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Tiotropium suppresses acetylcholine-induced release of chemotactic mediators in vitro

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Summary
The driving force in the progression of COPD is the development of exacerbations which are mostly the result of excessive inflammation. Bronchodilatators play an important role in the treatment of COPD. The reported reduction in exacerbation rates in COPD is due to the inhibition of vagal-mediated bronchoconstriction and mucus secretion. However, recent studies have highlighted the existence of muscarinic receptors on inflammatory cells and we have explored the possibility that tiotropium bromide might also inhibit neutrophil migration. We analysed the influence of tiotropium on the release of neutrophil chemotactic activity in response to acetylcholine (ACh) and the expression of muscarinic receptors on human alveolar macrophages (AM), A549 cells, MonoMac6 cells, and human lung fibroblasts. We found significant levels of all muscarinic receptor subtypes on all analysed cells except the fibroblasts. Fibroblasts expressed predominantly M2, receptors and did not release chemotactic activity. AM, A549 cells, and MonoMac6 cells released chemotactic active mediators after incubation with ACh. The secretion could be suppressed by more than 70% after coincubation with tiotropium. Tiotropium alone did not influence the granulocyte migration. Most of the chemotactic activity could be attributed to leukotriene B_4 (LTB_4). The release of interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) was not induced by ACh. From this, we suggest that the suppression of the Ach-mediated release of chemotactic substances like LTB_4 modulates

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the inflammatory reaction. This may contribute to the decreased rate of exacerbations in COPD, which was observed in clinical trials.

Introduction

Chronic obstructive lung disease (COPD) is the only major fatal disease which is increasing. At this time, COPD is the fourth most common disease in the world. Therefore, this disease will be one of the most important economical factors in public health.[1] The cause of COPD is attributed to the burden of toxic gases and small particles that individuals inhale during their lifetime. Although atmospheric pollution contributes to this burden, the smoking of tobacco products is the major risk factor.[2] Recent findings indicate that lung inflammation is present in all smokers. The reason why only a minority of smokers develops significant air flow obstruction is still unknown, but preliminary evidence suggests that the lung inflammatory response is amplified in this group. The chronic inflammation induces remodelling of the bronchial tissue and the up-regulation of protease activity leading to emphysematous lung destruction. Furthermore, an important factor in the course of COPD is the development of exacerbations as a result of excessive inflammation which is often caused by infectious agents. To date, there is no causal therapeutic approach which targets the chronic inflammatory processes.

Bronchodilatators play an important role in the treatment of COPD and anti-cholinergics have a long history as an effective and safe treatment.[3] The use of ipratropium was limited by the need for frequent dosing.[4,5] Tiotropium, a new anti-cholinergic agent exhibits a long duration of action due to prolonged muscarinic receptor antagonism. The compound dissociates from the M3-receptor subtype extremely slowly but rapidly from the M2 receptors.[5] This offers the therapeutic advantage of once-daily dosing combined with a kinetic selectivity towards the M3-receptor subtype. Clinical trials with tiotropium have shown its effectiveness as a bronchodilatator in COPD[6] with marked influence on dyspnea reduction and improvement of exercise tolerance.[7] Additionally, tiotropium reduces the exacerbation frequency in patients with COPD.[6] These effects are associated with a beneficial effect on the health status. The mechanisms that prevent the decline in lung function and that are responsible for the reduction in the exacerbation frequency after treatment with tiotropium but not after treatment using drugs such as ipatropium are unclear. It has been suggested that tiotropium may have beneficial properties beside its bronchodilatory efficiency resulting, e.g., in a decrease of chronic inflammation in COPD patients.[5] In general, the selectivity of tiotropium for M3-muscarinic receptors is higher compared to ipatropium and the duration of action is longer. If M3-receptor antagonists suppress the release of mediators that prevent inflammation or tissue remodelling, tiotropium will assure a steady suppression of these mediators.

Increased number of cells of the innate immune system is one important characteristic of chronic inflammation in COPD. These are mostly neutrophil granulocytes which are attracted as a result of mediator release by activated epithelial cells and resident cells of the monocyte/macrophage lineage at the air/blood barrier of the alveoli and the small conducting airways. The activation of these cells at the site of inflammation may trigger airway remodelling. In addition, it has been suggested that chemokines, which attract neutrophils act directly on fibroblasts and smooth muscle cells and thus promote airway remodelling.[8]

Parasympathetic nerves provide the dominant autonomic innervations of the airways. Release of acetylcholine (ACh) from parasympathetic nerves activates muscarinic receptors present on airway smooth muscles and submucosal glands causing, e.g., bronchoconstriction and mucus secretion which are important symptoms of COPD.[9] In addition to this signal transduction pathway of the parasymphathetic nervous system, it has been shown that other cell types, e.g., bovine bronchial epithelial cells and alveolar macrophages (AM) are responsive to ACh stimulation resulting in the release of chemotactic factors which trigger granulocyte migration.[8,10,11] This mechanism may maintain the chronic inflammation in the airways in addition to other physical and chemical triggers such as cigarette smoke.

We postulated that tiotropium may regulate the release of chemotactic factors from epithelial cells and macrophages because tiotropium was thought to have an additional, anti-inflammatory function. This effect should be mediated via muscarinic Ach-receptors on the cells involved in the regulation of innate immune response in the lung.

Methods

Cell culture

Tissue samples from patients without lung fibrosis or COPD were obtained from healthy tissue areas during pneumonectomy for tumour resection from a tumour-free area. The ethical committee of the University of Magdeburg approved the study.

Fibroblasts were obtained by mincing freshly excised lung parenchyma into $\sim$1 mm^3 pieces, followed by digestion with collagenase IV (1 mg/ml; Sigma, Deissenhofen, Germany) for 30 min at 37 °C. Fibroblasts were cultured in a 75-ml tissue culture flask containing Iscove's modified Dulbecco's medium with 10% (w/v) foetal calf serum (FCS), 10^{-3} M glutamine and antibiotics (growth medium, GM), at 37 °C and 5% (v/v) CO_2 until they reached confluence. Only fibroblasts between passages 3 and 8 were used for the experiments. The myelo-monocytic cell line MonoMac6 and the bronchial epithelial cell line A549 were cultured in GM and subdivided every 3 days.

Bronchoalveolar lavage (BAL) and preparation of alveolar macrophages (AM)

Only patients (non-smokers) without clinical signs of interstitial, obstructive or infectious lung disease, who underwent bronchoscopy for exclusion of lung tumour, were included in this study. We included 10 patients (seven males, three females, age between 48 and 71 years).

Bronchoscopy (Olympus model BF 20 D or the P 10 bronchoscope) was performed through the nasal orifice or endotracheal tube. Topical anaesthesia (10 ml 2% lidocaine) was applied and the bronchoscope was advanced and brought into wedge position in a segmental bronchus. If no infiltration was evident on chest X-ray, bronchoalveolar lavage (100 ml of 0.9% saline solution at 37 °C sequentially instilled and suctioned in 20-ml portions) was performed in the right middle lobe.

BALF was filtered through sterile gauze filters, collected on ice and immediately centrifuged at 200*g* for 10 min. The cell pellets were resuspended in cell culture medium and counted. Differential cell counting was performed in May-Grünwald-Giemsa-stained cytocentrifuge preparations. Only preparations with $<3\%$ granulocytes and $<10\%$ lymphocytes were used for the experiments.

RT-PCR

For quantitative RT-PCR, total RNA was isolated from cells using RNeasy Mini kit (QIAGEN, Hilden, Germany). Remaining DNA was digested using DNAse as described by the manufacturer. The RNA was reverse transcribed by First Strand DNA Synthesis kit (Amersham Pharmacia Biotech, Freiburg, Germany). The PCR reaction was performed with the help of primers described in Buchli et al.[12] using the Quantitect SYBR Green PCR Kit (QIAGEN) and a ABI7000 PCR cycler (Applied Biosystems, Foster City, CA). The specificity of PCR reactions was verified by melting curve analyses. Additionally, the products were separated by agarose-gel electrophoresis. The receptor mRNA expression was normalised to β-actin mRNA compared between different cells using the $2^{-\Delta\Delta CT}$ method.[13]

Conditioned media

Fibroblasts (2×10^5 cells) and A549 cells (2×10^5 cells) were seeded in 24-well plates and grown to confluence in RPMI containing 10% foetal calf serum and antibiotics. MonoMac6 cells and AM were incubated at a concentration of 3.2×10^5 and 1×10^6 cells/ml, respectively. We used three independent wells for each time period and each concentration of ACh and tiotropium. For generation of conditioned medium, the growth medium was replaced by Ham's F12 medium. The cells were incubated for different time periods (4–72 h) with different concentrations of ACh (1–100 μM, Sigma, Deissenhofen, Germany) as indicated in the respective figures. Tiotropium bromide (20 nM; Boehringer Ingelheim) was added as indicated in the respective figures. The supernatants were removed after the indicated time periods, centrifuged to remove remaining cells and snap-frozen until further use.

The viability of the cells was measured after the removal of the supernatants at the end of the incubation period using propidium iodide and flow cytometry. The viability was higher than 90% in all experiments.

Separation of granulocytes

Heparinised blood (10 IU/ml blood) from healthy donors was used. PBMC were isolated by density gradient centrifugation with Ficoll/Paque (Pharmacia) for 30 min at $500 \times g$.[14] Supernatant, mononuclear cells and separation medium were removed and the cell pellet containing the erythrocytes and the granulocytes was resuspended in equal volume of polyvinylalcohol in 15-ml polypropylene tubes (BD, Heidelberg, Germany). The suspension was incubated at 37 °C for 30 min to allow the sedimentation of erythrocytes. The granulocyte-rich supernatant was removed and remaining erythrocytes were lysed using ice-cold 0.8% ammonium chloride solution (3 min). The purity of the isolated granulocytes was determined by flow cytometry after staining with anti-CD62L, anti-CD3, anti-CD19 and anti-CD14 antibodies (BD Biosciences, Heidelberg, Germany). Only cell population which contained more than 95% CD62L-positive and CD3/CD19/CD14-negative cells were used for the experiments. The cells were resuspended in ice-cold RPMI at a concentration of 2×10^7 cells/ml.

Migration

Chemotaxis was assayed in a 24-well transwell chamber (pore diameter 3 μm, BD). The bottom chamber was filled one by one with conditioned medium, which has been generated as described before. Isolated granulocytes (100 μl, 2×10^6 cells) were placed in the upper chamber. The transwells were incubated in a humidified atmosphere with 5% CO_2 for 30 min at 37 °C. Non-migrated cells were wiped away from the upper surface of the filter and the filter was formalin fixed. The fixed cells were stained using crystal violet solution (0.1% in 0.1 M borate buffer with 2% ethanol) for 30 min. Unbound stain was removed and the bound crystal violet was extracted using 10% acidic acid (10 min, room temperature). The amount of bound stain as a measure of the number of migrated cells was determined photometrically at 570 nm. Spontaneous migration was determined by adding GM alone. fMLP (1×10^{-6} M) was added to the bottom chamber and used as positive control. The values of the spontaneous migration were set to 100% and used for the normalisation of different experiments. The leukotriene B_4 receptor was blocked by the selective LTB_4-receptor antagonist BIIL260 (100 nM; Boehringer Ingelheim) which was added to the upper compartment of the migration chamber together with the isolated granulocytes.[15]

Measurement of cytokine concentration

Commercially available ELISA kits were used (R&D Systems; Minneapolis, MN, USA) for the detection of human interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) in culture supernatants.

Statistical analysis

All statistical analyses were performed with SPSS 10.0 for Windows (SPSS, Chicago, IL). Results were presented as mean values ±S.E. Mean values were compared by Student's *t*-test. In addition, the data were analysed using the non-parametric Mann–Whitney *U*-test. Differences were considered to be significant if the *p*-values were below 0.05 in both tests.

Results

Expression of muscarinic ACh receptors

First, we analysed the expression of muscarinic ACh receptors on the isolated cells. The expression of M1, M2, M3, M4, and M5-receptor mRNA was investigated by quantitative RT-PCR in lung tissue homogenates, isolated lung fibroblasts, A569 cells, and MonoMac6 cells. In the lung tissue, A549 and MonoMac6 cells we found a nearly similar pattern of expression of all five muscarinic ACh receptors with a predominance of the M3 receptors. In contrast, isolated lung fibroblasts expressed predominantly M2-receptor mRNA. The M2 receptor mRNA expression was 226-fold higher than the M3-receptor mRNA expression. Fibroblasts expressed 35-times more M2-receptor mRNA compared to A549 cells. The expression of the M3-receptor mRNA was significantly lower in fibroblasts compared to A549 cells (10% of the expression in A549 cells) and MonoMac6 cells (Table 1). M5-receptor mRNA was expressed at low amount and therefore not quantified. Compared to the isolated cells, we found high ACh-receptor concentration in tissue homogenates that indicates the expression of muscarinic ACh receptors in cells such as neuronal cells or airway smooth muscle cells.

ACh-induced release of chemotactic mediators

The release of chemotactic mediators was analysed with the help of a two-step model described before by Sato et al.[11]; Alveolar macrophages, MonoMac6 cells, A549 cells, and isolated fibroblasts were incubated for different time periods with increasing concentrations of ACh. The conditioned supernatants were removed and tested for their chemotactic activity on isolated granulocytes of healthy donors. First, we defined the optimal time course and concentration of ACh in different cells types. A dose-dependent release of chemotactic activity was found in MonoMac6 cells and in A549 cells after incubation with ACh at concentrations of 10^{-6}–10^{-4} M. The release reached a maximum after 24 h in MonoMac6 cells and after 48 h in A549 cells (Fig. 1A and B). Due to the limited cell number in the BALF of single patients, only a concentration of 10^{-4} M ACh has been used for stimulation of AM. We found a more rapid response. The release was increased after 4 h and reached a significant level after 8 h (Fig. 1C). Fibroblasts did not release chemotactic substances (data not shown). From this, it can also be concluded that ACh in the conditioned medium did not induce migration of granulocytes.

Suppression of ACh action by tiotropium

Based on these findings, we hypothesised that the long-acting antimuscarinic tiotropium may block the pro-inflammatory action of ACh. Therefore, the cells were incubated with ACh in the presence and absence of tiotropium (20 nM).

Table 1 Real-time RT-PCR quantification of muscarinic ACh receptors.

Receptor	Cells	Fold-expression (compared to M3-receptors)	Fold-expression (compared to A549)
M1	A549	0.04±0.02	1
	MonoMac6	0.14±0.02	110±59
	Fibroblasts	0.08±0.04	2.6±2.5
	Lung tissue	0.22±0.03	4467±751
M2	A549	0.76±0.02	1
	MonoMac-6	0.93±0.07	17±6
	Fibroblasts	226±16	35±4
	Lung tissue	0.46±0.24	264±18
M3	A549	1	1
	MonoMac-6	1	49±19
	Fibroblasts	1	0.1±0.03
	Lung tissue	1	903±28
M4	A549	0.84±0.02	1
	MonoMac-6	0.84±0.05	15±5
	Fibroblasts	0.48±0.16	0.04±0.02
	Lung tissue	0.77±0.13	902±142

Quantitative RT-PCR was performed as described in section "Methods". The expression of different ACh-receptors was compared to the expression of the M3 receptors in the respective cells (third column; for example, the expression of M1 receptors in A549 cells was compared to the M3-receptor expression in A549 cells). The expression of distinct receptors was compared between different cells and A549 cells (fourth column; for example, the expression of M3 receptor in fibroblasts was compared to the M3 receptor expression in A549 cells. Values >1 mean increased expression. Values between 0 and 1 mean decreased expression.

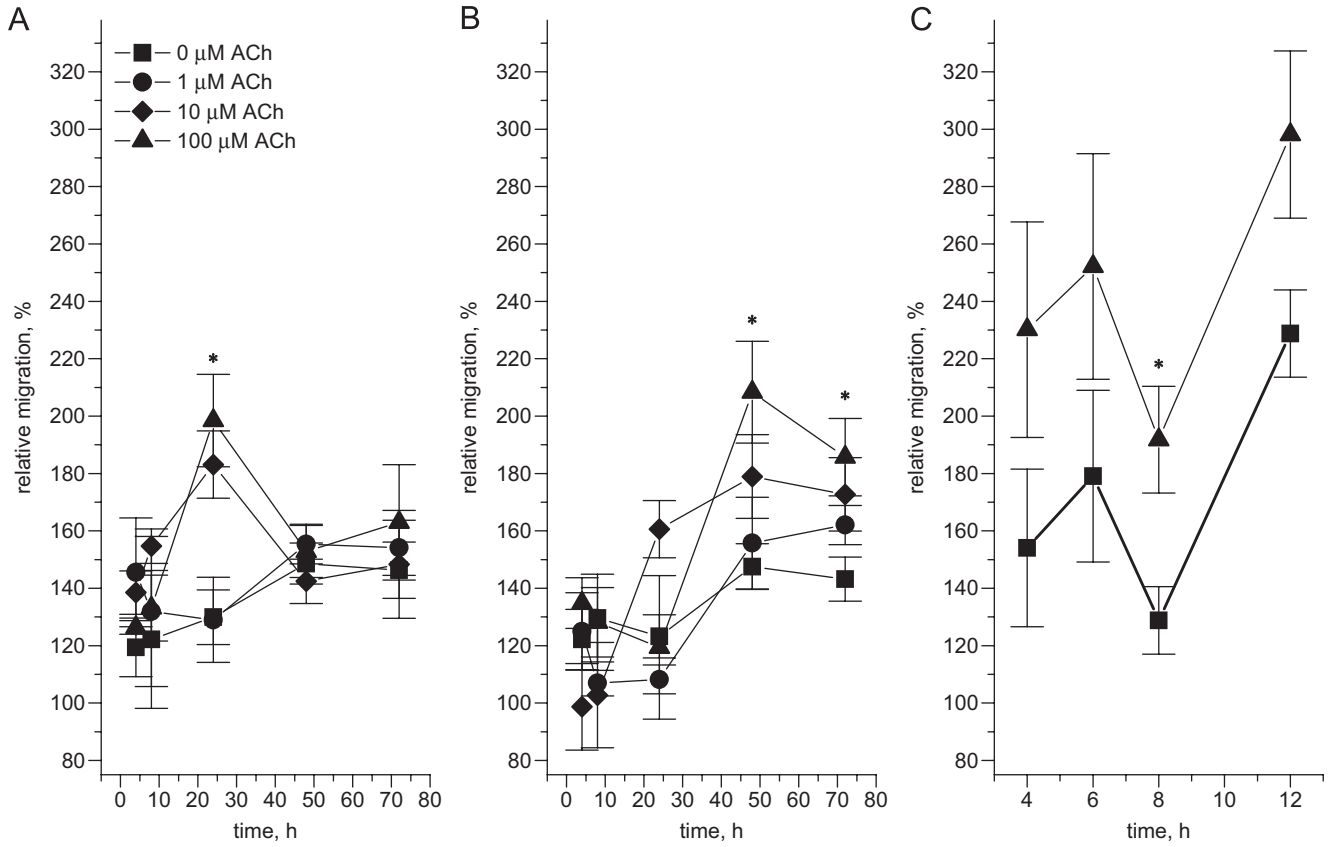

Figure 1 Release of chemotactic activity after incubation with acetylcholine (ACh). MonoMac6 cells (A), A549 cells (B), and AM (C) were incubated with ACh at concentrations of 1 μmol/l (circle), 10 μmol/l (diamond), 100 μmol/l (triangle) or without ACh (square). The chemotactic activity was assayed as described in section "Methods". In control experiments, granulocytes were incubated with fMLP. The spontaneous migration of granulocytes which were incubated with native culture medium was set to 100%. The results of five experiments are presented as mean ± SEM. Asterisks indicate significant ($p < 0.05$) differences between the chemotactic activity of unstimulated and ACh-stimulated cells at the given time point.

Previous experiments investigating the effect on human bronchi have shown that this concentration effectively inhibited constrictions induced by electric field stimulations.[16] Fig. 2 shows that tiotropium significantly inhibited the release of chemotactic substances by AM, MonoMac6 and A549 cells. Tiotropium alone did not change the chemotactic activity, arguing for a direct competition with ACh for binding to the muscarinic receptors. Furthermore, we have demonstrated that preincubation with tiotropium did not increase the observed effect (not shown). Release of chemotactic mediators was not detected in fibroblasts incubated with ACh.

Considering the different receptor expression in fibroblasts compared to all other cell types which were included in our study, the absence of the pro-inflammatory action of ACh and, consequently, the inhibition by tiotropium on fibroblasts may be contributed to the low M3-receptor expression in comparison to the M2 receptor. Overall, the results demonstrate one mechanism of a possible anti-inflammatory role of tiotropium.

Influence of ACh on chemokine release

No effect of cholinergic stimulation on the concentration of IL-8 or MCP-1 was found (data not shown).

Sato and co-workers[10,11,17] have shown that the eicosanoid leukotriene B_4 (LTB_4) is released by bovine bronchial epithelial in response to Ach. Consequently, we used the specific LTB_4-receptor antagonist BIIL260 to test whether LTB_4 mediated the ACh-induced chemotactic activity. Addition of the LTB_4 inhibitor caused an inhibition of the migration of the granulocytes in response to the conditioned supernatants from AM, MonoMac6 cells, and A549 cells (Fig. 3). Interestingly, BIIL260 inhibited both the ACh-induced migration and the unspecific migration induced by the supernatants of cells incubated without ACh. We suggest that the unspecific migration was the result of the spontaneous release of growth factors by the cultured cells.

Discussion

The development of COPD is associated with chronic inflammation of the central airways, obstruction of small airways and emphysematous destruction of the lung's elastic recoil force.[2] Inflammatory cells from both the innate and the adaptive immune response participate in this process.[18] The accumulation of these cells seems to be mediated by limitations in the capillary blood flow and, in addition, by

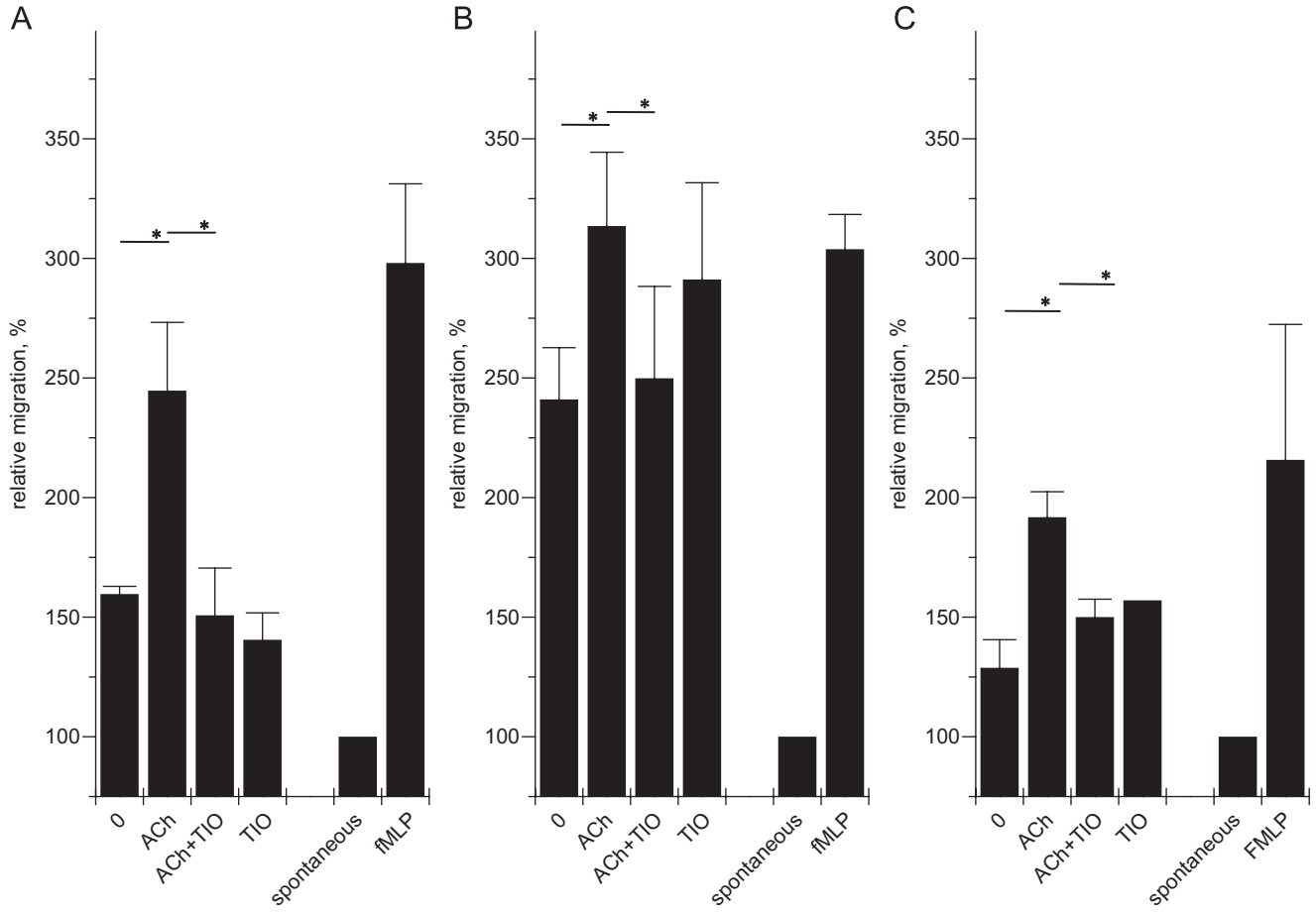

Figure 2 Inhibition of chemotactic activity by tiotropium. MonoMac6 cells (A), A549 cells (B), and AM (C) were stimulated without (0) or with ACh (100 μmol/l) and tiotropium (TIO, 20 nM) for 24, 48, or 8 h. The conditioned supernatants were used to stimulate granulocyte migration. Tiotropium alone did not significantly influence the migration of human neutrophilic granulocytes. In control experiments, granulocytes were incubated with fMLP. The spontaneous migration of granulocytes which were incubated with native culture medium was set to 100%. The results of four experiments are presented as mean ± SEM. Asterisks indicate significant ($p < 0.05$) differences between the indicated samples.

chemotactic factors which are released by activated macrophages, epithelial cells and fibroblasts.

As COPD progresses, the increased frequency and severity of exacerbations become notable. Increased exacerbation frequency has been associated with worsening the quality of life and is predictive of an earlier mortality.[6] One of the most important health outcomes of the studies evaluating tiotropium in COPD was the alteration in exacerbation frequency as well as prolonging the time to the first exacerbation.[4,6] Therefore, it was suggested that tiotropium has the potential to affect broader issues beyond symptomatic improvement. The mechanism of these effects remained unclear.

Focussing on the extraneuronal actions of Ach,[15] which were described before, we have shown that tiotropium decreases the release of chemotactic mediators from epithelial cells and macrophages. This may suppress the inflammatory reaction in the lung and thus contribute to the prevention of exacerbations. Furthermore, the study of Gosens et al. has shown that tiotropium prevented the allergen-induced remodelling of airway smooth muscle in vivo.[19]

In detail, we have shown that various lung cells express different patterns of muscarinic ACh receptors which may point to a variable action of ACh on different lung cells. The presence of muscarinic ACh receptors especially of the M2-type has been demonstrated before on human lung fibroblasts.[20,21] We have found that fibroblasts expressed predominantly M2-receptor mRNA and only lower amount of M3-receptor mRNA. Similar findings have been published recently by Matthiesen et al.[22] A549 cells and MonoMac6 cells expressed more M3-receptor mRNA which could explain the different responsiveness to ACh of A549 and MonoMac6 cells compared to fibroblasts.

Non-neuronal functions of ACh have been discussed before by different authors.[11,23] Salari and Chan-Yeung have shown that ACh potentates the release of the pro-inflammatory 15-HETE and of PGE_2 at concentrations of 10^{-7}–10^{-4} M.[24] Sato et al.[9] have described that ACh induces the release of chemotactic agents by bovine AM. Using specific inhibitors they determined that muscarinic M3 receptors and not nicotinic or muscarinic M1 or M2 receptors were involved in this process. In contrast to these results it has been described by Tracey and co-workers that ACh

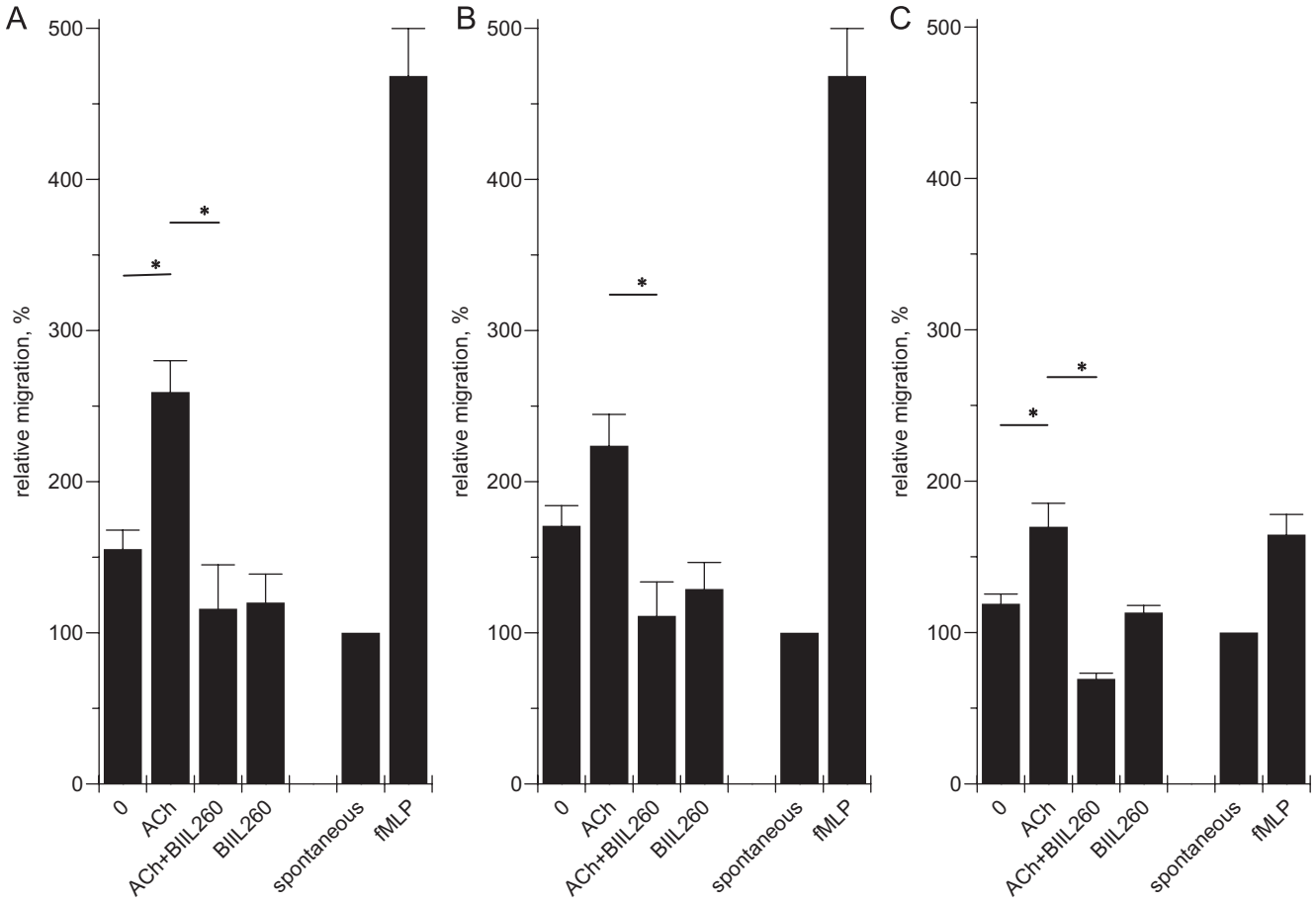

Figure 3 Inhibition of chemotactic activity by the selective leukotriene B_4 receptor antagonist BIIL260. MonoMac6 cells (A), A549 cells (B), and AM (C) were stimulated with ACh (100 μm/l) for 24, 48, or 8 h. Neutrophil chemotactic activity was analysed in the presence and absence of BIIL260. In control experiments, granulocytes were incubated with BIIL260 or fMLP. The spontaneous migration of granulocytes which were incubated with native culture medium was set to 100%. The results of four experiments are presented as mean ± SEM. Asterisks indicate significant ($p < 0.05$) differences between the indicated samples.

initiates the "systemic anti-inflammatory pathway".[25–27] They have shown that central muscarinic M1 and M2 receptors and the nicotinic ACh-receptor alpha7 subunit are involved in the regulation of this pathway. One explanation of these contrasting results could be the predominant action of different receptors in defined cell types.

We have shown that ACh at concentrations between 10^{-6} and 10^{-4} M induced a significant release of chemotactic substances from human AM and from a human myeloid and a lung epithelial cell line. Klapproth et al.[28] have found significant amounts of ACh (33 pmol/g) at bronchial epithelial surface. Wessler et al.[29] have shown that freshly dissected tracheal tissue contains considerable amount of Ach. Electrical stimulation increased the ACh release. The concentration of ACh at the neuromuscular junction was reported to be 10^{-4}–10^{-3} M.[30] There are no reported data concerning the release of ACh in inflamed tissues or after cytokine stimulation. Therefore, we suggest that the concentrations used in our experiments are within the patho-physiological range of human lung tissue. We cannot exclude that there is an additional suppressive action of nicotinic ACh receptors on the chemokine release because we have not used an antagonist of these receptors and have not investigated their expression on our cells.

The time course of the release of chemotactic activity varied between the different cells types. We found increased chemotactic activity in the supernatant of macrophages after 6–8 h and in A549 cells and MonoMac6 cells after 24–48 h. Interestingly enough, recent studies found very similar differences between macrophages, monocytes, and epithelial cells.[10,11,17] The subcellular mechanisms which regulate the kinetic of the synthesis of chemotactic cytokines and especially of leukotrienes are not fully identified. It has been shown that the efficiency and the capacity of the leukotriene synthesis depend largely on the membrane organisation of the components of the synthetic machinery which is different in different cell types.[30,31] This could be one explanation for the variations in the time course of the release of chemotactic activity.

Different cytokines such as IL-8 and LTB_4 have been shown to be increased in the sputum and the exhaled breath condensate of COPD patients.[32,33] Beeh et al.[23] have published that anti-IL-8 antibodies and the LTB_4 antagonist, SB201146, inhibited neutrophil chemotaxis which was evoked by sputum from patients with COPD. We have

investigated whether ACh enhances the release of IL-8 and MCP-1 because we have shown before that they are released from epithelial and myeloid cells.[34] ACh did not induce the secretion of these chemokines. This is in accordance with data published by Sato et al.[9] They have shown that anti-IL-8 antibodies did not suppress the chemotactic acivity induced by human AM in response to ACh. However, using the specific LTB_4 inhibitor BIIL260, we could suppress both the complete ACh-induced chemotactic activity and part of the unstimulated chemotactic activity induced by the cells incubated without ACh. From this, we conclude that LTB_4 is an important chemotactic mediator, released upon unspecific stimuli and, in higher concentrations, after M3-receptor-dependent stimulation, by ACh. We suggest, therefore, that the LTB_4 release is suppressed by tiotropium. This may contribute to its potential anti-inflammatory action. Against a cytotoxic or unspecific action of the antagonist on granulocytes argues the fact that there was no inhibition of the spontaneous migration of granulocytes. Interestingly, these data would also suggest that LTB_4 inhibitors should influence the course of COPD. This hypothesis should be further investigated in clinical studies.

In conclusion, we have shown for the first time that tiotropium suppresses the ACh-induced release of chemotactic agents from primary human macrophages as well as from myeloid cell and epithelial cell lines via the blockade of muscarinic receptors. The chemotactic activity was mainly mediated by leukotriene B_4. This mechanism may possibly explain an anti-inflammatory action of tiotropium which may contribute to a reduced exacerbation frequency observed in clinical studies. Further studies confirming these findings in vivo are necessary.

Conflict of interest

The authors declare that they have no conflict of interest.

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