Acetylcholine leads to signal transducer and activator of transcription 1 (STAT-1) mediated oxidative/nitrosative stress in human bronchial epithelial cell line

Mirella Profita [a,*], Giusy Daniela Albano [a,b], Angela Marina Montalbano [a], Caterina Di Sano [a], Giulia Anzalone [a], Rosalia Gagliardo [a], Loredana Riccobono [a], Anna Bonanno [a], Liboria Siena [a], 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

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ABSTRACT

The induction of nitric oxide synthase (iNOS) expression via the signal transducer and activator of transcription 1 (STAT-1) is involved in the mechanism of oxidative/nitrosative stress. We investigated whether acetylcholine (ACh) generates oxidative/nitrosative stress in bronchial epithelial cells during airway inflammation of COPD and evaluated the effects of Tiotropium, a once-daily antimuscarinic drug, and Olodaterol, a long-acting β2-agonist on these mechanisms. Human bronchial epithelial cells (16-HBE) were stimulated (4 h, 37 °C) with induced sputum supernatants (ISSs) from healthy controls (HC) (n = 10), healthy smokers (HS) (n = 10) or COPD patients (n = 10), as well as with ACh (from 1 μM to 100 μM). The activation of STAT-1 pathway (STAT-1Ser727 and STAT-1Tyr701) and iNOS was evaluated in the cell lysates by Western blot analysis as well as nitrotyrosine levels by ELISA, while reactive oxygen species (ROS) were evaluated by flow cytometry. Finally, the effect of Tiotropium (Spiriva®) (100 nM), alone or in combination with Olodaterol (1 nM), was tested in this model. ISSs from COPD patients significantly increased the phosphorylation of STAT-1Ser727 and STAT-1Tyr701, iNOS and ROS/Nitrotyrosine when compared with ISSs from HC or HS subjects in 16-HBE cells. Furthermore, synthetic ACh increased all these parameters in stimulated 16HBE when compared with untreated cells. Tiotropium and Olodaterol reduced the oxidative/nitrosative stress generated by ACh and ISSs. We concluded that ACh mediated the oxidative/nitrosative stress involving the STAT-1 pathway activation in human bronchial epithelial cells during COPD. β2-Long acting and antimuscarinic drugs, normally used in the treatment of COPD as bronchodilator, might be able to control these cellular events.

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1. Introduction

Oxidative stress, defined as an increase in oxidants and/or a decrease in antioxidant capacity, is associated with inflammatory airway diseases, including asthma and Chronic Obstructive Pulmonary Disease (COPD). Airway cells, especially eosinophils and epithelial cells, under inflammatory conditions, produce more superoxide anions (O_2^-) and release reactive nitrogen compounds (NO) and reactive oxygen species (ROS) than normal airway cells [1]. NO and O_2^- react producing peroxynitrite anions promoting the nitrosylation of tyrosine residue of proteins to form nitrotyrosine in the airway of asthma and COPD patients [2]. Recent studies showed a strong immunoreactivity to Nitrotyrosine in the epithelium, lung parenchyma, and inflammatory cells (eosinophils, neutrophils) in patients with inflammatory airway diseases such as COPD [3]. Nitrotyrosine and ROS production often co-exists within the cells [4].

The inducible nitric oxide synthase (iNOS) expression plays a prominent role in the mechanism of oxidative/nitrosative stress of various cell types [5]. Among signaling pathways implicated in controlling the activation of iNOS transcription factors and subsequent iNOS expression, NF-κB pathway, mitogen-activated protein kinases (MAPK), and Janus kinase (JAK)/STAT-dependent signal transduction appear to have a relevant role [6,7].

Acetylcholine (ACh) is synthesized by choline acetyltransferase (ChAT) in different cell types such as macrophages, T lymphocytes, fibroblasts and epithelial cells [8]. Recent findings described ACh as

a new emerging factor of airway inflammation and remodeling processes in COPD, targeting the cells via muscarinic acetylcholine receptors (mAChRs) [9–11]. Further, we have shown that ACh was increased in induced sputum supernatants from COPD patients compared with healthy controls [12]. ACh affects ROS production by activation of Phosphoinositide 3-kinase (PI3K), Src-kinases or the extracellular signal-regulated kinase (ERK) pathway in adult rabbit ventricular myocytes [13] suggesting its involvement in the mechanism of oxidative stress in the cells.

The current guidelines recommend the regular use of β2-AR-agonists and anticholinergics alone or as concurrent therapy in COPD to maximize bronchodilation [14,15]. It was demonstrated that Tiotropium is able to counteract the proinflammatory activity of ACh on different cell types [10,16–19] and is involved in the control of oxidative stress regulating "ex vivo" the release of ROS in peripheral blood neutrophils from COPD subjects [20]. Further recent findings showed that β2-AR-agonists and antimuscarinic additively control TGF-β1-mediated neutrophilic inflammation in COPD [12]. However, the antioxidant efficacy of this combination is not yet widely studied.

Since the oxidative/nitrosative stress and ACh are implicated in the activation of bronchial epithelial cells in COPD, in this study we aimed to investigate whether ACh is able to generate oxidative/nitrosative stress during airway inflammation. Finally, we evaluated the effects of the once-daily anticholinergic Tiotropium and the long-acting β2-agonist Olodaterol, alone or in combination, on the cellular oxidative and nitrosative activities of ACh.

2. Materials and methods

2.1. Subjects

We included three groups of subjects: healthy non-smoking subjects (HC) (n = 10), asymptomatic smokers with normal lung function (HS) (n = 10), and patients with COPD (n = 10). COPD was defined and classified according to the criteria reported by Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines as stage ≥1 [21]. 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. COPD patients who had routine chest X-rays and computed tomographic scans that showed emphysema were excluded.

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 (Table 1). 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

antimuscarinics within 3 months before the study and treatment with long acting β2-adrenergic agonists 15 days before the study. The local Ethics Committee approved the study, and participating subjects gave their informed consent.

2.2. Pulmonary function tests

Pulmonary function tests included measurements of FEV1, FVC, and PEF. The reversibility of the airway obstruction in subjects with a baseline FEV1% is less than 80% of predicted. To assess the reversibility, the FEV1 measurement was repeated 15 min after the inhalation of 200 μg of salbutamol. Data are expressed as percentage of predicted values.

2.3. Sputum induction and processing

Sputum induction and processing were performed with the plug method as previously described [10] according to the methods of Hargreave et al. [22]. The volume of the induced sputum was previously determined, and an equal volume of Dithiothreitol (DTT) (0.1% in saline; Sigma, St. Louis, MO) was added. After homogenization, sputum samples were centrifuged at 800 g for 10 min to separate the supernatants from the cell pellet. The supernatants were then aspirated and frozen at −20 °C in separate aliquots. The cells obtained from induced sputum were then cytocentrifuged (Cytospin 2; Shandon, Runcorn, United Kingdom) and stained with May–Grunwald–Giemsa. Cytospins were used for differential cell counts.

2.4. Human bronchial epithelial cell culture

The SV40 large T antigen-transformed 16-HBE cell line (16-HBE) was used for these studies. 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 [23]. 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 aminoacids, Euroclone), 2 mM L-glutamine and gentamicin 250 μg/ml at 37 °C in a humidified 5% CO2 atmosphere.

2.5. Human bronchial epithelial cell stimulation with ACh

16-HBE were plated until confluence with MEM 10% FBS (5% CO2 at 37 °C); after 24 h under FBS-free conditions, 16-HBE were stimulated with various concentrations of ACh (from 1 μM to 100 μM) (Sigma St. Louis, MO) for 4 h to evaluate the production of ROS and Nitrotyrosine. The iNOS expression was evaluated in 16-HBE stimulated with ACh

Table 1
Demographic characteristics of patients.

	HC	HS	COPD	p Value		
				HC vs HS	HC vs COPD	HS vs COPD
Age, yr	63 ± 7	60.5 ± 7	64 ± 13	ns	ns	ns
Gender, male/female	5/5	6/4	3/7	–	–	–
FEV1, % predicted	111.6 ± 10.6	103.2 ± 10	60.7 ± 55	ns	<0.005	<0.02
FVC, % predicted	108.4 ± 16.2	107.1 ± 15	70.6 ± 112	ns	<0.03	<0.03
FEV1 post β2 (% from baseline)	–	–	4 ± 2	–	–	–
FEV1/FVC (%)	94.6 ± 3.0	89.3 ± 4.9	64.4 ± 9.0	ns	<0.001	<0.01
FEV1/FVC post β2 (% from baseline)	–	–	6 ± 2	–	–	–
Smoking, pack/yr	0	62.5 ± 17.0	62 ± 21.0	<0.002	<0.002	ns

Data are presented as mean ± SD. Abbreviations: HC = asymptomatic nonsmoking subjects with normal lung function; HS = asymptomatic smokers with normal lung function; COPD = subject 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.

10 μM for 2 and 4 h. Furthermore, the cells were stimulated with ACh 10 μM for 15 and 30 min at 37 °C to study the STAT-1 pathway activation. To determine the involvement of the mAChRs on oxidative/nitrosative stress generated in 16-HBE by ACh, Tiotropium (100 nM) (Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany) was added to 16-HBE 1 h before the stimulation with ACh 10 μM.

2.6. Human bronchial epithelial cell stimulation with ISSs

16-HBE (70,000 cells/well) were plated in standard 6-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 COPD (n = 10) patients, HS (n = 10) and HC (n = 10) subjects for 4 h at 37 °C to evaluate ROS, Nitrotyrosine and iNOS production and for 30 min to evaluate the STAT-1 pathway activation. 16HBE were stimulated with DTT (0.1% in saline solution) to exclude the nonspecific effect of DTT present in ISSs used in the experiments on iNOS, ROS, NT production and on STAT-1 pathway activation.

Tiotropium Spiriva® (100 nM) (Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany) and Olodaterol (1 nM) (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.

Hemicholinium-3 (HCh-3) (50 μM, Sigma–Aldrich), a potent and selective choline uptake blocker was added to the 16-HBE 1 h before the stimulation with ISSs from COPD patients. After the stimulation, the cells were recovered and analyzed for ROS, Nitrotyrosine, iNOS and STAT-1 pathway activation. Cell viability was confirmed by light microscopy and trypan blue exclusion.

2.7. Detection of intracellular ROS

16-HBE cells, stimulated via the above methods, were detached by trypsin, washed in PBS and collected in FACS tubes. Cells were then incubated with the oxidant-sensitive dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA, 1 mM) in PBS for 10 min in the dark at room temperature. After washing, cells were resuspended in PBS and then analyzed by flow cytometry for fluorescence-positive cells. The intracellular ROS generation was evaluated by flow cytometry analysis using a FACSCalibur™ flow cytometer (Becton Dickinson, Mountain View, CA, USA). Negative controls consisted of 16-HBE cells cultured without DCFH-DA. Gating on the cells, excluding debris, was performed using forward and sideways scatter patterns.

2.8. Nitrotyrosine detection

Nitrotyrosine levels were assessed in duplicate in cell lysates, using the Nitrotyrosine ELISA kit purchased by Hycult Biotechnology b.v. (HK-501, Uden, the Netherlands) according to the manufacturer's instructions. Absorbance was read at 450 nm by a Wallac 1420 Victor2 multilabel counter (Perkin–Elmer Life Sciences, Turku, Finland). The minimum detection level was 0.5 nM. We run as positive control 16-HBE protein extract nitrated with tetranitromethane (dissolved in methanol) (4% v/v) (TNM) (Sigma–Aldrich, Italy) for 2 h and as negative control 16-HBE protein extract or bovine serum albumin (BSA, 1 mg/ml) (Sigma–Aldrich, Italy).

2.9. RNA interference of the STAT-1 pathway

To confirm that the increase of iNOS, ROS and Nitrotyrosine production is associated with ACh or with ISSs dependent STAT-1 activation, we tested the effect of p91/STAT-1 silencing in human bronchial epithelial cells using a specific siRNA transfection [24]. 16-HBE cells were plated in six well tissue culture plates and grown in medium containing 10% FBS without antibiotics until 60 to 80% confluency. p91/STAT-1α

siRNA (10 μM; Santa Cruz Biotechnology, Inc.) was then added to 100 μl of siRNA transfection medium, and the reaction was performed according to the manufacturer's instructions until complete cell transfection (7 h at 37 °C). For optimal siRNA transfection efficiency, siRNA (10 μM; Santa Cruz Biotechnology, Inc.) containing a scrambled sequence that did not lead to the specific degradation of any known cellular mRNA was used to control non-specific effects. Finally, cells were stimulated with ISSs from COPD or ACh 10 μM for 4 h. The silencing efficacy of RNA interference in p91/STAT-1α was assessed by Western blot analysis.

2.10. Western blotting

Total proteins were extracted from stimulated 16HBE cells using a lysis buffer (NaCl 50 mM, Tris–HCl 10 mM, EDTA 5 mM, NP-40 1%) containing a protease and phosphatase inhibitor. Protein concentration was assessed using the Bradford method. The total protein extracts were separated by SDS-PAGE on 10% gradient gels followed by electroblotting onto nitrocellulose membranes. The following antibodies were used: rabbit polyclonal anti-phospho-STAT1 (Ser727 and Y710) (Upstate Biotechnology, Temecula CA), rabbit polyclonal anti-STAT-1α/p91 (C-24) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and rabbit polyclonal anti-iNOS (Abcam, Cambridge, UK). β-Actin (Sigma-Aldrich) was used as a housekeeping protein to control the total amount of protein in each sample. Cell extracts were transferred to microcentrifuge tubes, left on ice for 45 min, and centrifuged at 15,000 *g* for 20 min at 4 °C. Forty micrograms of protein was subjected to SDS-polyacrylamide gel electrophoresis on 8 to 10% gradient gels and blotted onto nitrocellulose membranes. After blocking with PBS containing 5% milk and 0.1% Tween 20, membranes were probed with specific antibodies, washed, and incubated with peroxidase-conjugated secondary antibodies. Detection was performed with an enhanced chemiluminescence system (Ambion, Austin, TX) followed by autoradiography. Gel images were acquired using an Epson GT-6000 scanner (Epson America, Torrance, CA) and then imported into the National Institutes of Health Image analysis 1.61 program to determine band intensity. Data are expressed as arbitrary densitometric units corrected against the density of β-actin bands.

2.11. Statistical analysis

Statistical analysis was calculated using Kruskal Wallis followed by Bonferroni Dunn correction for multiple comparisons. Nonparametric Kruskal Wallis test was chosen to verify that the population means are equal. 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.

3. Results

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

The patients' characteristics are summarized in Table 1. The differential cell counts of induced sputum samples showed a significant increase of the cell number in COPD. In COPD and HS the percentage of macrophages was significantly lower and the percentage of neutrophils was significantly higher than in HC. The percentage of lymphocytes, eosinophils, and epithelial cells did not show significant differences among the three study groups (Table 2).

3.2. ACh and ISSs increased the levels of oxidative/nitrosative stress in human bronchial epithelial cells

ACh activated the oxidative and nitrosative stress in 16-HBE cells promoting ROS and Nitrotyrosine production in a concentration-

Table 2
Total and differential cell count from induced sputum.

	HC	HS	COPD	p Value		
				HC vs HS	HC vs COPD	HS vs COPD
Macrophages (%)	83.2 (67.9–88.4)	52.6 (42.1–69)	20.6 (10.0–58.2)	<0.02	<0.002	ns
Neutrophils (%)	18.3 (10–28.1)	45.0 (30.2–45.2)	72.0 (42.2–83.5)	<0.02	<0.002	ns
Lymphocytes (%)	0.3 (0–1.2)	0.6 (0.4–1.3)	1.3 (0–1.5)	ns	ns	ns
Eosinophils (%)	0.1 (0–0.7)	0.8 (0–1.4)	0.9 (0.2–2.4)	ns	ns	ns
Epithelial cells (%)	0.6 (0.5–2.0)	0.7 (0–0.9)	0.6 (0–1.8)	ns	ns	ns
Total cells (10^6/g ISS)	3.5 (2.1–7.8)	3.3 (3.5–6)	5.6 (3–14.8)	ns	ns	ns

Results are expressed as median (25th to 75th percentiles) Statistical analysis for multiple comparisons was calculated using Kruskal–Wallis and nonparametric ANOVA tests followed by Bonferroni Dunn correction. Abbreviations: HC = healthy asymptomatic nonsmoking subjects with normal lung function; HS = asymptomatic smokers with normal lung function; COPD = subjects with Chronic Obstructive Pulmonary Disease; ns = not significant.

dependent manner, reaching higher levels at the ACh concentration of 10 μM (Fig. 1, A) compared to untreated cells. Tiotropium 100 nM reduced both ROS and Nitrotyrosine levels generated by ACh stimulation (Fig. 1, B).

ISSs from COPD patients and from HS subjects increased ROS and Nitrotyrosine production in stimulated 16-HBE cells when compared to untreated cells and to the cells stimulated with ISSs from HC subjects (Fig. 2, A). The pretreatment of the cells with Tiotropium 100 nM reduced the levels of both ROS and Nitrotyrosine production in 16-HBE cells stimulated with ISSs from COPD patients. Furthermore, the pretreatment of the cells with Olodaterol (1 nM) reduced the levels of ROS and Nitrotyrosine production in the cells. The pretreatment of the cells with both Tiotropium and Olodaterol did not have an additive or synergistic effect when the drugs were used in combination in the experimental conditions (Fig. 2, B).

3.3. ACh and ISSs increased the iNOS expression in human bronchial epithelial cells

ACh increased the expression of iNOS in 16-HBE cells, in a time dependent manner reaching the higher levels after 4 h of stimulation. The iNOS control value for the longer stimulation period of 4 h of ACh exposure, is nearly identical to the values obtained in the 2 h groups but different from the other control value (Fig. 3, A). The pretreatment of the cells with Tiotropium 100 nM reduced the levels of iNOS expression generated in 16-HBE cells stimulated with ACh 10 μM compared to untreated cells (Fig. 3, B).

ISSs from COPD patients significantly increased the expression of iNOS in 16-HBE cells, in comparison to untreated cells or stimulation with ISSs from HC and HS subjects (Fig. 3, C). The pretreatment of the cells with Tiotropium or Olodaterol reduced the levels of iNOS
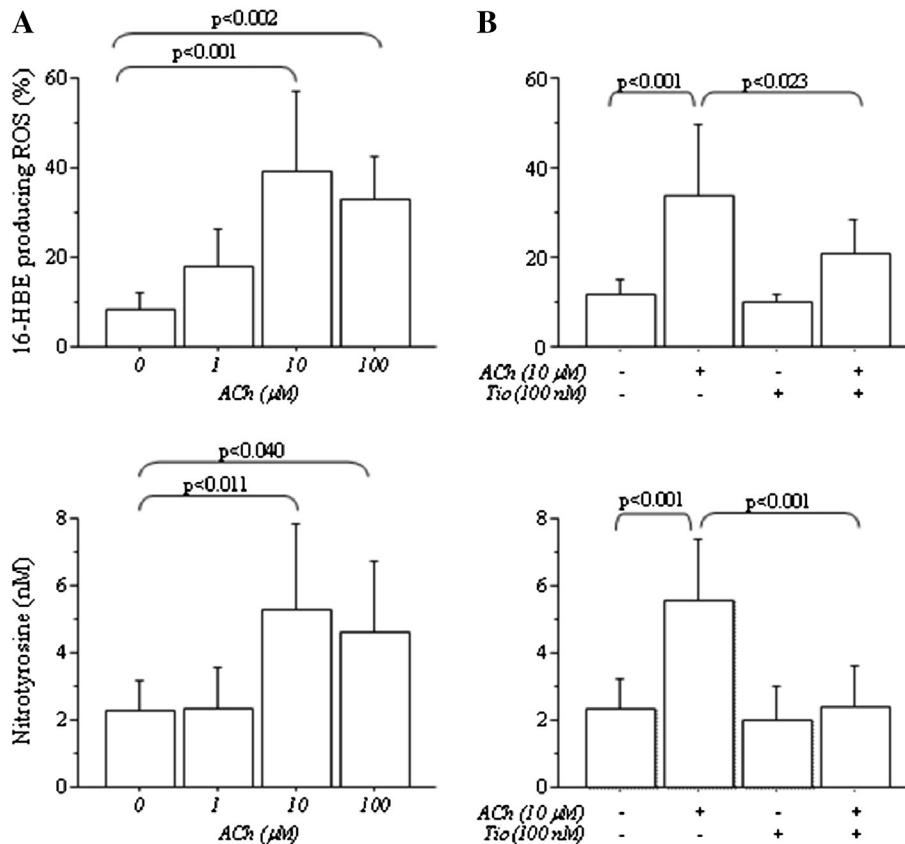

Fig. 1. ACh activates the oxidative/nitrosative stress in 16-HBE cells. A) The 16-HBE were stimulated with different concentrations of ACh (0 to 100 μM) for 4 h; B) Tiotropium (100 nM) was added to 16-HBE 1 h before the stimulation with ACh 10 μM for 4 h. At the end of the stimulation, we evaluated intracellular ROS production (as percentage of fluorescent positive cells) by flow cytometry and Nitrotyrosine (nM) in cell lysates by ELISA. The bars represent the data from six independent experiments. The results were expressed as mean ± S.D. ANOVA with Fisher's test correction was used for the analysis of the data.
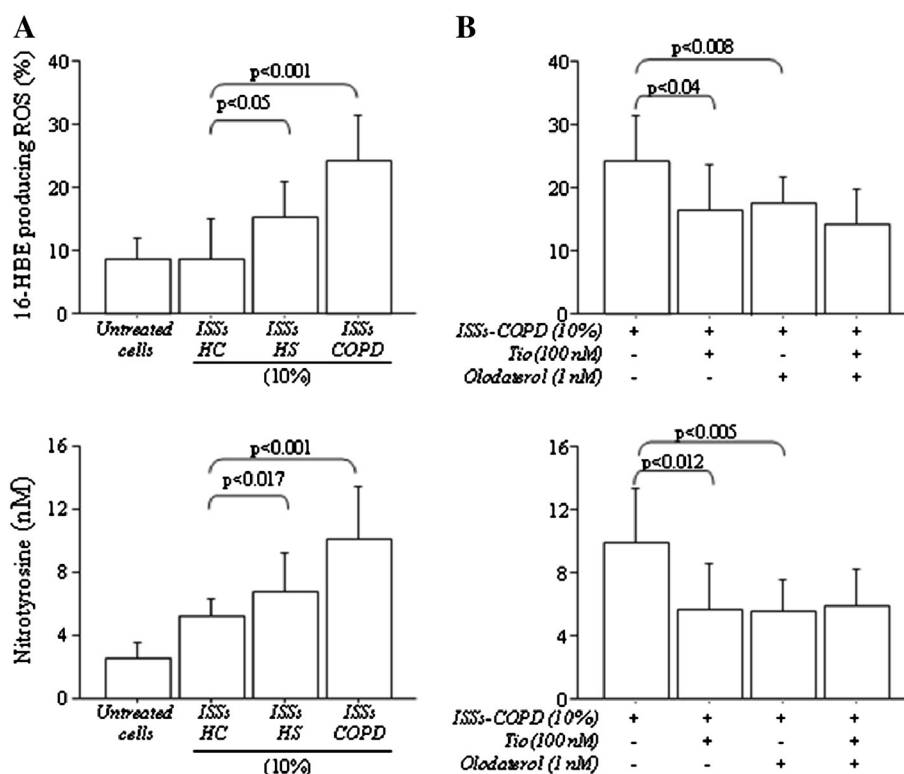
Fig. 2. ISSs activate the oxidative/nitrosative stress in 16-HBE cells. A) The 16-HBE were stimulated with ISSs from COPD patients (n = 10) and from HS (n = 10) and HC (n = 10) subjects for 4 h; B) Tiotropium (100 nM) and Olodaterol (1 nM) alone or in combination were added to 16-HBE 1 h before the stimulation with ISSs for 4 h. At the end of the stimulation, ROS production was evaluated in the cells by flow cytometry and Nitrotyrosine in cell lysates by ELISA. The results were expressed as mean ± S.D. ANOVA with Fisher's test correction was used for the analysis of the data.

expression generated in 16-HBE stimulated with ISSs from COPD patients. The pretreatment of the cells with both Tiotropium and Olodaterol did not exert any additive or synergistic effects (Fig. 3, D).

3.4. ACh and ISSs activated the STAT-1 pathway

ACh increased in 16-HBE cells the pSTAT-1 Tyr701 and pSTAT-1 Ser727 phosphorylation in a time-dependent manner, reaching higher levels after 30 min of stimulation compared to untreated cells. The pSTAT-1 Tyr701 and pSTAT-1 Ser727 phosphorylation control value for the longer stimulation period of 30 min of ACh exposure, is nearly identical to the values obtained in the 15 min groups but different from the other control value (Fig. 4, A). The pretreatment with Tiotropium reduced the levels of STAT-1 pathway phosphorylation generated by ACh (Fig. 4, B).

ISSs from COPD patients activated the STAT-1 pathway in 16-HBE cells, promoting increased levels of pSTAT-1 Tyr701 and pSTAT-1 Ser727 compared to untreated cells and to the cells stimulated with ISSs from HC subjects. Further, ISSs from HS subjects increased in 16-HBE cells the level of pSTAT-1 Ser727 compared to untreated cells and to the cells stimulated with ISSs from HC subjects (Fig. 5, A). The pretreatment of 16-HBE with Tiotropium or Olodaterol reduced the levels of pSTAT-1 Tyr701 and pSTAT-1 Ser727 generated by ISSs from COPD patients. The pretreatment with Tiotropium and Olodaterol in combination showed an additive effect in the reduction of STAT-1 pathway activation (Fig. 5, B). Finally, we observed that the stimulation of 16HBE with DTT (0.1% saline solution) did not increase the pSTAT-1 Tyr701 and pSTAT-1 Ser727 when compared with untreated cells (Fig. 5).

The HCh-3, a potent and selective choline uptake blocker, was able to reduce the levels of pSTAT-1 Tyr701 and pSTAT-1 Ser727 (Fig. 6, A), ROS (Fig. 6, B), Nitrotyrosine production (Fig. 6, C), and iNOS expression

(Fig. 6, D) in untreated 16-HBE cells and in 16HBE stimulated with ISSs from COPD patients. Finally, we observed that the stimulation of 16HBE with DTT (0.1% saline solution) did not increase the ROS, iNOs and Nitrotyrosine production when compared with untreated cells (Fig. 6).

3.5. Effect of STAT-1 silencing on ROS, NT and iNOS in 16-HBE cells

Temporary cell transfection with p91/STAT-1α siRNA significantly decreased the synthesis of STAT-1 protein and iNOs in 16-HBE. Furthermore, the temporary cell transfection with control siRNA (scrambled sequence) was not different than untreated cells showing a specific effect of transfection with p91/STAT-1α siRNA (Fig. 7, A). The silencing of p91/STAT-1α reduced the levels of iNOS, ROS and Nitrotyrosine production (Fig. 7, B, C and D) in 16-HBE stimulated with synthetic ACh or ISSs from COPD patients when compared with stimulated unsilenced 16-HBE. Furthermore, in a few number of experiments (n = 2) we obtained that the response to ACh or ISSs from COPD patients was not affected in the cells transfected with control siRNA when compared with untreated cells (data not shown). Finally, we observed that the stimulation of 16HBE with DTT (0.1% saline solution) did not increase the ROS, and Nitrotyrosine production in silenced and unsilenced cells when compared with untreated cells (Fig. 7, C, D).

These findings support the newly described role of STAT-1 activation in the transduction pathway leading to the enhanced oxidative/nitrosative stress in 16-HBE cells.

4. Discussion

We studied oxidative/nitrosative stress markers, in terms of ROS and Nitrotyrosine production, in an in vitro model of human bronchial epithelial cells, in response to ACh or airway inflammation present in induced sputum supernatants from COPD patients. We observed that
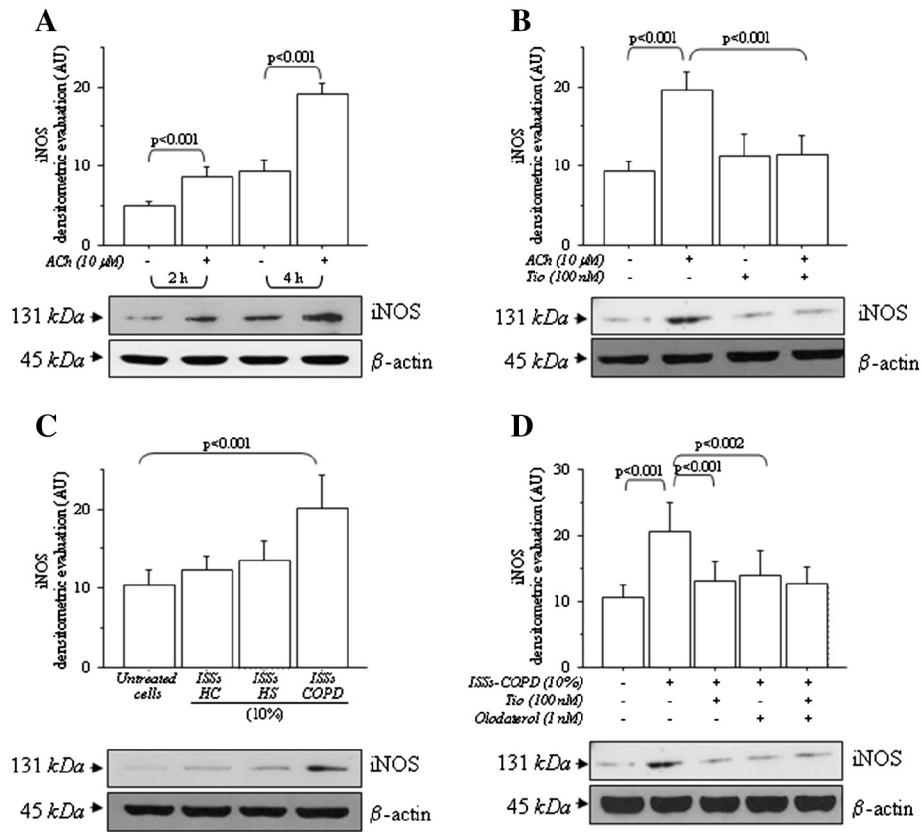
Fig. 3. ACh and ISSs activate the iNOS expression in 16-HBE cells. A) The 16-HBE were stimulated with ACh 10 μM for 2 and 4 h; B) Tiotropium (100 nM) was added to 16-HBE 1 h before the stimulation with ACh 10 μM for 4 h. C) The 16-HBE were stimulated with ISSs from COPD patients (n = 10) and from HS (n = 10) and HC (n = 10) subjects for 4 h; D) Tiotropium (100 nM) and Olodaterol (1 nM) alone or in combination were added to 16-HBE 1 h before the stimulation with ISSs from COPD patients for 4 h. At the end of stimulation, the iNOS expression was evaluated in cell lysates by Western blot. The bars of A, B and D represent the densitometric analysis of iNOS from six independent experiments. The bars of C represent the densitometric analysis of iNOS from ten independent experiments. Representative gel images of iNOS expression were shown. The results were expressed as mean ± S.D. ANOVA with Fisher's test correction was used for the analysis of the data.
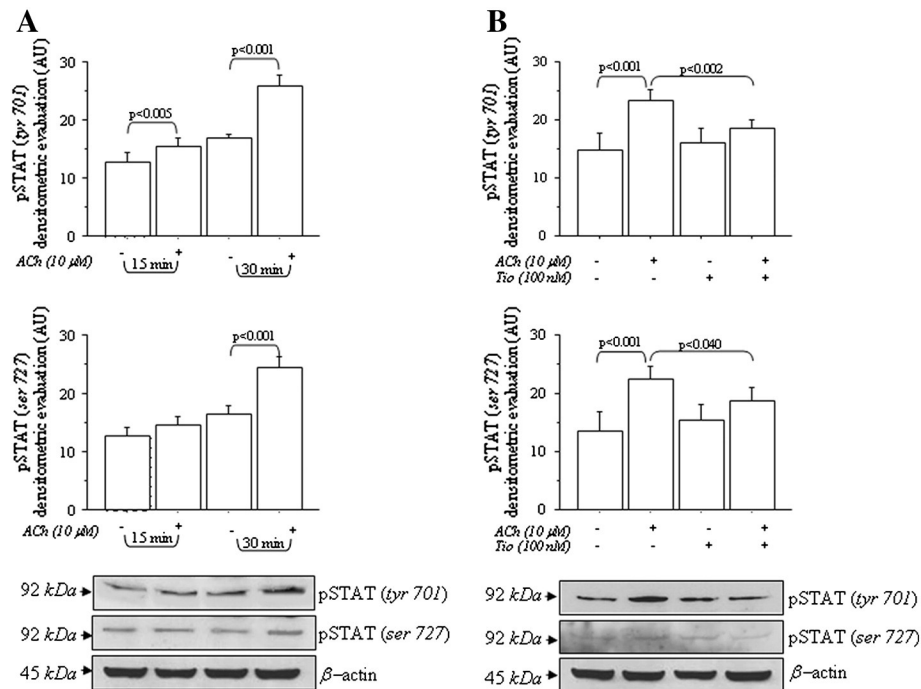

Fig. 4. ACh activates the STAT-1 pathway in 16-HBE cells. A) The 16-HBE were stimulated with ACh 10 μM for 15 and 30 min; B) Tiotropium (100 nM) was added to 16-HBE 1 h before the stimulation with ACh 10 μM for 30 min. At the end of the stimulation the cell extracts were separated with 10% SDS-polyacrylamide gel electrophoresis, and Western blot analyses were carried out sequentially with anti-phosphoSTAT-1Ser727, anti-phosphoSTAT-1Tyr701, and anti-β-actin antibodies. Representative Western blot of anti-phosphoSTAT-1Ser727, anti-phosphoSTAT-1Tyr701and relative β-actin are shown. The bars represent the densitometric analysis of six separate experiments. The results were expressed as mean ± S.D. ANOVA with Fisher's test correction was used for the analysis of the data.
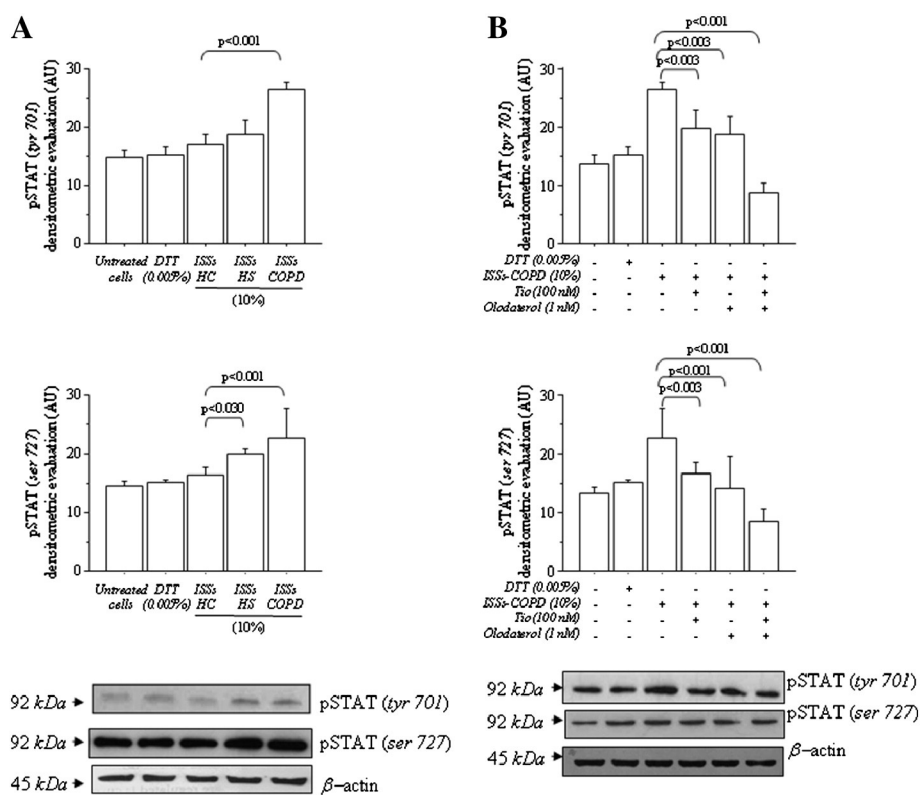
Fig. 5. ISSs activate the STAT-1 pathway in 16-HBE cells. A) The 16-HBE cells were stimulated with ISSs from COPD patients (n = 10), HS (n = 10), HC (n = 10) subjects or DTT for 30 min. B) Tiotropium (100 nM) and Olodaterol (1 nM) alone or in combination were added to 16-HBE 1 h before the stimulation with ISSs for 30 min. At the end of the stimulation the cell extracts were separated with 10% SDS-polyacrylamide gel electrophoresis, and Western blot analyses were carried out sequentially with anti-phosphoSTAT-1Ser727, anti-phosphoSTAT-1Tyr701, and anti-β-actin antibodies. Representative Western blot of anti-phosphoSTAT-1Ser727, anti-phosphoSTAT-1Tyr701 and the relative β-actin are shown. The bars of A represent the densitometric analysis of pSTAT-1Ser727, pSTAT-1Tyr701 from ten independent experiments. The bars of B represent the densitometric analysis of pSTAT-1Ser727, pSTAT-1Tyr701 from six independent experiments. The results were expressed as mean ± S.D. ANOVA with Fisher's test correction was used for the analysis of the data.

this pathological event may be associated with an increased expression of iNOS due to STAT-1 downstream signaling activation. Finally in our in vitro model, showing that Tiotropium or Olodaterol might be able to control the oxidative and nitrosative stress markers generated in bronchial epithelial cells of inflammation, we support the concept of long term beneficial effects of β2-adrenoceptoragonists and muscarinic antagonist treatment during airway inflammation.

Inflammation is considered of primary pathogenic importance in COPD. The current therapeutic approach suggests the use of anticholinergic and long-acting β2 agonists in the treatment of COPD to maximize bronchodilatation [14,15]. The utility of the treatment of both, anti-inflammatory and bronchodilatatory drugs has relatively recently been included in the current guidelines at least in certain stages of the disease. Demonstrating that antimuscarinic drugs and β2 long-acting might exert an anti-inflammatory or an antioxidant/ antinitrosative action, we suggest an additional long-term beneficial effect of these drugs in the treatment of COPD.

Various studies in myocytes emphasize the stimulating effect of cholinergic stimulation on ROS production which is caused by activation of PI3K, Src-kinases or the ERK pathway at least leading to the opening of mitochondrial K_{ATP} channels and ROS release [25,26]. ROS by themselves trigger chemokine production in inflammatory cells thus enhancing the inflammatory process [26,27]. The involvement of the cholinergic system in ROS and ROS-mediated cytotoxicity, makes it an ideal system to test muscarinic receptor inhibitors for their cell protective function [28]. Today, more than one finding describe the immunomodulatory effects of ACh, potentially influencing the inflammatory processes in COPD [10,11,18,19]. In accordance with these observations, we showed that "in vitro" synthetic ACh is able to increase the ROS production and

the antimuscarinic Tiotropium is able to inhibit this effect in 16 HBE cell line. Our previous results showed that the induced sputum from COPD patients showed higher levels of ACh compared with smokers and healthy control subjects [12]. In this study, we showed that ISSs from COPD patients induced an increased production of ROS and that Tiotropium is able to inhibit this effect in 16 HBE. These findings support the presence and action of ACh in ISSs from COPD or a de novo ACh synthesis during 16HBE stimulation. Based on the observations that β2AR-agonists can have an antiinflammatory activity in primary lung epithelial cells [29], we identified the antioxidant effect of Olodaterol on ROS production in 16-HBE stimulated with ISS from COPD. In this scenario, our findings support the concept that β2AR-agonists and antimuscarinic drugs besides to maximize bronchodilatation in the treatment of COPD, are able to reduce the oxidative/nitrosative stress produced during airway inflammation from bronchial epithelial cells. Finally, since β2 adrenoceptor agonist is able to mediate opposite effect of muscarinic receptors, reducing endotelin-1 production in human lung fibroblasts [30] and is able to modulate inflammation in COPD [20], accordingly our findings suggest that Olodaterol is able to control ACh activity present or generated by ISS samples from COPD patients reducing ROS production in 16-HBE.

Airway inflammatory cells especially eosinophils and epithelial cells under inflammatory conditions, produce more superoxide anions (O_2^-) and release reactive nitrogen compounds (NO) and reactive oxygen species (ROS) than normal airway cells [1]. An excessive production of NO, derived from the inducible type of NOS (iNOS, NOS2), reacts with superoxide anion O_2^- and produces peroxynitrite able to nitrate the tyrosine residues of proteins to form the stable product of Nitrotyrosine [2], a compound playing a pivotal role in the airway and lung parenchymal
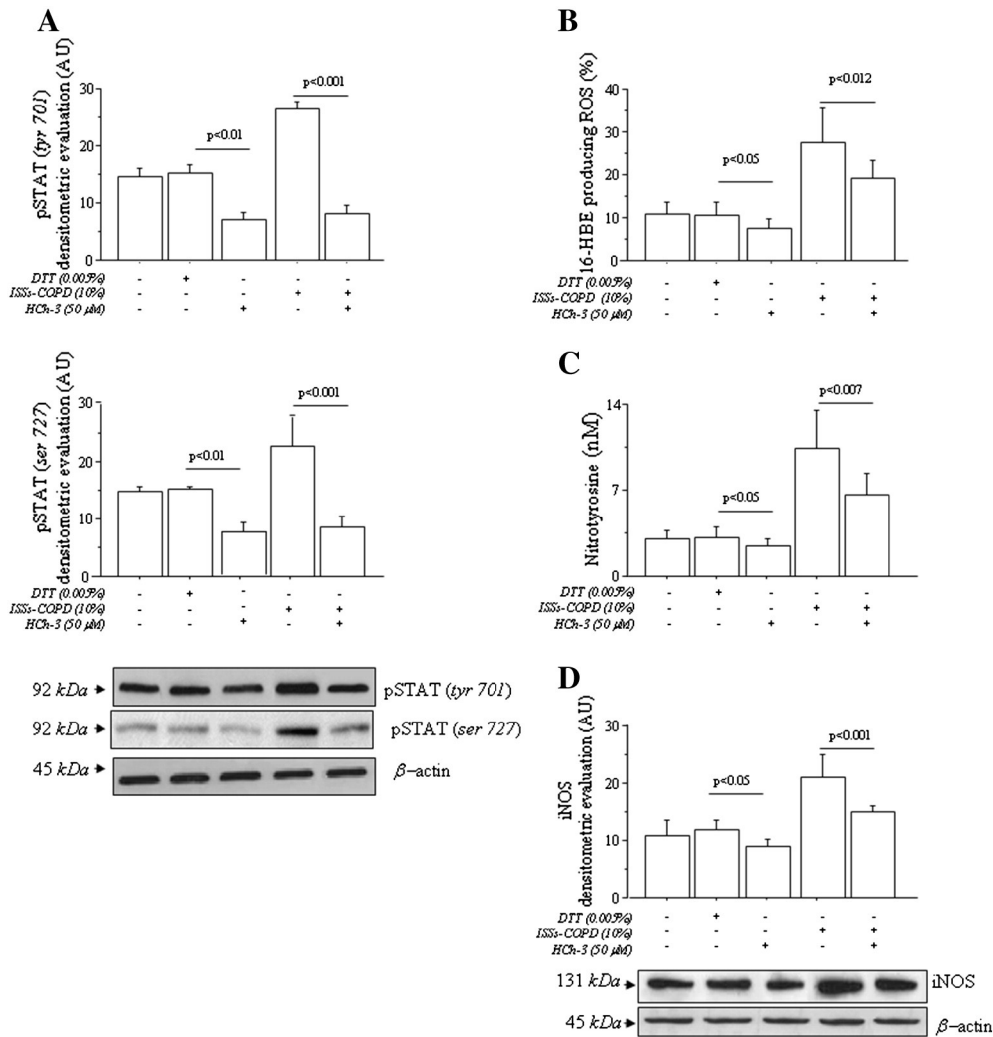
Fig. 6. Hemicholinium (HCh-3) reduces the oxidative/nitrosative stress generated by ISSs from COPD patients in 16-HBE cells. The 16-HBE cells were stimulated for 30 min with ISSs from COPD patients in the presence and absence of Hemicholinium (a potent choline uptake blocker). A) Western blot analyses were carried out sequentially with anti-phosphoSTAT-1Ser727, anti-phosphoSTAT-1Tyr701, and anti-β-actin antibodies. Representative Western blot of phosphoSTAT-1Ser727, anti-phosphoSTAT-1Tyr701 and the relative β-actin is shown. The bars represent the densitometric analysis of 3 separate experiments. At the end of the stimulation for 4 h B) ROS production was evaluated in the cells by flow cytometry, C) Nitrotyrosine in cell lysates by ELISA and D) iNOS expression by Western blot. Representative gel image of iNOS expression is shown. The results were expressed as mean ± S.D. ANOVA with Fisher's test correction was used for the analysis of the data.

inflammation [31–33]. A strong immunoreactivity to Nitrotyrosine was observed in the epithelium, lung parenchyma, and inflammatory cells (eosinophils, neutrophils) of patients with COPD [3]. Nitrotyrosine and ROS production often coexists within the cells during airway inflammation [4]. Accordingly, we showed that synthetic ACh and ISSs from COPD patients are able to increase Nitrotyrosine production and iNOS expression in cell extract of stimulated 16-HBE. In light of these findings we suggest that synthetic ACh and mediators of inflammation present in ISSs from COPD patients, are able to generate nitrosative stress and iNOS expression. Finally, we showed that Tiotropium controls the effect of synthetic ACh and that both Tiotropium and Olodaterol reduce the effect of ISSs on Nitrotyrosine and iNOS expression in bronchial epithelial cells. In this scenario, we suggest that β_2-adrenoceptors are able to oppose their activity to ACh present or de novo generated by ISS samples from COPD patients during stimulation of 16HBE. However, further molecular study will be necessary to better understand whether Olodaterol directly inhibit cholinergic activity in bronchial epithelial cells.

NF-κB pathway, mitogen-activated protein kinases (MAPK), and Janus kinase (JAK)/STAT-dependent signaling pathways are involved in iNOS expression in various cell types [7,34]. Particularly, upon

activation STATs become tyrosine phosphorylated, translocate to the nucleus, and induce expression of target genes. Concurrently, Ser727 of STAT-1 is phosphorylated independently of tyrosine phosphorylation and the full transcriptional activity of STAT-1 is also related to serine phosphorylation [7]. One of the purposes of this study was to elucidate the molecular mechanisms of ACh promotion of iNOS, emphasizing the critical role of the key transcription factor STAT-1 on iNOS gene expression in 16-HBE cells. We found that the synthetic ACh and ISSs from COPD promote the activation of STAT-1 Ser727 and Tyr701 phosphorylation suggesting that the increased expression of iNOS is related to the downstream signaling activation involving the STAT-1 pathway. However, the control value for the longer stimulation period with ACh for iNOS and STAT-1 pathway activation is nearly identical to the stimulated values in lower period stimulation groups, respectively. Since these values are different from the other control value, we suggest the involvement of low levels of autocrine production of ACh (time dependent) in the regulation of iNOS/STAT-1 activity in cultured 16HBE. Furthermore we observed that the antimuscarinic drug Tiotropium controls the effect of ACh and both Tiotropium and β2AR-agonist Olodaterol reduce the effect of ISSs on STAT-1 pathway activation. These data confirm that β2-adrenoceptors as the antimuscarinic drugs
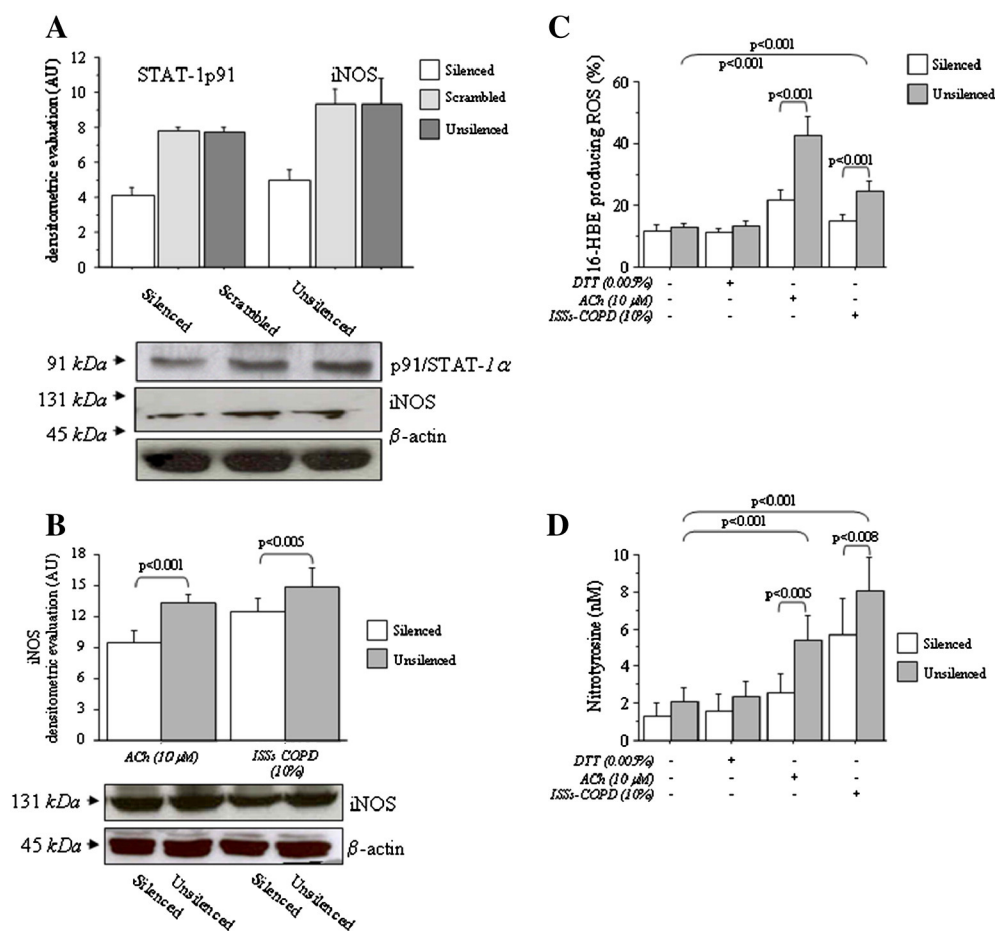
Fig. 7. p91/STAT-1α silencing and oxidative/nitrosative stress in 16-HBE cells. A) Expression of p91/STAT-1α protein and iNOS in control cells and cells transfected with scrambled siRNA or siRNA for p91/STAT-1α (a representative Western blot of p91/STAT-1α protein and iNOS is shown). The bars represent the densitometric analysis of 4 separate experiments; B) expression of iNOS in control cells and cells transfected with siRNA for p91/STAT-1α stimulated with synthetic ACh and ISSs from COPD patients for 4 h was evaluated by Western blot. A representative Western blot of iNOS and relative β-actin is shown. The bars represent the densitometric analysis of 6 separate experiments; C) intracellular ROS production was evaluated by flow cytometry; D) Nitrotyrosine levels were evaluated in cell extract by ELISA. The results were expressed as mean ± S.D. Statistical analysis was performed by using ANOVA with Fisher's test correction.

are able to oppose their activity to ACh and to mediators of inflammation eventually present in ISSs. Finally, since HCh a potent uptake choline blocker, added to the untreated or stimulated with ISS bronchial epithelial cells, reduced the ROS, iNOs, Nitrotyrosine production and STAT-1 pathway phosphorylation, we suggest the involvement of a de novo ACh synthesis which generates the mechanism of oxidative/nitrosative stress by STAT-1 downstream signaling activation in bronchial epithelial cells. These findings support the role of ACh in the mechanism of airway inflammation during COPD.

However, the experiments of RNA interference of the STAT-1 pathway clarify the role of ACh in the iNOS expression, ROS and Nitrotyrosine production obtained by STAT-1 pathway activation. Furthermore, we observed that the pretreatment of 16-HBE with Tiotropium and Olodaterol in combination additively reduced STAT-1 Ser727 and Tyr701 phosphorylation generated by ISSs from COPD, which was not observed for ROS, Nitrotyrosine production and iNOS expression. These findings suggest that antimuscarinic drugs and β2AR-agonist might be able to reduce other inflammatory mechanism generated by STAT-1 pathway activation. However further molecular studies will be necessary to better understand the inhibition of STAT-1 pathway activation.

In conclusion, we demonstrated that the nonneuronal ACh present in the airway of COPD might be cause of the degree of oxidative/nitrosative stress generated by STAT-1 pathway activation in bronchial epithelial cells. Furthermore, in this in vitro study we support the

potential therapeutic approach using antimuscarinics or long-acting β2-agonists in patients with COPD to control the oxidative/nitrosative stress generated in human bronchial epithelial cells by ACh. However, further clinical study will be necessary to support the results and the pharmacological approach described in our in vitro model of airway inflammation.

Disclosure statements

The author of the manuscript MPP is an employee of Boehringer Ingelheim Pharma GmbH & Co. KG. There is no other conflict of interest for this study.

Authors' contributions

The author MP conceived the study, designed the experiments and wrote the manuscript. GD Albano, AM Montalbano, C Di Sano, G Anzalone, R Gagliardo, A Bonanno, L Riccobono and L Siena performed the technical procedures. M Gjomarkaj gives us some suggestions about the data analysis and together with MP Pieper revised the final draft of

the manuscript. All authors read and approved the final version of the manuscript.

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