Biochimica et Biophysica Acta 1822 (2012) 1079–1089

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

β_2 long-acting and anticholinergic drugs control TGF-β1-mediated neutrophilic inflammation in COPD

Mirella Profita [a,*], Anna Bonanno [a], Angela Marina Montalbano [a], Giusy Daniela Albano [a,b], Loredana Riccobono [a], Liboria Siena [a], Maria Ferraro [a], Paola Casarosa [c], Michael Paul Pieper [c], Mark Gjomarkaj [a]

[a] Unit: "Ex vivo/In vitro models to study the Immunopathology and the Pharmacology of airway diseases", Institute of Biomedicine and Molecular Immunology (IBIM), Italian National Research Council (CNR), Palermo, Italy
[b] Dipartimento Biomedico di Medicina, Interna e Specialistica (Di.Bi.M.I.S.), Sezione di Pneumologia, University of Palermo, Palermo, Italy
[c] Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany

ARTICLE INFO

Article history:
Received 12 August 2011
Received in revised form 13 February 2012
Accepted 6 March 2012
Available online 13 March 2012

Keywords:
TGF-β1
COPD
Neutrophilic inflammation
Beta$_2$ long acting
Anticholinergic drug

ABSTRACT

We quantified TGF-β1 and acetylcholine (ACh) concentrations in induced sputum supernatants (ISSs) from 18 healthy controls (HC), 22 healthy smokers (HS) and 21 COPDs. ISSs from HC, HS and COPD as well as rhTGF-β1 were also tested in neutrophil adhesion and in mAChR2, mAChR3 and ChAT expression experiments in human bronchial epithelial cells (16-HBE). Finally, we evaluated the effects of Olodaterol (a novel inhaled β_2-adrenoceptor agonist) and Tiotropium Spiriva®, alone or in combination, on neutrophil adhesion and mAChRs and ChAT expression in stimulated 16-HBE. The results showed that 1) TGF-β1 and ACh concentrations are increased in ISSs from COPD in comparison to HC and HS, and TGF-β1 in HS is higher than in HC; 2) ISSs from COPD and HS caused increased neutrophil adhesion to 16-HBE when compared to ISSs from HC. The effect of ISSs from COPD was significantly reduced by TGF-β1 depletion or by the pretreatment with Olodaterol or Tiotropium alone or in combination, while the effect of ISSs from HS was significantly reduced by the pretreatment with Olodaterol alone; 3) mAChR2, mAChR3 and ChAT expression was increased in 16-HBE stimulated with ISSs from COPD and TGF-β1 depletion significantly reduced this effect on mAChR3 and ChAT expression; 4) rhTGF-β1 increased mAChR2, mAChR3 and ChAT expression in 16-HBE; 5) Olodaterol did not affect the expression of mAChRs and ChAT in 16-HBE. Our findings support the use of β_2 long-acting and anticholinergic drugs to control the bronchoconstriction and TGF-β1-mediated neutrophilic inflammation in COPD.

1. Introduction

β_2-Adrenoceptor (AR)-agonists are the most potent known airway smooth muscle relaxants and they have been targeted for decades to treat asthma and COPD. β_2-AR-agonists are present in inflammatory cells and inhibit their activation, the release of mediators, and the recruitment and the survival of these cells [1]. In human alveolar epithelial cells stimulated with TNF-α, β_2-AR agonists exert a negative regulatory control in the secretion of CXCL8/IL-8, GMCSF and VEGF [2] and reduce neutrophilic airway inflammation in patients with mild asthma [3]. The current guidelines recommend the regular use of β_2-AR-agonists and anticholinergic-bronchodilator agents alone or as concurrent therapy in COPD to maximize bronchodilation [1,4] and, although studies suggest a potential antiinflammatory role of β_2-AR-agonists and anticholinergic drugs, the antiinflammatory efficacy of this combination is not yet widely studied.

Airway epithelial cells are central cells in the pathogenesis of COPD and the function of the airway epithelium is further modified by local inflammatory/immune signals often promoting neutrophilic inflammation in COPD [5,6]. Transforming growth factor beta (TGF-β1) is a cytokine that affects many different cell processes in COPD lungs and in cells importantly involved in the pathogenesis of COPD including bronchial epithelial cells, macrophages, and fibroblasts isolated from COPD specimens, suggesting an impact of TGF-β1 signaling in the development and progression of this disease [7]. TGF-β1 is also involved in a variety of inflammatory processes in COPD which are mediated by T cells and neutrophils [8]. Thus, targeting TGF-β1 signaling may represent a suitable therapeutic option in COPD in the light that TGF-β1 signaling is not well controlled by the use of corticosteroid treatment [9].

ACh, synthesized by choline acetyltransferase (ChAT) in different cell types (macrophages, T lymphocytes, fibroblasts and epithelial cells) [10], is also a new emerging actor of airway inflammation and

* Corresponding author at: Institute of Biomedicine and Molecular Immunology "A. Monroy", Italian National Research Council, Palermo, Italy. Tel.: +39 0916809121; fax: +39 0916809122.
E-mail address: profita@ibim.cnr.it (M. Profita).

0925-4439/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2012.03.002

remodeling processes in COPD targeting the cells via muscarinic receptors (mAChRs) [11–14]. Tiotropium is able to counteract the proinflammatory activity of acetylcholine (ACh) on different cell types [13] and reduces the activity of ACh in a guinea pig model of neutrophilic inflammation and remodeling in COPD. It has been also showed that Tiotropium is able to inhibit TGF-β1-induced matrix metalloproteinases (MMPs) production from lung fibroblasts [15] as well as that aclidinium inhibits TGF-β1 mediated human lung fibroblast to myofibroblast transition [16]. The inhibitory effect of anticholinergic drugs on ACh signaling and on TGF-β1 might suggest a link between this cytokine and non cholinergic system components such as mAChRs and ChAT in COPD.

We hypothesized that the levels of TGF-β1 are increased in induced sputum from COPD patients and that this cytokine is involved in the pathogenesis of this disease promoting neutrophil adhesion to bronchial epithelial cells. Since the increase in TGF-β1 was shown to persist despite the use of corticosteroid treatment in *in vivo* and *in vitro* studies [9,17], we investigated whether the combination of Olodaterol (a novel generation of inhaled β_2-AR-agonist) with Tiotropium Spiriva® (an anticholinergic bronchodilator) might have more effectiveness in the control of airway inflammation associated with TGF-β1 and non neuronal cholinergic system components such as mAChR2, mAChR3 and ChAT in neutrophil adhesion to human bronchial epithelial cells.

2. Materials and methods

2.1. Patients

We recruited three groups of subjects: healthy asymptomatic non-smoking subjects with normal lung function (HC) (n = 18), symptomatic smokers with normal lung function (HS) (n = 22), and COPD (n = 21). COPD subjects were defined and classified according to the criteria reported by Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [18] and were classified as stage ≥1. Reversibility test to bronchodilator was performed to exclude an asthmatic component, and the increase in forced expiratory volume in the 1st second (FEV1) after salbutamol was lower than 12% and 200 ml compared with basal values, in all COPD subjects. All COPD patients were in stable conditions. All COPD patients who had routine chest X-rays and computed tomographic scans that showed obvious emphysema were excluded.

COPD former smokers (n = 13), included in the group of COPD patients, had quit smoking for at least 1 yr. Patients selected for induced sputum (IS) collection underwent an initial 2-week run-in period. All patients were characterized with respect to gender, age, smoking history, COPD symptoms, comorbidity, and current history of treatment. Exclusion criteria included the following: other systemic diseases, chronic bronchitis, chronic spontaneous, sputum production, other lung diseases, upper and lower respiratory tract infections, treatment with glucocorticoids or anticholinergics within 3 months before the study and treatment with long acting beta adrenergic agonists 15 days before the study.

The local Ethics Committee approved the study, and participating subjects gave their informed consent.

2.2. Sputum induction and processing

Sputum induction and processing were performed according to the method of the plugs as previously described [12]. Briefly after the collection of the sputum, the selected plugs were diluted with 4 volumes of phosphate-buffered saline (PBS 1×; Gibco). The resulting suspension was vortexed for 30 s and then centrifuged at 1000 *g* for 20 min. The induced sputum supernatants (ISSs) were then aspirated and frozen at −80 °C in separate aliquots for the subsequent biochemical analyses. The cells obtained from induced sputum were then cytocentrifuged (Cytospin 2; Shandon, Runcorn, United Kingdom) and stained with May–Grunwald–Giemsa. For differential cell counts, the slides were read blindly by 2 independent investigators who counted at least 400 cells per slide.

2.3. Measurements of TGF-β1 and ACh in ISSs

TGF-β1 was measured in the ISSs by ELISA (R&D Systems Europe Ltd, Abingdon, UK) after acidification with HCl and neutralization with NaOH to pH 7.0–7.4 to measure biologically active TGF-β1. The detection limit of the assay was 50 pg/ml.

ACh was measured in the ISSs by a commercial kit (Biovision, catalog K615-100). The kit can detect choline (Ch) and total choline (TCh) (by adding acetylcholinesterase to the reaction that converts ACh to Ch) with a sensitivity until 50 pmol/well (1 μM/well) by plotting fluorescence readings (Ex/Em 535/587 nm) against the standard curve. This sensitivity is correspondent to the concentration of 1 μM of TCh or Ch. Absorbance was read using a Wallac 1420 Victor^2 multilabel counter (Perkin-Elmer Life Sciences, Turku, Finland). We quantified TCh and free Ch in ISSs from HC (n = 18), HS (n = 22) and COPD (n = 21) subjects. ACh was evaluated as differences between TCh and Ch. Results were expressed as μM (1 μM/l). Three samples of each group (HC, HS, and COPD) were spiked with a known concentration of ACh (1 μM) to confirm that the analyzed samples did not contain substances that would interfere with the bioassay. The recovery of ACh in spiked ISSs was 82.3% ± 2.

2.4. Epithelial cell cultures

The SV40 large T antigen-transformed 16-HBE cell line (16-HBE) was used for these studies. 16-HBE is a cell line that retains the differentiated morphology and function of normal airway epithelial cells. 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. 16-HBE cells were cultured as adherent monolayers in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS), 1% MEM (non-essential amino acids, Euroclone), 2 mM L-glutamine and gentamicin 250 μg/ml at 37 °C in a humidified 5% CO_2 atmosphere. Evidences showed that 16-HBE are similar to primary normal human bronchial epithelial (NHBE) cells (Lonza, Brussels, Belgium), and bronchial epithelial cells (BECs) from bronchial brushings in regard to the response to TGF-β1 and antiinflammatory drugs [19].

2.5. 16-HBE stimulation for neutrophil adhesion

16-HBE (70,000 cells/well) were plated in standard 24-well culture plates in MEM 10% FCS and grown to confluence. After 24 h with MEM 1% FCS, the medium was replaced and 16-HBE cells were stimulated with ISSs (10% in fresh MEM 1% FCS) from HC (n = 6), HS (n = 6) and COPD (n = 6) subjects for 24 h. The ISS samples were chosen by selecting the samples with the TGF-β1 and the ACh concentrations closest to the median of each group. To determine the contribution of TGF-β1 present in ISSs to the observed effect on neutrophil adhesion, the ISSs were pretreated with a monoclonal anti-TGF-β1 antibody (4 μg/ml) (R&D Systems Europe Ltd, Abingdon, UK) for 1 h at 4 °C to neutralize sputum TGF-β1 activity before the addition to 16-HBE. The anti-TGF-β1 antibody was selected for its ability to neutralize the biological activity of TGF-β1 as previously described [19]. To determine the effects of β_2-AR-agonist and anticholinergic bronchodilator compounds on neutrophil adhesion to 16-HBE, Olodaterol (1 nM) (Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany) and Tiotropium Spiriva® (0.1 μM) (Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany) were added, alone or in combination, to 16-HBE 1 h before the stimulation with

ISSs from 8 COPD patients. Additionally, to verify the contribution of TGF-β1 and ACh on neutrophil adhesion to 16-HBE, the cells were stimulated with and without rhTGF-β1 (5 ng/ml) alone or in combination with ACh (1 μM) (Sigma St. Louis, MO) for 24 h (5% CO_2 at 37 °C) in the presence or absence of Olodaterol (1 nM) alone or in combination with Tiotropium (0.1 μM).

2.6. 16-HBE stimulation for mAChR2, mAChR3 and ChAT expression

16-HBE (150,000 cells/well) were plated into six-well culture plates until confluence with MEM 10% FBS, and grown to confluence followed by an additional 24 h under FBS-free conditions (5% CO_2 at 37 °C). After 24 h, the medium was replaced with fresh MEM 1% FCS and 16-HBE cells were stimulated with ISSs (10% in fresh MEM 1% FCS) from 8 COPD patients or with rhTGF-β1 (eight separate experiments) (5 ng/ml) for 24 h (5% CO_2 at 37 °C). For 16-HBE stimulation, ISSs were concentrated 10× (Vivaspin 6 10,000 MWC0, Vivascience, Sartorius Group Hannover, Germany) and added to the culture medium. The concentration of ISSs was modified to eliminate the molecules with low molecular weight such as ACh that might interfere with the detection of mAChR expression on 16-HBE.

To determine the contribution of TGF-β1 present in ISSs to the observed effects on mAChR2, mAChR3 and ChAT expression, ISSs from 8 COPDs were pretreated with a monoclonal anti-TGF-β1 antibody (4 μg/ml) (R&D Systems Europe Ltd, Abingdon, UK) for 1 h at 4 °C to neutralize sputum TGF-β1 activity before the addition to 16-HBE.

To determine whether the β_2-AR-agonist can affect the mAChR2, mAChR3 and ChAT expression on 16-HBE, the cells were also pretreated with Olodaterol (1 nM) 1 h before the addition of ISSs from 8 COPD patients for 24 h at 37 °C.

2.7. Adhesion assay

Neutrophils from normal donors were purified as previously described [20], resuspended in PBS (2×10^6/ml), labeled for 45 min at 37 °C with an equal volume of the fluorochromic dye SFDA at the final concentration of 50 μg/ml (Molecular Probes), washed and resuspended in PBS (0.4×10^6/ml). Immediately before the addition of neutrophils, the medium with ISSs was removed from the 16-HBE cultures and the cells were washed with warm PBS. Labeled neutrophils were added in a final volume of 0.5 ml (0.2×10^6/well). The plates were incubated at 37 °C for 25 min to allow neutrophils to contact and to adhere to the confluent 16-HBE, and total fluorescence was measured using an excitation wavelength of 485 nm and monitoring emission at 530 nm in a Wallac 1420 Victor multilabel counter (PerkinElmer Life and Analytical Sciences-Wallac OY, Turku, Finland). Nonadherent cells were then removed by washing and fluorescence was measured to evaluate bound cells. Adhesion was expressed as percentage of the fluorescence ratio of bound cells on total cells. All test points were performed in triplicate. The baseline values represent the adhesion of neutrophils to unstimulated 16-HBE. The levels of non-specific fluorescence were calculated in 16-HBE cells (3% ± 5.1%) and in the neutrophils (2.3% ± 7%). The % of unlabelled cells was subtracted as background in all experimental conditions. The efficiency of SDFA incorporation, evaluated using the flowcytometry analysis, was 87% ± 8% in neutrophils. At the end of incubation time (25 min) with 16-HBE the necrosis of neutrophils was calculated (3% ± 1.8).

2.8. Flow cytometry of mAChR2, mAChR3, ChAT and MAC-1 expression

The expression of mAChR2, mAChR3 and ChAT in 16-HBE cells was evaluated by flow-cytometry analyses using indirect label immunofluorescence by a FACSCalibur™ flow cytometer (Becton Dickinson, Mountain View, CA, USA) as previously described [14]. The cells (4×10^5/ml) were permeabilized using a commercial fix-perm cell and incubated in the dark for 30 min at 4 °C with the following primary antibodies: a rabbit polyclonal antibody directed against the human M_2 (H-170 Santa Cruz Biotechnology, Inc., Santa Cruz CA), a rabbit polyclonal antibody directed against the human M_3 (H-210 Santa Cruz Biotechnology, Inc., Santa Cruz CA), a mouse monoclonal antibody directed against ChAT (Chemicon International, USA) followed by a fluorescein isothiocyanate conjugated anti-rabbit immunoglobulin G (Dako, Glostrup, Denmark) for mAChR2 and mAChR3 and by a fluorescein isothiocyanate conjugated anti-mouse immunoglobulin G (Dako, Glostrup, Denmark) for ChAT. FITC-conjugated rabbit and mouse IgG1 were used as isotype negative control antibodies (Dako LSAB, Glostrup, Denmark). Results were obtained as percentage of positive cells by flowcytometry. Since the baseline values of mAChRs and ChAT expression are high, to better represent the significant changes, the results of the experimental conditions were plotted as fold-change compared to untreated cells which were chosen as the reference sample.

The expression of MAC-1 was evaluated using (RPE)-conjugated mouse monoclonal antibody direct against an anti-human CD11b/CD18 (DAKO A/S, Glostrup, Denmark). Results of flowcytometry were expressed as percentage of positive cells.

2.9. Real-time quantitative RT-PCR of mAChR2, mAChR3 and ChAT expression

We measured the levels of mAChR2, mAChR3 and ChAT mRNA by real-time quantitative RT-PCR in the 16-HBE cells stimulated with ISSs from six COPD patients untreated and pretreated with a monoclonal anti-TGF-β1 antibody and in the 16-HBE cells stimulated with TGF-β1. Briefly, total cellular RNA was extracted from 16-HBE according to the method of Chomczynski and Sacchi, using the RNAzol kit (Biotech Italia, Rome, Italy) as previously described [21], and was reverse-transcribed into first-strand complementary DNA, using Moloney-murine leukemia virus-reverse transcriptase (M-MLV-RT) and oligo$(dT)_{12-18}$ primer (Invitrogen). Quantitative real-time PCR of transcripts was carried out on an ABI PRISM 7900 HT Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA) using specific FAM-labeled probe and primers (prevalidated TaqMan Gene expression Assay for mAChR2 Hs00265208m1; mAChR3 Hs00327458m1 and ChAT, Hs00253141m1; Applied Biosystems). Gene expression was normalized to glyceraldehyde-3 phosphate dehydrogenase (GAPDH) endogenous control gene. GAPDH gene expression was used as endogenous control. Gene expression levels were expressed as threshold cycle crossover points considered as previously described [22].

2.10. Neutrophils stimulation for MAC-1 expression

Neutrophils were resuspended and cultured at the concentration of 4×105/ml in vials with fresh RPMI 1% FCS and were stimulated for 2 h with concentrated ISSs from HC (n = 6), HS (n = 6) and COPD (n = 6). At the end of the incubation time, neutrophils were assessed as described below for macrophage-1 antigen (MAC-1) CD11b/CD18 expression. In addition we tested the effect of Tiotropium and Olodaterol, alone or in combination, on MAC-1 expression generated to ISSs from 6 COPDs.

2.11. Statistical analysis

Statistical analysis for multiple comparisons was calculated using Kruskal–Wallis with ANOVA tests followed by Bonferroni–Dunn correction for multiple comparisons. ANOVA with Fisher test correction was used for the analysis of the data obtained from *in vitro* experiments. A p value <0.05 was considered as statistically significant.

Table 1
Demographic characteristics of patients.

	HC (n = 18)	HS (n = 22)	COPD (n = 21)	*p* Value		
				HC *vs* HS	HC *vs* COPD	HS *vs* COPD
Age, yr	66 ± 7	65.5 ± 3	64 ± 13	ns	ns	ns
Gender, male/female	7/11	10/11	12/10	–	–	–
FEV1, % predicted	109.2 ± 10.6	102 ± 15	63.7 ± 23.5	ns	<0.0001	<0.01
FVC, % predicted	101.2 ± 13.7	114.1 ± 15	70.6 ± 21.2	ns	<0.02	<0.03
FEV1/FVC (%)	90.5 ± 2	84.3 ± 5.0	63.4 ± 10.0	ns	<0.002	<0.03
Smoking, pack/yr	0	65.7 ± 18.3	60 ± 24.8	<0.001	<0.001	ns

Data are presented as mean ± SD. Abbreviations: HC = healthy asymptomatic nonsmoking subjects with normal lung function; HS = asymptomatic smokers with normal lung function; COPD = subjects with Chronic Obstructive Pulmonary Disease; FEV1 = forced expiratory volume in one second; FVC = forced vital capacity. Statistical analysis for multiple comparisons was calculated using Kruskal–Wallis and nonparametric ANOVA tests followed by Bonferroni–Dunn correction for multiple comparisons.

3. Results

3.1. Demographic characteristic of patients and differential cell counts of ISS

The patients' characteristics are summarized in Table 1. The results of the differential cell counts of induced sputum samples showed a significant increase in the number of cells in HS and COPD subjects. In HS, this increase reflected an increase in the number of both macrophages and neutrophils, whereas in COPD subjects neutrophils showed a large increase with the number of macrophages being significantly lower than in HS and in HC. Eosinophils resulted significantly higher in COPD subjects than in HC, whereas lymphocytes did not show significant differences among the three study groups (Table 2).

3.2. Levels of TGF-β1 and ACh in ISSs

The evaluation of TGF-β1 concentrations in ISSs showed significantly increased levels in HS subjects and COPD patients when compared with HC subjects, while no statistically significant differences were observed between HS subjects and COPD patients (Fig. 1A). ACh concentrations were significantly increased in ISSs from COPD patients when compared with HS and HC subjects. No statistically significant differences were observed between HS and HC (Fig. 1B).

3.3. Effect of ISSs on neutrophil adhesion to 16-HBE cells

The stimulation of 16-HBE cells with ISSs from HC, HS and COPD subjects promoted different degrees of adhesion between neutrophils and 16-HBE cells. ISSs from COPD patients and from HS subjects generated higher levels of adhesion in comparison to ISSs from HC subjects (Fig. 2A). Interestingly, the adhesion generated by ISSs from COPD subjects was significantly inhibited by the addition of an anti-TGF-β1 antibody demonstrating that TGF-β1 is involved, at least partly, in this phenomenon. No effects on the 16-HBE–neutrophil adhesion were seen by the addition of the same monoclonal antibody in the cultures with ISSs from HS and HC (Fig. 2B). These findings suggest that TGF-β1 might be responsible of the higher levels of neutrophil adhesion and recruitment into the airways of COPD patients. The neutrophil adhesion to 16-HBE stimulated with ISSs from HS subjects was not significantly reduced by Tiotropium while was inhibited by Olodaterol (Fig. 3A). Finally, the neutrophil adhesion to 16-HBE stimulated with ISSs from COPD patients was significantly reduced by both Olodaterol and Tiotropium alone showing an additional effect when added to the cultures in combination (Fig. 3B). These results suggest that the neutrophil adhesion generated by TGF-β1 might be associated with higher levels of ACh activity present in ISSs from COPD patients than in HS where the levels of ACh are lower than in COPD. ACh promoting the activation of mAChRs expressed in 16-HBE regulates neutrophil adhesion via mAChRs. Olodaterol plays an additional role in this phenomenon.

3.4. Effects of ACh and rhTGF-β1 on neutrophil adhesion to 16-HBE

To further investigate the role of both ACh and TGF-β1 in this phenomenon, we performed *in vitro* experiments aimed to mimic the airways environment in COPD. ACh increased the neutrophil adhesion to 16-HBE when compared with the baseline conditions and the use of both Tiotropium and Olodaterol alone reduced this effect. The combined use of Tiotropium and Olodaterol did not further reduce neutrophil adhesion when compared to Tiotropium or Olodaterol alone (Fig. 4 left panel). The stimulation of 16-HBE with TGF-β1 increased the levels of neutrophil adhesion when compared to the baseline conditions and the addition of TGF-β1 to the ACh cultured cells further increased the levels of adhering neutrophils. The use of both Tiotropium and Olodaterol alone significantly reduced the levels of neutrophil adhesion to 16-HBE stimulated with TGF-β1 plus ACh and, interestingly, the addition of the two drugs in combination further reduced the neutrophil adhesion to 16-HBE (Fig. 4 right panel). These findings suggest that TGF-β1 signaling regulates the neutrophil adhesion to 16-HBE promoting the mAChR expression and increasing the response to ACh stimulation and that this phenomenon may be

Table 2
Total and differential cell count from induced sputum.

	HC (n = 18)	HS (n = 22)	COPD (n = 21)	*p* Value		
				HC *vs* HS	HC *vs* COPD	HS *vs* COPD
Macrophages (%)	85 ± 9	54 ± 7.2	24.6 ± 3	<0.01	<0.002	ns
Neutrophils (%)	21.3 ± 2.0	48 ± 5	73 ± 10.2	<0.01	<0.002	ns
Lymphocytes (%)	0.6 ± 03	0.7 ± 09	1.2 ± 0.4	ns	ns	ns
Eosinophils (%)	0.1 ± 0.2	0.9 ± 06	0.8 ± 0.3	ns	ns	ns
Epithelial cells (%)	0.7 ± 0.8	0.5 ± 0.9	0.4 ± 0.6	ns	ns	ns
Total cells (10^6/g ISS)	3.8 ± 3.2	4.3 ± 0.5	6.6 ± 7.0	ns	ns	ns

Data are presented as mean ± SD.
Abbreviations: HC = healthy asymptomatic nonsmoking subjects with normal lung function; HS = asymptomatic smokers with normal lung function; COPD = subjects with Chronic Obstructive Pulmonary Disease. Statistical analysis for multiple comparisons was calculated using Kruskal–Wallis and nonparametric ANOVA tests followed by Bonferroni–Dunn correction for multiple comparisons.

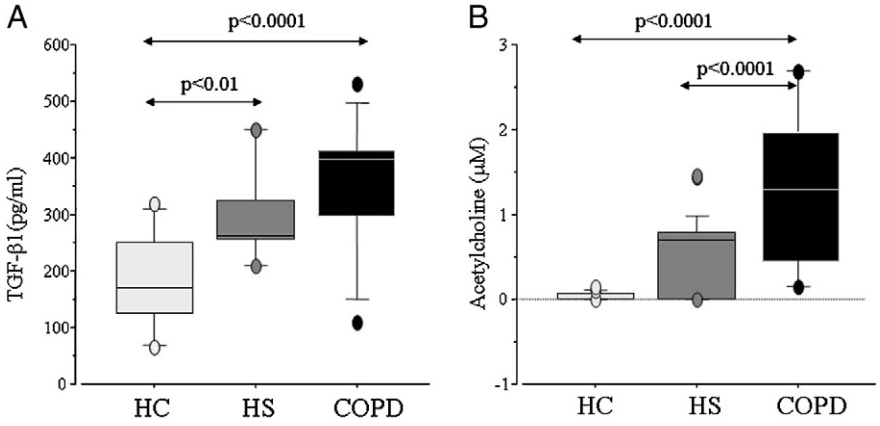

Fig. 1. TGF-β1 and ACh concentrations in ISS samples from HC (n = 18), HS (n = 22) and COPD subjects (n = 21). A) TGF-β1 and B) ACh levels were measured by specific commercial available kits as described in the Materials and methods section. Data are expressed as medians and 25th to 75th percentiles (box plot) of concentrations. Error bars represent 10th and 90th. Statistical analysis was performed by Kruskal–Wallis test and Bonferroni–Dunn procedures.

controlled by the use of the anticholinergic drugs alone or in combination with β_2-AR antagonists.

3.5. Effect of ISSs on the expression of mAChRs and ChAT in 16-HBE cells

To further clarify the role of TGF-β1 and the non neuronal cholinergic system components in COPD, we investigated the role of TGF-β1 present in ISSs from COPD on mAChRs and ChAT expression. The stimulation of 16-HBE with ISSs from COPD patients increased both mAChRs and ChAT expression when compared to baseline. Interestingly, the pre-treatment of ISSs from COPD patients with an anti-TGF-β1 antibody significantly reduced the COPD ISSs generated mAChR3 and ChAT expression while no effects were exerted on the COPD ISSs generated expression of mAChR2 (Fig. 5). These findings suggest that the levels of TGF-β1 present in the airways of COPD patients, although do not affect mAChR2, might be responsible of the induction of mAChR3 and ChAT expression in the epithelial cells during airway inflammation in COPD. However other factors present in the ISSs, apart from TGF-β1, might be involved in the increased expression of mAChR2.

The preincubation of 16-HBE with Olodaterol before the addition of ISSs from COPD patients did not reduce the mAChRs and ChAT expression (Fig. 6). These results suggest that the use of Olodaterol alone is not sufficient to efficiently counteract the TGF-β1 related proinflammatory activity (expression of mAChRs and ChAT in epithelial cells) present in ISSs from COPD patients.

3.6. Effect of rhTGF-β1 on the expression of mAChRs and ChAT in 16-HBE

The stimulation of 16-HBE with rhTGF-β1 showed increased mAChR2, mAChR3 and ChAT protein expression in a dose dependent manner (data not shown) with the highest levels of induced expression reached at 5 ng/ml. The preincubation of 16-HBE with Olodaterol (1 nM), both in the presence and absence of rhTGF-β1 (5 ng/ml) did not affect the expression of mAChR3 and ChAT, while further increased the expression of mAChR2 generated by rhTGF-β1 (Fig. 7).

RT-PCR analysis of mAChR2, mAChR3 and ChAT showed that the 16-HBE increased the levels of mAChR2, mAChR3 and ChAT mRNA (as indicated by the lower number of amplification cycles required) after the treatment with ISSs from COPD. The levels of mAChR3 and ChAT mRNA were reduced after the depletion of TGF-β1 from the tested samples (Fig. 8A). However the stimulation of 16-HBE with higher concentrations of rhTGF-β1 generated increased levels of mAChR2, mAChR3 and ChAT mRNA (Fig. 8B) further supporting a probable contribution of TGF-β1 on the expression of the non neuronal component of cholinergic system in bronchial epithelial cells.

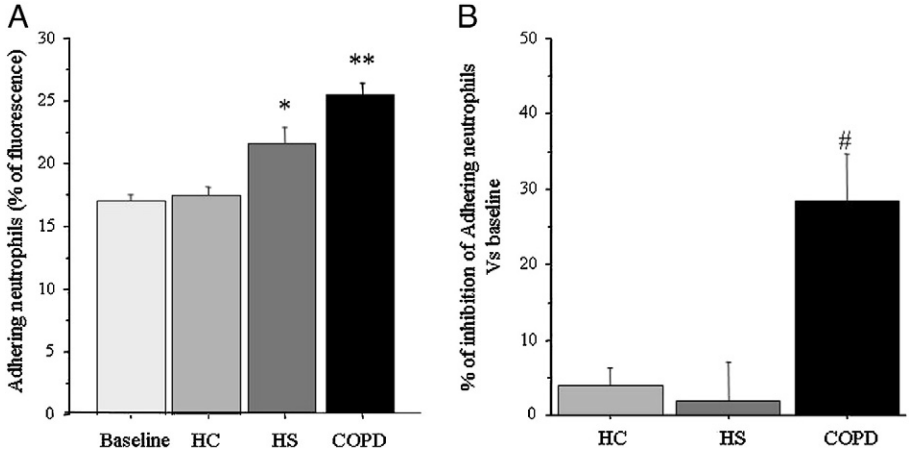

Fig. 2. Adhesion of neutrophils to 16-HBE stimulated with ISSs. A) 16-HBE were stimulated with ISSs obtained from HC (n = 6), HS (n = 6) and COPD patients (n = 6) for 24 h and then co-incubated with neutrophils, obtained from normal donors, for 25 min at 37 °C before the neutrophil adhesion assay. Results are expressed as percentage of adhering neutrophils (% of fluorescence). B) 16-HBE were stimulated with ISSs obtained from HC (n = 6), HS (n = 6) and COPD patients (n = 6) which are treated with and without a monoclonal anti-TGF-β1 antibody for 1 h, before their addition to 16-HBE for 24 h. 16-HBE cells were then incubated with neutrophils obtained from normal donors for 25 min at 37 °C before the neutrophil adhesion assay. Results are expressed as percentage of inhibition of neutrophil adhesion *vs* untreated ISSs. The bars represent the means ± SD of values. ANOVA with Fisher test correction was used for the analysis of the data. *$p<0.05$ *vs* HC and baseline; **$p<0.01$ *vs* HC and baseline; #$p<0.001$ *vs* HC and HS.

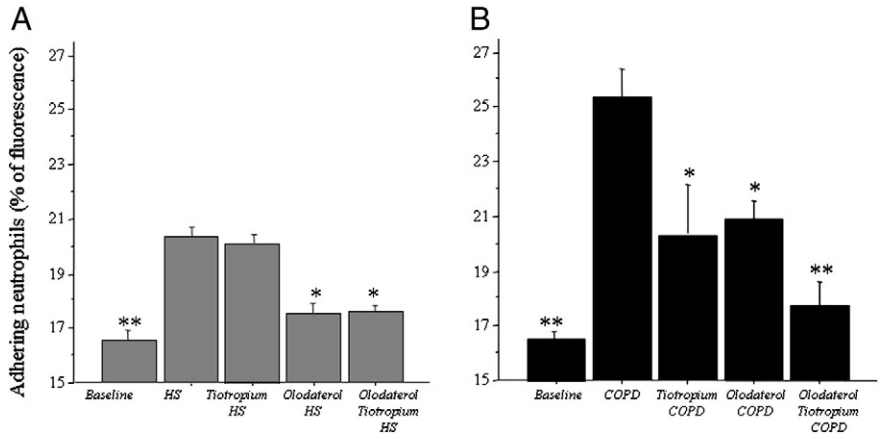

Fig. 3. Effect of Olodaterol and Tiotropium on neutrophil adhesion induced by ISSs from HS (n = 6) subjects A) and COPD patients (n = 8) B). 16-HBE stimulated with ISSs, in the presence of Olodaterol (1 nM), alone or in combination with Tiotropium (0.1 μM), were incubated with neutrophils obtained from normal donors for 25 min at 37 °C. Results are expressed as mean ± SD of adhering neutrophils (% of fluorescence). ANOVA with Fisher test correction was used for the analysis of the data. A)*$p<0.001$ *vs* HS; **$p<0.001$ *vs* HS; B) *$p<0.001$ *vs* COPD; **$p<0.0001$ *vs* COPD.

3.7. Effects of ISSs on the expression of MAC-1 in neutrophils

Since the adherence between bronchial epithelial cells and neutrophils may be modulated by different expression of adhesion molecule on the surface of neutrophils, we next evaluated the expression of MAC-1 on the surface of neutrophils. MAC-1 expression was increased in the neutrophils stimulated with ISSs from HS and COPD when compared to HC. The preincubation of neutrophils with Tiotropium or Olodaterol alone significantly reduced the expression of MAC-1 in neutrophils stimulated with ISSs from COPD patients and this expression was further reduced by the use of the two drugs in combination (Fig. 9). These results suggest that the increased adhesion generated by ISSs from COPD and related with TGFβ1 increased expression of mAChRs and ChAT might also involve the adhesion molecules MAC-1.

4. Discussion

This study demonstrates that, inflammatory components present in the airways of COPD patients, are involved in molecular mechanisms promoting a deregulation of mAChRs expression by TGF-β1 and their activation by ACh generating in turn neutrophils adhesion to bronchial epithelial cells. It provides also evidences that Tiotropium (non-selective anticholinergic drug) attenuates neutrophilic inflammation in COPD and that the combined use with Olodaterol (novel long acting β_2AR-agonists) may increase this effect.

Neutrophilic airway inflammation is associated to the pathogenesis and to disease severity in COPD [23]. Inhaled corticosteroid therapy remains somewhat controversial since it is not able to control symptoms and neutrophilic inflammation. Today it is important to identify new molecules and pathways involved in regulating and controlling neutrophil inflammation with the aim to identify new mechanisms and new pharmacologic approaches that could better control this universal and sometimes debilitating inflammatory condition in COPD. The current therapeutic approach suggests the use of anticholinergic and β_2 long-acting drugs in the treatment of this disease. Although the use of these drugs in combination does not influence the progression of the disease, demonstrating that anti-cholinergic drugs and β_2 long-acting might exert also an anti-inflammatory effect, we may provide some additional information on the effects of this drug in the treatment of COPD.

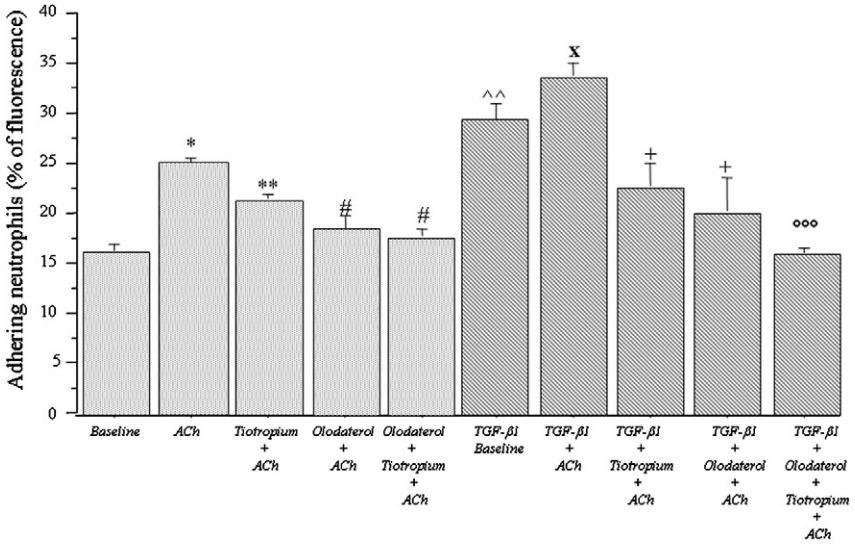

Fig. 4. Adhesion of neutrophils to 16-HBE stimulated with ACh (1 μM) alone (left panel) or with ACh (1 μM) in combination with TGF-β1 (5 ng/ml) (right panel). Results are expressed as adhering neutrophils (% of fluorescence) *vs* baseline values. The bars represent the means ± SD of values. ANOVA with Fisher test correction was used for the analysis of the data. *$p<0.01$ *vs* baseline; **$p<0.05$ *vs* ACh; #$p<0.03$ *vs* ACh; ^^$p<0.01$ *vs* baseline; X$p<0.05$ *vs* TGF-β1; +$p<0.001$ *vs* TGF-β1 ACh; °°°$p<0.001$ *vs* TGF-β1 ACh.

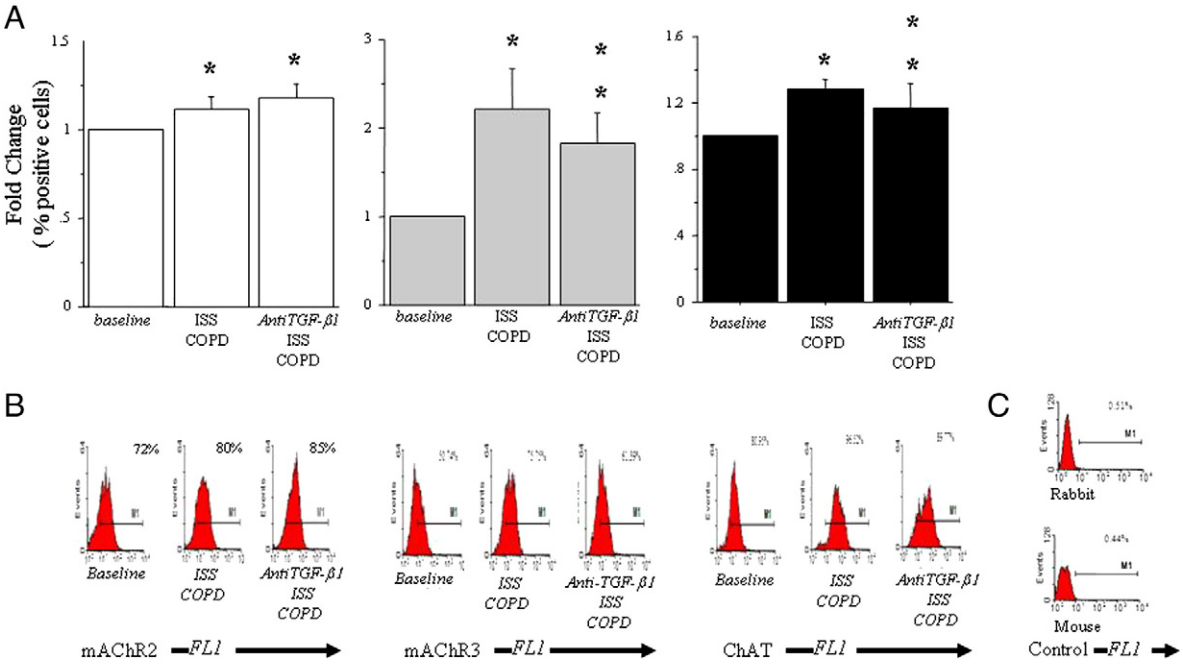

Fig. 5. Expression of mAChR2, mAChR3 and ChAT proteins in 16-HBE stimulated with ISSs from COPD patients. A) 16-HBE were stimulated for 24 h at 37 °C with ISSs from COPD patients (n = 8) pretreated with and without an anti-TGF-β1 antibody for 1 h. B) Representative flow cytometry of mAChR2 and mAChR3 and ChAT proteins of each experimental conditions; C) representative flow cytometry of FITC-conjugated rabbit and FITC-conjugated mouse (used as isotype negative control) antibodies (Dako Denmark A/S). Results were obtained as percentage of positive cells by flowcytometry and were plotted as fold-change compared to untreated cells which were chosen as the reference sample. ANOVA with Fisher test correction was used for the analysis of the data. *$p<0.05$ *vs* baseline; **$p<0.01$ *vs* ISS COPD.

The utility of the use of both, anti-inflammatory and bronchodilatatory drugs has relatively recently been included in the current guidelines at least in certain stages of the disease. Moreover, inflammation is considered to be of primary pathogenic importance in COPD but the evidence on which current understanding is based does not distinguish between cause and effect, and no single mechanism can account for the complex pathology. In this scenario, the control of inflammation may play an important role. Accordingly, it has been shown that anticholinergic drugs may control the activity of ACh in a guinea pig model of neutrophilic inflammation and remodeling of COPD [24] both mechanisms involved in

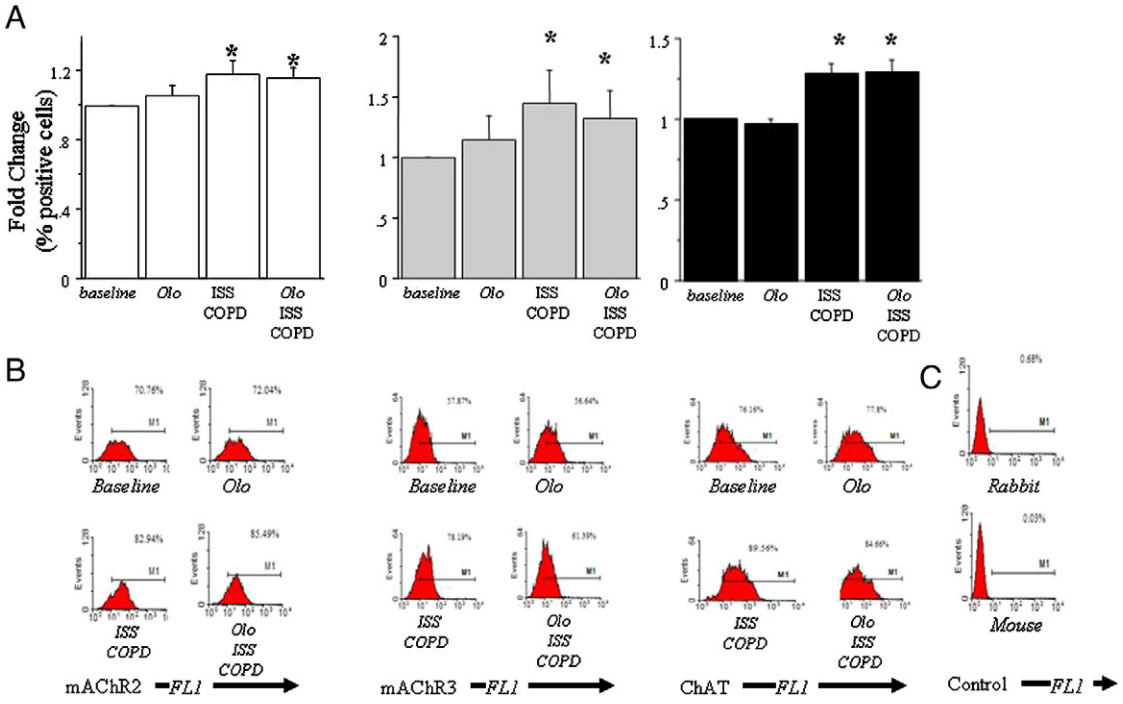

Fig. 6. Expression of mAChR2, mAChR3 and ChAT proteins in 16-HBE stimulated with ISSs from COPD patients. A) 16-HBE were stimulated in the presence or absence of Olodaterol (1 nM) for 1 h and then with ISSs from 8 COPD patients for 24 h at 37 °C. The protein analysis was performed by flow cytometry; B) representative flow cytometry of mAChR2, mAChR3 and ChAT proteins of each experimental condition; C) representative flow cytometry of FITC-conjugated rabbit and FITC-conjugate mouse (used as isotype negative control) antibodies (Dako Denmark A/S). Results were obtained as percentage of positive cells by flowcytometry and were plotted as fold-change compared to untreated cells which were chosen as the reference sample. ANOVA with Fisher test correction was used for the analysis of the data. *$p<0.01$ *vs* baseline.

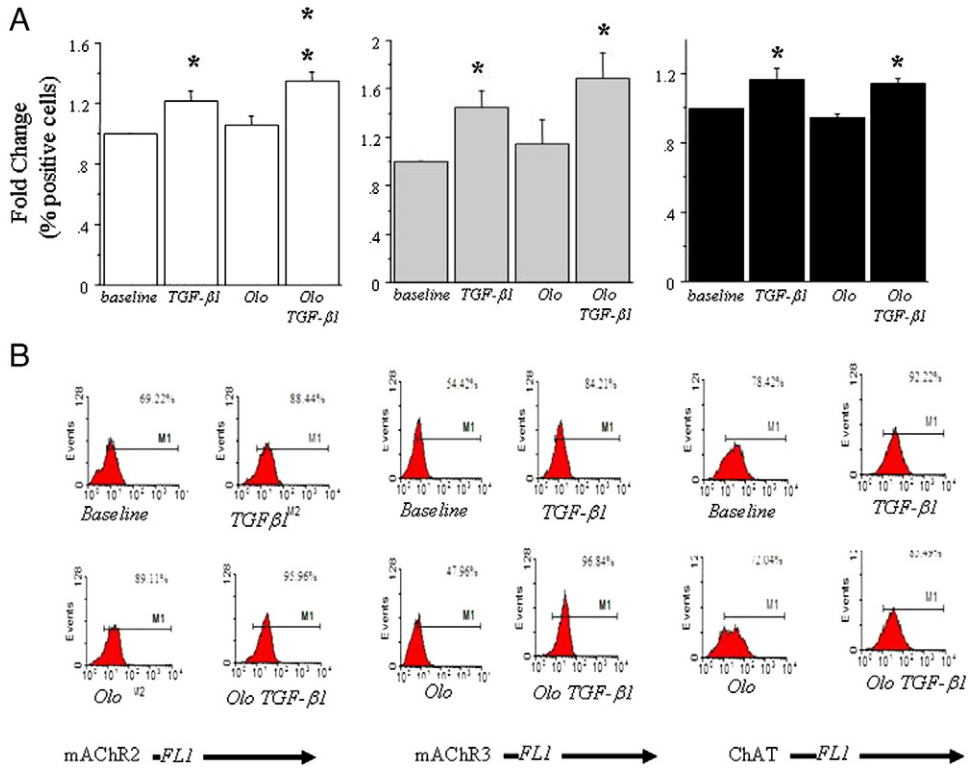

Fig. 7. Expression of mAChR2, mAChR3 and ChAT proteins in 16-HBE stimulated with TGF-β1. A) 16-HBE were stimulated in the presence or absence of Olodaterol (1 nM) for 24 h at 37 °C with TGF-β1 (5 ng/ml). The protein analysis was performed by flow cytometry. The bars represent mean ± SD of the % of positive fluorescent cells of eight separate experiments; B) representative flow cytometry of mAChR2, mAChR3 and ChAT proteins of each experimental condition. Results were obtained as percentage of positive cells by flowcytometry and were plotted as fold-change compared to untreated cells which were chosen as the reference sample. ANOVA with Fisher test correction was used for the analysis of the data. $^{*}p<0.05$ *vs* baseline; $^{**}p<0.01$ *vs* baseline.

the progression of the disease. Although with an only putative implication in the progression of the disease and actually without a practical use, our findings provide new information on the mechanism by which anticholinergic drugs, alone or in combination with β_2 long-acting, might control neutrophilic inflammation in COPD.

Neutrophils take part in specific and highly regulated mechanisms for controlling the expression of adhesion molecules that allow their tethering and migration into airway sites by epithelial cell signals generated in turn by inflammatory mediators [25]. Based on the observations that β_2AR-agonists can have an anti-inflammatory activity in primary lung epithelial cells that is independent of glucocorticoid receptors [26] and they can reduce neutrophilic airway inflammation in patients with mild asthma [3], we tested and identified the antiinflammatory effect of Olodaterol on neutrophil adhesion to bronchial epithelial cells in an *in vitro* model of neutrophilic airway inflammation using ISSs from COPD patients and the human bronchial epithelial cell line 16-HBE. Our findings show that Tiotropium and Olodaterol, well control the neutrophil adhesion to epithelial cells in COPD and that their use in combination has an additional effect.

We identify here increased levels of TGF-β1 and ACh in the induced sputum of COPD patients rather than in the airways of HC and HS confirming that these two pathways are importantly involved in COPD

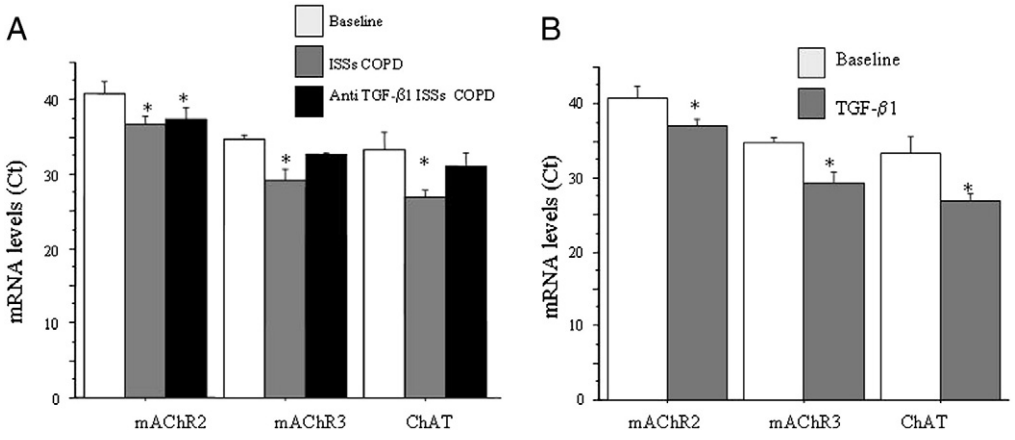

Fig. 8. Real-time quantitative RT-PCR of mAChR2, mAChR3 and ChAT was performed in A) 16-HBE stimulated with and without hr TGF-β1 (2 ng/ml) and in B) 16-HBE treated with ISSs from six COPD patients treated with and without antiTGF-β1. Gene expression levels are expressed as CT (crossover cycle at the threshold of PCR amplification; the higher the number of cycles required to reach the CT, the lower the abundance of target mRNA in the reaction). The results for panel A and B represent the means ± SD of six experiments. $^{*}p<0.01$ *vs* baseline.

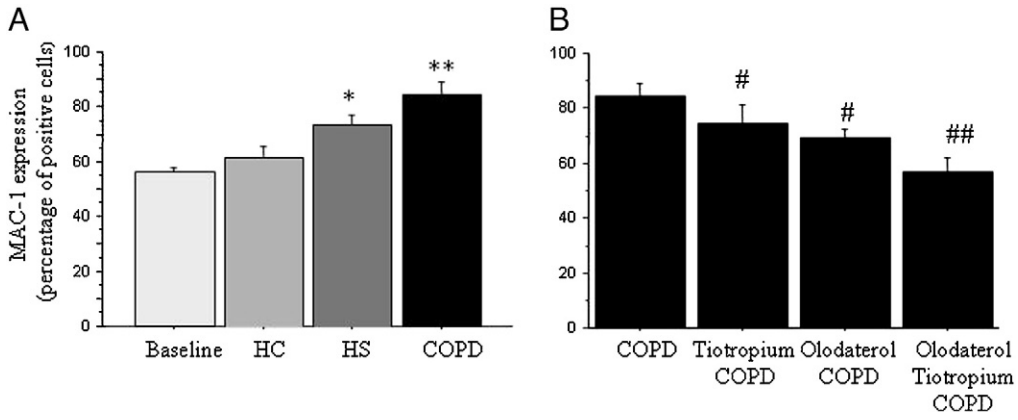

Fig. 9. Expression of MAC-1 in neutrophils stimulated with ISSs. A) Neutrophils were stimulated for 2 h at 37 °C with ISSs from HC (n = 6), HS (n = 6) and COPD patients (n = 6) ISSs from COPD patients (n = 6); B) Neutrophils stimulated with ISSs from COPD (n = 6) patients in the presence of Olodaterol (1 nM), alone or in combination with Tiotropium (0.1 μM) for 2 h at 37 °C. The expression of MAC-1 was evaluated using (RPE)-conjugated mouse monoclonal antibody direct against an anti-human CD11b/CD18 (Dako Denmark A/S). Results are expressed as mean ± SD of % fluorescent positive cells. ANOVA with Fisher test correction was used for the analysis of the data. *$p<0.01$ *vs* neutrophils; **$p<0.001$ *vs* neutrophils; #$p<0.01$ *vs* COPD, ##$p<0.001$ *vs* COPD.

pathogenesis and suggesting that, also at the bronchial epithelium level, they may exert a pro-inflammatory activity. Moreover, the evidences that the amount of ACh present in ISSs from HS has a trend toward higher levels, not statistically significant when compared with HC and statistically different from COPD patients, suggest that the immune inflammatory mechanisms originated by cigarette smoke and involved in the production of ACh are maintained and amplified during the cellular and molecular mechanisms of COPD [27]. Emerging evidences have shown increased expression of TGF-β1 in COPD lungs and in primary cells, such as epithelial cells, macrophages, cells of the immune system or fibroblasts isolated from COPD specimens, suggesting an impact of TGF-β1 signaling on the development and progression of neutrophilic inflammation in COPD. Moreover, TGF-β1 signaling regulates degranulation and oxidant release by adherent human neutrophils underlining its contribution to neutrophilic inflammation [28]. According to these findings, we demonstrate here that ISSs from COPD and from HS, are able to induce neutrophil adherence to bronchial epithelial cells in a significantly higher manner than HC and that this phenomenon is driven, at list partly, by the presence of TGF-β1 supporting the important role of this molecule in the neutrophilic inflammation in COPD. Increased levels of TGF-β1 persist despite oral corticosteroid treatment in asthma [29] and the *in vitro* TGF-β1 induced epithelial–mesenchymal transition (EMT) in human bronchial epithelial cells is enhanced by IL-1β and not abrogated by corticosteroids [9]. Thus, new targeted interventions may represent a suitable therapeutic approach in COPD to control activity and production of TGF-β1 signaling [7]. Our findings show that the use of Tiotropium and Olodaterol is able to strongly and significantly inhibit the COPD ISSs mediated neutrophil–epithelial cell adhesion strongly supporting the concept that these two drugs, other than being strong bronchodilators, are also important anti-inflammatory drugs. The findings that they exert an additional effect in the down-regulation of the COPD ISSs mediated neutrophil–epithelial cell adhesion further support the new rising concept of their anti-inflammatory activity. These results are strongly supported by the fact that ACh is considered an emerging marker of COPD. The ACh-mediated mAChR activation involves the release of chemotactic mediators for eosinophils and neutrophils from bronchial epithelial cells and alveolar macrophages [11,12,30,31]. ACh is synthesized by choline acetyltransferase (ChAT) in different cell types (macrophages, T-lymphocytes, fibroblasts, and epithelial cells) [10] and often increased levels of ChAT expression are associated with increased production and activity of ACh [32]. The proinflammatory activity of ACh can be counteracted by the use of anticholinergic drugs such as Tiotropium [13]; in addition recent evidences identified a direct relationship between Tiotropium bromide and TGF-β1 showing that, interfering with Smad and MAPK pathways *in vitro*, Tiotropium is able to inhibit TGF-β1 induced MMP production from lung fibroblasts [15].

The depletion of TGF-β1 reduced the neutrophil adhesion to 16-HBE in the cells stimulated with ISSs from COPD rather than in HS despite there is no significant difference in the amount of TGF-β1 in the HS and COPD and despite, in *in vivo* conditions, in COPD patients, insensitivity to TGF-β1 has been observed. These findings suggest that high levels of TGF-β1 together with higher levels of ACh present in ISSs from COPD are additively involved in the regulation of neutrophil adhesion to bronchial epithelial cells since TGF-β1 promotes the expression of muscarinic receptors and ACh promotes their activation in bronchial epithelial cells. The inhibition of neutrophil adhesion exerted by Tiotropium in the presence of TGF-β1 might support these explanations. It is also interesting to note that the % of inhibition of neutrophil adhesion obtained by TGF-β1 antibody is evident in COPD and not in HS. This lack of effect might be due to the lower levels of ACh present in the airways of HS since, despite the increased expression of mAChR due to the TGF-β1, they are unable to be activated by the low levels of ACh. These concepts are further supported by the uncomplete effects of Tiotropium on the neutrophil adhesion generated with HS ISSs also in the presence of Olodaterol. Moreover, the finding that the use of Olodaterol well controls the adhesion of neutrophils generated by ISSs from both HS and COPD, suggests the involvement of other factors in this phenomenon independently from mAChRs.

We decided to use neutrophils from normal donors rather than neutrophils recovered from the same subjects in order to by-pass the bias due to the potential different expression of adhesion molecules as well as to the effects of different activating factors in neutrophils recovered from peripheral blood of HC, HS and COPD subjects. We were thus able to focus only on the effects on the neutrophil adhesion of the mediators present in the sputum from each group of subjects. Therefore the 16-HBE, activated with ISSs from HC, HS and COPD, during the incubation with neutrophils for the adhesion assay, differentially modify the adhesion of neutrophils from normal donors due to the increased expression of adhesion molecules generated from *epithelial–neutrophil interactions* in the airways of COPD patients as previously described [25]. These observations support the translation of our *in vitro* model to an *in vivo* situation of airway inflammation in COPD. Particularly we show that ISSs from COPD patients increase the expression of MAC-1 on the neutrophils from normal donors. This effect was controlled *in vitro* by the use of Olodaterol and Tiotropium supporting the concept that the β2 agonist together with anticholinergic drug may be involved in the inhibition

of neutrophil adhesion regulating the expression of MAC-1. Future study is necessary to better examine the cellular and molecular mechanisms of neutrophil adhesion and their inhibition by anticholinergic drugs or by β-2 agonists [33,34].

TGF-β1 present in the ISSs from COPD patients might be involved in the induction of mAChR3 and ChAT expression in epithelial cells from these patients promoting an increased production and activity of ACh in these cells during airway inflammation in COPD. Our findings that 16-HBE stimulated with ISSs from COPD patients display an upregulation of the expression of mAChR3 and ChAT not controlled by Olodaterol but significantly reduced by the depletion of TGF-β1 strongly support this hypothesis and the concept of the direct contribution of TGF-β1 in the induction of non cholinergic system components in epithelial cells. These observations are further supported by the induction of mAChRs and ChAT expression by higher levels of rhTGF-β1 in 16-HBE and by the fact that this phenomenon is not controlled by Olodaterol.

Smooth muscle bronchoconstriction is activated via mAChR3, and mucus secretion via mAChR1 and mAChR3, while prejunctional mAChR2 autoreceptors possess a significant inhibitory activity, providing a negative feedback to ACh release [35]. The expression of mAChR2 is reduced in ISS cells from COPD and increased levels mAChR3 are present in these patients suggesting a defective inflammatory response to ACh in the airways of COPDs [12]. The increased expression of mAChR2 might represent a defense mechanism of the cells with the aim to downregulate the response to ACh. These cellular events can generate mechanisms of cross-talk between the activities of the different mAChRs [36] and between mAChRs and β_2-adrenoceptors [4,34,37]. The exposure to proinflammatory cytokines, such as TGF-β1 and TNF-α, can modulate mAChRs [38]. Accordingly we have shown that rhTGF-β1 and Olodaterol increase mAChR2 expression that may be the cause of a defective resolution of ACh mediated response in the epithelial cells. Therefore we observed that the use of Olodaterol alone was able to significantly reduce the adhesion of neutrophils generated by ISSs from COPD with higher levels of ACh. On the other hand, *in vivo* Olodaterol other than being able to potently reverse contraction induced by different stimuli in isolated human bronchi, is able to potently reverse contraction induced by ACh challenges in anesthetized guinea pigs and dogs [39]. These observations together with our findings support the translation *in vivo* of the use of anticholinergic drugs and β-2 agonist to well control the bronchoconstriction and inflammation in COPD.

In conclusion, to our knowledge, this is the first paper demonstrating anti-inflammatory effects exerted by Olodaterol. It provides also evidences on additive anti-inflammatory effects generated by Olodaterol and Tiotropium in combination. The combined use of β_2AR-agonists with anticholinergic drugs might be a useful alternative therapy to the combination of β_2AR-agonists and inhaled corticosteroid to control inflammation in COPD. Since our *in vitro* studies are obtained with a limited number of samples, further studies in a larger population of subjects with COPD performing adequate clinical trials are required to confirm the relevant contribution of this new therapeutic approach.

Funding source

The study was supported by an unrestricted educational grant from Boehringer Ingelheim Pharma GmbH & Co. KG (contract number: 43028659).

Disclosure statements

The authors of the manuscript PC and MPP are employees of Boehringer Ingelheim Pharma GmbH & Co. KG. There are no other conflicts of interest for this study.

Authors' contributions

The authors MP, AB, LR conceived the study and designed the experiments. LS, MF GDA and AMM performed the technical procedures. MP provided the interpretation of data. MG together with PC and MPP revised the final draft of the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgements

The authors thank the Italian National Research Council and Boehringer Ingelheim Pharma GmbH & Co. KG for the support.

References

[1] M. Johnson, Combination therapy for asthma: complementary effects of long-acting beta2-agonists and corticosteroids, Curr. Allergy Clin. Immunol. 15 (2002) 16–22.
[2] J.C. Chiu, J.Y. Hsu, L.S. Fu, J.J. Chu, C.S. Chi, Comparison of the effects of two long-acting beta2-agonists on cytokine secretion by human airway epithelial cells, J. Microbiol. Immunol. Infect. 40 (5) (Oct. 2007) 388–394.
[3] K. Maneechotesuwan, S. Essilfie-Quaye, S. Meah, C. Kelly, S.A. Kharitonov, I.M. Adcock, P.J. Barnes, Formoterol attenuates neutrophilic airway inflammation in asthma, Chest 128 (4) (Oct. 2005) 1936–1942.
[4] K.E. Belmonte, Cholinergic pathways in the lungs and anticholinergic therapy for chronic obstructive pulmonary disease, Proc. Am. Thorac. Soc. 2 (4) (2005) 297–304 (discussion 11–2).
[5] A.B. Thompson, R.A. Robbins, D.J. Romberger, J.H. Sisson, J.R. Spurzem, H. Teschler, S.I. Rennard, Immunological functions of the pulmonary epithelium, Eur. Respir. J. 8 (1995) 127–149.
[6] E. Puchelle, J.M. Zahm, J.M. Tournier, C. Coraux, Airway epithelial repair, regeneration, and remodeling after injury in chronic obstructive pulmonary disease, Proc. Am. Thorac. Soc. 3 (2006) 726–733.
[7] M. Königshoff, N. Kneidingerb, O. Eickelberg, TGF-β signalling in COPD: deciphering genetic and cellular susceptibilities for future therapeutic regimens, Swiss Med. Wkly. 139 (39–40) (2009) 554–563.
[8] B. Moore, R.F. Murphy, D.K. Agrawal, Interaction of TGF-beta with immune cells in airway disease, Curr. Mol. Med. 8 (5) (2008) 427–436.
[9] A.M. Doerner, B.L. Zuraw, TGF-beta1 induced epithelial to mesenchymal transition (EMT) in human bronchial epithelial cells is enhanced by IL-1beta but not abrogated by corticosteroids, Respir. Res. 10 (2009) 100.
[10] I. Wessler, C.J. Kirkpatrick, K. Racke, The cholinergic 'pitfall': acetylcholine, a universal cell molecule in biological systems, including humans, Clin. Exp. Pharmacol. Physiol. 26 (3) (Mar. 1999) 198–205.
[11] R. Gosens, J. Zaagsma, M. Grootte Bromhaar, A. Nelemans, H. Meurs, Acetylcholine: a novel regulator of airway smooth muscle remodelling? Eur. J. Pharmacol. 500 (1–3) (Oct. 1 2004) 193–201.
[12] M. Profita, R. Di Giorgi, A. Sala, A. Bonanno, L. Riccobono, F. Mirabella, M. Gjomarkaj, G. Bonsignore, J. Bousquet, A.M. Vignola, Muscarinic receptors, leukotriene B4 production and neutrophilic inflammation in COPD patients, Allergy 60 (11) (Nov. 2005) 1361–1369.
[13] E.D. Bateman, S. Rennard, P.J. Barnes, P.V. Dicpinigaitis, R. Gosens, N.J. Gross, J.A. Nadel, M. Pfeifer, K. Racke, K.F. Rabe, B.K. Rubin, T. Welte, I. Wessler, Alternative mechanisms for tiotropium, Pulm. Pharmacol. Ther. 22 (6) (Dec. 2009) 533–542.
[14] M. Profita, A. Bonanno, L. Siena, A. Bruno, M. Ferraro, A.M. Montalbano, G.D. Albano, L. Riccobono, P. Casarosa, M.P. Pieper, M. Gjomarkaj, Smoke, choline acetyltransferase, muscarinic receptors, and fibroblast proliferation in chronic obstructive pulmonary disease, J. Pharmacol. Exp. Ther. 329 (2) (May 2009) 753–763.
[15] K. Asano, Y. Shikama, N. Shoji, K. Hirano, H. Suzaki, H. Nakajima, Tiotropium bromide inhibits TGF-beta-induced MMP production from lung fibroblasts by interfering with Smad and MAPK pathways *in vitro*, Int. J. Chron. Obstruct. Pulmon. Dis. 5 (2010) 277–286.
[16] J. Milara, A. Serrano, T. Peiró, A. Gavaldà, M. Miralpeix, E.J. Morcillo, J. Cortijo, Aclidinium inhibits human lung fibroblast to myofibroblast transition, Thorax 67 (3) (Sep. 28 2011) 229–237.
[17] M. Yamaguchi, A. Niimi, H. Matsumoto, T. Ueda, M. Takemura, H. Matsuoka, M. Jinnai, K. Otsuka, T. Oguma, T. Takeda, I. Ito, K. Chin, M. Mishima, Sputum levels of transforming growth factor-beta1 in asthma: relation to clinical and computed tomography findings, J. Investig. Allergol. Clin. Immunol. 18 (3) (2008) 202–206.
[18] R.A. Pauwels, A.S. Buist, P.M. Calverley, C.R. Jenkins, S.S. Hurd, Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary, Am. J. Respir. Crit. Care Med. 163 (5) (Apr. 2001) 1256–1276.
[19] A. Bruno, E. Pace, P. Chanez, D. Gras, I. Vachier, G. Chiappara, M. La Guardia, S. Gerbino, M. Profita, M. Gjomarkaj, Leptin and leptin receptor expression in asthma, J. Allergy Clin. Immunol. 124 (2009) 230–237.
[20] M. Profita, A. Sala, A. Bonanno, L. Riccobono, M. Ferraro, S. La Grutta, G.D. Albano, A.M. Montalbano, M. Gjomarkaj, Chronic obstructive pulmonary disease and

neutrophil infiltration: role of cigarette smoke and cyclooxygenase products, Am. J. Physiol. Lung Cell. Mol. Physiol. 298 (2) (Feb. 2010) L261–L269.
[21] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[22] N. Steuerwald, J. Cohen, R.J. Herrera, C.A. Brenner, Quantification of mRNA in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT-PCR, Mol. Hum. Reprod. 6 (5) (May 2000) 448–453.
[23] P.K. Jeffery, Structural and inflammatory changes in COPD: a comparison with asthma, Thorax 53 (2) (Feb. 1998) 129–136.
[24] T. Pera, A. Zuidhof, J. Valadas, M. Smit, R.G. Schoemaker, R. Gosens, H. Maarsingh, J. Zaagsma, H. Meurs, Tiotropium inhibits pulmonary inflammation and remodelling in a guinea pig model of COPD, Eur. Respir. J. 38 (4) (Feb. 24 2011) 789–796.
[25] C.A. Pettersen, K.B. Adler, Airways inflammation and COPD: epithelial–neutrophil interactions, Chest 121 (5 Suppl.) (May 2002) 142S–150S.
[26] J. Loven, N. Svitacheva, A. Jerre, A. Miller-Larsson, S.H. Korn, Anti-inflammatory activity of beta2-agonists in primary lung epithelial cells is independent of glucocorticoid receptor, Eur. Respir. J. 30 (5) (Nov. 2007) 848–856.
[27] P.J. Barnes, S.D. Shapiro, R.A. Pauwels, Chronic obstructive pulmonary disease: molecular and cellular mechanisms, Eur. Respir. J. 22 (4) (Oct. 2003) 672–688.
[28] K.J. Balazovich, R. Fernandez, V. Hinkovska-Galcheva, S.J. Suchard, L.A. Boxer, Transforming growth factor-beta1 stimulates degranulation and oxidant release by adherent human neutrophils, J. Leukoc. Biol. 60 (6) (1996) 772–777.
[29] J. Chakir, J. Shannon, S. Molet, M. Fukakusa, J. Elias, M. Laviolette, L.P. Boulet, Q. Hamid, Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression, J. Allergy Clin. Immunol. 111 (2003) 293–1298.
[30] S. Koyama, E. Sato, H. Nomura, K. Kubo, S. Nagai, T. Izumi, Acetylcholine and substance P stimulate bronchial epithelial cells to release eosinophil chemotactic activity, J. Appl. Physiol. 84 (5) (May 1998) 1528–1534.
[31] E. Sato, S. Koyama, Y. Okubo, K. Kubo, M. Sekiguchi, Acetylcholine stimulates alveolar macrophages to release inflammatory cell chemotactic activity, Am. J. Physiol. 274 (6 Pt 1) (1998) L970–L979.
[32] B.J. Proskocil, H.S. Sekhon, Y. Jia, V. Savchenko, R.D. Blakely, J. Lindstrom, E.R. Spindel, Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells, Endocrinology 145 (5) (May 2004) 2498–2506.
[33] P.G. Bloemen, M.C. van den Tweel, P.A. Henricks, F. Engels, M.H. Kester, P.G. van de Loo, F.J. Blomjous, F.P. Nijkamp, Increased cAMP levels in stimulated neutrophils inhibit their adhesion to human bronchial epithelial cells, Am. J. Physiol. 272 (4Pt 1) (Apr. 1997) L580–L587.
[34] M. Johnson, Effects of b2-agonists on resident and infiltrating inflammatory cells, J. Allergy Clin. Immunol. 110 (2002) S282–S290.
[35] R.W. Costello, D.B. Jacoby, A.D. Fryer, Pulmonary neuronal M2 muscarinic receptor function in asthma and animal models of hyperreactivity, Thorax 53 (7) (Jul. 1998) 613–616.
[36] D.C. Hornigold, R. Mistry, P.D. Raymond, J.L. Blank, R.A. Challiss, Evidence for cross-talk between M2 and M3 muscarinic acetylcholine receptors in the regulation of second messenger and extracellular signal-regulated kinase signalling pathways in Chinese hamster ovary cells, Br. J. Pharmacol. 138 (7) (Apr. 2003) 1340–1350.
[37] B.J. Proskocil, A.D. Fryer, Beta2-agonist and anticholinergic drugs in the treatment of lung disease, Proc. Am. Thorac. Soc. 2 (4) (2005) 305–310 (discussion 11–2).
[38] E.B. Haddad, J. Rousell, J.C. Mak, P.J. Barnes, Transforming growth factor-beta 1 induces transcriptional down-regulation of m2 muscarinic receptor gene expression, Mol. Pharmacol. 49 (5) (May 1996) 781–787.
[39] T. Bouyssou, P. Casarosa, E. Naline, S. Pestel, I. Konetzki, P. Devillier, A. Schnapp, Pharmacological characterization of olodaterol, a novel inhaled beta2-adrenoceptor agonist exerting a 24-hour-long duration of action in preclinical models, J. Pharmacol. Exp. Ther. 334 (1) (2010) 53–62.