Pulmonary Pharmacology & Therapeutics 23 (2010) 283–291

Contents lists available at ScienceDirect

Pulmonary Pharmacology & Therapeutics

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

A phosphodiesterase 4 inhibitor, roflumilast N-oxide, inhibits human lung fibroblast functions *in vitro*

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ARTICLE INFO

Article history:
Received 28 July 2009
Received in revised form
27 January 2010
Accepted 28 February 2010

Keywords:
Roflumilast N-oxide
Human lung fibroblasts
ICAM-1 expression
Eotaxin
Myofibroblast transition
Extracellular matrix

ABSTRACT

The PDE4 inhibitor roflumilast mitigates bleomycin-induced lung fibrotic remodeling in rodents. In the current study it was explored whether roflumilast N-oxide, the active metabolite of roflumilast influences functions of cultured lung fibroblasts. Cells of the human foetal lung fibroblast strain GM06114 were stimulated with TNF-α (5 ng ml^{-1}) and cell surface ICAM-1 and eotaxin release were assessed. [*methyl*-^{3}H] thymidine incorporation was measured following stimulation with bFGF (10 ng ml^{-1}). α-Smooth muscle actin (protein), CTGF (mRNA) and fibronectin (mRNA) were determined secondary to TGFß1 (1 ng ml^{-1}). In the presence of PGE_2 (1 nM), roflumilast N-oxide reduced TNF-α-induced ICAM-1 and eotaxin by about 70% and >90% with half-maximum inhibition at 0.9 nM and 0.5 nM, respectively. Roflumilast N-oxide also attenuated [*methyl*-^{3}H] thymidine incorporation secondary to bFGF by about 75% with half-maximum inhibition at 0.7 nM when cells were co-incubated with IL-1ß (10 pg ml^{-1}). In the presence of this cytokine roflumilast N-oxide (1 μM) diminished TGFß1-induced expression of α-smooth muscle actin and transcripts of CTGF and fibronectin. In addition, IL-1ß up-regulated PDE4 activity in the lung fibroblasts. Taken together, these findings indicate that roflumilast N-oxide directly targets human lung fibroblasts, which may at least partially explain the efficacy of roflumilast to mitigate a pulmonary fibrotic response *in vivo*.

1. Introduction

A mixture of small airways disease and lung parenchymal destruction causes airflow limitation in chronic obstructive pulmonary disease (COPD) [1]. These abnormalities are characterized by the presence of an inflammatory cell infiltrate and a remodeling that thickens the airway wall and reduces the airway diameter [2]. A current hypothesis is that aberrantly activated lung fibroblasts are involved in inflammation as well as tissue repair and remodeling [2–4].

Inhibitors of phosphodiesterase 4 (PDE4) are an emerging perspective in respiratory disorders such as COPD [5]. PDE4 inhibitors act by increasing intracellular concentrations of cyclic adenosine [3,5] monophosphate (cAMP), which has a broad range of effects on numerous cell types involved in these ailments [5,6]. Aside from targeting inflammatory cells, PDE4 inhibitors may address lung structural cells (fibroblasts, vascular smooth muscle cells, endothelial and epithelial cells) [6]. As a corollary, PDE4 inhibitors might potentially mitigate pulmonary inflammation, architectural changes, mucociliary malfunction or vascular remodeling [6–8].

A PDE4 inhibitor currently in clinical development for COPD is roflumilast [8,9]. The potential of roflumilast to mitigate an inflammatory response is now established *in vitro*, *in vivo* and in clinical studies [9–15].

Extending its marked anti-inflammatory profile evidence is accumulating that roflumilast may favorably influence lung structural changes *in vivo*. The PDE4 inhibitor prevents the development of airspace enlargement in mice exposed to tobacco smoke [14], reduces muscularization of pulmonary arterioles secondary to chronic hypoxia or monocrotaline in rats [16] and attenuates subepithelial collagen deposition in murine airways following repeated ovalbumin challenge [17]. Finally, roflumilast mitigates the bleomycin-induced lung fibrotic response not only in a preventive but also in a therapeutic protocol in mice or rats while glucocorticoids remained inactive in the latter condition [18]. These studies suggest that roflumilast may influence lung fibroblasts. Indeed, the PDE4 inhibitor reduces contraction of collagen gels

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doi:10.1016/j.pupt.2010.02.004

governed by embedded human lung fibroblasts, their chemotaxis or release of fibronectin triggered by TGFß1 [19].

The objective of the current study was to expand on these findings by exploring the effects of roflumilast N-oxide, the active metabolite of roflumilast [20], on markers of an inflammatory response (ICAM-1, eotaxin), proliferation ([*methyl*-^{3}H] thymidine incorporation), myofibroblast transition (expression of α-smooth muscle actin) and extracellular matrix formation (connective tissue growth factor, fibronectin) in human lung fibroblasts.

2. Materials and methods

2.1. Fibroblast culture

The human foetal lung fibroblasts cell line GM06114, acquired from the Human Genetic Cell Repository of the Coriell Institute for Medical Research (Camden, NJ), was used. Fibroblasts were expanded in Dulbecco's Modified Eagle Medium (DMEM) (InVitrogen, Paisley, Scotland) supplemented with 10% foetal bovine serum (FBS) (InVitrogen, Paisley, Scotland), 2 mM L-glutamine and 50 U ml^{-1} penicillin/50 μg ml^{-1} streptomycin (InVitrogen, Paisley, Scotland) at 37 °C and 5% CO_2.

2.2. Roflumilast N-oxide

Roflumilast N-oxide (Nycomed GmbH) selectively inhibits PDE4 up to concentrations of 1 μM [11]. Dilutions were prepared from a 10 mM stock in 100% DMSO. A final DMSO concentration of 0.2% was identical in all incubations and did not interfere with the addressed fibroblast functions. Neither roflumilast N-oxide nor the solvent affected viability of the lung fibroblasts.

2.3. Surface expression of ICAM-1

Cell surface ICAM-1 expression was determined by flow cytometry as previously described with minor modifications [21,22]. Briefly, GM06114 human foetal lung fibroblasts were grown to confluence in 24-well plates and serum-deprived for 48 h. Cells were preincubated with roflumilast N-oxide (0.1 nM–1 μM), 1 nM PGE_2 or vehicle (DMSO, adjusted to 0.2% in all incubations) for 15 min and then stimulated with TNF-α at 5 ng ml^{-1} for 18 h. At the end of the incubation period cells were detached by trypsinization, washed twice in DMEM and placed into a round-bottom 96-well microtiter plate in a 100 μl volume (DMEM). GM06114 cells were incubated for 30 min at 4 °C with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human ICAM-1 monoclonal antibody (10 μl per 10^6 cells) (InVitrogen, Paisley, Scotland), washed twice in DMEM, fixed in 0.5% paraformaldehyde, and ICAM-1 expressed at the cellular membrane was determined by flow cytometry (Becton Dickinson Immunocytometry Systems, Mountain View, CA). To compare fluorescence intensities of different samples from the same experiment, identical settings of the logarithmic amplifier and compensation were used and listmode files were analysed with CELLQuest Software (Becton Dickinson). All experiments were performed in triplicates. The intensity of fluorescence was expressed as the mean fluorescence channel (mfc) [22].

2.4. Eotaxin release

Eotaxin was determined in cell culture supernatants (obtained from incubations for measurements of ICAM-1), using a commercially available enzyme-linked immunosorbent assay (ELISA) (InVitrogen, Paisley, Scotland).

2.5. [methyl-^{3}H] thymidine incorporation

GM06114 cells were grown to subconfluence in 24-well cell culture plates precoated with 30 μg ml^{-1} collagen (INAMED Biomaterials, Fremont, CA), 10 μg ml^{-1} fibronectin (from human plasma, Sigma–Aldrich, St Louis, MO) and 10 μg ml^{-1} bovine serum albumin (Sigma–Aldrich, St Louis, MO), and growth-arrested for 48 h in serum-free DMEM. Following preincubation with roflumilast N-oxide (1 pM–1 μM), IL-1ß (1–10,000 pg ml^{-1}) (R&D Systems, Wiesbaden-Nordenstadt, Germany), indomethacin (10 μM), PGE_2 (0.01–1000 nM) (Sigma–Aldrich, Taufkirchen, Germany) or vehicle (DMSO, adjusted to 0.2% in all incubations) for 15 min fibroblasts were stimulated with 10 ng ml^{-1} bFGF [21]. [*methyl*-^{3}H] thymidine (1 μCi per well) was added over the last 6 h of a 24 h incubation period. Culture supernatants were removed and adherent cells washed twice in PBS. Nucleic acids were precipitated by ice-cold 10% trichloroacetic acid and dissolved in 0.2 N NaOH (0.5 ml per well). Incorporated radioactivity was counted and expressed as cpm per well.

2.6. Expression of alpha-smooth muscle actin (α-SMA)

Confluent and growth-arrested GM06114 fibroblasts were preincubated with 1 μM roflumilast N-oxide, 10 or 100 pg ml^{-1} IL-1ß, 10 μM indomethacin or vehicle (DMSO, adjusted to 0.2% in all incubations) for 15 min before 1 ng ml^{-1} TGFß1 was added over 48 h. At the end of this incubation time cells were collected by trypsinization, washed twice in PBS, resuspended in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris HCl pH 7.4, 0.1% NaN_3, 0.5% Triton X-100, 5 μM pepstatin A, 10 μM leupeptin, 50 μM phenylmethylsulfonyl fluoride, 10 μM soybean trypsin inhibitor, 2 mM benzamidine) and left on ice for 30 min. A 1000 × g (10 min) supernatant of the lysate was supplemented with gel loading buffer (Rothi-Load, Carl Roth GmbH, Karlsruhe, Germany) and samples boiled at 95 °C for 10 min. Proteins (5 μg per lane) were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel under reducing conditions and electroblotted on PVDF membranes (Immobilon-P, Millipore, Bedford, MA). The membrane was blocked in 5% low-fat milk and then incubated with a mouse monoclonal antibody to human α-SMA (1:2000 in 0.05% Tween 20; Sigma–Aldrich, St Louis, MO) overnight at 4 °C. A goat anti-mouse IgG antibody conjugated to horseradish peroxidase (1:100.000 in 0.05% Tween 20; Dianova mbH, Hamburg, Germany) served as a secondary antibody. Horseradish peroxidase activity bound to the membrane was visualized with Amersham ECL™ Advance Western Blotting Detection Kit (GE Healthcare, Chalfont, Buckinghamshire, UK) using the LAS1000 Fuji Imaging system. Densitometric analyses were performed with AIDA 3.52 software [21].

2.7. Real-time quantitative PCR to assess mRNA of fibronectin and connective tissue growth factor (CTGF)

Growth-arrested confluent GM06114 cells in 12-well cell culture plates were preincubated with 1 μM roflumilast N-oxide, 10 pg ml^{-1} IL-1ß, 10 μM indomethacin or vehicle (DMSO, adjusted to 0.2% in all incubations) for 15 min followed by stimulation with 1 ng ml^{-1} TGFß1. After an incubation period of 12 h the medium was removed, cells were washed twice with PBS and immediately lysed in *buffer RLT* (Qiagen, Hilden, Germany) supplemented with 1 mM ß-mercaptoethanol, 350 μl per well. RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Reverse transcription was performed with approximately 0.5 μg RNA using avian myeloblastosis virus (AMV) reverse transcriptase (Roche Diagnostics, Mannheim, Germany). Quantitative PCR for CTGF and fibronectin mRNA was performed