Acetylcholine mediates the release of IL-8 in human bronchial epithelial cells by a NFkB/ERK-dependent mechanism

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Abstract

Acetylcholine may play a role in cell activation and airway inflammation. We evaluated the levels of both mRNA and protein of muscarinic M1, M2, M3 receptors in human bronchial epithelial cell line (16HBE). 16HBE cells were also stimulated with acetylcholine and extracellular signal-regulated kinase1/2 (ERK1/2) and NFkB pathway activation as well as the IL-8 release was assessed in the presence or absence of the inhibitor of Protein-kinase (PKC) (GF109203X), of the inhibitor of mitogenic activated protein-kinase kinase (MAPKK) (PDO9805), of the inhibitor of kinaseB-α phosphorilation (pIkBα) (BAY11-7082), and of muscarinic receptor antagonists tiotropium bromide, 4-Diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), telenzepine, gallamine. Additionally, we tested the IL-8-mediated neutrophil chemotactic activity of 16HBE supernatants stimulated with acetylcholine in the presence or absence of tiotropium. 16HBE cells expressed both protein and mRNA for muscarinic M1, M2 and M3 receptors with levels of muscarinic M1 receptor > muscarinic M2 receptor > muscarinic M3 receptor. Acetylcholine (10 μM) significantly stimulated ERK1/2 and NFkB activation as well as IL-8 release in 16HBE cells when compared to basal values. Furthermore, while the use of tiotropium, 4-DAMP, GF109203X, PDO9805, BAY11-7082 completely abolished these events, the use of telenzepine and gallamine were only partially able to downregulate these effects. Additionally, acetylcholine-mediated IL-8 release from 16HBE cells significantly increased chemotaxis toward neutrophils and this effect was blocked by tiotropium. In conclusion, acetylcholine activates the release of IL-8 from 16HBE involving PKC, ERK1/2 and NFkB pathways via muscarinic receptors, suggesting that it is likely to contribute to IL-8 related neutrophil inflammatory disorders in the airway. Thus, muscarinic antagonists may contribute to control inflammatory processes in airway diseases.

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1. Introduction

Acetylcholine is the primary parasympathetic neurotransmitter in the airways and exerts its physiological effects by muscarinic receptors (Costello et al., 1998). Acetylcholine-mediated effects are involved in the airway smooth muscle contraction and mucus secretion during a variety of airway diseases (Proskocil and Fryer, 2005). This traditional point of view is now changing and acetylcholine is also considered as a factor able to stimulate airway inflammation (Proskocil and Fryer, 2005; Gosens et al., 2004) by promoting the activation of bronchial epithelial cells (Koyama et al., 1998), alveolar macrophages (Sato et al., 1998), and of inflammatory cells (macrophages, neutrophils, eosinophils and lymphocytes) of induced sputum in patients with Chronic Obstructive Pulmonary Disease (COPD) (Profita et al., 2005), leading to the release of mediators with chemotactic activity for both eosinophils and neutrophils.

The muscarinic M1, M2 and M3 receptor subtypes are expressed in the airway smooth muscle and in nerves controlling
the tone of the airway smooth muscle as well as in bronchial epithelial cells (Koyama et al., 1998). The priming of muscarinic M1, M2 and M3 receptors promotes the activation of Protein-Kinase C (PKC) and Mitogenic Activated Protein-kinase/extracellular signal-regulated kinase (MAPK/ERK1/2) in transfected Chinese hamster ovary cells (Budd et al., 2001) and intestinal smooth muscle cells (Zhou et al., 2003). Moreover, the carbachol-induced activation of PKC mediates ERK1/2 activation via sequential activation of Ras, Raf and MAPK kinase (MAPKK) in human neuroblastoma SK-N-BE2 cells (Kim et al., 1999). The activation of MAPKK appears to be strongly associated to muscarinic receptor activity inducing cell proliferation and protein synthesis in human breast cancer cells (Jimenez and Montiel, 2005) as well as promoting cellular adhesion in muscarinic M3 receptor transfected human embryonic kidney cells (Slack, 2000).

Epithelial cells can contribute to chronic inflammatory disorders, synthesizing and secreting a variety of pro-inflammatory cytokines, such as IL-8, which regulate neutrophil accumulation in the airways of COPD subjects (Profita et al., 2003a,b; Beeh et al., 2003). In response to pro-inflammatory stimuli, IL-8 production is dependent on MAPK and NFkB in epithelial cells (Oudin and Pugin, 2002). In addition, in human bronchial epithelial cells, the NFkB activation may regulate IL-8 release via ERK/MAP kinase-dependent (Li et al., 2002) or -independent (Williams, 2003) processes. However, the intracellular signalling mechanisms by which acetylcholine activation of muscarinic receptors promotes inflammatory responses, in particular IL-8 release, are not yet completely defined.

In the present study, we assessed the potential role of acetylcholine in the release of IL-8, a pro-inflammatory mediator inducing neutrophils chemotaxis, from human bronchial epithelial cells. We also investigated the role of PKC, ERK1/2 and NFkB, as well as of muscarinic M1, M2, M3 receptors, in the acetylcholine-mediated release of IL-8. Finally, the acetylcholine blockade was assessed to identify its potential anti-inflammatory properties.

2. Materials and methods

2.1. Epithelial cell cultures

SV40 large T antigen-transformed human airway epithelial cell line (16HBE) were used for these studies. The 16HBE cell line was cultured as adherent monolayers in Eagle’s minimum essential medium (MEM) supplemented with 10% heat-inactivated (56 °C, 30 min) foetal calf serum + 100 U/ml penicillin and 100 mg/ml streptomycin. The cells represent a clonal diploid (2n=6) cell line isolated from human lung previously used to study the functional properties of bronchial epithelial cells in inflammation and repair processes (Merendino et al., 2002). Unstimulated cells were used for the extraction of protein and mRNA and in slide preparation.

2.2. Flow cytometry

Quantitative expression of ChAT and muscarinic M1, M2, M3 receptors was determined in 16HBE cells using indirect label immunofluorescence with a FACStar Plus analyzer (Becton Dickinson, Mountain View, CA). The cells were previously permeabilized using a commercially available kit (Caltag Laboratories Bioresearch GmbH, Austria). The following antibodies were used: rabbit polyclonal human muscarinic M1 receptor (H-120 Santa Cruz Biotechnology, Inc., Santa Cruz CA), rabbit polyclonal anti-human muscarinic M2 receptor (H-170 Santa Cruz Biotechnology); rabbit polyclonal anti-human muscarinic M3 receptor (H-120 Santa Cruz Biotechnology). Anti-rabbit IgG FITC swine F(ab′)2 (Dako LSAB, Glostrup, Denmark) was used as a secondary. FITC-conjugated rabbit IgG1 was used as an isotype negative control antibody (Dako LSAB, Glostrup, Denmark).

2.3. Immunocytochemistry of muscarinic receptors

Immunostaining of muscarinic M1, M2 and M3 receptors in 16HBE cells was performed using anti-muscarinic M1, M2, and M3 receptor antibodies. The slides were stained by the immunoalkaline-phosphatase method (Dako LSAB, Glostrup, Denmark). Negative controls were performed using an isotope control IgG (Dako LSAB, Glostrup, Denmark). To verify whether the anti-muscarinic M1, M2 and M3 receptor antibodies were specific for each muscarinic receptor subtype and did not cross-react with other subtypes or with nicotinic receptors (Tice et al., 1996), we tested the specificity of the staining of M1, M2 and M3 as previously described by Campbell et al. (modified) (Campbell et al., 1998). Polyclonal antibodies were incubated with soluble recombinant peptides muscarinic M1 receptor (Santa Cruz Biotechnology, 100 μg/ml), muscarinic M2 receptor (Sigma St. Louis, MO, 100 μg/ml) and muscarinic M3 (Sigma St. Louis, MO, 100 μg/ml) receptors for 30 min prior to performing the staining procedure.

2.4. Real-time quantitative RT-PCR of muscarinic M1, M2, M3 receptors

Total cellular RNA was extracted from cells accordingly to the method of Chomczynski and Sacchi, using the RNAzol kit (Biotech Italia, Rome, Italy). Four μg of total RNA was reverse-transcribed to cDNA, using M-MLV-RT and oligo(dT)22–18 primers (Invitrogen) in a 25 μl reaction mixture. Real-time quantitative PCR of muscarinic M1, M2, and M3 receptor subtypes of human muscarinic acetylcholine receptors was carried out using the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) using specific FAM-labelled probes and primers (Applied Biosystems, TaqMan Assays on Demand). GAPDH gene expression was used as endogenous control. Gene expression levels were expressed as C_T (Steuerwald et al., 2000).

2.5. Stimulation of 16HBE with acetylcholine

Viable 16HBE cells (5×10^5) were seeded into 75 cm² flasks with RPMI 10% FBS and cultured for 72 h. Cells were then made quiescent by culturing for 24 h in RPMI without serum. After selection of the optimal concentration of acetylcholine...
(Sigma St. Louis, MO) for cell stimulation (0.1–100 μM) (30 min to test the activation of the intracellular signal pathways and 18 h to evaluate the IL-8 release), the cells were tested for the time course for intracellular signal pathway activation (0, 15, 30 and 45 min) and for IL-8 release (0, 2, 8 and 18 h) using acetylcholine at the concentration of 10 μM. Furthermore, to identify whether the effect of acetylcholine on 16HBE was mediated by the activation of Protein-Kinase C (PKC) or by ERK1/2 or by NFκB pathways, the cells were pretreated for 1 h with Bisindolylmaldeide (GF109203X a PKC inhibitor, 10 μM) (Sigma Aldrich s.r.l, Milan Italy), 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PDO98059 a MAPKK inhibitor, 50 μM) (Sigma St. Louis, MO) or (E)-3-(4-Methylphenylsulfonyl)-2-propenetrile (BAY11-7082 an inhibitor of IkB phosphorylation, 50 μM) (Sigma St. Louis, MO) before the addition of acetylcholine (10 μM). Subsequently, we assessed the activation of muscarinic receptors using the following anticholinergic drugs: tiotropium bromide (0.1 μM) (Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany), telenzepine (described as muscarinic M1 receptor antagonist, 20 μM) (Sigma St. Louis, MO), gallamine (described as muscarinic M2 receptor antagonist, 1 μM) (Sigma Aldrich s.r.l, Milan Italy), and 4-diphenylacetoxymethylpiperidin methiodide (4-DAMP) (described as muscarinic receptor antagonist with selectivity for M3, 1 μM) (Sigma St. Louis, MO), 1 h before the addition of acetylcholine (10 μM).

2.6. ERK1/2 and NFκB activation

To assess ERK1/2 and NFκB activation in cell lysates from 16HBE cells stimulated with acetylcholine, we evaluated ERK1/2 and NFκB activation using two commercially available ELISA kits (SuperArray Bioscience, Frederick, MD) that measure phosphorylated and total ERK1/2, as well as phosphorylated and total NFκB. Results are expressed respectively as pERK1/2/tERK1/2 ratio and pNFκB/tNFκB ratio. Additionally, we performed Western blot analysis, using two rabbit monoclonal antibodies directed against anti-phospho ERK1/2 and against anti-phospho IkBα rabbit antibody, respectively (Cell Signaling Technology, Beverly, MA), and anti-β-actin (Sigma St. Louis, MO).

2.7. Gel image evaluation

Gel images were taken with an EPSON GT-6000 scanner and then imported into a National Institutes of Health Image analysis 1.61 program to determine band intensities. Data are expressed as arbitrary densitometric units corrected against the density of β-actin bands.

2.8. Measurement of IL-8 release in 16HBE cells

The release of IL-8 was determined in the culture supernatants of 16HBE cells with an enzyme-linked immunosorbent assay (ELISA) (Amersham Biosciences, UK Limited). All commercial reagents were used according to the manufacturers’ specifications. The lower limit of detection was 5 pg/ml.

2.9. Purification of peripheral blood neutrophils and neutrophil chemotaxis assay

Peripheral polymorphonuclear leukocytes were isolated from normal donors by the use of dextran sedimentation and centrifugation over Ficoll cushions, followed by adhesion, as previously described (Pace et al., 1999). Normal neutrophils were incubated with supernatants from 16HBE cells stimulated with acetylcholine 10 μM in the presence or absence of the tiotropium, to perform neutrophil chemotaxis assay. To demonstrate that IL-8 was responsible for neutrophil migration, blocking experiments were performed by incubation of the neutrophils with an anti-IL-8 mAb (1 μg/ml, for 30 min at 37 °C) prior to loading the chemotaxis chamber as previously described.

2.10. Statistics

Data are expressed as mean counts±standard deviation (S.D.). Analysis of variance (ANOVA) corrected with the Bonferroni test.
and t-test are used for comparisons. *P < 0.05 was accepted as statistically significant.

3. Results

3.1. Muscarinic M1, M2 and M3 receptor expression in 16HBE cells

Flow cytometric analysis showed that 16HBE cells express all three muscarinic M1, M2, and M3 receptors. Immunofluorescent cell staining showed higher levels of muscarinic M3 and M1 receptors than muscarinic M2 receptor and higher levels of muscarinic M3 receptor than muscarinic M1 receptor (Fig. 1A). Immunocytochemistry analysis of 16HBE cells confirmed, as evaluated by the intensity of the stainings, the levels of expression of muscarinic M1, M2, and M3 receptors (Fig. 1B). Additionally, the use of the polyclonal anti-muscarinic M1, M2 and M3 receptor antibodies incubated with each soluble recombinant peptides muscarinic M1, M2 and M3 receptors, clearly showed a negative staining of the cells, supporting the specificity of the antibodies used for immucytochemistry (data not shown). The analysis of muscarinic M1, M2, and M3 receptor mRNAs subtypes by Real-time quantitative PCR showed higher levels of muscarinic M1 and M3 receptor mRNA transcripts than muscarinic M2 receptor and higher levels of muscarinic M3 receptor than muscarinic M1 receptor, showing the same trend of muscarinic M1, M2 and M3 receptor protein expression (Fig. 1C).

3.2. Dose response and time course of acetylcholine-induced ERK1/2 and NFkB activation and IL-8 release from 16HBE cells

The stimulation of 16HBE cells with different concentrations of acetylcholine (0.1–100 μM) generated a concentration-dependent activation of ERK1/2 and NFkB after 30 min of incubation. For ERK1/2, activation reached statistic significance, in comparison to baseline values, at the acetylcholine concentration of 10 μM, as indicated by both ELISA and Western blot analyses (Fig. 2A,B). Time course experiments showed that the activation of both ERK1/2 and of NFkB pathways were maximal after 30 min of incubation (Fig. 2C,D). Evaluation of Western blot data for pERK1/2 and pIkB α, expressed as arbitrary densitometric units corrected against the density of β-actin bands, strongly supported the values obtained by ELISA (data not shown). Additionally, the stimulation of 16HBE cells with different concentrations of acetylcholine (from 0.1 to 100 μM) for 18 h generated a significantly increased release of IL-8 at the acetylcholine concentration of 10 μM (Fig. 3A). The time course release test performed using this acetylcholine concentration demonstrated that a significant release of IL-8 was reached after 18 h of incubation (Fig. 3B).

3.3. Acetylcholine activates ERK1/2 and NFkB pathways and IL-8 release via independent mechanisms

In order to understand the selectivity of the pathway activation during acetylcholine stimulation of 16HBE cells and
the relationships between PKC, ERK1/2 and NFkB pathways activation and the IL-8 release, we pre-incubated 16HBE cells with GF109203X, PDO98059 and BAY11-7082 before the addition of acetylcholine (10 μM) (Fig. 4). We detected the ERK1/2 and NFkB pathway activation after 30 min and IL-8 release after 18 h. All these inhibitors completely abolished the acetylcholine-mediated activation of ERK1/2 (Fig. 5A,B) and NFkB (Fig. 5C,D). They were also able to completely abolish acetylcholine-mediated IL-8 release (Fig. 5E). These results suggest a direct involvement of the PKC, ERK1/2 and NFkB pathways in acetylcholine-mediated IL-8 release. Interestingly, pre-incubation of 16HBE cells with the receptor antagonists tiotropium and 4-DAMP, completely abolished acetylcholine-mediated activation of ERK1/2 and NFkB pathways, while the use of Telenzepine and Gallamine completely abolished acetylcholine-mediated activation of ERK1/2 (Fig. 5A,B) and partially inhibited NFkB activation (Fig. 5C,D). Evaluation of Western blot data for pERK1/2 and pIkBα, expressed as arbitrary densitometric units corrected against the density of β-actin bands, strongly supported the values obtained by ELISA (data not shown). Additionally, pre-incubation of the cells with tiotropium and 4-DAMP completely abolished acetylcholine-mediated IL-8 release, while the use of telenzepine and gallamine only partially inhibited acetylcholine-mediated IL-8 release (Fig. 5E).

3.4. Acetylcholine stimulated 16HBE cells induce the chemotaxis of peripheral blood neutrophils via the release of IL-8

Culture supernatants recovered from acetylcholine-stimulated 16HBE cells showed a significantly increased chemotactic activity for neutrophils when compared to supernatants from cells untreated with acetylcholine. This increase was not observed in 16HBE cells treated with acetylcholine in the presence of tiotropium (Fig. 6), clearly showing that the increase in chemotactic activity was secondary to muscarinic receptor activation. Interestingly, the increase of neutrophil chemotaxis due to the stimulation of the cells with acetylcholine

Fig. 4. Acetylcholine stimulated ERK1/2 and NFkB activation and IL-8 release through different mechanisms. 16HBE cells were stimulated with acetylcholine 10 μM for 30 min and 18 h in the presence or absence of GF109203X (PKC inhibitor, 10 μM), PDO98059 (ERK1/2 inhibitor, 50 μM), BAY11-7082 (IkBα inhibitor, 50 μM). The activation of ERK1/2 for each experimental condition was tested for the pERK1/2/ERK1/2 ratio by ELISA (A) and for phospho-ERK1/2 and β-actin by Western blot (B) after 30 min of acetylcholine stimulation. The activation of NFkB for each experimental condition was tested for the pNFkB/tNFkB ratio by ELISA (C) and for phospho-IkBα by Western blot (D) after 30 min of acetylcholine stimulation. The IL-8 concentration was measured by ELISA (E) after 18 h of acetylcholine stimulation. The values shown are the mean ± S. D. of 8 separate experiments. *P<0.05 was accepted as statistically significant, compared with the response to medium alone baseline values. The gel images shown are representative of 8 separate experiments.
was also inhibited by pre-treating the cells with an anti-IL-8 monoclonal antibody, suggesting that IL-8 is the factor responsible for neutrophil chemotaxis (Fig. 6). This finding provides an important link between acetylcholine release, inflammatory cell activation, IL-8 release and neutrophilic influx within the airways of COPD subjects.

4. Discussion

Epithelial cells contribute to chronic inflammatory disorders by synthesizing and secreting a variety of pro-inflammatory cytokines, including IL-8, which regulate neutrophil accumulation in the airways of COPD subjects (Beeh et al., 2003; Profita et al., 2003a,b). Acetylcholine is involved in the activation of bronchial epithelial cells, leading to the release of chemotactic mediators for both eosinophils and neutrophils (Koyama et al., 1998). In the present study, we show that acetylcholine, via PKC, ERK1/2 and NFkB pathways, increases the release of IL-8 in the 16HBE cells. These activities are mediated by the binding of acetylcholine to the muscarinic M1, M2 and M3 receptors. Furthermore, IL-8 released after acetylcholine stimulation of 16HBE cells is able to actively induce neutrophil chemotaxis. Finally, muscarinic receptor inhibitors including tiotropium, the inhaled anticholinergic drug used in the treatment of COPD, are able to counteract these phenomena.

We have previously demonstrated that induced sputum cells from COPD subjects express higher levels of muscarinic M1 and M3 receptors and lower levels of muscarinic M2 receptor compared to controls (Koyama et al., 1998). We show here that a similar expression profile of muscarinic receptors is evident in 16HBE cells, confirming that these cells can be considered a valid experimental model to study the role of muscarinic receptors in airway inflammation of COPD subjects. Moreover, both the protein and mRNA level for these receptors are constantly correspondent and always reproducible at different cell passages. This further supports the usefulness of the 16HBE cell line for in vitro experiments (Merendino et al., 2002; Kassel et al., 2001) avoiding problems that occurred in other studies in which the activation of muscarinic receptors subtypes showed a variability among the single individuals and cell lines (Nicke et al 1999; Pereira et al., 2003; Bany et al., 1999; Sato et al., 1999). Similar observations are supported by preliminary results of Blass et al on the effect of carbachol on IL-8 secretion observed in primary bronchial epithelial cells. The findings by Blass that only 5 out of 12 patients release IL-8 after carbachol

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**Fig. 5.** Acetylcholine stimulated ERK1/2 and NFkB activation and IL-8 release through different muscarinic receptors in 16HBE cell line. 16HBE cells were stimulated with acetylcholine 10 μM for 30 min and 18 h in the presence or absence of tiotropium (0.1 μM), telenzepine (described as muscarinic M1 receptor antagonist 20 μM), gallamine (described as muscarinic M2 receptor antagonist; 1 μM), and 4-DAMP (described as muscarinic M3 receptor antagonist; 1 μM) 1 h before the acetylcholine stimulation. The activation of ERK1/2 for each experimental condition was tested for the pERK1/2/tERK1/2 ratio by ELISA (A) and for phospho-ERK1/2 by Western blot (B) after 30 min of acetylcholine stimulation. The activation of NFkB for each experimental condition was tested for the pNFkB / tNFkB ratio by ELISA (C) and for phospho-IkBα and β-actin by Western blot (D) after 30 min of acetylcholine stimulation. The release of IL-8 for each experimental condition was tested by ELISA (E) after 18 h of acetylcholine stimulation. The values shown are the mean±S.D. for 8 separate experiments. *P<0.05 was accepted as statistically significant, compared with the response to medium alone baseline values. The gel images shown are representative of 8 separate experiments.

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**Fig. 6.** Supernatants of acetylcholine stimulated 16HBE cell line induced the chemotaxis of peripheral blood neutrophils. Neutrophil chemotactic activity of 16HBE cell line supernatants obtained from the experiments performed with acetylcholine (10 μM) for 18 h at 37 °C in the presence or absence of tiotropium. Chemotaxis was also assessed on neutrophils pretreated with an anti-IL-8 mAb (1 μg/ml, for 30 min at 37 °C). Results are expressed as mean±S. D. of 8 experiments. Statistical analysis of variance (ANOVA) corrected with the Bonferroni test and t-test was used for comparisons. *P<0.05 was accepted as statistically significant, compared with the response to medium alone baseline values.
stimulation, might probably due a bias related to subjects variability not present in our in vitro model (Blias et al., 2007). The activities of muscarinic M1, M2 and M3 receptors have been well described in the smooth muscle. The smooth muscle is activated via the muscarinic M3 receptor and the activation of both muscarinic M1 and M3 receptors leads to mucus secretion. In parallel, the stimulation of prejunctional muscarinic M2 autoreceptors possesses a significant inhibitory activity via a negative feedback towards acetylcholine release. It is now accepted that acetylcholine is not only a neurotransmitter but also an autocrine/paracrine hormone synthesized and secreted from non-neuronal cells including airway bronchial epithelial cells, fibroblasts and lymphocytes (Proskocil et al., 2004; Wessler et al., 2003; Wessler et al., 1999). Since patients with atopic dermatitis have been shown to have a substantially increased acetylcholine content in the skin, acetylcholine has been suggested to possess a possible pro-inflammatory role (Luger, 2002). Accordingly, in asthma and in COPD, neuronal acetylcholine might diffuse into the alveolar spaces and bronchial lumens where it could exert a pro-inflammatory effect (Wessler et al., 1999). Additionally, the involvement of non-neuronal sources of acetylcholine in inflammatory diseases has also recently been postulated as some pro-inflammatory and immunocompetent cells are able to release acetylcholine (Wessler et al., 1999).

Most chronic airway diseases, including COPD, are characterized by activation of the bronchial epithelium and by an influx of pro-inflammatory cells. Increased neutrophil numbers and activation in the airways has been demonstrated in COPD where the progression of the disease is related to activation of the airway epithelium (Glader et al., 2006; de Boer et al., 2000). In this context, in response to pro-inflammatory stimuli, the production of IL-8 is dependent on activation of MAPK and NFkB in epithelial cells (Oudin and Pugin, 2002; Holtmann et al., 1999). In unstimulated cells, NFkB is sequestered in the cytoplasm by IkBa proteins. However, phosphorylation and degradation of IkBa allows translocation of NFkB into the nucleus where it regulates gene transcription. IkBa may be phosphorylated by IkB kinase, which may be activated in turn by a number of kinases including MAPK/ERK1 (MEKK) and PKC (Karin and Delhase, 1998; Lallena et al., 1999; Zhao and Lee, 1999). In addition, in human bronchial epithelial cells, NFkB activation may regulate IL-8 release by an ERK-independent (Li et al., 2002) or dependent (Williams et al., 2007) process. The acetylcholinergic muscarinic receptors are G-protein coupled receptors involved in cell proliferation and protein synthesis via MAPK s activation (Jimenez and Montiel, 2005). Additionally, NFkB activation is dependent on ERK-dependent mechanism for the regulation of IL-8 release, in association to G-coupled receptor such as the bradykinin (Hayashi et al., 2000) and tachykinin (Williams et al., 2007) receptors. Furthermore, we demonstrated that muscarinic receptors are differently expressed in 16HBE cells and that acetylcholine promotes the release of increased concentrations of IL-8 by the phosphorylation of ERK1/2 and the activation of NFkB pathways via the IkBa phosphorylation and via a PKC and ERK1/2 dependent mechanism. Taken together, this demonstrates that the cascade of events which lead to the acetylcholine-mediated IL-8 release is strictly dependent on the subsequent PKC-ERK1/2-NFkB activation and strongly support the concept of a possible role played by acetylcholine in the airway inflammation of COPD patients (Proskocil and Fryer, 2005; Gosens et al., 2004).

Using the antagonists of muscarinic M1, M2, and M3 receptors, we show that the events described above are preferentially related to the activation of muscarinic receptors. The findings that the addition of muscarinic receptor antagonists are able to effectively counteract acetylcholine-mediated IL-8 release, demonstrate that the activity of this anticholinergic drug, despite its wide range of action, is related, for this specific effect, to the inhibition of the muscarinic receptors. In particular, the present evaluation of the antagonizing effects of tiotropium (Hansel and Barnes, 2002), telenzepine, gallamine and 4-DAMP (Gosens et al., 2003) on the release of IL-8, shows that tiotropium and 4-DAMP are able to completely abrogate the IL-8 release by 16HBE cells while telenzepine and gallamine only partially and not at all, respectively, interfere with this phenomenon. Despite it should be emphasized that the subtype selectivity of the “selective” muscarinic receptor antagonists is currently limited at the higher concentration, our results on the effects of acetylcholine stimulation, along with the partial inhibitory effect of telenzepine and gallamine, might suggest that the muscarinic M3 receptor is particularly involved in the activation of the airway epithelium.

Supernatants from 16HBE cells incubated with acetylcholine caused increased neutrophil chemotaxis suggesting that acetylcholine contributes to regulate neutrophil infiltration. Although we did not test the potential contribution of other neutrophils chemoattractants, such as LTB4, the finding that when the supernatants are pre-incubated with IL-8 antibodies the neutrophil chemotaxis is almost completely inhibited, strongly suggests that this acetylcholine-mediated effect is preferentially due to release of IL-8. The fact that this effect is abrogated when tiotropium is added to the acetylcholine stimulated 16HBE cells, demonstrates the involvement of the muscarinic receptors in the recruitment of neutrophils, and suggests that acetylcholine and muscarinic receptors are involved in the pro-inflammatory events occurring in COPD and supports the concept of an anti-inflammatory activity of anticholinergic drug in COPD (Profita et al., 2005).

The kinetic subtype selectivity of muscarinic receptor antagonists used in the treatment of COPD is of clinical importance and is still debatable. It may be expected that non-selective anticholinergic drug such as tiotropium causes not only a significant block of the muscarinic M3 receptor but also of the muscarinic M2 autoreceptor in the lung under clinical conditions. In the experimental model with 16HBE cells it appears that the commercially available anticholinergic drug might play a role in the prevention of inflammatory disorders overall in the presence of lower levels of the expression of muscarinic M2 receptor in comparison to M3 (Disse, 2001; Gosens et al., 2006), a condition similar to what detected in inflammatory cells of induced sputum of COPD subjects (Profita et al., 2005).
The main factors which lead to bronchial obstruction in COPD include mucus hypersecretion and an increase in bronchial muscle tone, which is triggered mainly by a cholinergic mechanism. Indeed, anticholinergic drugs are the bronchodilators of choice in the management of COPD, as the cholinergic tone appears to be the only partially reversible component in COPD. This clearly highlights the potential contribution of an altered reactivity due to an altered expression of the cholinergic system in the airway inflammation of COPD subjects (Profita et al., 2005). A number of factors may potentially contribute to the observed differences in the expression of muscarinic receptors. In particular, exposure to cytokines such as transforming growth factor-1 (TGF-β1) and tumor necrosis factor (TNF-α), known to be involved in the inflammatory process of COPD (Takizawa et al., 2001) as well as to be able to modulate muscarinic receptors expression (Haddad et al., 1996a,b), can certainly play a major role and may contribute to a vicious inflammatory circle within the airways of COPD subjects. It is presently unknown whether muscarinic M1, M2, and M3 receptors are differentially expressed in the airway epithelium during airway inflammation, but on the basis of previous evidences it is possible to hypothesize an altered expression of the levels of muscarinic receptors in the airway epithelium during inflammatory diseases such as asthma and COPD.

In this regard, the different expression profile of muscarinic receptors that we show in 16HBE cells and the ability of these cells to respond to higher concentrations of acetylcholine to produce IL-8, clearly suggest a potential involvement of acetylcholine in the release of neutrophil chemoattractant factor(s) by airway epithelial cells, and support the hypothesis that the cholinergic system, or non-neuronal acetylcholine, may be involved in regulating the synthesis of inflammatory mediators, such as IL-8, during airway inflammation in COPD. Further studies are needed to clarify these hypotheses.

The ability of muscarinic receptor antagonists, to modulate the release of a potent chemotactic factor such as IL-8 by the airway epithelium opens up new perspectives in the control of inflammatory cell migration into the airways of COPD patients, especially in light of new potent and selective muscarinic receptors antagonists becoming available. The effects of non-selective anticholinergic drug on the release of inflammatory mediators also provide support to the hypothesis that bronchodilators might also contribute to the control of inflammation in pulmonary diseases, including COPD and asthma.

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References


Acetylcholine and substance P stimulate bronchial epithelial cells to release eosinophil chemotactic activity. J. Appl. Physiol. 84, 1528–1534.


