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Roflumilast N-oxide inhibits bronchial epithelial to mesenchymal transition induced by cigarette smoke in smokers with COPD

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ABSTRACT

Background: Epithelial to mesenchymal transition (EMT) is under discussion as a potential mechanism of small airway remodelling in COPD. In bronchial epithelium of COPD and smokers markers of EMT were described. *In vitro*, EMT may be reproduced by exposing well-differentiated human bronchial epithelial cells (WD-HBEC) to cigarette smoke extract (CSE). EMT may be mitigated by an increase in cellular cAMP.
Objective: This study explored the effects of roflumilast N-oxide, a PDE4 inhibitor on CSE-induced EMT in WD-HBEC and in primary bronchial epithelial cells from smokers and COPD *in vitro*.
Methods: WD-HBEC from normal donors were stimulated with CSE (2.5%) for 72 h in presence of roflumilast N-oxide (2 nM or 1 μM) or vehicle. mRNA and protein of EMT markers αSMA, vimentin, collagen-1, E-cadherin, ZO-1, KRT5 as well as NOX4 were quantified by real-time quantitative PCR or protein array, respectively. Phosphorylated and total ERK1/2 and Smad3 were assessed by protein array. cAMP and TGFβ1 were measured by ELISA. Reactive oxygen species (ROS) were determined by DCF fluorescence, after 30 min CSE (2.5%). Apoptosis was measured with Annexin V/PI labelling. In some experiments, EMT markers were determined in monolayers of bronchial epithelial cells from smokers, COPD *versus* controls.
Results: Roflumilast N-oxide protected from CSE-induced EMT in WD-HBEC. The PDE4 inhibitor reversed both the increase in mesenchymal and the loss in epithelial EMT markers. Roflumilast N-oxide restored the loss in cellular cAMP following CSE, reduced ROS, NOX4 expression, the increase in TGFβ1 release, phospho ERK1/2 and Smad3. The PDE4 inhibitor partly protected from the increment in apoptosis with CSE. Finally the PDE4 inhibitor decreased mesenchymal yet increased epithelial phenotype markers in HBEC of COPD and smokers.
Conclusions: Roflumilast N-oxide may mitigate epithelial–mesenchymal transition in bronchial epithelial cells *in vitro*.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a preventable and treatable lung disease characterized by airflow limitation that is progressive and not fully reversible. The airflow limitation is the consequence of abnormal chronic inflammation of the lungs and manifests as small airway disease (obstructive bronchiolitis) and

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parenchymal destruction (emphysema). Small airways (smaller than 2 mm of internal diameter) are responsible of the 60% of the total resistance in COPD [1–4]. Cigarette smoking is the most commonly recognized risk factor for COPD, increasing and perpetuating lung inflammation and remodelling. As part of lung remodelling, airway wall thickening, peribronchial fibrosis, luminal inflammatory exudates and the consequent airway narrowing in small airways are correlated with disease severity [5]. Recent studies have shown that proliferation of bronchial smooth muscle cells and bronchial accumulation of myofibroblasts critically determines small airway narrowing in COPD [6,7]. Myofibroblasts may have different origins such as resident bronchial smooth muscle or fibroblast to myofibroblast transition, epithelial to mesenchymal transition (EMT) or the recruitment of fibrocytes [8].

Small airway disease likely elicited by cigarette smoking has been considered as a therapeutic target in COPD [6]. Besides inflammation, cigarette smoke promotes lung fibroblasts to myofibroblast transformation [9–11]. Furthermore, recent studies indicate that EMT may be induced by cigarette smoke in bronchial epithelial cells contributing to small bronchial narrowing in COPD [12–14]. In this regard, we recently showed that EMT is present in primary bronchial epithelial cells from small bronchi of smokers and COPD patients and induced by cigarette smoke [12]. EMT may be controlled by a number of intracellular pathways such as the generation of reactive oxygen species (ROS), activation of mitogen-activated protein kinase (MAPK) and Smad signalling, as well as by the increase of adenosine 3′,5′-cyclic monophosphate (cAMP) degrading phosphodiesterase (PDE) 4 [15,16]. The PDE4 family is composed of four subtypes (PDE4A-D) encoded by different genes that by alternative splicing are expressed as multiple variants differing in their N-terminal domains [17,18]. PDE4 plays a role in almost all cells related to COPD including lung fibroblasts and airway epithelial cells [19]. In particular, inhibition of PDE4 by rolipram reduced epithelial to mesenchymal transition (EMT) secondary to TGF-β1 in the alveolar epithelial cell line A549 [16].

The PDE4 inhibitor roflumilast has been approved to reduce the risk of acute exacerbations and improve lung function in patients with severe COPD associated with chronic bronchitis and a history of exacerbations [20,21]. While anti-inflammatory effects are discussed as key to understand the clinical efficacy of roflumilast [21,22] *in vivo* and *in vitro* studies indicate that the PDE4 inhibitor could also influence lung architectural remodelling. For example roflumilast mitigates airspace enlargement in mice exposed to tobacco smoke over several months or alleviates bleomycin-induced lung fibrotic remodelling in therapeutic protocols *in vivo*. In cell culture experiments, roflumilast N-oxide and other PDE4 inhibitors were shown to inhibit myofibroblast transition, migration or proliferation of human lung fibroblasts [23–25]. However, whether roflumilast curbs cigarette smoke-induced EMT in well-differentiated human bronchial epithelial cells *in vitro* has not previously been investigated.

The current study was designed to explore the effects of the PDE4 inhibitor roflumilast N-oxide on EMT induced by cigarette smoke in well-differentiated human bronchial epithelial cells (HBECs) of small bronchi *in vitro*. We also analysed the effects of roflumilast N-oxide on mesenchymal and epithelial markers of primary HBECs from smokers and COPD patients. Roflumilast N-oxide is the active metabolite of roflumilast largely accounting for its clinical efficacy in man [19].

2. Materials and methods

2.1. Patients

A total of 10 non-smoker controls, 8 smokers without COPD and 9 smokers with COPD were included in the study. COPD patients were diagnosed according to the GOLD guidelines [26]. All lung tissues studied in this work were taken from uninvolved lung tissue during lobectomy/wedge resection for malignant lesions in the thoracic Surgery and Respiratory Unit, University General Hospital Consortium, Valencia, Spain, between 2009 and 2012. Samples of distal lung, located as far away as possible from the tumour, were chosen for the study. All pulmonary function tests were performed within 3 months before surgery. Clinical data of all patients were examined for possible co-morbidity and medication use. Inclusion criteria were defined as: non-smokers, smokers without and with COPD (free of symptoms of upper respiratory tract infections, none received antibiotics perioperatively). After selection based on lung function, all lung tissue samples used for the study were checked histologically by using the following exclusion criteria: (1) presence of tumour, (2) presence of post-stenotic pneumonia, and (3) fibrosis of lung parenchyma. The protocol was approved by the local research and independent ethics committee of the University General Hospital of Valencia. Informed written consent was obtained from each participant.

2.2. Isolation of primary bronchial epithelial cells and incubations with roflumilast N-oxide

Isolation of human bronchial epithelial cells from small bronchi was performed as previously outlined [27]. Small pieces of human bronchi (0.5–1 mm internal diameter) were excised from microscopically normal lung areas, carefully dissected free from lung parenchyma and plated on collagen-coated culture dishes (10 μg/cm^2 rat type I collagen (Sigma Aldrich, Madrid, Spain)) in bronchial epithelial growth medium (BEGM, comprising bronchial epithelial basal medium (BEBM) supplemented with bovine pituitary extract (52 μg/ml), hydrocortisone (0.5 μg/ml), human recombinant epidermal growth factor (EGF) (25 ng/ml), epinephrine (0.5 μg/ml), transferrin (10 μg/ml), insulin (5 μg/ml), retinoic acid (50 nM), triiodo-L-thyronine (6.5 ng/ml), gentamycin (40 μg/ml), amphotericin B (50 ng/ml), and bovine serum albumin (1.5 μg/ml). Small bronchi were oriented with the epithelial layer to be in contact with the culture plate. After a period of about 7–12 days, bronchial epithelial cells were observed around bronchi and used to measure mRNA and protein expression of epithelial and mesenchymal markers of different subjects in presence or absence of roflumilast N-oxide (2 nM) or vehicle (0.1% DMSO) for 24 h. The identity of the monolayer as bronchial epithelial cells was affirmed by morphological criteria and immunofluorescence for cytokeratin 5 (KRT5) as well as the later *in vitro* differentiation in air–liquid interface as pseudo-stratified bronchial epithelium with basal cells, ciliated cells, columnar and goblet cells. Cell viability was assessed by vital trypan blue exclusion analysis using the Countness® automated cell counter (Life technologies, Madrid, Spain). Cell viability was >98% in all cell cultures tested in this work. Roflumilast N-oxide (Takeda) was diluted from a 10 mM stock in DMSO to the final concentration of 2 nM in 0.1% DMSO. A concentration of 2 nM of roflumilast N-oxide corresponds to plasma concentrations (unbound to plasma proteins) of this active metabolite of roflumilast maintained over the 24 h dosing interval following repeated dosing of roflumilast at its approved clinical dose of 500 μg once daily *per os* [19].

2.3. Culture of air–liquid interface bronchial epithelial cells

Differentiated HBECs for *in vitro* experiments were obtained from non-smoking subjects. Primary HBECs were trypsinized and subpassaged on 12-well polyester Transwell inserts (Millipore) at 1.5×10^5 cells per insert. Cells were left for 7 days submerged in BEGM/DMEM (1:1) culture medium. From day 7 the air–liquid interface culture was initiated by removing the medium from the

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upper well leaving the apical side of the cells exposed to air and changing the final epidermal growth factor (EGF) concentration to 0.5 ng/ml (differentiation medium). In general air–liquid interface cultures were pursued until about 80–90% of cells were ciliated by microscopic inspection (about 3–4 weeks after initiation) before experiments were commenced [27]. Cells were maintained at 37 °C with 5% CO_2 and medium changed every other day. At this stage a pseudo-stratified bronchial epithelium comprising basal cells, ciliated cells and goblet cells was obtained and considered as "differentiated".

2.4. Preparation of cigarette smoke extract

Cigarette smoke extracts (CSE) were obtained as previously outlined [28,29]. Briefly, the smoke of a research cigarette (2R4F; Tobacco Health Research, University of Kentucky, KY, USA) was generated by a respiratory pump (Apparatus Rodent Respirator 680; Harvard, Germany) through a puffing mechanism related to the human smoking pattern (3 puff/min; 1 puff 35 ml; each puff of 2 s duration with 0.5 cm above the filter) and was bubbled into a flask containing 25 ml of pre-warmed (37 °C) BEGM/DMEM medium. The CSE solution was sterilized by filtration through a 0.22-μm cellulose acetate sterilizing system (Corning, NY). The resultant CSE solution was considered to be 100% CSE and was used for experiments within 30 min of preparation. CSE 10% corresponds approximately to the exposure associated with smoking two packs per day [30]. The quality of the prepared CSE solution was assessed based on the absorbance at 320 nm, which is the specific absorption wavelength of peroxynitrite. Stock solutions with an absorbance value of 3.0 ± 0.1 were used.

2.5. In vitro stimulation of differentiated HBECs

For *in vitro* studies, differentiated HBEC were stimulated with CSE 2.5% for the indicated times (in general 72 h unless indicated otherwise), replacing culture medium and stimulus every 24 h. Roflumilast N-oxide (2 nM and 1 μM; RNO) or vehicle (0.1% DMSO) was added 30 min before stimulus. Both test compounds and CSE were added to the basolateral media (500 μl) and at the apical surface (25 μl). As manipulations at the apical surface may affect pseudo-stratified epithelium, all incubations with vehicle controls were run under identical conditions as with CSE and test compounds (for example identical volumes of medium were added to the apical surface throughout all conditions, same for the basolateral compartment). In control experiments where vehicle/medium (for test compounds/CSE) were added to the apical surface of differentiated HBECs over a maximum of 3 days (including daily replacement procedures, as indicated) the number of ciliated cells and expression of cilia markers were found to be not different from cultures of differentiated human bronchial epithelial cells in the absence of the manipulations at the apical surface.

As previously shown by us and others, the *in vitro* incubation of well-differentiated human bronchial epithelial cells with CSE recapitulates changes in markers of EMT as found in bronchial epithelium of smokers with and without COPD [12,13,15,31] and may therefore be useful to dissect molecular mechanisms involved in this pathological process.

2.6. Real-time RT-PCR

Total RNA was isolated from primary HBEC and differentiated bronchial epithelial cells in air–liquid interface by using TriPure® Isolation Reagent (Roche, Indianapolis, USA). The integrity of the extracted RNA was confirmed with Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA). The reverse transcription was performed in 300 ng of total RNA with TaqMan reverse transcription reagents kit (Applied Biosystems, Perkin–Elmer Corporation, CA, USA). cDNA was amplified with specific primers and probes predesigned by Applied Biosystems for α-SMA (Hs00559403_m1), α_1(I)-collagen (collagen type I; cat. n°: Hs00164004_m1), vimentin (cat. n°: Hs 00958116_m1), E-cadherin (cat. n°: Hs01023894_m1), zona occludens-1 (ZO-1; cat. n°: Hs01551861_m1), KRT5 (cat. n°: Hs00361185_m1) and NOX4 (cat. n°: Hs00276431_m1), and GAPDH (cat. n°: 4352339E) as a housekeeping gene in a 7900HT Fast Real-Time PCR System (Applied Biosystem) using Universal Master Mix (Applied Biosystems). Relative expression of these different transcripts was determined with the $2^{-\Delta\Delta Ct}$ method using GAPDH as endogenous control (Applied Biosystems; 4352339E) and normalized to non-smoker or control group.

2.7. Protein array

Protein expression in primary isolated HBEC from non-smokers, smokers and COPD patients and *in vitro* differentiated HBECs was analysed with Zeptosens (Division of Bayer [Schweiz], Switzerland) protein array technology as previously outlined [12]. Cells were lysed with CeLyA Lysis Buffer CLB1 (Zeptosens, Division of Bayer (Schweiz), Switzerland), incubated during 30 min at room temperature and centrifuged (5 min at 15,000× *g*) in order to remove debris. The supernatants were collected, frozen in liquid nitrogen and stored at −80 °C.

Protein concentration was determined using a Bradford-Coomassie Plus Assay Kit (Pierce). Protein content was adjusted to 2 mg/ml and samples were subsequently diluted using spotting buffer (PBS + 10% DMSO + 5% Glycerol) to obtain four different protein concentrations corresponding to 100, 75, 50 and 25% (0.2 mg/ml, 0.15 mg/ml, 0.1 mg/ml and 0.05 mg/ml) of the primary spotting solution. For each of these four dilutions, duplicate spots were arrayed onto ZeptoMARK chips (Zeptosens) as single sample droplets of about 400 pl, using a Micro Pipetting System Nanoplotter™ (NP2.1, GeSiM, Großerkmannsdorf, Germany). After spotting, the chips were dried for 1 h at 37 °C and blocked in an ultrasonic nebulizer (ZeptoFOG Blocking Station, Zeptosens) with CeLyA Blocking Buffer (BB1, Zeptosens). Blocked chips were rinsed with water (Milli-Q quality), dried and stored at 4 °C in the dark until further use. Antibody incubations were done in CeLyA Assay Buffer CAB1 based on BSA according to standard protocols (Zeptosens). The chips were assembled with chip fluidic structures in a ChipCARRIER (Zeptosens) and were incubated with primary antibodies (1:500 dilution in CAB1) overnight at room temperature. After rinsing the system with assay buffer, the chips were incubated with secondary fluorescence-labelledlabelled anti-species antibodies (Zenon Alexa Fluor 647 Rabbit IgG and mouse IgG LabellingLabelling Kit (cat. n°: Z25308 and Z25008, Molecular Probes) (1:500 dilution in CAB1) for 1 h at room temperature. After rinsing the system with assay buffer to remove the excess secondary antibody, the fluorescence readout was performed with the ZeptoREADER instrument (Zeptosens), at an extinction wavelength of 635 nm and an emission wavelength of 670 nm. The fluorescence signal was integrated over a period of 1–10 s, depending on the signal intensity. Array images were stored as 16-bit TIFF files and analysed with the ZeptoView Pro software package (version 2.0, Zeptosens). Relative intensities were obtained by plotting net spot intensities against protein concentrations of the spotted samples determined by a Bradford assay as described above. Briefly, the eight data points for each sample were fitted using a weighted linear least squares fit [33]. The relative intensity was then interpolated at the median protein concentration. The SD calculated from the fit is indicative for the linearity of the dilution series. Subsequently the data were renormalized to correct for small variations in protein content using mouse anti-human β-actin (cat n°: A1978;

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Sigma), total rabbit anti-human ERK1/2 (1:1000) antibody (monoclonal antibody; Cell Signalling, Boston, Massachusetts, USA; catalogue no. 4695) or total rabbit anti-human Smad3 (cat n°: 566414; Calbiochem) as internal standards. Primary antibodies used were mouse anti-human α-SMA (cat. n°: A5228; Sigma), rabbit anti-human collagen type I antibody (cat. n°: PA1-26204; Affinity Bioreagents), mouse anti-human vimentin (cat. n°: V6389; Sigma), mouse anti-human E-cadherin (cat. n°: CM1681; ECM BioScience), rabbit anti-human ZO-1 (cat. n°: ab59720; Abcam), mouse anti-human phospho-ERK1/2 (cat. n°: M-9692; Sigma), rabbit anti-human phospho-Smad3 (cat. n°: PS1023; Calbiochem), and NOX4 (cat. n°: NB110-58849; Novus Biologicals).

2.8. DCF fluorescence measurement of reactive oxygen species

2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Molecular Probes, UK) is a cell-permeable compound that following intracellular ester hydrolysis is oxidized to fluorescent 2′, 7′-dichlorofluorescein (DCF) by O_2^- and H_2O_2, and can therefore be used to monitor intracellular generation of ROS [34]. To quantify ROS levels, differentiated human bronchial epithelial cells were washed twice with PBS and incubated for 30 min with 50 μM H_2DCF-DA diluted in Opti-MEM in presence of roflumilast N-oxide or vehicle. Then, cells were again washed twice with PBS to remove remaining H_2DCF-DA and stimulated with CSE for 30 min in presence of roflumilast N-oxide or vehicle. Five randomly selected fields per condition were measured for fluorescent intensity using an epifluorescence microscope (Nikon Eclipse TE 200, Tokio, Japan) with filter set for FITC. Subsequent image capture and analysis was performed using Metafluor® 5.0 software (Analytical Technologies, US). Results were expressed as DCF fluorescence in relative fluorescence units.

2.9. Enzyme-linked immunosorbent assays for TGF-β1 and cAMP

Quantitative ELISA for TGF-β1 was performed with supernatants of differentiated HBECs following 72 h of stimulation with CSE in presence or absence of roflumilast N-oxide using quantikine human TGF-β1 immunoassay (R&D Systems; catalogue no. 891124). To measure latent complexes of TGF-β1, activation was accomplished by acid treatment. Therefore, 50 μl of cell culture supernatants were treated with 10 μl of 1 M HCl, incubated for 10 min, and then neutralised with 10 μl of 1.2 M NaOH/0.5 M HEPES.

Intracellular cAMP content was determined in differentiated HBEC as previously outlined [35]. Following incubations with CSE in presence or absence of roflumilast N-oxide as indicated, culture medium was removed and cells were washed with phosphate-buffered saline (PBS). Then, cells were lysed and the intracellular cAMP content was determined with the cAMP Biotrak Enzyme Immunoassay (EIA) system according to manufacturer's instructions (Amersham, UK). Results were expressed as fmol cAMP per mg of protein (per insert).

2.10. Apoptosis assay

To determine apoptosis/necrosis, phosphatidylserine flipping from the inside to the outside surface of the plasma membrane was examined by the annexin V assay according to the manufacturer's instructions (Roche, Applied Science, UK). Differentiated HBECs were incubated with CSE (2.5%) in the presence of roflumilast N-oxide (2 nM or 1 μM) or vehicle for 72 h. In separate experiments, apoptosis/necrosis in primary HBECs from healthy, smokers and COPD patients was also evaluated. At the end of the incubations cells were washed three times with phosphate-buffered saline (PBS) and then labelled *in situ* with 5 μl each of annexin V-FITC and propidium iodide (PI) for 15 min at room temperature as previously outlined [32]. Six randomly selected fields were counted for fluorescent cells using an epifluorescence microscopy (Spectramaster System, Perkin Elmer, Life Sciences, Cambridge, UK) with dual filter set for FITC and rhodamine. Subsequent image capture and analysis was performed using Metafluor® 5.0 software (Analytical Technologies, US). Apoptotic (Annexin V + / PI −) and necrotic (Annexin V + / PI +) cells were counted and expressed as % of total cells (counted by phase contrast microscopy of the same field.

2.11. Statistics

Statistical analysis of results was carried out by parametric or non-parametric analysis as appropriate. Data from parametric analysis (Figs. 1–4) were expressed as mean ± SD. Data of *in vitro* mechanistic experiments in differentiated HBECs from non-smoker patients were performed by Student's *t*-test, one-way or two-way analysis of variance followed by Bonferroni post hoc test. Data from non-parametric analysis (Figs. 5–7) was expressed as median, interquartile range and minimum and maximum values. Mann–Whitney *U* test or Kruskal–Wallis test followed by Dunn's post hoc test for multiple comparisons were used to compare mesenchymal and epithelial data from non-smokers, smokers and COPD patients. Correlations were analysed using the Spearman (ρ) correlation analysis. Significance was accepted when $P < 0.05$.

3. Results

3.1. Roflumilast N-oxide inhibits cigarette smoke-induced epithelial to mesenchymal transition in differentiated HBECs

Differentiated HBECs were obtained from bronchial epithelial cells of non-smokers following air–liquid interface culture. As previously described [12], CSE at 2.5% increased gene and protein expression of mesenchymal markers α-SMA, vimentin and collagen type I (Fig. 1A and C) and decreased the expression of epithelial markers E-cadherin, ZO-1 and KRT5 (Fig. 1B and D). Furthermore, under phase contrast microscopy, differentiated HBEC exposed to CSE adopted a flattened and elongated morphology characteristic of a myofibroblast-like phenotype (Fig. 2). Incubation with roflumilast N-oxide at 1 μM (corresponding to complete and selective inhibition of PDE4) and 2 nM (corresponding to about half-maximum inhibition of PDE4 [19] and free plasma concentrations in man following clinical dosing of roflumilast [36]) largely abolished the increase of mesenchymal markers, reversed the loss of epithelial markers induced by cigarette smoke and conserved the epithelial cell phenotype (Fig. 1A–D and Fig. 2).

In other experiments effects of medium (72 h) or CSE (2.5% over 72 h) in the presence of roflumilast N-oxide (2 nM, 1 μM over 72 h) or vehicle (0.1% DMSO) on apoptosis or necrosis (assessed by Annexin V-FITC/PI labelling) of differentiated HBECs were explored. Necrosis (Annexin V+ / PI+ cells) was (invariably) almost absent. In medium control cells apoptosis (Annexin V+ / PI−) was found in 17.5 ± 3.7% of total cells that may reflect turnover in the differentiated bronchial epithelial cell cultures. CSE about doubled apoptosis to 35.3 ± 2.62% of total cells and the increment was partially prevented (by about 50%) in the additional presence of the PDE4 inhibitor (to 26.1 ± 1.2% at 2 nM and 25.3 ± 1.8% at 1 μM of roflumilast N-oxide (Fig. 2)).

3.2. Roflumilast N-oxide inhibits intracellular pathways involved in cigarette smoke induced epithelial to mesenchymal transition in differentiated HBECs

Exposure of well-differentiated human bronchial epithelial cells to CSE impairs cellular cAMP content and enhances levels of

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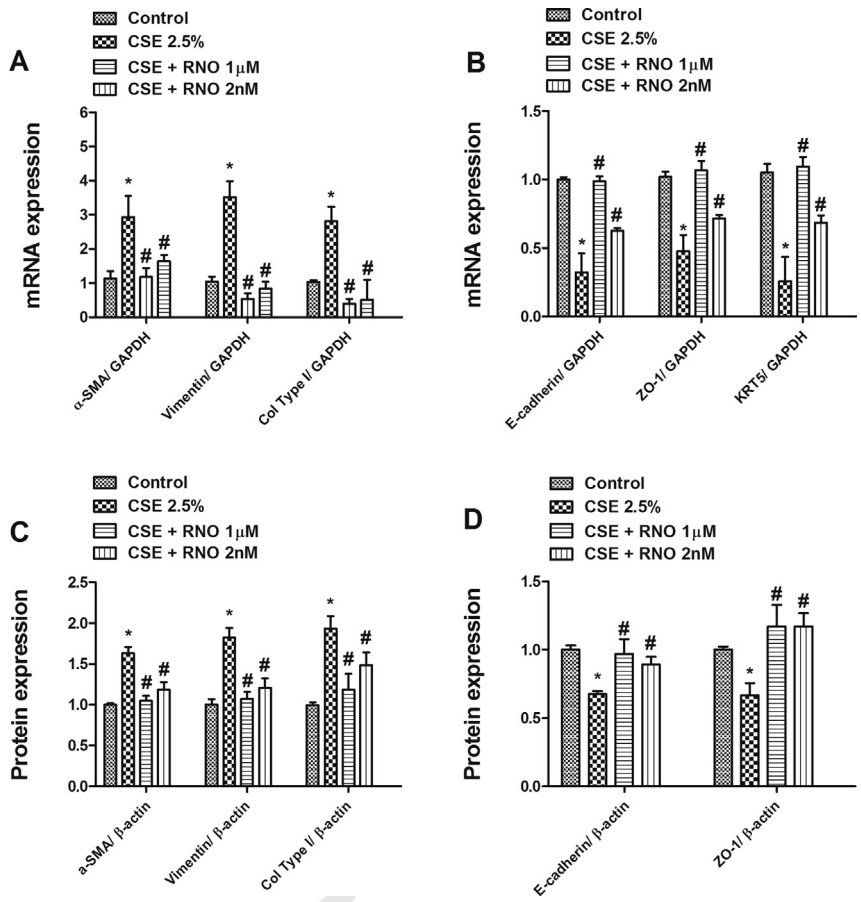

Fig. 1. Roflumilast N-oxide (RNO) inhibits epithelial to mesenchymal transition induced by cigarette smoke extract (CSE) in differentiated human bronchial epithelial cells (HBECs). Differentiated HBECs were incubated with RNO (2 nM, 1 μM) for 30 min before CSE (2.5%) stimulation for 72 h (*A, B*) Total RNA and (*C, D*) protein were isolated for real-time PCR and protein array Zeptosens analysis respectively. CSE-induced upregulation of (*A*) mRNA and (*C*) protein expression of mesenchymal markers α-SMA, vimentin and collagen type I (col type I) was inhibited by RNO. CSE-induced downregulation of (*B*) mRNA and (*D*) protein expression of epithelial markers E-cadherin, ZO-1 and KRT5 was reversed by RNO. Data are expressed as the ratio to GAPDH for mRNA levels and to β-actin for protein levels and normalized to the vehicle control group. Results are expressed as means ± SD of $n = 3–4$ (two to four cell non-smoker population) experiments per condition. Two-way ANOVA followed by post hoc Bonferroni tests. $^{*}P < 0.05$ related to solvent controls; $\#P < 0.05$ related to CSE.

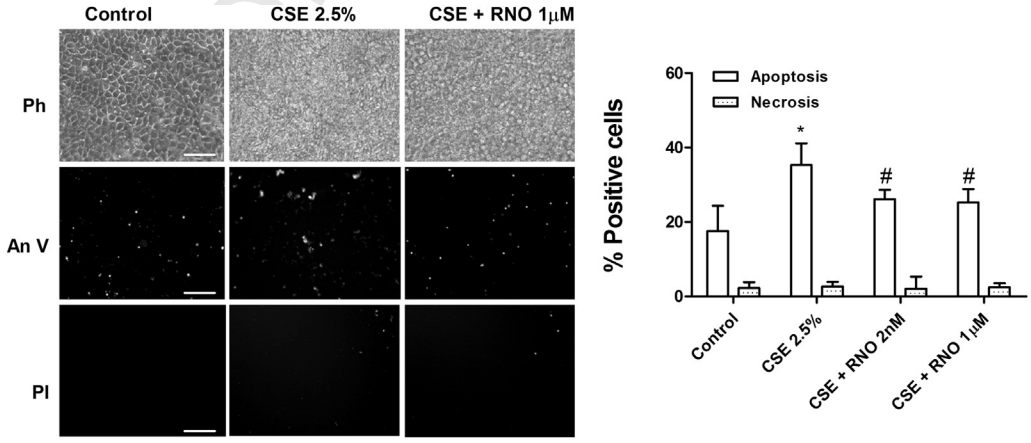

Fig. 2. Roflumilast N-oxide (RNO) reduces apoptosis induced by cigarette smoke extract (CSE) in differentiated human bronchial epithelial cells (HBECs). Differentiated HBEC were exposed to CSE (2.5%) over 72 h or medium (control) in the presence of RNO at 2 nM or 1 μM, or vehicle (0.1% DMSO) (30 min pre-incubation). CSE changes the morphology from epithelial to mesenchymal with reduced cell-to-cell contact that was prevented by RNO (1 μM) (phase contrast light microscopy). Apoptosis/Necrosis were assessed by Annexin V-FITC and propidium iodide (15 min) labeling. Annexin V+/PI- (apoptotic) and Annexin V+/PI+ (necrotic) cells were counted (epifluorescence microscope in a total of six fields per condition) and results expressed as percentage of total cells. RNO partly reduced the CSE-induced increment in apoptosis. Results are the mean ± SD of n = 3 experiments from three different patients. Scale bar: 50 μm.

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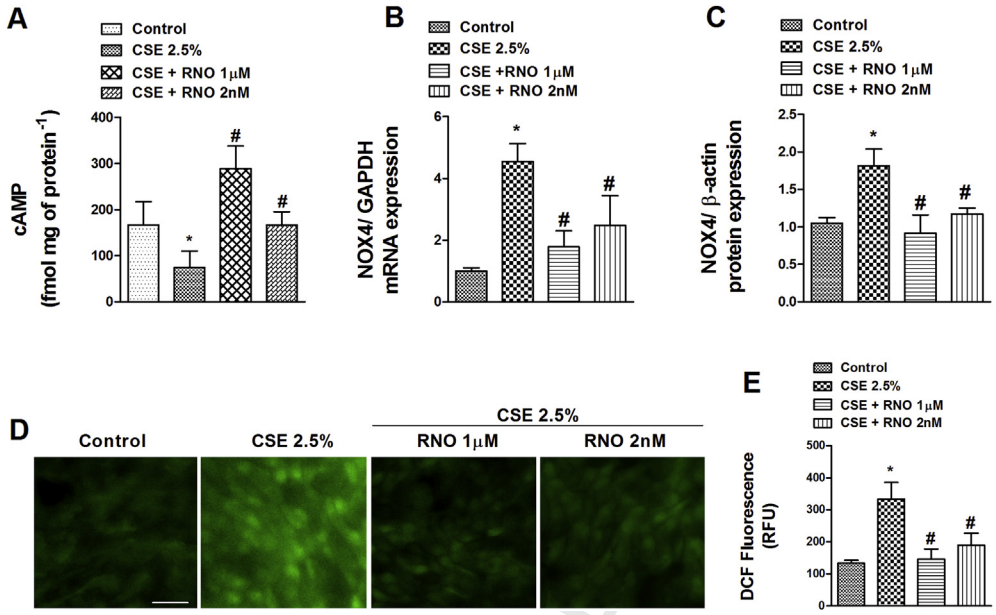

Fig. 3. Roflumilast N-oxide (RNO) reversed a cigarette smoke extract (CSE)-induced decrease in cyclic adenosine monophosphate (cAMP) and increase in intracellular reactive oxygen species (ROS) and NOX4 expression in differentiated human bronchial epithelial cells (HBECs). Differentiated HBECs were incubated with RNO (2 nM, 1 μM) for 30 min before stimulation with CSE (2.5%) for (A, B, C) 72 h or (*D, E*) 30 min. (A) The loss in intracellular cAMP following CSE was prevented by RNO. (B, C) CSE-induced mRNA and protein expression of NOX4, which were significantly inhibited by RNO. Data are expressed as the ratio to GAPDH for mRNA levels and to β-actin for protein levels and normalized to vehicle control group. (D, E) RNO reduced ROS following CSE determined by means of DCF fluorescence intensity. (D) Representative images of positive DCF fluorescence in differentiated HBECs. Scale bar: 10 μm. (B, C) Results are expressed as means ± SD of $n = 3$ (two to three cell non-smoker population) experiments per condition. Two-way ANOVA followed by post hoc Bonferroni tests. $^*P < 0.05$ related to solvent controls; $\#P < 0.05$ related to CSE. RFU: relative fluorescence units.

oxidative stress and both are involved in EMT secondary to cigarette smoke [12]. In the current study, pre-incubation with roflumilast N-oxide concentration-dependently rescued from a CSE (2.5%) induced loss in intracellular cAMP levels after 72 h of exposure. In fact, at 2 nM the PDE4 inhibitor reversed compromised cAMP levels to baseline and even higher levels were found at 1 μM concentration (Fig. 3A). When well-differentiated HBEC were exposed to CSE 2.5% over 30 min intracellular ROS increased that was concentration-dependently reduced by roflumilast N-oxide at 2 nM and 1 μM (Fig. 3D, E). Long-term stimulation of well-differentiated HBEC with CSE 2.5% for 72 h resulted in an increase in NOX4 mRNA and protein that both were curbed by roflumilast N-oxide (Fig. 3B and C).

As previously reported cigarette smoke may increase the release of TGF-β1 and then result in ERK1/2 and Smad3 phosphorylation playing a key role in CSE-induced EMT [12]. We now report that roflumilast N-oxide reduced this increase in TGF-β1 release and that of ERK1/2 and Smad3 phosphorylation secondary to CSE (2.5%, 72 h) (Fig. 4A, B and 4C).

3.3. EMT markers in primary HBECs from smokers and COPD and lung function

Previously we have shown enhanced mesenchymal yet compromised epithelial markers of EMT in primary cultured human bronchial epithelial cells from smokers or COPD if compared with non-smokers, non-COPD [12]. Here it has been explored whether markers of EMT may relate to lung function (FEV1 (post), % of predicted). To this end, human bronchial epithelial cells obtained from small bronchi (<2 mm of internal diameter) of smokers ($n = 8$) and COPD patients (current smokers) ($n = 9$) (who were under lung surgery for lung carcinoma) where analysed for the

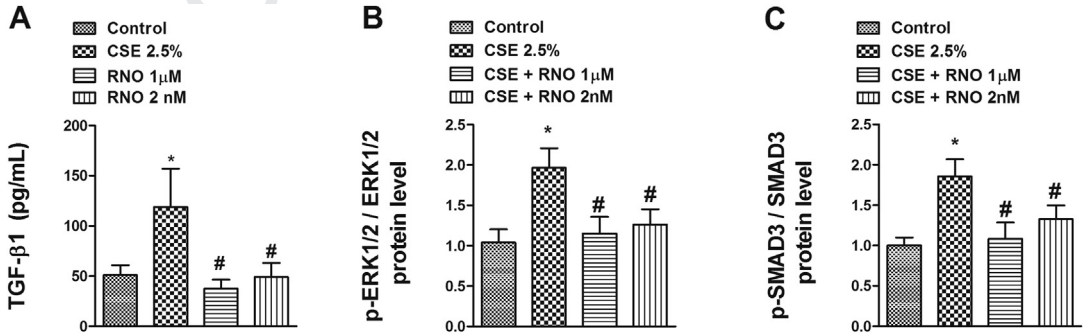

Fig. 4. Roflumilast N-oxide (RNO) inhibits cigarette smoke extract (CSE)-induced TGF-β1 secretion and phosphorylation of ERK1/2 and Smad3 in differentiated human bronchial epithelial cells (HBECs). Differentiated HBECs were incubated with RNO for 30 min before CSE (2.5%) stimulation for 72 h. Cell culture supernatant (A) and protein content (B, C) were extracted to quantify (A) TGF-β1 release, (B) ERK1/2 phosphorylation and (C) Smad3 phosphorylation. (B, C) Phospho-Smad3 and phospho-ERK1/2 protein levels were expressed as the ratio to total ERK1/2 and Smad3 respectively and normalized to the vehicle control group. Results are expressed as means ± SD of $n = 3$ (two to four cell non-smoker population) experiments per condition. Two-way ANOVA followed by post hoc Bonferroni tests. $^*P < 0.05$ related to solvent controls; $\#P < 0.05$ related to CSE.

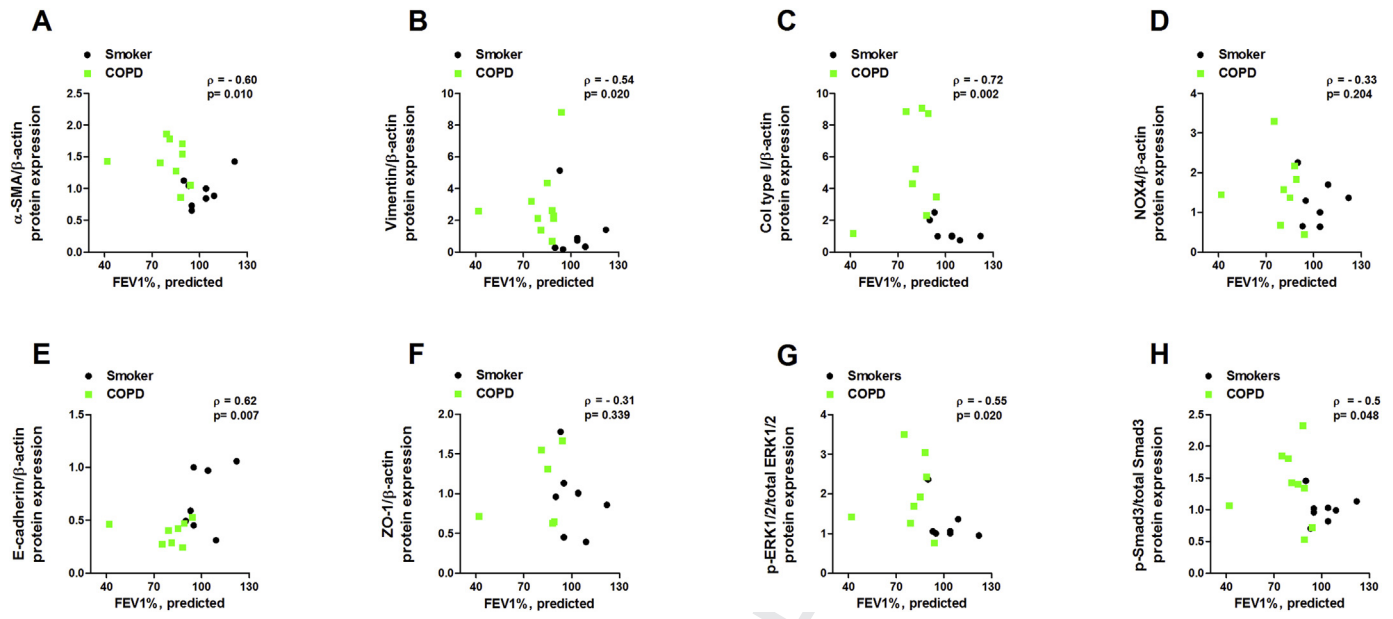

Fig. 5. The expression of epithelial to mesenchymal transition markers is correlated with lung function. Primary human bronchial epithelial cells (HBECs) from smokers ($n = 9$) and COPD patients ($n = 9$) were isolated from small bronchi and lysed to extract proteins. Protein expression of mesenchymal markers (A) α-SMA, (B) vimentin, (C) collagen type I (col type I) and (D) NOX4; Epithelial markers (E) E-cadherin, (*F*) ZO-1; intracellular signals (G) phospho-ERK1/2 and (H) phospho-Smad3 was measured (Zeptosens). Data are expressed as the ratio of target protein to house-keeping β-actin, normalized to a control group (non smoker, non COPD; Table 1). Phospho-Smad3 and phospho-ERK1/2 protein levels were expressed as the ratio to total Smad 3 and ERK1/2, normalized to a control group (non smoker, non COPD; Table 1), respectively. Data was correlated with $\%FEV_1$ lung function. Spearman (ρ) correlation analysis. $P < 0.05$ value showed significant correlation.

expression of EMT markers. Clinical data of the investigated cohort are depicted in Table 1.

α-SMA, vimentin and collagen type I protein in primary HBECs were inversely correlated with $\%FEV_1$ (Fig. 5A, B and 5C; Spearman $\rho = -0.60$, $\rho = -0.54$ and $\rho = -0.72$ respectively, and $P = 0.010$, $P = 0.020$ and $P = 0.002$ respectively). E-cadherin was directly correlated with $\%FEV_1$ (Fig. 5E; Spearman $\rho = 0.62$; $P = 0.007$). ZO-1 expression and NOX4 were not correlated with $\%FEV_1$. Both, phospho-ERK1/2 and phospho-Smad3 were inversely correlated with $\%FEV_1$ (Fig. 5G and H; Spearman $\rho = -0.55$, $\rho = -0.50$ and $P = 0.020$, $P = 0.048$ respectively).

3.4. *In vitro* treatment of primary HBECs from smokers and COPD patients with roflumilast N-oxide attenuates EMT markers

Finally it was asked whether *in vitro* incubation of human bronchial epithelial cells from current smokers with and without COPD compared to non-smokers, non COPD with roflumilast N-oxide (2 nM) or vehicle over 24 h influenced the expression of EMT markers. In HBEC of current smokers with or without COPD, roflumilast N-oxide significantly reduced the mRNA expression of α-SMA, vimentin and collagen type I as mesenchymal markers and NOX4 but increased the expression of the epithelial markers E-cadherin and ZO-1 compared to vehicle (Fig. 6 C–F). In contrast, the PDE4 inhibitor did not significantly influence these mRNA transcripts in HBEC from non smokers, non COPD (Fig. 6 A, B). There were no differences in the proportion of apoptotic (or necrotic) cells between non-smoking healthy controls, smokers without COPD and smokers with COPD (Fig. 7).

4. Discussion

The current study demonstrates for the first time that roflumilast N-oxide, a PDE4 inhibitor mitigates epithelial to mesenchymal transition (EMT) elicited by cigarette smoke extract in well-differentiated human bronchial epithelial cells (HBEC) *in vitro*. Adding roflumilast N-oxide at 2 nM (a concentration corresponding to plasma concentrations of this active metabolite following repeated dosing of roflumilast at its clinically approved dose of 500 μg/d *per os*), to HBEC primary cultures from smokers with and without COPD (but not from non-smokers, non COPD) attenuated EMT markers. Finally, markers of EMT correlated to FEV1 (% of predicted) in smokers with and without COPD. Collectively, addressing EMT could hypothetically represent another mechanistic pathway in the mode of action of the PDE4 inhibitor roflumilast to target COPD pathophysiology [37,38].

EMT as part of the airway remodelling in COPD patients has recently been studied by our group and others [12,39,40]. Indeed, one may postulate that EMT is initiated in small bronchi of smokers and patients with COPD by chronic oxidative stress from cigarette smoking. Hypothetically, transition of basal bronchial epithelial cells into a mesenchymal, myofibroblast-like phenotype may allow their migration to the smooth muscle layer, increases peribronchiolar fibrosis and bronchial smooth muscle thickness, finally supporting small bronchi narrowing and airway resistance. In our previous study immunohistochemical analyses in small airways revealed that in comparison to non-smokers, non-COPD mesenchymal markers of EMT are increased in basal bronchial epithelial cells of smokers and patients with COPD while epithelial markers are decreased [12]. Furthermore, primary HBECs isolated from smokers and COPD patients maintained such increased mesenchymal and decreased epithelial cell markers [12]. Complementing these findings we now observed that across the subjects (current smokers with and without COPD) included in this study protein expression of EMT mesenchymal markers such as αSMA or type I collagen in primary HBEC cultures may inversely correlate to their FEV1 (% of predicted) while there was a direct correlation to the epithelial marker E-cadherin. A limitation may be that the evaluation included subjects with mostly mild (COPD) or no (smoker) impairment of lung function. In this context, it has recently been confirmed that EMT occurs likely as an early response to smoking [41]. Furthermore, EMT can also be observed in small bronchi of

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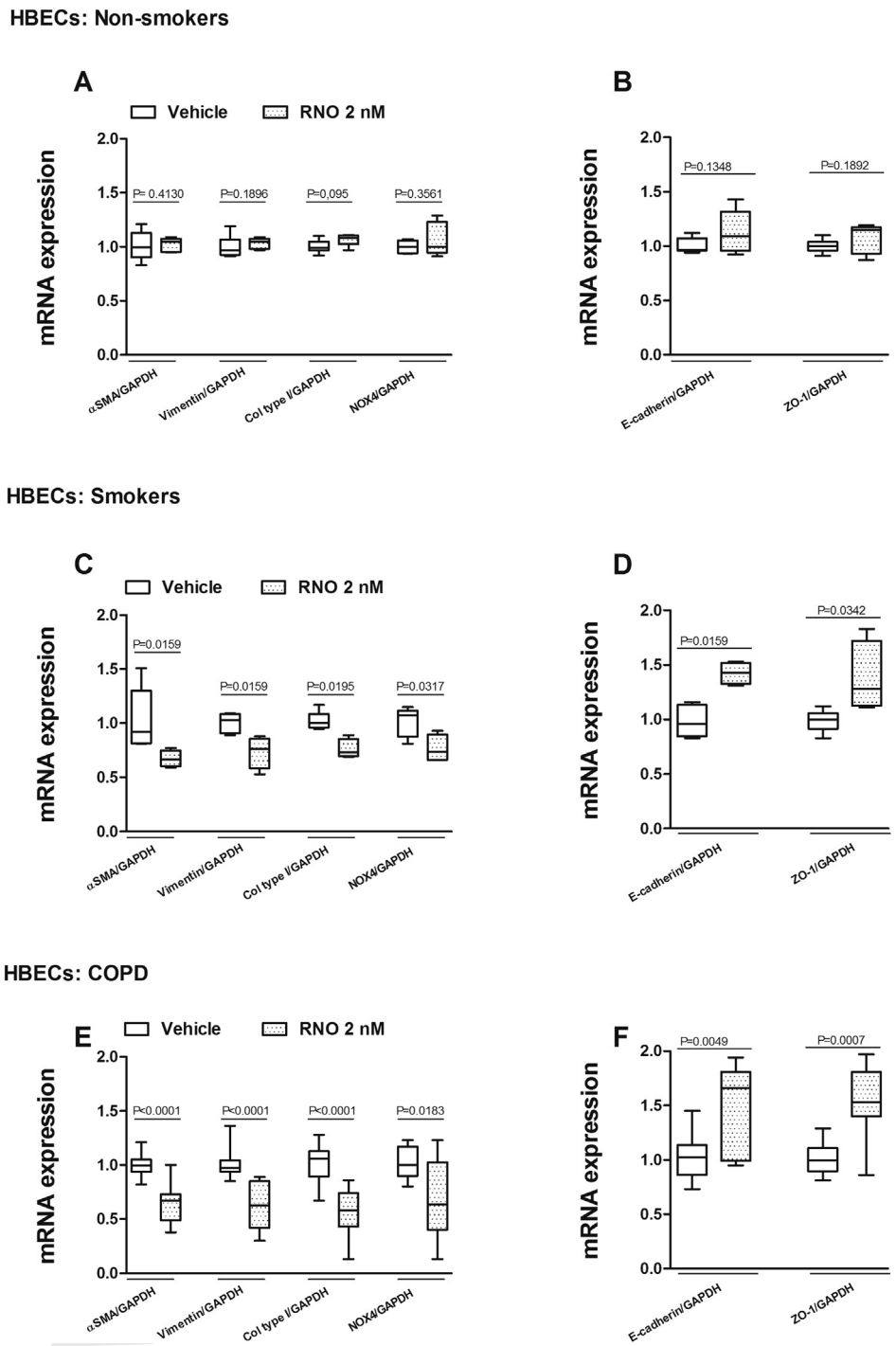

Fig. 6. Roflumilast N-oxide (RNO) decreased mesenchymal markers and increased epithelial markers in primary human bronchial epithelial cells (HBECs) isolated from small bronchi of smokers and COPD patients. Human small bronchi from non-smokers ($n = 3$), smokers ($n = 4$) and smokers with COPD ($n = 6$) patients were plated with the epithelial cell layer in contact with culture plates. After approximately 10 days when HBECs were observed around bronchi at about confluency bronchial tissue was removed and primary HBECs were incubated with vehicle or RNO (2 nM) for 24 h in cells from non-smokers, non COPD (A, B) or smokers without (C, D) or with COPD (E, F). Total mRNA was isolated and quantified by real-time PCR with appropriate primers. Results from mRNA measurements of the mesenchymal markers α-SMA, vimentin, collagen type I (col type I) or NOX4, as well as the epithelial markers E-cadherin or ZO-1 are shown. Data are expressed as the ratio to GAPDH for mRNA levels and normalized to the vehicle group. Data are presented as a box and whisker plot with median, IQR and minimum and maximum values. "*P*" exact values were obtained following Mann–Whitney *U* test.

more severe COPD (GOLD stage 3) [31] reflecting a sustained EMT remodelling process during the progression of COPD.

Previously we described that both an increased expression of the mesenchymal EMT markers αSMA, vimentin and collagen type I and a reduction of the epithelial EMT markers E-cadherin and ZO-1 following exposure of well-differentiated HBECs to CSE (2.5%) over 72 h was reversed by the cAMP analogue dibutyryl cAMP (1 mM). Given the earlier observation that CSE (however at 10% and over 24 h) enhanced PDE4 activity and expression (PDE4B) in well-differentiated HBEC along with the finding that roflumilast N-oxide reversed a potentially resulting CSE-induced loss in cellular cAMP content it was reasonable to assume that the PDE4 inhibitor

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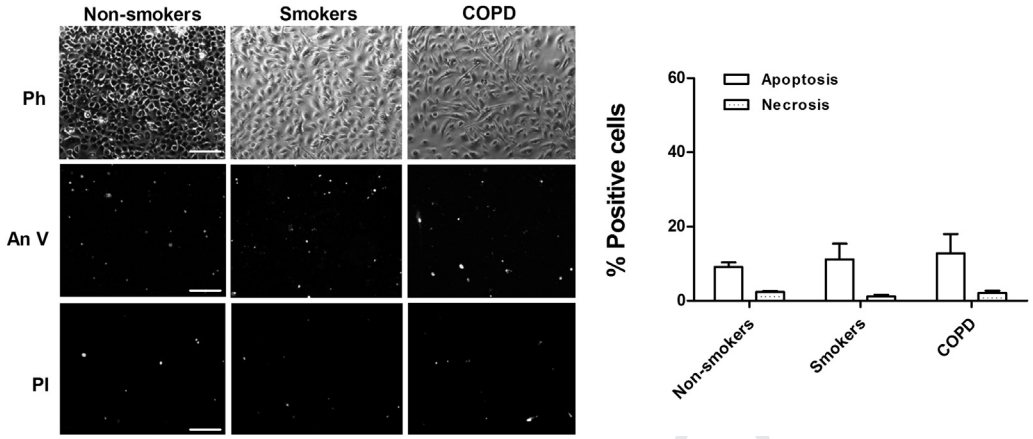

Fig. 7. Analysis of spontaneous apoptosis/necrosis in human primary bronchial epithelial cells (HBECs) from healthy non-smokers, smokers and patients with COPD. Primary human bronchial epithelial cells from healthy non-smokers ($n = 3$), smokers ($n = 4$) and smokers with COPD ($n = 4$) were isolated and cultured without further passaging. When cells reached confluency, they were washed three times and labeled with annexin V-FITC and propidium iodide for 15 min. Annexin V+/PI- (apoptotic) and Annexin V+/PI+ (necrotic) cells were counted (epifluorescence microscope in a total of six fields per condition) and results expressed as percentage of total cells. Results are the mean ± SD of $n = 3$ experiments from three different patients. Scale bar: 50 μm.

was capable to curb CSE-induced EMT in these cells. While in general, the ability of PDE4 inhibitors to mitigate EMT has already been demonstrated by others with human alveolar adenocarcinoma A549 cells and TGFβ1 as stimulus [16], the novelty in the current study is based on the use of well-differentiated HBECs obtained from air–liquid interface culture considered to well reflect the composition of the pseudo-stratified human bronchial epithelium and CSE extract to imitate smoking as a key risk factor in COPD directly targeting bronchial epithelium of those who smoke. Further, roflumilast N-oxide as the active metabolite of roflumilast in use for the treatment of COPD served as the PDE4 inhibitor not only at 1 μM where PDE4 is completely and selectively inhibited but also at 2 nM which corresponds to plasma concentrations in man (unbound to protein) following repeated, once daily dosing of roflumilast at the approved dose of 500 μg/d. In line with its ability to fully restore the loss in cellular cAMP following CSE attributed to inhibition of PDE4, roflumilast N-oxide at 2 nM revealed as rather effective to reverse CSE-induced EMT based on the mRNA and protein expression of mesenchymal and epithelial markers. For comparison the IC50 values for roflumilast N-oxide to inhibit human PDE4B2, D3 and A4 splice variants expressed in well-differentiated human bronchial epithelial cells [12] are at 1.1, 0.8 and 2.3 nM respectively [19]. As cellular effects of a PDE4 inhibitor are contingent on the extent of cAMP synthesis amongst others one may speculate that a considerable adenylyl cyclase activity at baseline or enhanced by autocrine mediators may enable roflumilast N-oxide (2 nM) to reduce CSE-induced EMT. Notably, though, in contrast to effects on mesenchymal EMT marker transcripts and proteins where the increase by CSE was largely abolished with roflumilast N-oxide at 2 nM this concentration of the PDE4 inhibitor did only partially (40–45%) reverse the CSE-induced loss in E-cadherin and ZO-1 epithelial marker transcripts while their protein levels were fully restored. This may indicate that E-cadherin and ZO-1 transcripts *versus* proteins are differentially regulated by cAMP. In fact it has been shown that TGFβ1-induced E-cadherin loss representing an early event in EMT may occur secondary to MMP-induced proteolytic E-cadherin disruption in renal tubular epithelial cells [42].

Table 1
Clinical features of patients. % pred, % predicted; COPD, chronic obstructive pulmonary disease (current smokers); FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; Pack-yr, 1 year smoking 20 cigarettes-day; PaO_2, oxygen tension in arterial blood; $PaCO_2$, carbon dioxide tension in arterial blood; Post, post-bronchodilator; Pre, pre-bronchodilator; LABA, long-acting β_2-adrenoceptor agonists; LAMA, long-acting muscarinic receptor antagonists. Data is the median [interquartile range].

	Non-smokers ($n = 10$)	Smokers ($n = 8$)	COPD ($n = 9$)
Sex (female/male)	4/6	2/6	3/6
Age (years)	65 [58–77]	67 [58–72]	66 [56–71]
Tobacco consumption, pack-year	0	40 [35–47]	37 [32–43]
FEV1 (pre), % predicted	98 [96–102]	92 [90–96]	60 [51–69]
FVC (pre), % predicted	101 [96–105]	96 [92–101]	83 [87–99]
FEV1/FVC (pre) %	94 [92–100]	84 [79–92]	56 [52–61]
FEV1 (post), % predicted	101 [98–105]	94 [90–97]	68 [52–70]
FVC (post), % predicted	103 [95–106]	97 [92–102]	90[87–100]
FEV1/FVC (post) %	96 [92–102]	87 [78–91]	60 [50–65]
PaO_2, mmHg	92 [86–94]	88 [85–94]	80 [76–87]
$PaCO_2$ mmHg	38 [37–41]	36 [34–40]	42 [38–44]
Subjects on inhaled steroids, (N)	0	0	2
Subjects on theophyllines, (N)	0	0	0
Subjects on LABA, (N)	0	0	6
Subjects on LAMA, (N)	0	0	5

How may roflumilast N-oxide by enhancing cellular cAMP alleviate CSE-induced EMT in well-differentiated HBECs? Previously, we observed that the loss in E-cadherin and ZO-1 and the increase in αSMA, collagen I and vimentin following exposure of well-differentiated HBEC to CSE are all reversed by a neutralizing antibody to TGFβ1 [12]. In fact, exposure of HBEC to CSE (2.5%) over 72 h resulted in an increase in TGFβ1 into the culture medium. Such increase was abolished by roflumilast N-oxide at both 2 nM and 1 μM concentrations along with down-stream signalling exemplified by the reduced phosphorylation of ERK1/2 and Smad3.

Reactive oxygen species (ROS) are intimately related to EMT and an increase in cellular cAMP is well capable to mitigate stimulus (e.g. TGFβ1, CSE)-induced cellular ROS formation. For example in renal tubular epithelial cell lines TGFβ1-induced a rapid increase in ROS and anti-oxidants (such as apocynin, diphenyliodonium, rotenone, N-acetylcysteine) were all able to prevent EMT [47]. In human alveolar epithelial A549 cells TGFβ1 enhanced ROS formation in parallel to the occurance of EMT [16]. Recent work from this laboratory [12] documented in well-differentiated HBECs stimulated with CSE (2.5%) (i) a rapid (at 30 min) increase in intracellular ROS (assessed as DCF fluorescence), (ii) a later (measured at 72 h) up-regulation of NOX4 mRNA and protein, (iii) a prevention of EMT based on mesenchymal and epidermal markers with the antioxidants apocynin or NAC. In renal tubular epithelial cell lines an

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increase in cAMP by prostaglandin E2, forskolin or the non-specific PDE inhibitor IBMX largely abolished the TGFβ1-induced increase in the rapid (30 min) accumulation of intracellular ROS and EMT [48]. Further, TGFβ1 induced ROS in A549 cells (24 h) was partially reduced by rolipram that curbs EMT. In well-differentiated HBECs we previously found that dibutyryl cAMP as well as roflumilast N-oxide (1 μM, 2 nM) prevented the increment in ROS accumulating following a 30 min exposure to CSE [12,27] that was confirmed in the current work. Whilst the mechanistic background for the inhibition of the rapid, stimulus-triggered ROS formation by the PDE4 inhibitor remains to be explored the notion may be raised that this effect is caused by an inhibition of NOX2 or NOX1 assembly (well expressed in human bronchial epithelial cells [27]) perhaps by reducing active (GTP-bound) Rac1 [49].

Expanding on our previous findings that exposing well-differentiated HBECs to CSE over longer time periods (i) enhances NOX1 and 2 transcripts which is suppressed by roflumilast N-oxide [27], (ii) increases NOX4 transcripts and protein which is reduced by dibutyryl cAMP [12] it has now been shown that roflumilast N-oxide curbed NOX4 expression. However, the functional role of the NOX isoenzymes in CSE-induced EMT remains to be dissected. NOX4 represents a constitutively active, mainly hydrogen peroxide producing NADPH oxidase that is independent from cytosolic proteins (such as p67phox) for its operation (hence, from rac1) with its activity largely regulated by expression. NOX4 is functionally involved in myofibroblast transition [50] and up-regulated in IPF lung fibroblasts [51]. It has been described that genetic knock-down of NOX4 reduced TGFβ1-induced fibronectin expression in human breast epithelial cells that was considered as a marker of EMT [52]. On the other hand, NOX4 was dispensable for TGFβ1-induced EMT in hepatocytes [53].

Taken together, an inhibition of CSE-induced, autocrinously acting TGFβ1, likely the most prominent inductor of EMT and that of ROS could explain why roflumilast N-oxide prevents well-differentiated HBEC from CSE-induced EMT. Indeed, emanating from an increase in cAMP a PDE4 inhibitor would be capable to suppress ROS at least at three different levels (i) by a short-term reduction of NOX1 or NOX2 activity likely caused by reduction of active (GTP bound) Rac1, (ii) by long term inhibition of NADPH oxidase expression (e.g. NOX1, 2, 4), (iii) by improving the anti-oxidative armamentarium through enhancing Nrf2 [54,55].

Hypothetically, such mechanisms may also explain why roflumilast N-oxide has been able to alleviate enhanced mesenchymal and reduced epithelial markers of EMT maintained in primary cultured HBEC from smokers with or without COPD, while not affecting these measures in cultures from non-smokers, non COPD subjects. Of interest in this context it was recently shown that PGE_2 acting via the EP2/EP4-cAMP signalling pathway might reverse established lung myofibroblast differentiation to TGFβ1 [56].

In parallel to EMT, CSE may entail other effects such as epithelial cell apoptosis/necrosis which largely depend of the concentration and time of exposure. Thus, high concentrations of CSE (for example 10% over 24 h) may induce bronchial epithelial cell apoptosis [43] while low concentrations do not show such effect [44,45]. In our *in vitro* model exposure of well-differentiated human bronchial epithelial cells to CSE at 2.5% over a period of 72 h about doubled apoptosis at medium control conditions (to about 35% or total cells). Roflumilast N-oxide partly reduced the increment in apoptosis secondary to CSE indicating to a protective effect in parallel to the inhibition of EMT. A common denominator of these findings may be that CSE is reported to enhance ER stress resulting in apoptosis or EMT contingent on the experimental conditions in airway epithelial cells [57]. Necrosis was negligible and unchanged under the explored conditions.

5. Conclusions

Roflumilast N-oxide, a PDE4 inhibitor protects from EMT emanating from exposing well-differentiated HBECs to cigarette smoke extracts (2.5%) over 72 h *in vitro*. Reducing the burden of reactive oxygen species and suppressing the release of autocrinously acting TGFβ1 from the HBEC cultures may account for these protective effects. These effects of roflumilast N-oxide were also found at 2 nM, a concentration corresponding to plasma concentrations following clinical dosing of roflumilast and were reproduced in primary HBECs from smokers and COPD. Finally, prior observations that EMT markers were enhanced in smokers and patients with COPD were extended by showing their correlation to FEV1 (% of predicted).

Conflict of interest statement

JC received a research grant from Nycomed. HT is a full time employee of Takeda.

Other authors declare no conflict of interest.

Uncited reference

[46]. Q4

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