Smoke, Choline Acetyltransferase, Muscarinic Receptors, and Fibroblast Proliferation in Chronic Obstructive Pulmonary Disease

Mirella Profita, Anna Bonanno, Liboria Siena, Andreina Bruno, Maria Ferraro, Angela Marina Montalbano, Giusy Daniela Albano, Loredana Riccobono, Paola Casarosa, Michael Paul Pieper, and Mark Gjomarkaj

Institute of Biomedicine and Molecular Immunology, Italian National Research Council, Palermo, Italy (M.P., A.BO., L.S., A.Br., M.F., A.M.M., G.D.A., L.R., M.G.); and Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany (P.C., M.P.P.)

Received September 18, 2008; accepted February 2, 2009

ABSTRACT

Acetylcholine (ACh), synthesized by choline acetyltransferase (ChAT), and muscarinic M1, M2, and M3 receptors (MRs) are involved in fibroblast proliferation. We evaluated ChAT, MRs, and extracellular signal-regulated kinase (ERK) 1/2 and nuclear factor (NF) κB activation in lung fibroblasts from patients with chronic obstructive pulmonary disease (COPD), control smokers, and controls. Human fetal lung fibroblasts (HFL-1) stimulated with interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and cigarette smoke extracts (CSEs) were evaluated for ChAT and MR expression. We tested the effects of ACh on fibroblast proliferation and its ability to bind fibroblasts from patients with COPD, control smokers, and controls, and HFL-1 stimulated with IL-1β, TNF-α, and CSE. ChAT, M1, and M3 expression and ERK1/2 and NFκB activation were increased, whereas M2 was reduced, in COPD and smoker subjects compared with controls. IL-1β increased the ChAT and M3, TNF-α down-regulated M2, and CSE increased ChAT and M3 expression while down-regulating the expression of M2 in HFL-1 cells. ACh stimulation increased fibroblast proliferation in patients with COPD, control smokers, and controls, with higher effect in control smokers and patients with COPD and increased HFL-1 proliferation only in CSE-treated cells. The binding of ACh was higher in patients with COPD and in control smokers than in controls and in CSE-treated than in IL-1β- and TNF-α-stimulated HFL-1 cells. Tiotropium (Spiriva; [1α,2β,4β,5α,7β-7-hydroxydi-2-thienylacetyl][oxo]-9,9-dimethyl-3-oxa-9-azoniatrcyclo[3.3.1.02,4]H22NO3S2Br2H2O), gallamine triethiodide (C19H22N4O2S), telenzepine [4,9-D-dihydro-3-methyl-4-(4-methyl-1piperazinyl)acetyl]-10H-thieno[3,4-b][1,5]benzodiazepine-10-one dihydrobromide, C30H60I3N3O3; 4-diphenylacetoxy-N-methylpyrrolidin-4H-1benzopyran-4-one, C14H15NO3; and BAY 11-7082 [(E)-3-(4-methylphenylsulfonyl)-2-propenitrile, C15H13NO2C], down-regulated the ACh-induced fibroblast proliferation, promoting the MRs and ERK1/2 and NFκB pathways involvement in this phenomenon. These results suggest that cigarette smoke might alter the expression of ChAT and MRs, promoting airway remodeling in COPD and that anticholinergic drugs, including tiotropium, might prevent these events.

This work was supported by a research grant from Boehringer Ingelheim Pharma GmbH and Co. KG (Biberach, Germany).

ABBREVIATIONS:
M1, muscarinic M1 receptor; M2, muscarinic M2 receptor; M3, muscarinic M3 receptor; MR, muscarinic M1, M2, and M3 receptor; MAPK, mitogen-activated protein kinase; ACh, acetylcholine; ChAT, choline acetyltransferase; COPD, chronic obstructive pulmonary disease; IL, interleukin; TNF, tumor necrosis factor; CS, cigarette smoke; ERK, extracellular signal-regulated kinase; NF, nuclear factor; CSE, cigarette smoke extract; C, asymptomatic nonsmoking subject(s) with normal lung function; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay; pNFκB, phosphorylated NFκB; TNFκB, total NFκB; tiotropium, Spiriva, [1α,2β,4β,5α,7β-7-hydroxydi-2-thienylacetyl][oxo]-9,9-dimethyl-3-oxa-9-azoniatrcyclo[3.3.1.02,4]H22NO3S2Br2H2O; telenzepine, 4,9-D-dihydro-3-methyl-4-(4-methyl-1piperazinyl)acetyl]-10H-thieno[3,4-b][1,5]benzodiazepine-10-one dihydrobromide, C30H60I3N3O3; gallamine triethiodide, C19H22N4O2S2Br2H2O; 4-DAMP, 4-diphenylacetoxy-N-methylpyrrolidin-4H-1benzopyran-4-one, C15H13NO3; BAY 11-7082 [(E)-3-(4-methylphenylsulfonyl)-2-propenitrile, C15H13NO2C]; hemicolinium-3, 2-(4,4′-biphenylene)bis[2-hydroxy-4,4′-dimethylmorpholinium bromide, C20H35BrN2O4; PBS, phosphate-buffered saline; ANOVA, analysis of variance; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; pERK, phospho-ERK1/2; tERK, total ERK1/2.
muscarnic M₁, M₂, and M₃ receptor (MR) proteins and that these receptors are involved in fibroblast proliferation (Matthiesen et al., 2006) via the activation of mitogenic-activated protein kinase kinase pathway (Profita et al., 2005). In addition, the MAPK may be associated with MR activity, inducing cell proliferation and protein synthesis in human breast cancer cells (Jiménez and Montioli, 2005) and promoting the cellular adhesion in M₃-transfected human embryonic kidney cells (Slack, 2000).

Acetylcholine (ACh) is the primary parasympathetic neurotransmitter in the airways and is traditionally associated with the induction of airway smooth muscle contraction and mucus secretion in airway diseases (Belmonte, 2005). ACh has also recently been demonstrated to be involved in airway inflammation and remodeling processes (Koyama et al., 1998; Sato et al., 1998; Gosena et al., 2004; Profita et al., 2005). It is synthesized by choline acetyltransferase (ChAT) in different cell types (macrophages, T lymphocytes, fibroblasts, and epithelial cells) (Wessler et al., 1999) and acts as an autocrine or paracrine growth factor in small-cell lung cancer by an increase of ChAT expression and ACh release (Song et al., 2003).

The ACh activity is mediated by MRs within the lung (Helme et al., 1990). It is involved in the activation of bronchial epithelial cells and alveolar macrophages and in the release of chemotactic mediators for eosinophils and neutrophils (Koyama et al., 1998; Sato et al., 1998; Gosena et al., 2004; Profita et al., 2005). In accordance, the ACh-mediated activation of MRs might contribute to promote the inflammatory processes involved in the pathogenesis of chronic obstructive pulmonary disease (COPD). The muscarinic M₃ receptor expression was increased, whereas muscarinic M₂ receptor (M₂) resulted decreased, in induced sputum cells (macrophages, neutrophils, eosinophils) from COPD subjects compared with controls and control smokers (Profita et al., 2005). In this context, it has been observed that cytokines involved in COPD, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α (Chung, 2001), are able to downregulate synergistically the M₂ expression in human embryonic lung fibroblasts (HeLa 299 cells), whereas there is no evidence on the role of cytokines in the modification of muscarinic M₃ receptor (M₃), M₄, and ChAT expression (Haddad et al., 1996). Moreover, the role of cigarette smoke (CS) on the expression of ChAT and MRs has to be clarified. In the present study, we assessed the expression of ChAT and of the MRs and the levels of the activation of extracellular signal-regulated kinase (ERK) 1/2 and nuclear factor (NF) κB pathways in human lung fibroblasts recovered from patients with COPD, control smokers, and controls. In addition, we tested the involvement of MRs and of ERK1/2 and NFκB pathway activation in the phenomenon of fibroblast proliferation induced by ACh in COPD. Finally, we evaluated whether the inflammatory cytokines IL-1β and TNF-α and cigarette smoke extracts (CSEs) affect the expression of ChAT and MRs and increase the binding of ACh promoting human lung fibroblast proliferation.

Materials and Methods

Study Population. Three groups of subjects who underwent lung resection for a solitary peripheral lung cancer were recruited: patients with COPD (n = 15), asymptomatic smokers with normal lung function (n = 10), and asymptomatic nonsmoking subjects with normal lung function (n = 10). Patients COPD were defined and classified according to the criteria reported by the GOLD guidelines (Pauwels et al., 2001) and were classified as stages I to II (mild to moderate COPD). Patients with COPD and control smokers had a smoking history of 10 pack years or more. Reversibility tests to bronchodilator were performed to exclude an asthmatic component, and the increase in FEV₁ was lower than 12% and 200 ml, compared with basal values, in all COPD subjects. All patients were characterized with respect to sex, age, smoking history, COPD symptoms, comorbidities, and current treatment. Exclusion criteria included the following: other systemic diseases, other lung diseases apart from COPD and lung tumors, upper respiratory tract infections, and treatment with glucocorticoids or anticholinergics within the 3 months before the study. The local region ethic committee approved the study, and participating subjects gave their informed consent.

Pulmonary Function Tests. Pulmonary function tests included measurements of blood gas analysis, FEV₁, and FVC. To assess the reversibility of the airway obstruction in subjects with a baseline FEV₁ less than 80% of predicted, the FEV₁ measurement were repeated 15 min after the inhalation of 200 µg of salbutamol.

Isolation of Human Lung Fibroblasts. Tumor free material and nontraumatic tissue of surgical specimens were used. Human lung fibroblasts were isolated from surgical specimens of human bronchus, as described previously by Jordana et al. (1988) with minor modifications. To confirm the purity of the cultured fibroblasts, the recovered cells were identified by their morphology, adherent nature, expression of vimentin and types I and III collagen, and lack of expression of cytokeratin, α-smooth muscle actin, factor VIII, and CD45. Immunoreactivity for these markers was revealed using the LSAB method (Dako Denmark A/S, Glostrup, Denmark) following the manufacturer’s instructions.

Cultures of Human Lung Fibroblasts. Purified human lung fibroblasts from patients with COPD and control smoker and control subjects were grown in a complete medium [RPMI 1640 plus 10% fetal calf serum, 25 mM, HEPES, 2 mM, penicillin (100 ng/ml), and streptomycin (100 ng/ml)] (Invitrogen, Paisley, UK) in a humidified atmosphere containing 5% CO₂ and passed by trypsinization at nearly confluence owing to 10-cm² culture plates. Subsequently, after an additional 24 h under FBS-free conditions (5% CO₂ at 37°C), the human lung fibroblasts were detached and treated for the protein extraction, flow cytometry analysis, and evaluation of the ACh binding. In addition, fibroblasts were plated onto six-well culture plates for the proliferation studies and cellular stimulation. Only early passage cells (1–3) were used for each experiment to avoid problems that may occur in the higher number of passage cells and the expression of MRs (Bany et al., 1999; Nicke et al., 1999; Pereira et al., 2003).

Western Blot Analyses of ChAT and MRs. Total protein extracts from cultured fibroblasts of patients with COPD and control smoker and control subjects were resuspended in 2× Laemmlli buffer and separated by SDS-PAGE followed by electroblotting onto nitrocellulose membranes for the ChAT and MR proteins. The following monoclonal antibodies were used: a rabbit monoclonal antibody directed against choline acetyltransferase (Millipore Bioscience Research Reagents, Temecula, CA), a rabbit polyclonal antibody directed against the human M₁ (H-120; Santa Cruz Biotechnology, Inc., Santa Cruz CA; recognizes human, mouse, and rat mAChR M₁), a rabbit polyclonal antibody directed against the human M₂ (H-170; Santa Cruz Biotechnology; recognizes human, mouse, and rat mAChR M₂), and a rabbit polyclonal antibody directed against the human M₃ (H-120; Santa Cruz Biotechnology, Inc.; recognizes human, mouse, and rat mAChR M₃). Primary antisera were visualized with horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO) and detected with an enhanced chemiluminescence system (GE Healthcare, Chalfont St. Giles, UK). Approximate molecular masses were determined using calibrated prestained standards (GE Healthcare). Negative controls were performed in the
absence of primary antibody or including an isotype control antibody. β-Actin (Sigma-Aldrich) was used as a housekeeping protein to control the quantity of proteins included in the Western blot analyses. To check whether the anti-M1, M2, or M3 Abs were specific for the single MR subtype and did not cross-react with other subtypes or with nonicotic receptors (Tice et al., 1996) and to check the molecular weight of ChAT and MRs, soluble recombinant peptides M1 (Santa Cruz Biotechnology, Inc.; 50 μg), M2 (Sigma-Aldrich; 30 μg), and M3 (Sigma-Aldrich; 50 μg) were used.

**Flow Cytometry Analyses.** The quantitative expression of ChAT and MRs was determined in human lung fibroblasts from patients with COPD and control smoker and control subjects using indirect label immunofluorescence by a FACStar Plus analyzer (BD Biosciences, San Jose, CA). Cells were permeabilized previously using a commercial available kit (Invitrogen, Carlsbad, CA). The primary antibodies were the same used for Western blot analyses. Anti-rabbit IgG FITC swine F(ab)2 (Dako Denmark A/S) was used as secondary Ab. FITC-conjugated rabbit was used as an isotype negative control antibody (Dako Denmark A/S).

**ERK1/2 and NFκB Activation in Human Lung Fibroblasts.** To assess ERK1/2 and NFκB activation in cell lysates of human lung fibroblasts from patients with COPD and control smoker and control subjects, Western blot analyses were performed using two rabbit monoclonal antibodies directed against anti-phospho-ERK1/2 and against anti-phospho-IκBα rabbit antibody (Cell Signaling Technology Inc., Danvers, MA), and anti-β-actin antibody (Sigma-Aldrich). In addition, the ERK1/2 and NFκB activation were evaluated using two commercially available ELISA kits (Superarray Bioscience Corporation, Frederick, MD) that measure the phosphorylated ERK1/2 and the total ERK1/2 and the phosphorylated NFκB (pNFκB) and the total NFκB (tNFκB). Results are expressed as pERK1/2/ERK1/2 and pNFκB/tNFκB ratios, respectively.

**Gel Image Evaluation.** Gel images were taken with an EPSON GT-6000 scanner and then imported to a NIH Image analyses 1.61 program to determine band intensity. Data are expressed as arbitrary densitometric units corrected against the density of β-actin bands.

**ACH Stimulation of Human Lung Fibroblasts.** To investigate the functional role of MRs in the proliferation of fibroblasts from patients with COPD and control smoker and control subjects, cells were cultured for 24 h in the presence of 10% FBS, followed by an additional 24 h under FBS-free conditions (5% CO2 at 37°C). Fibroblasts were then incubated for 24 h with different concentration of ACh (1 nM–10 μM) [acetylcholine chloride, (CH3)3N–CH2–OCCH3Cl] (Sigma-Aldrich), and their proliferation was then evaluated. The kinetics of ERK1/2 and NFκB activation were tested in fibroblasts stimulated with ACh (10 nM) (15 min–1 h). The effects of the following anticholinergic compounds on fibroblast proliferation were next evaluated: tiotropium bromide (Spiriva) (0.1 μM) (Boehringer Ingelheim Pharma GmbH and Co. KG, Biberach, Germany), telenzepine (described as M1 antagonist, 20 μM) (Sigma-Aldrich), gallamine triethiodide (described as M2 antagonist, 1 μM) (Sigma-Aldrich), and 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) methiodide (described as M3 antagonist, 1 μM) (Sigma-Aldrich). The drugs were added to fibroblasts 1 h before the stimulation with acetylcholine (10 nM) to understand whether the activation of MRs was related to M1, M2, or M3. In addition, to identify whether ACh affects fibroblast proliferation via ERK1/2 and NFκB pathways, the cells were preincubated (1 h, 37°C, 5% CO2) with PD98059, an inhibitor of mitogen-activated protein kinase kinase activation, 50 μM (Sigma-Aldrich) or with BAY 11-7082, an inhibitor of IκBα phosphorylation, 50 μM (Sigma-Aldrich) before the addition of ACh (10 nM) for 24 h for cell proliferation and for 30 min for ERK1/2 and NFκB activation. Cell viability was determined by light microscopy and trypan blue exclusion. Cell numbers were measured by directly counting the cells using a hemocytometer. To clarify the role of ChAT and endogenous ACh synthesis in fibroblast proliferation, we tested the effect of hemicholinium-3, a potent and selective choline uptake blocker (50 μM) (Sigma-Aldrich) added to fibroblasts immediately before ACh (10 nM).

**Preparation of CSE.** Commercial cigarettes (Marlboro; Philip Morris USA, Richmond, VA) were used in this study. CSE was prepared as described previously (Su et al., 1998), with some modifications. Each cigarette was smoked for 5 min, and two cigarettes were used per 25 ml of PBS to generate a CSE-PBS solution. The CSE-PBS solution was filtered through a 0.22-μm pore size to remove bacteria and large particulates. The smoke solution was then adjusted to pH 7.4. To prevent possible inactivation of compounds present in the filtered CSE-PBS solution, it was kept in the dark and used within 30 min from the preparation. This solution was considered as a 100% CSE and subsequently diluted to obtain the desired concentrations in each experiment. The concentration of CSE was calculated spectrophotometrically, measuring the OD between 270 and 280 as described previously (Luppi et al., 2005). The pattern of absorbance among different batches showed very low differences. The concentration, expressed as arbitrary units per milliliter, was calculated based on the following formula: ODmax × 2 × dilution factor. The CSE was further diluted to the required concentration in fresh culture medium. Ten arbitrary units per milliliter was found to correspond to a mean of 5% (v/v) CSE. HFL-1 cells were exposed to various concentrations of CSE.

**HFL-1 Stimulation with IL-1β, TNF-α, and CSE.** To evaluate whether cytokines IL-1β and TNF-α and CS were affecting fibroblast proliferation by altering the expression of ChAT and MRs, human fetal lung fibroblasts (HFL-1) (American Type Culture Collection, Manassas, VA) were cultured at 1 × 106 ml for 24 h in the presence or absence of human recombinant IL-1β (0.1–2.5 ng/ml) (R&D Systems Europe Ltd, Abingdon, Oxfordshire, UK), human recombinant TNF-α (0.1–5 ng/ml) (R&D Systems Europe Ltd), and CSE (2.5–10%). The protein expression subsequently was measured by Western blot and flow cytometry analyses as described above. Additional experiments were performed with HFL-1 stimulated with IL-1β (5 ng/ml), TNF-α (5 ng/ml), and CSE (5%) in the presence or absence of tiotropium (0.1 μM), followed by an additional 6-h culture under FBS-free conditions (5% CO2, 37°C). These cell cultures were then further incubated with and without ACh (10 nM) for 24 h, and then cell proliferation analyses were performed as described above. In addition, to clarify the role of ChAT and endogenous ACh synthesis in fibroblast proliferation, we tested the effect of hemicholinium-3 (50 μM), added to the cells immediately before the ACh (10 nM).

**Binding of ACh to Fibroblasts from COPD Patients, Control Smokers, Controls, and to HFL-1 Cells.** Fibroblasts from patients with COPD, control smokers, and controls and HFL-1 cells [cultured at 1 × 105 for 24 h in the presence or absence of IL-1β (2.5 ng/ml), TNF-α (5 ng/ml), and CSE (10%)], were studied for the binding of ACh in the presence or absence of tiotropium (0.1 μM), telenzepine (20 μM), galamine (1 μM), and 4-DAMP (1 μM). Approximately 5 × 105 cells were treated with trypsin, washed in cold (37°C) PBS, and incubated for 1 h at 4°C with ACh (10 nM) (Sigma-Aldrich). The cells were washed in cold (37°C) PBS and incubated for 1 h at 4°C with a rabbit polyclonal anti-ACh antibody (Abcam plc, Cambridge, UK). Anti rabbit IgG was used as an isotype-negative control antibody (Dako Denmark A/S). No specific binding and background fluorescence were detected by analyzing negative control samples. After washing cells with cold (37°C) PBS, an FITC-conjugated polyclonal swine anti-rabbit IgG (Dako Denmark A/S) was added to the cells and incubated for 30 min at 4°C. Fluorescence-positive cells were analyzed by using a FACStar Plus (BD Biosciences) analyzer. The percentage of positive cells was determined from forward and sideways scatter patterns.

**Statistical Analysis.** Data are expressed as mean ± S.D. Statistical analysis for multiple comparison were calculated using one-way analyses of variance (ANOVA) or Kruskal-Wallis and nonparametric ANOVA tests followed by Fisher’s PLSD correction for multiple comparison. Student’s t test was used for the cell proliferation experiments, for ERK1/2 and NFκB activation, and
for ACh binding experiments. A p value < 0.05 was considered as statistically significant.

Results

Demographic Characteristic of the Patients. The patients’ characteristic are summarized in Table 1. The differences in the median ages or smoking history of the controls, control smokers, and subjects with COPD were not statistically significant. In accordance with the used classification of the FEV1% of patients after bronchodilators was significantly lower in patients with COPD than control smokers and controls (p < 0.001) and FEV1%/FVC (p < 0.001).

Expression of ChAT and M1, M2, and M3 in Human Lung Fibroblasts. ChAT, M1, and M3 expression was significantly higher in patients with COPD and control smokers than in controls, whereas M2 expression was lower in patients with COPD and control smokers than in controls when evaluated by semiquantitative analyses of cell lysates (Fig. 1A). These results were confirmed by flow cytometric analyses (Fig. 1, B and C). The described pattern of MR expression was stable under the culture conditions because a similar pattern was observed until the third data (part not shown).

Levels of ERK1/2 and NFkB Activation in Cultured Human Lung Fibroblasts. The analyses of cell lysates of human lung fibroblasts showed a significantly increased activation of ERK1/2 and NFkB pathways in patients with COPD and control smokers compared with controls. These results were obtained by Western blot evaluating the presence of phospho-ERK1/2 and phospho-IκBα proteins (Fig. 2A) and by ELISA measuring the molar ratio between pERK1/2 and tERK1/2 as well as between pNFκB and tNFκB (Fig. 2B).

ACh Induces Fibroblast Proliferation. ACh was able to stimulate fibroblast proliferation in a dose-dependent manner. Human lung fibroblasts cultured in the presence of increasing concentrations of ACh (1 nM–10 μM) reached the maximal proliferation at the dose of 10 nM, whereas further higher doses of ACh induced a reduction of the proliferation that reaches a plateau (Fig. 3A). Having identified the optimal ACh concentration (10 nM), we evaluated whether ACh was able to differently stimulate fibroblast proliferation in patients with COPD, control smokers, and controls. It is interesting that ACh was able to significantly increase fibroblast proliferation in all the three study groups and significantly higher levels of cell proliferation were observed in patients with COPD (p < 0.0001) and control smokers (p < 0.001) than in controls (p < 0.05) compared with each basal value. In addition, statistical significant differences were observed between the effect of ACh on fibroblast proliferation of patients with COPD compared with the effect of ACh on fibroblast proliferation of control smokers (p < 0.05) and of controls (p < 0.0001). Statistical differences were also observed between the effect of ACh on proliferation of control smokers and controls (p < 0.05) and between control smokers and patients with COPD (p < 0.05). The addition of tiotropium (0.1 μM) was able to prevent the pro-proliferative effect of ACh in a significant manner in patients with COPD (p < 0.0001), in control smokers (p < 0.001), and in controls (p < 0.05) (Fig. 3B). Tiotropium (0.1 μM) alone failed to reduce the basal values of fibroblast proliferation. The viability of the cells in the presence of tiotropium ranged from 89 to 98%.

ACh Stimulates Fibroblast Proliferation through Different Mechanisms. To verify which MRs and intracellular signal pathways were involved in the ACh-induced fibroblast proliferation in COPD, we performed additional experiments using specific inhibitors of MRs and intracellular pathways. Tiotropium (0.1 μM), telenzepine (20 μM), and 4-DAMP (1 μM) were able to completely abolish the ACh-mediated fibroblast proliferation, whereas gallamine (1 μM) exerted a less potent down-regulation of this effect (Fig. 4A). These results might suggest that all MRs mediated ACh-induced fibroblast proliferation. Furthermore the preincubation of fibroblasts with PD098059 (50 μM) and BAY 11-7082 (50 μM) was also able to inhibit the ACh-induced proliferation, demonstrating that in COPD, M1, M2, and M3 mediate fibroblast proliferation via the activation of ERK1/2 and NFkB pathways (Fig. 4B). The use of tiotropium (0.1 μM), telenzepine (20 μM), gallamine (1 μM), 4-DAMP (1 μM), PD098059 (50 μM), and BAY 11-7082 (50 μM) alone did not affect the fibroblast proliferation in the absence of ACh stimulation (data not shown). In addition, the hemicholinium-3 did not affect the basal values of cell proliferation, whereas it inhibited the effect of exogenous ACh (10 nM) on fibroblast proliferation of patients with COPD (Fig. 4C). These findings suggest that the autocrine release of ACh generated by ChAT is not involved in fibroblast proliferation. The viability of the cells in the presence of tiotropium ranged from 90 to 98%; in the presence of telenzepine, gallamine, 4-DAMP, and hemicholinium-3, from 89 to 97%; and in the presence of PD098059 and BAY 11-7082, from 90 to 98%.

ACh Stimulates ERK1/2 and NFkB Pathways. The kinetics of ERK1/2 and NFkB activation by ACh stimulation of fibroblasts from patients with COPD showed the maximal activation of phospho-ERK1/2 and phospho-IκBα after 30 min of incubation with ACh (10 nM), evaluated by Western blot and by the molar ratio between pERK1/2 and tERK1/2 as well as between pNFκB and tNFκB evaluated by the ELISA analyses (Fig. 5, A and B). In addition, the preincubation of

---

**TABLE 1**

Characteristics of patients

<table>
<thead>
<tr>
<th>Statistical analysis was performed with Kruskal-Wallis test. Mean ± S.D. values are shown.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>Subject no.</td>
</tr>
<tr>
<td>Sex (male/female)</td>
</tr>
<tr>
<td>Mean age (years)</td>
</tr>
<tr>
<td>FEV1% predicted</td>
</tr>
<tr>
<td>FEV1/FVC%</td>
</tr>
<tr>
<td>Pack years</td>
</tr>
</tbody>
</table>

* Statistical differences between the groups.
fibroblasts with tiotropium (0.1 μM) and PD098059 (50 μM) was able to inhibit the ACh-mediated activation of both pathways, whereas, as expected, the preincubation with BAY 11-7082 (50 μM) was able to inhibit only the activation of NFκB (Fig. 5, C and D). Finally, the use of tiotropium (0.1 μM), telenzepine (20 μM), gallamine (1 μM), 4-DAMP (1 μM), PD098059 (50 μM), and BAY 11-7082 (50 μM) alone did not affect the basal values of ERK1/2 and NFκB pathway activation (data not shown). The viability of the cells in the presence of tiotropium ranged from 90 to 98%; in the presence of telenzepine, gallamine, and 4-DAMP, the presence of tiotropium ranged from 87 to 98%; and in the presence of PD098059 and BAY 11-7082, the presence of tiotropium ranged from 89 to 97%.

**IL-1β, TNF-α, and CSE Alter the Expression of ChAT, M1, M2, and M3 Proteins and HFL-1 Proliferation.** We next wondered whether an inflammatory environment, including the presence of CS, was able to affect the expression of ChAT, M1, M2, and M3 proteins in HFL-1 cells. The incubation of HFL-1 with IL-1β was able to significantly and dramatically increase the expression of ChAT protein at all the tested concentrations and to significantly increase the expression of M3 protein only at the highest concentration. No effects were exerted on the expression of the other proteins. The incubation with TNF-α generated a down-regulation of M2 protein only at the highest tested concentration, and no effects were exerted on the expression of the other proteins. It is interesting that the incubation with CSE significantly and dramatically increased the expression of ChAT protein at all tested concentrations and also increased the expression of the M3 protein at the two highest tested concentrations. Moreover, CSE was also able to down-regulate the expression of the M2 protein at the highest tested concentration. No effects were exerted by CSE on the M1 protein (Fig. 6A). These results, generated by flow cytometric analyses, were also confirmed by Western blot analyses of ChAT and MRs (Fig. 6B) when the protein concentrations are evaluated as arbitrary densitometric units corrected against the density of β-actin bands (data not shown). The incubation with ACh (10 nM) significantly increased HFL-1 cell proliferation, and this effect was significantly further increased by presence of CSE. It is interesting that these effects were completely counteracted by the presence of tiotropium (0.1 μM). IL-1β and of TNF-α did not exert any effects on the
Fig. 3. ACh-induced cell proliferation in human lung fibroblasts from patients with COPD (n = 15), control smokers (n = 15), and controls (n = 8). A, cells were stimulated with different concentrations of ACh (1 nM–10 μM) at 37°C for 24 h, and fibroblast proliferation was evaluated (for details, see Materials and Methods). B, molar ratio between pERK1/2 and tERK1/2 as well as between pNFκB and tNFκB by ELISA. Box plots, mean ± S.D. of the values. *, p < 0.05 (values are calculated by Kruskal-Wallis and ANOVA with Fisher’s PLDS correction for multiple comparison).

Fig. 2. Levels of ERK1/2 and NFκB activation from human lung fibroblasts from patients with COPD (n = 15), control smokers (n = 15), and controls (n = 8). A, box plots, evaluation of pERK1/2 and pIκBα proteins by Western blot analyses expressed as arbitrary densitometric units corrected against the density of β-actin bands. Representatives Western blot analyses of pERK1/2 and pIκBα are shown. B, molar ratio between pERK1/2 and tERK1/2 as well as between pNFκB and tNFκB by ELISA. Box plots, mean ± S.D. of the values. *, p < 0.05 (values are calculated by Kruskal-Wallis and ANOVA with Fisher’s PLDS correction for multiple comparison).
HFL-1 cell proliferation (Fig. 7A). In addition, the use of hemicholinium-3 did not affect the values of cell proliferation in HFL-1 stimulated with IL-1 or CSE, whereas it inhibited the effect of ACh (10 nM) on HFL-1 stimulated with CSE (Fig. 7B). These findings suggest that the autocrine release of ACh generated by ChAT in HFL-1 cells stimulated with IL-1 and CSE is not involved in fibroblast proliferation. The viability of the cells in the presence of tiotropium ranged from 92 to 98%, whereas in the presence of hemicholinium-3, it ranged from 89 to 97%.

**Binding of ACh to Human Lung Fibroblasts from COPD Patients and to CSE-Treated HFL-1 Cells.** To further get insight the ACh-mediated proinflammatory mechanisms described above, we tested the ability of ACh to bind fibroblasts. The additional advantage of this technique consists of the fact that the natural agonist can bind and recognize just receptors correctly folded at the plasma membrane, whereas antibodies in principle could also recognize and quantify misfolded proteins. Higher levels of the ACh binding to human lung fibroblasts were detected in patients with COPD and control smoker subjects compared with controls. Moreover, higher levels of ACh binding to HFL-1 cells were detected in the presence of CSE compared with the basal condition or with the presence of IL-1β or TNF-α (Fig. 8B). Results obtained with ACh binding confirm up-regulation of muscarinic receptors in patients with COPD. No statistically significant differences were observed in the experiments using both the selective MR antagonists and tiotropium (data not shown). These data suggest that, although ACh exerts the pro-proliferative activity through MRs, it might bind both nicotinic and muscarinic receptors in fibroblasts (Carlisle et al., 2004).

**Discussion**

This study describes an increased expression of ChAT, M₁, and M₃ and a decreased expression of M₂ in human lung fibroblasts isolated from patients with COPD and control smoker subjects compared with controls. Moreover, higher levels of activation of the ERK1/2 and NFκB pathways in human lung fibroblasts from patients with COPD and control smokers compared with controls were detected, suggesting higher fibroblast activation in patients with COPD than in control smokers (Fig. 8A). In addition, higher levels of ACh binding to HFL-1 cells were detected in the presence of CSE compared with the basal condition or with the presence of IL-1β or TNF-α (Fig. 8B). Results obtained with ACh binding confirm up-regulation of muscarinic receptors in patients with COPD. No statistically significant differences were observed in the experiments using both the selective MR antagonists and tiotropium (data not shown). These data suggest that, although ACh exerts the pro-proliferative activity through MRs, it might bind both nicotinic and muscarinic receptors in fibroblasts (Carlisle et al., 2004).
control smokers than in control subjects. We also showed that ACh stimulation of human lung fibroblasts alters the normal status of cell proliferation in fibroblasts from patients with COPD, control smokers, and controls, with higher levels of fibroblast proliferation in COPD subjects because of ERK1/2 and NFκB pathway activation. CS alters the expression of ChAT and MRs in lung fibroblasts and increases the binding of ACh to fibroblasts. The addition of tiotropium counteracts most of these events. To our knowledge, this is the first study that describes a proinflammatory and pro-remodeling involvement of ACh-mediated events in airway fibroblasts of COPD subjects and shows the ability of tiotropium to counteract these events.

We show here higher levels of M1 and M3 proteins and lower levels of M2 proteins in fibroblasts from patients with COPD compared with controls, confirming that the airway inflammation may alter the expression of MRs in COPD (Profita et al., 2005). These findings support the presence of a dysregulation of MR expression that might be involved in inflammatory and pro-remodeling effects.

ACh is also secreted from non-neuronal cells, including bronchial epithelial cells, fibroblasts, and lymphocytes (Wessler et al., 1999, 2003; Proskocil et al., 2004). We demonstrate an increased expression of ChAT in lung fibroblasts from patients with COPD and control smokers, with a possible consequent overproduction of ACh in the airways of these subjects that might take part in airway inflammation and remodeling by targeting the expression of MRs in fibroblasts. Although in our ex vivo experiments the basal autocrine production of ACh seems not to be involved in fibroblast proliferation, the exogenous ACh added to the cultures targets fibroblasts from COPD subjects and control smokers, promoting their proliferation. The use of choline uptake blocker inhibits the effect of exogenous ACh on fibroblasts, supporting the concept that the extracellular ACh-esterases might hydrolyze exogenous ACh in acetate and choline, which in turn is taken up into the cells and serves as substrate of the overexpressed cellular ChAT, which generates further ACh production. Within the airways of COPD and smoker subjects, the effects exerted by the exogenous ACh used in our experiments might be exerted by the possible release of ACh by proinflammatory cells present in the airways of COPD and smoker subjects. Additional studies are necessary to further investigate this phenomenon.

The altered expression of MRs in fibroblasts from COPD subjects and control smokers associated with increased fibroblast proliferation supports the hypothesis that all MRs participate in the phenomenon of fibroblast proliferation in COPD and not only the M2 or the M3 as described previously (Matthiesen et al., 2006; Pieper et al., 2007). This is further supported by the findings that the addition of the MR antagonists tiotropium, telenzepine, gallamine, and 4-DAMP com-

![Fig. 5. ACh-induced ERK1/2 and NFκB activation in human lung fibroblasts from COPD subjects. The kinetic of ERK1/2 and NFκB activation was evaluated in fibroblasts incubated with 10 nM ACh for 15, 30, and 60 min and analyzed for ERK1/2 and NFκB activation by Western blot (A) and by ELISA (B). Fibroblasts were stimulated with ACh 10 nM at 37°C for 30 min in the presence of tiotropium, PD098059, and BAY 11-7082, added to the cultures 1 h before the addition of ACh by Western blot (C) and by ELISA (D). Evaluation of pERK1/2, pIκBα, and β-actin proteins by Western blot analyses. Representative experiment of SDS-PAGE (n = 12). Molar ratio between pERK1/2 and tERK1/2 as well as between pNFκB and tNFκB by the ELISA (for details, see Materials and Methods). Bars, mean ± S.D. of 12 different experiments. Student’s t test was used to compare the experimental conditions. *, p < 0.05 versus bars without asterisk.]
Fig. 7. ACh-induced cell proliferation in HFL-1 cells. A, HFL-1 cells were incubated with IL-1β (0.1–2.5 ng/ml), TNF-α (0.1–5 ng/ml), and CSE (2.5–10%) in the presence or absence of tiotropium (0.1 μM) (added to the cultures 1 h before ACh) and then stimulated with ACh (10 nM) for 24 h at 37°C (for details, see Materials and Methods) or in the presence of hemicholinium-3 (50 μM) added to the cultures immediately before the addition of ACh (B). Values are expressed as mean ± S.D. of cell number (×10⁵). Student’s t test was used to compare the experimental conditions. *, p < 0.05 versus baseline without IL-1β, TNF-α, and CSE; **, p < 0.001 versus all the other conditions.

Fig. 6. Expression of ChAT, M₁, M₂, and M₃ proteins from HFL-1 cells stimulated with IL-1β (0.1–2.5 ng/ml), TNF-α (0.1–5 ng/ml), and CSE (2.5–10%) for 24 h. A, evaluation of the levels of ChAT, M₁, M₂, and M₃ proteins by flow cytometry. Data are expressed as mean ± S.D. of fluorescence intensity. Student’s t test was used to compare the experimental conditions. *, p < 0.05 was accepted as statistically significant. B, representative Western blot analyses of ChAT, M₁, M₂, and M₃ proteins from lysates of HFL-1 cells. Representative experiment of SDS-PAGE (n = 6). *, p < 0.001 versus baseline.
completely or partially (gallamine) counteracts the ACh-mediated fibroblast proliferation from COPD subjects. Moreover, these inhibitory effects exerted by anticholinergic drugs demonstrate that these events are preferentially related to the activation of MRs and not of nicotinic receptors in human lung fibroblasts (Carlisle et al., 2004). The ability of the specific MR antagonists used here (telenzepine, gallamine, and 4-DAMP) to affect ACh-mediated fibroblast proliferation demonstrates that all the MRs are involved in this phenomenon, and the ability of tiotropium to completely inhibit the ACh-mediated fibroblast proliferation suggests that ACh, although unable to selectively bind MRs, exerts an anti-inflamatory activity in patients with COPD. On the basis of these observations, it is conceivable that tiotropium causes not only a significant block of the $M_3$ receptor but also the block of $M_1$ and $M_2$ in the lung under clinical conditions (Disse, 2001), interfering also with the proinflammatory activities of MRs.

NFkB is sequestered in the cytoplasm by IxBa proteins. The IxBa phosphorylation and degradation by the IxB kinase allow the translocation of NFkB into the nucleus, where it regulates gene transcription. IkB kinase may be activated by MAPK/ERK1 (mitogen-activated protein kinase kinase kinase) in association with the activation of G-coupled receptors (Ye, 2001). MRs are G-protein-coupled receptors involved in cell proliferation and protein synthesis via MAPKs (Jiménez and Montiel, 2005). In this context, ACh stimulation of human lung fibroblasts from patients with COPD promotes the ERK1/2 and NFkB pathway activation, suggesting that the ACh-mediated fibroblast proliferation might involve these pathways. This is further supported by the findings that tiotropium, PD098059, and BAY 11-7082 inhibit ACh effects on fibroblast proliferation in patients with COPD together with the down-regulation of ERK1/2 and NFkB activation. In addition, the complete abrogation of ACh-induced IxBa phosphorylation by the use of PD098059 clearly demonstrates that the activation of NFkB is dependent on the ERK1/2 pathway.

IL-1β and TNF-α are involved in COPD (Chung, 2001) and have been demonstrated to generate a down-regulation of $M_2$ in human embryonic lung fibroblasts (HeLa 299 cells) (Haddad et al., 1996). Our findings that $M_2$ expression is reduced in fibroblasts from patients with COPD, compared with control smoker and control subjects, and that TNF-α is able to contribute to the $M_2$ down-regulation are consistent with these observations and support the concept of the involvement of ACh and MRs in the inflammatory processes in COPD. In addition, the observed altered expression of ChAT and MRs in control smokers, compared with control subjects, suggests that the expression of the noncholinergic system components in human lung fibroblasts may be affected by CS.

The experiments with HFL-1 cells studying the effects of IL-1β, TNF-α, and CSE on MRs and ChAT expression and on fibroblast proliferation and on the ACh binding, performed here to understand whether the observed altered expression of ChAT and MRs in patients with COPD, and their functional consequences, are affected by the presence of cytokines and of CS, demonstrate that cells preincubated with CSE show a significant increased proliferation and significant increased levels of ACh binding. CSE-mediated cell proliferation is completely abolished by the use of tiotropium, whereas the binding of ACh was not reduced (data not shown), in accordance with the presence of nicotine receptors on fibroblasts (Carlisle et al., 2004). These results are consistent with the findings of a dysregulation of MRs because of CSE (increased expression of $M_2$ associated with a down-regulation of $M_3$) and with our previous observations showing a proinflammatory response of the cells to ACh in the presence of high levels of $M_3$ associated with low levels of $M_2$ (Profita et al., 2005, 2008). This MR dysregulation concept is further supported by the absence of pro-proliferative effects in the presence of IL-1β and TNF-α; the observed effect of IL-1β to increase the expression of ChAT in HFL-1 cells is not sufficient, in the absence of MR dysregulation, to generate...
any ACh-mediated effects on fibroblast proliferation. In accordance, the TNF-α incubation of HFL-1, generating only a down-regulation of M₂, also is unable to exert any effects on cell proliferation.

In the airways of patients with COPD, the dysregulation of the M₂ and M₃ expression associated with the presence of ACh, may generate a consistent fibroblast proliferation, contributing to airway remodeling. It is interesting that we also observed an increased activation of fibroblast proliferation after ACh stimulation in control subjects, and these results are consistent with those obtained by Matthiesen et al. (2006, 2007), but the stimulation with ACh of fibroblasts from COPD subjects and smoker subjects generated higher levels of cell proliferation. These different levels of response to ACh between fibroblasts from patients with COPD and control smokers in comparison with controls may be due to the phenomenon of cross-talk associated with M₂ and M₃ and to the ERK1/2 activity (Hornigold et al., 2003). The cross-talk phenomenon provides novel insights into the involvement of G(i/o) proteins in the positive modulation of ERK responses evoked by G protein-coupled receptors and may shift the cellular response to lower levels of the agonist, promoting higher levels of ACh bound to the cells with a consequent more consistent response (Hornigold et al., 2003). In accordance, our results show that higher levels of ACh were bound to fibroblasts with an altered expression of M₂ and M₃, and higher levels of proliferation were present in fibroblasts from COPD subjects and in HLF-1 stimulated with CSE. Additional studies are needed to further clarify the effects of inflammation on the cross-talk phenomenon associated with M₂ and M₃ expression in fibroblasts from patients with COPD.

In conclusion, our results support the hypothesis that the cholinergic system, or non-neuronal ACh, may be involved in the regulation of airway inflammation and remodeling in COPD. The ability of MR antagonists, including tiotropium, to modulate fibroblast proliferation opens up new perspectives for the control of structural changes of the airways in patients with COPD, especially in light of new potent and selective MR antagonists becoming available. The effects of tiotropium on the proliferative activity of human lung fibroblasts also support the hypothesis that bronchodilators may contribute to control inflammation in chronic pulmonary diseases, including COPD.

References


Pieper M, Bonanno A, Siena L, Ferraro M, Montalbano AM, Pompeo F, Riccobono L, Profita M, Bonanno A, Siena L, Ferraro M, Montalbano AM, Pompeo F, Riccobono L, Malfa 153, 90146 Palermo, Italy. E-mail: profita@ibim.cnr.it

Address correspondence to: Dr. Mirella Profita, Institute of Biomedicine and Molecular Immunology, Italian National Research Council, Via Ugo La Malfa 153, 90146 Palermo, Italy. E-mail: profita@ibim.cnr.it

Dr. Mirella Profita, Institute of Biomedicine and Molecular Immunology, Italian National Research Council, Via Ugo La Malfa 153, 90146 Palermo, Italy. E-mail: profita@ibim.cnr.it