosphatase method (Dako LSAB, Glostrup, Denmark) as previously described (18). Negative controls were performed using an irrelevant IgG anti-rabbit of the same isotype and at the same concentration of the primary Ab.

Effects of ACh on monocytes

Normal donors peripheral blood monocytes were stimulated with ACh (100 μ M), in the presence or absence of OB (10 μ M; 1 h before ACh treatment), or of the specific inhibitor of ERK1/2 pathways PD98059 (25 μ M; 1 h before). At the end of incubation, supernatants and cells were collected for LTB₄ measurements and western blot analysis, respectively.

Western blot analysis of total and phosphorylated ERK1/2 MAP kinases

50 μ g of total proteins from monocyte lysates were separated by SDS-PAGE, transferred onto nitrocellulose membranes and probed with anti-phospho-ERK1/2 rabbit antibody, anti-ERK antibody (New England Biolabs), and anti- β -actin antibody (Sigma). Primary antisera were visualized with HRP-conjugated secondary antibody (Sigma) and developed with an enhanced chemiluminescence system (Amersham Life Sciences).

Effect of ACh on induced sputum cells

The SC from three COPD patients was incubated with increasing concentrations of ACh (1–100 μ M) (Sigma St. Louis, MO) for 4 h according to Sato (10). Cells were also preincubated with oxitropium bromide (OB) at various concentrations (1–100 μ M) for 1 h before stimulation with ACh 100 μ M. Based on these results, SC recovered from six controls, six smokers and six COPD subjects were stimulated with ACh (100 μ M), in the absence or presence of OB (10 μ M), and supernatants evaluated for the presence of LTB₄ and for their neutrophil chemotactic activity.

Purification of peripheral blood neutrophils and monocytes, and neutrophil chemotaxis assay

Peripheral polymorphonuclear leukocytes and monocytes were prepared from normal donors with the use of dextran sedimentation and centrifugation over Ficoll cushions, followed by adhesion, as previously described (16, 20).

Normal neutrophils were incubated with supernatants from SC stimulated with ACh 100 μ M (4 h at 37°C), in the presence or absence of the LTB₄ receptor antagonist LY 223982 (10 μ M; Eli Lilly, Basingstoke, UK) and chemotaxis was evaluated as previously described (16).

Statistical analysis

Statistical analysis was performed using nonparametric Kruskal–Wallis test and Dunn *post hoc* test with Bonferroni correction for multiple comparisons or Wilcoxon's signed rank-sum test, as appropriate. Correlations were evaluated using the Spearman's rank test. A probability level of $P \leq 0.05$ values was selected as statistically significant.

Results

Demographic characteristics of patients

As expected FEV₁ after bronchodilators was significantly lower in COPD patients than smoker and control subjects ($P < 0.0001$) (Table 1).

Total and differential cell counts in induced sputum

Total cell count, viability and percentage of squamous cells were not significantly different among groups. The percentage of macrophages and neutrophils were not significantly different between COPD and smokers without airflow obstruction, but neutrophils resulted higher ($P < 0.001$) and macrophages lower ($P < 0.01$) in COPD when compared to control subjects (Table 2).

LTB₄ measurement in induced sputum supernatant

The concentrations of LTB₄ in induced sputum samples of COPD patients (2.7, 1.1–4.9 ng/ml; median and 25–75%) were higher than controls (0.74, 0.3–1.1 ng/ml) and smokers (1.2, 1–2 ng/ml), but the difference was statistically significant only between COPD and controls ($P < 0.01$) (Fig. 1, Panel A). Furthermore, when analyzed together with the number of neutrophils collected in induced sputum, LTB₄ concentrations in COPD patients showed a statistically significant direct correlation ($P < 0.05$; $\rho = 0.65$) (Fig. 1, Panel B).

Immunocytochemistry of muscarinic receptors in sputum cells

The immunostaining of induced SC showed expression of all three muscarinic receptor subtypes in control, smoker, or COPD subjects (Fig. 2).

The percentage of total SC expressing M3 receptors was significantly increased in COPD when compared to controls and smokers ($P < 0.0001$ and 0.05, respec-

Table 2. Total and differential cell count in SC

	Control	Smoker	COPD bronchitis
Squamous cells (%)	3 (1.8–4.7)	3.5 (1.2–6)	1.2 (0.1–3.5)
Total cell counts (10 ⁶ cells/g sputum)	4.8 (2.2–8.7)	4.3 (3.6–6)	7.6 (3–15.9)
Differential cell counts (%)			
Macrophages	79.5 (67.8–88.1)	50.6 (48.5–68.3)	24.6 (12.4–72.2)*
Neutrophils	18.5 (9.1–29.1)	46.1 (30.2–48.2)	72.8 (22.2–85.5)*
Lymphocytes	0.7 (0–1.2)	0.7 (0.4–1.2)	0 (0–0.5)
Eosinophils	0 (0–0.6)	0.8 (0–1.4)	0.8 (0.2–2.3)
Epithelial cells	0.7 (0.5–2.2)	0 (0–0.4)	0.2 (0–1.7)
Viability (%)	77.8 (63.9–86.2)	76 (74.2–85.3)	79.7 (67.5–88.7)

Results are expressed as median (25–75%) of total number of cells or of the percentages observed in the differential cell count. COPD, chronic obstructive pulmonary disease. Statistical analysis was carried out by nonparametric Bonferroni Dunn's *post hoc* test.

* $P < 0.01$ vs control.

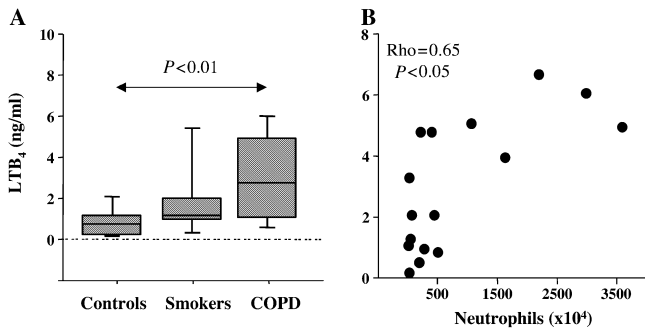


Figure 1. Panel A: LTB₄ levels in induced SS of control, smoker, and COPD subjects. Results are expressed as median, 25–75% (box) and minimum-maximum (error bars). Kruskal–Wallis test and Dunn *post hoc* test with Bonferroni statistical analysis were performed, with correction for multiple comparisons. Panel B: Correlation between LTB₄ concentrations in induced SS of COPD patients and absolute values of neutrophils in induced sputum. Statistical analysis was performed using Spearman’s rank correlation test.

tively), while the expression of M2 receptors concomitantly decreased ($P < 0.01$ and 0.05 vs controls and smokers, respectively) (Fig. 3, Panels B and C).

Similarly to what observed for M3 receptor, the percentage of total SC expressing M1 receptors was higher in COPD than in control and smoker subjects, but

statistical significance was only reached vs controls ($P < 0.05$) (Fig. 3, Panel A).

Macrophages appeared to be the main cell type expressing M2 and M3 receptor subtypes in all subject studied, while, in COPD subjects only, neutrophils were the cell type that exquisitely expressed M1 receptors (Table 3).

M2 and M3 protein expression was also found in peripheral blood neutrophils monocytes, while monocytes only expressed M3 subtype (data not shown).

Release of LTB₄ following ACh stimulation in sputum cells

Based on the dose-responses obtained in a limited number of SC obtained from COPD subjects (data not shown), ACh 100 μ M and OB 10 μ M were selected as optimal concentrations for maximal effect in subsequent studies.

A spontaneous release of LTB₄ over the 4 h incubation time was observed in SC isolated from control (479 ± 254.4 pg/ml; mean $\pm$ SD) and smoker subjects (387.5 ± 193.4 pg/ml). In the presence of treatment with ACh (100 μ M), no statistically significant increase of LTB₄ release was observed (505.2 ± 238.8 and 428.2 ± 151.6 pg/ml, respectively) (Fig. 4, Panels A and B, respectively).

The average spontaneous release of LTB₄ in SC from COPD was 526 ± 283 pg/ml, and exposure to ACh

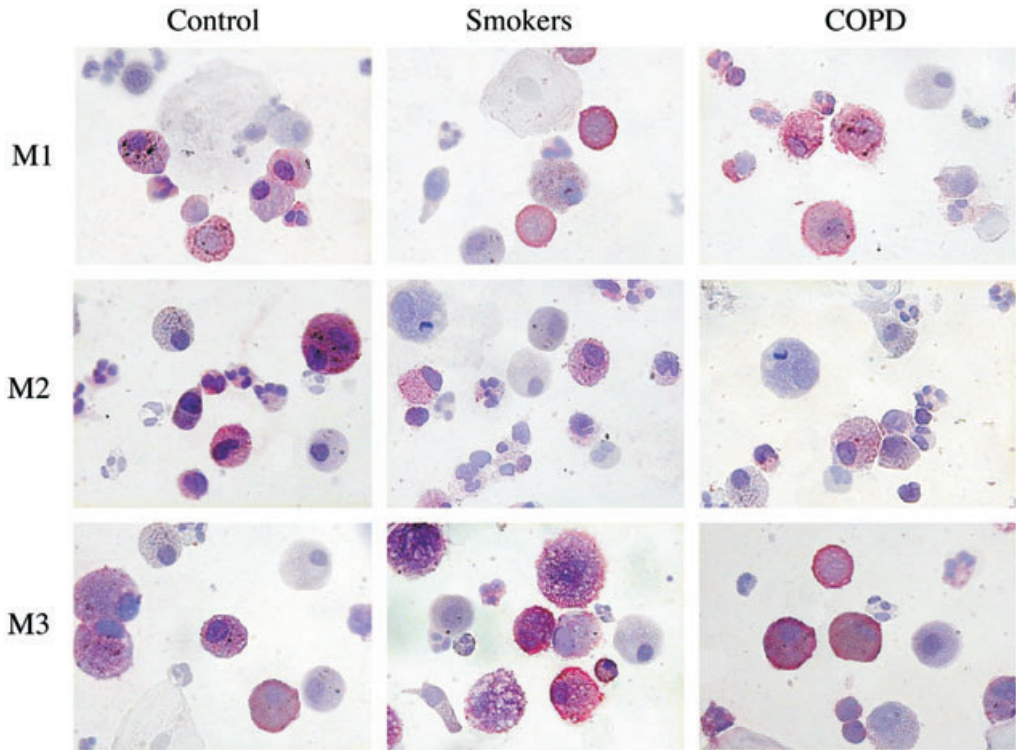


Figure 2. Expression of muscarinic receptors M1, M2, and M3 of cells isolated from induced sputum obtained from control, smoker, and COPD subjects. Immunocytochemistry was performed on cytospin preparations using the immuno-alkaline-phosphatase method, as described in Methods (Original magnification 400 $\times$).

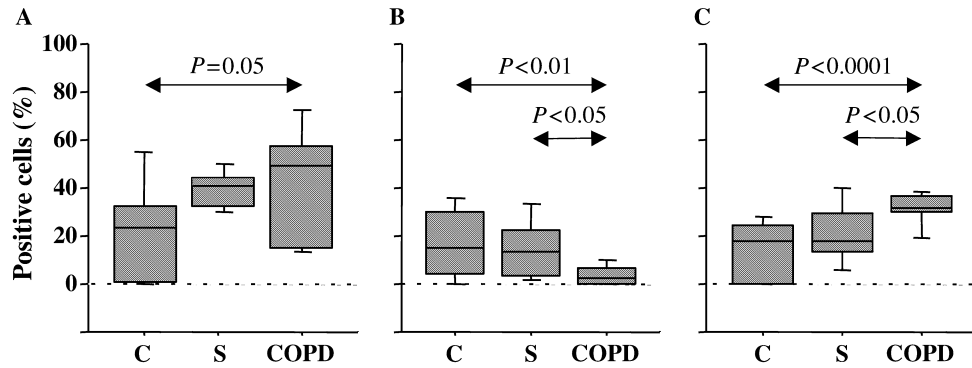


Figure 3. Quantitative analysis of the expression of muscarinic receptors in SC of control, smoker, and COPD subjects. Immunocytochemistry was performed using the immuno-alkaline-phosphatase method, as described in Methods. Panel A: Percentage of induced SC expressing M1 receptor in control (C), smoker (S), and COPD, respectively. Panel B: Percentage of induced SC expressing M2 receptor in C, S, and COPD, respectively. Panel C: Percentage of induced SC expressing M3 receptor in C, S, and COPD, respectively. Results are expressed as median, 25–75% (box) and minimum-maximum (error bars). Statistical analysis was performed by Dunn *post hoc* test with Bonferroni correction for multiple comparisons.

Table 3. Expression of M1, M2, and M3 subtype mAChRs in SC

	Control	Smoker	COPD
M1 receptors			
Macrophages	3.7 (0–20.9)	26.2 (23.5–30.7)	13.2 (6.5–31.4)
Neutrophils	2.6 (0–11.4)	10.8 (7.6–14.8)	16 (11.5–28.6)
Eosinophils	0	0 (0–1.3)	1.7 (0–2.7)
Lymphocytes	0	0	0
Epithelial cells	0	0	0
M2 receptors			
Macrophages	11.7 (0–22.1)	9 (2–20.1)	2.3 (2–3)**
Neutrophils	3.9 (2.8–6.9)	3.2 (0–3.7)	0.3 (0–2)*
Eosinophils	0	0	0
Lymphocytes	0	0	0
Epithelial cells	0	0	0
M3 receptors			
Macrophages	18 (0–21.3)	14 (11.1–30.7)	28.3 (27.1–32.9)**
Neutrophils	0 (0–2.2)	2.1 (4.2–4.3)	2.8 (0–6.9)
Eosinophils	0	0	0
Lymphocytes	0	0	0
Epithelial cells	0	0	0

Results are expressed as median (25–75%) of different cell expressing mAChRs over the total number of cells (%). COPD, chronic obstructive pulmonary disease. Statistical analysis was carried out by nonparametric Bonferroni Dunn's *post hoc* test.

* $P < 0.05$ vs control.

** $P < 0.01$ vs control.

(100 μ M), caused a statistically significant increase to 781 ± 360 pg/ml ($P < 0.05$). Pretreatment with OB (10 μ M, 1 h before ACh stimulation) was able to prevent the ACh-induced release of LTB₄ (569 ± 264 pg/ml) ($P < 0.05$) (Fig. 4, Panel C).

Release of LTB₄ and ERK1/2 activation in ACh-stimulated peripheral blood monocytes

A spontaneous release of LTB₄ was observed in peripheral blood monocytes upon incubation for 4 h (184 ± 70 pg/ml, mean $\pm$ SD); this release was

increased when cells were incubated in the presence of ACh (100 μ M) (229 ± 74 pg/ml), and, albeit modest in absolute value (+25%), the increase resulted statistically significant ($P < 0.05$).

To investigate the intracellular mechanism of ACh-induced LTB₄ release we analyzed the activation of ERK1/2 pathways following ACh challenge and we found that ACh indeed causes significant phosphorylation of ERK1/2. Preincubation with either the specific ERK pathway inhibitor PD98059 (25 μ M) (21) or the nonselective muscarinic antagonist (OB 10 μ M; 1 h before ACh) fully prevented both the observed increase in LTB₄ release (170 ± 60 pg/ml, n.s. vs basal) and the phosphorylation of ERK1/2 (Fig. 5).

Neutrophil chemotaxis assay

The supernatants of COPD subject SC stimulated with ACh in the presence or absence of the specific ERK pathway inhibitor PD98059 (25 μ M) were tested for their ability to induce neutrophils chemotaxis. Supernatants recovered from ACh-stimulated cells showed a significantly increased chemotactic activity for neutrophils (149.2 ± 22.4 cells/high power field; mean $\pm$ SD) when compared to supernatants from ACh-untreated cells (111.5 ± 11.2 ; $P < 0.05$). The increase was not observed in SC treated with ACh in the presence of PD98059 (113.5 ± 23 ; $P < 0.05$ vs ACh) (Fig. 6), clearly showing that the increase in chemotactic activity was secondary to muscarinic receptor activation of ERK phosphorylation. The observed increase was also inhibited by neutrophil pretreatment with the LTB₄ receptor antagonist LY 223982 (104 ± 32.6 ; $P < 0.01$ vs ACh), suggesting that LTB₄ was the chemotactic factor released, and providing a link between ACh release, inflammatory cell activation, LTB₄ and neutrophilic influx within the airways of COPD subjects.

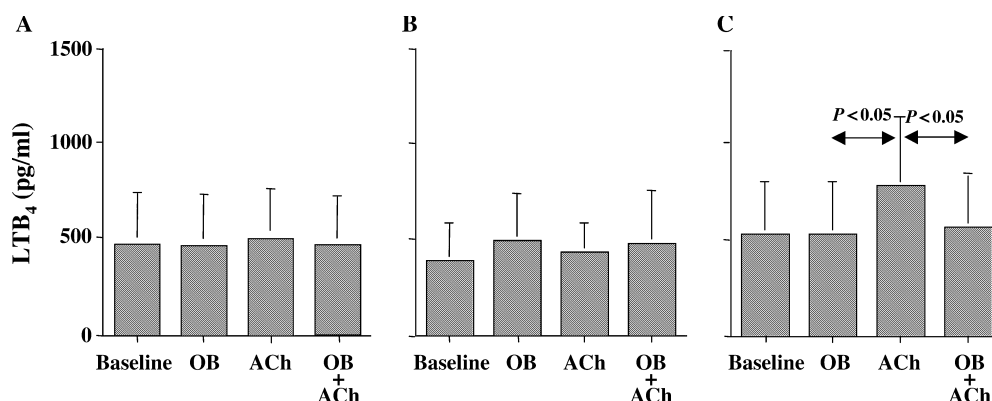


Figure 4. LTB₄ release by SC isolated from the airways of control (Panel A), smoker (Panel B) and COPD (Panel C) patients incubated for 4 h with ACh (100 μ M) at 37°C in the presence or absence of OB (10 μ M). Results are expressed as mean and SD. Statistical analysis was performed by Wilcoxon *U* signed rank-sum test with Bonferroni's correction.

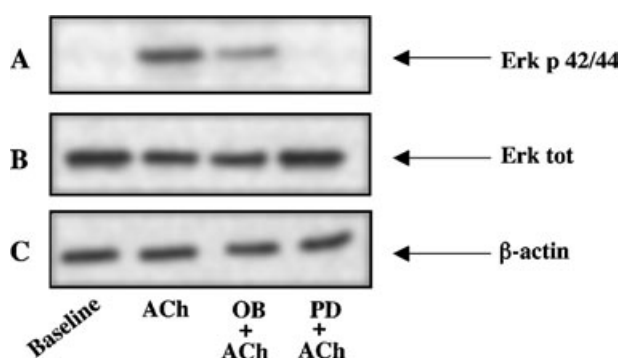


Figure 5. The ACh induced ERK1/2 activation in monocytes obtained from normal donors. Monocytes were incubated for 15 min with ACh (100 μ M) at 37°C in the presence or absence of OB (10 μ M) or PD 98059 (25 μ M; PD). Cells were then analyzed for the expression of the activated forms of ERK1/2 (A), total ERK1/2 (B), and β -actin (C) by western blots as described in methods.

Discussion

It is now accepted that COPD is characterized by a chronic inflammation and that neutrophils, macrophages and cytotoxic T-lymphocytes are the inflammatory cells mainly involved (1). Neutrophils can contribute to chronic inflammatory process leading to lung injury by the release of proteases, degrading extracellular matrix, and reactive oxygen species (22). Many factors can control the recruitment of neutrophils in the lungs, including the unique and complex structure of the pulmonary capillary bed, local haemodynamic factors, neutrophil deformability, neutrophil-endothelial interactions (2) and the release of mediators such as LTB₄ (23).

In the present study we showed that LTB₄ concentrations were higher in induced sputum obtained from

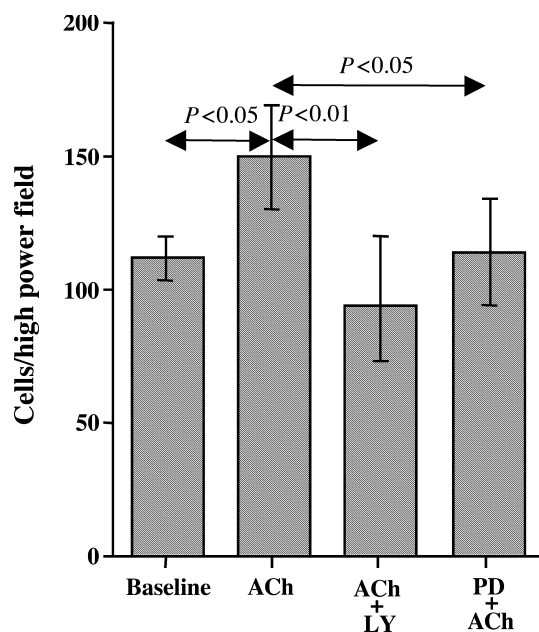


Figure 6. Neutrophil chemotactic activity of supernatants of SC obtained from COPD subjects stimulated with ACh (100 μ M) for 4 h at 37°C in the presence or absence of PD98059 (25 μ M; PD). Chemotactic activity was also assessed on neutrophils pretreated with the selective LTB₄ receptor antagonist LY223982 (10 μ M; LY). Results are expressed as median and SD. Statistical analysis was performed by Wilcoxon *U* test signed rank-sum test with Bonferroni's correction.

COPD subjects when compared to controls, and well correlated with the number of neutrophils present within the same induced sputum samples. These data are in agreement with previous studies showing that LTB₄ may have an important role in airway inflammation of COPD patients (4) contributing to the increased influx and activation of neutrophils into the airways and to the progression of the disease (23).

Factors leading to bronchial obstruction in COPD include mucus hypersecretion and an increase in bronchial muscle tone, which is triggered mainly by cholinergic mechanisms. Indeed, anticholinergic drugs are the bronchodilators of choice in the management of COPD, as the cholinergic tone appears to be the only reversible component of COPD. This is clearly pointing out to the potential contribution of an altered reactivity of the cholinergic system in the airways of COPD subjects. Based on data suggesting a potential involvement of ACh in the release of LTB₄ by alveolar macrophages (10), we studied the expression of muscarinic receptors on cells recovered with induced sputum. Among the five muscarinic receptor subtypes only M1, M2, and M3 are known to exert physiological effects on the lungs (11) and their action has been well described. Smooth muscle bronchoconstriction is activated via M3, and mucus secretion via M1 and M3 muscarinic receptors, while prejunctional M2 autoreceptors possess a significant inhibitory activity, providing a negative feedback to ACh release (12). Using immunocytochemistry, we found that in inflammatory cells obtained from COPD subjects, M2 expression is reduced and M3 expression is increased when compared to SC from smokers without airflow obstruction or controls.

Together with these differences in M receptor expression, ACh-stimulated SC obtained from COPD subjects produced increased amounts of LTB₄ when compared to cells obtained from controls and smokers without airflow obstruction, an effect that could be inhibited by pre-treatment of SC with the muscarinic antagonist OB, a nonselective anticholinergic drug blocking M2 and M3 receptors. Supernatant from ACh-stimulated SC obtained from COPD subjects also caused increased neutrophil chemotaxis, suggesting that ACh may contribute to regulate neutrophilic infiltration and activation through enhanced production of chemotactic factors such as LTB₄. Although we cannot rule out a potential contribution of other chemoattractant factors, such as IL-8, as well as the potential presence of significant chemotactic activity related to LTB₄ in control and smoker SC, the evidence that the increase in chemotactic activity observed in COPD was abolished when neutrophils were pretreated with a specific LTB₄ receptor antagonist, strongly suggest that the additional chemotactic activity resulting from ACh challenge may refer to the increased concentration of LTB₄ in SC supernatants.

A number of factor may potentially contribute to the observed differences in M receptor expression profiles and SC responsiveness between COPD and smokers or controls; in particular, exposure to cytokines such as transforming growth factor-1 (TGF- β 1), and tumor necrosis factor (TNF- α), known to be involved in the inflammatory process of COPD (24), as well as to modulate muscarinic expression (25), can certainly have

a major role and may contribute to install a vicious inflammatory circle within the airways of COPD subjects.

The RT-PCR expression of muscarinic receptor in blood leukocytes from different groups of subjects have been reported by several groups (26–28), with results showing a quite significant variability among study groups as well as among different subjects within the same study (28). Immunocytochemistry of peripheral blood neutrophils showed expression of both M2 and M3 receptors, while monocytes only appeared to express the M3 subtype; activation of PBM with ACh led to significant release of LTB₄ associated with, and depending upon, enhanced phosphorylation of ERK1/2, providing evidence of the potential relevance of phosphorylation processes in receptor-mediated activation of 5-LO. 5-Lipoxygenase activation and synthesis of leukotrienes have always been linked to the increase of intracellular calcium in 5-LO possessing cells. On the other hand calcium ionophore activation in HL-60 cells is also known to cause tyrosine kinase-dependent 5-LO translocation and formation of its main metabolite 5-HETE (29) and phosphorylation of 5-LO by ERK1/2 has been proposed to have a key role in 5-LO activation by stimuli such as phorbol myristate acetate and arachidonic acid (30).

Although our results were obtained in normal donors PBM, and the underlying pathways may vary between sputum and blood cells, the use of a specific inhibitor of ERK1/2 pathway (21) showed that ACh might regulate LTB₄ release through the activation of ERK1/2 pathway.

As observed in peripheral blood monocytes, ACh was able to enhance the release of LTB₄ from SC obtained from COPD subjects, but was ineffective on SC obtained from controls and smokers without airflow obstruction. Interestingly, metacholine challenge in asthmatic subjects, but not in healthy controls, has been shown to induce the release of LTB₄ and PGD₂, as evaluated by measurements of eicosanoids in BAL fluids (31), suggesting that this mechanism of activation may indeed take place *in vivo* as well as we observed it *ex vivo*. The effect of ACh on SC was well matched by an increased neutrophil chemotactic activity in the supernatant of the same cells, an increase that was specifically sensitive to an LTB₄ receptor antagonist as well as to the inhibition of ERK1/2 phosphorylation, further supporting a close link between ACh challenge, ERK1/2 pathway activation and LTB₄ synthesis.

Taken together, the different expression profile of muscarinic receptors and the ability of COPD SC to respond to ACh with increased production of biologically active LTB₄, clearly suggest a potential involvement of ACh in the release of neutrophil chemotactic factor(s) from cells within the airways of COPD subjects, and support the hypothesis that the cholinergic system, or nonneuronal ACh, may be involved in regulating the synthesis of inflammatory mediators such as LTB₄.

Future research may address the specific role of different receptor subtypes in the observed effect of ACh.

The ability of muscarinic antagonists, such as OB, to modulate the release of a potent chemotactic factor such as LTB₄ by monocyte/macrophages opens up new perspectives as far as the control of inflammatory cells migration into the airways of COPD patients, especially in light of potent and selective muscarinic antagonists becoming available. The effects of OB on the release of inflammatory mediators also provide support to the hypothesis that bronchodilators may contribute to the

control of inflammation in pulmonary disease such as COPD and asthma, therefore providing improved management of the pathology.

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