Roflumilast n-oxide associated with PGE2 prevents the neutrophil elastase-induced production of chemokines by epithelial cells

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A B S T R A C T

Neutrophil chemotaxis is involved in the lung inflammatory process in conditions such as chronic obstructive pulmonary disease (COPD). Neutrophil elastase (NE), one of the main proteases produced by neutrophils, has an important role in the inflammatory process via the release of chemokines from airway epithelial cells. It was recently shown that roflumilast N-oxide has therapeutic potential in COPD. The aim of the present study was to investigate roflumilast N-oxide’s effect on NE-induced chemokine production and signaling pathways in A549 epithelial cells. A549 cells were incubated with NE for 30 min, washed with PBS and then cultured for 2 h (for measurement of mRNA expression) and 24 h (for chemokine release) or for 5 to 30 min (for protein phosphorylation assays). Prior to the addition of NE, cells were also pre-incubated with prostaglandin E2 (PGE2), alone and in combination with roflumilast N-oxide.

Addition of NE was associated with elevated chemokine production by A549 cells and induction of the p38 MAP-kinase pathway. In contrast, when combined with PGE2, the roflumilast N-oxide had an additive effect on the inhibition of NE-induced chemokine release and p38α and other kinases activation.

In conclusion, we demonstrated that NE is able to increase the release of chemokines from epithelial cells via the activation of p38α MAP-kinase and that roflumilast N-oxide when combined with PGE2 lowers NE-induced kinase activation and chemokine production.

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1. Introduction

In the respiratory tract, neutrophils have a major role in inflammatory conditions, such as chronic obstructive pulmonary disease (COPD) [1]. Neutrophil elastase (NE), one of the major proteases produced by neutrophils, is a serine protease involved in host defense against bacterial pathogens [2]. Extracellular NE activity is regulated by a number of endogenous protease inhibitors, including α1-antitrypsin, secretory leukoprotease inhibitor and α2-macroglobulin [3]. When the extracellular concentration of free NE exceeds the buffering capacity provided by endogenous inhibitors, the elastase starts to drive inflammatory processes in the lung. Hence, elevated concentration of active proteinases degrades the protein components of the extracellular matrix, which leads to destruction of the alveolar walls and thus airspace enlargement [4]. In COPD, elevated levels of NE and low levels of endogenous antiproteases in bronchoalveolar lavage (BAL) fluid are correlated with the severity of emphysema [5]. Neutrophil elastase’s elastolytic activity thus has a role in lung inflammation via the release of pro-inflammatory mediators from airway epithelial cells [6]. Besides its direct elastolytic activity, NE releases soluble epidermal growth factor receptor (EGFR) ligands and initiates EGFR/MEK/ERK signaling [7]. Furthermore, NE’s actions activate the Toll-like receptor 4 (TLR-4)/MyD88/TRAF6/NF-κB or/and PKC/EGFR/ERK signal transduction pathways and lead to IL-8/CXCL8 release and mucin5AC expression in epithelial cells [8,9]. In animal models, genetic deficiency or pharmacological intervention with small-molecule or physiological inhibitors of NE affords significant protection (i.e. a reduction of approximately 60%) against the pro-inflammatory and emphysematous effects of chronic exposure to cigarette smoke [10,11].

It was recently reported that phosphodiesterase 4 (PDE4) inhibitors have therapeutic value in treating exacerbations of COPD [12,13]. By preventing the degradation of cAMP, PDE4 inhibitors enhance the anti-inflammatory action of this secondary messenger. Epithelial cells

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in the lung and airways express PDE4 [14]. The inhibitory effect of PDE4 inhibitors on the release of cytokines and chemokines in vitro and in vivo was recently reviewed [15]. Roflumilast and its metabo-
lite roflumilast-N-oxide (RNO) are known to reduce the lipopolysac-
charide (LPS)-induced release of CCL-2, −3, −4, CXCL10 and tumor necrosis factors (TNF)-α from human lung parenchyma, human pul-
monary macrophages and monocyte-derived macrophages [16,17, 18]. We recently demonstrated that RNO is able to reduce the pro-
duction of cytokines and chemokines from A549 epithelial cells stim-
ulated with cigarette smoke extract [19]. However, RNO’s effect on NE-
activated epithelial cells has not yet been investigated. The ob-
jective of the present study was to investigate RNO’s effect on NE-
induced cytokine/chemokine production and signaling pathway ac-
tivation in A549 epithelial cells.

2. Materials and methods

2.1. Reagents

Roflumilast-N-oxide was provided by Nycomed (Konstanz, Germany). Human ME was obtained from Biocenterum (Krakow, Poland). F-12K Nutrient Mix (Kaihig’s Modification) cell culture medium, antibiotics, glutamine and trypsin-EDTA were purchased from Invitrogen (Eugene, OR, USA). Fetal calf serum (FCS) was obtained from Hyclone (Logan, UT, USA). Lipopolysaccharide from E. coli 055:B5, 3-
(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the EGFR inhibitor AG-1478 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Specific antibodies against phospho-(p44/42) ERK1/2, (p44/42) ERK1/2, phospho-p38 MAPK, p38 MAP kinase, phospho-SAPK/JNK and SAPK/JNK were from Cell Signaling Technology (Beverly, MA, USA), Acrylamide, SDS, Tris, HEPES, and bovine serum albumin (BSA) was from Eurobio (Les Ulis, France). A Bradford protein assay and Precision Plus Protein Dual Color Standards were pur-
blished by Bio-Rad (Hercules, CA, USA).

2.2. Cell culture

The human alveolar epithelial cell line (A549) was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in F-12K supplemented with 10% FCS, 10 units/mL penicillin, 10 μg/mL streptomycin, 2 mM -glutamine and 10 mM HEPES at 37 °C and with 5% CO2. For the experiments, aliquots of 1 × 105 cells were transferred to 24-well plates and grown to confluence.

2.3. Treatments

A549 cells were washed and cultured overnight in serum-free F-12 K medium supplemented with antibiotics, -glutamine and HEPES. The starved cells were incubated with NE for 30 min or vehicle (PBS), washed with PBS and then cultured in serum-free F-12 K. After stimulation, cell supernatants were collected at 24 h (for cytokine measure-
ments) and cell pellets were collected after 2 h (for mRNA expression analysis). Alternatively, A549 cells were pre-incubated for 2 h with PGE2 (10 nM) alone or in combination with RNO (at 0.1 μM, 0.3 μM and 1 μM), vehicle (DMSO 0.01%) or EGFR inhibitor AG-1478 prior to the addition of NE. All experiments were performed in serum-free media in triplicate and were repeated at least three times. At the end of the incubation period, culture supernatants were harvested and stored at −80 °C until further analysis.

2.4. Measurement of chemokine protein and mRNA levels

The concentrations of IL-8/CXCL8, MCP-1/CCL2, Gro-α/CXCL1 in the culture supernatants were measured using ELISAs (R&D Systems, Abingdon, UK). Total RNA was isolated from A549 cells using a commer-
cially available kit (Promega, Madison, WI, USA). RNA quantity and purity were assessed with a Nanodrop ND-1000 spectrophotometer (Nxyor Biotech, Paris, France). Total RNA (1 μg) was reverse-
transcribed into cDNA using the High-Capacity cDNA Archive Kit (Ap-
plied Biosystems, Foster City, CA). Real-time, quantitative RT-PCR was performed with the SYBR Green fluorescent dye methodology (SYBR Green PCR Master Mix, Applied Biosystems) and the StepOnePlus™ real-time PCR system (Applied Biosystems). Primer pairs for each tran-
script were chosen with NCBI software (http://www.ncbi.nlm.nih.gov), as follows. IL-8/CXCL8: forward 5′-AAG AAA CCA CCG GAA GGA AC-3′, reverse 5′-AAA TTT GGG GTG GAA AGG TT-3′; MCP-1/CCL2: forward 5′-TGG ACC AAA GAA GCT GTG ATC-3′, reverse 5′-ATT CTT CCG TGG TGG TGG AGT GAG-3′; Gro-α/CXCL1: forward 5′-AAC CGA ACT AGT ACC AAC CAC AC-3′, reverse 5′-CCT CCC TTC TCG TCA GTT G3′; GAPDH: forward 5′-GGC ATG TAC TGT GAG CAT GAC-3′, reverse 5′-TGC ACC ACC AAC TGC TTA GC-3′. Amplification curves were analyzed according to the comparative cycle threshold method, using StepOne software (version 2.1, Applied Biosystems). The steady-state mRNA levels for the genes of interest were normalized against those of GAPDH.

2.5. Evaluation of protein kinase phosphorylation by Western blotting

A549 cells were incubated with medium alone, PBS and NE for 5, 15 and 30 min. Next, the cells were washed with PBS and lysed in lysis buff-
er (Novagen, San Diego, CA, USA) containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche, Mannheim, Germany) on ice for 15 min. Equal amounts of cell lysate (40 μg) were separated on 5% or 10% SDS-PAGE gels and then transferred onto nitrocellulose mem-
branes. The membranes were blocked for 1 h with 5% BSA or 5% w/v non-fat powdered milk in TBS containing 0.1% Tween 20. Next, the membranes were incubated with a mouse monoclonal antibody against phospho-p44/42 ERK1/2 (1/2000), a rabbit monoclonal antibody against phospho-p38 MAPK (1/1000), a mouse monoclonal antibody against phospho-JNK (1/1000), or a rabbit monoclonal antibody against total-p38 MAP Kinase, a rabbit monoclonal antibody against total-phospho-JNK (1/1000), a rabbit monoclonal antibody against phospho-STAT3 (1/1000), a rabbit monoclonal antibody against total-STAT3 (1/1000), a rabbit monoclonal antibody against phospho-

2.6. Human phosphoprotein array

Cells were also pre-incubated with PGE2 (10 nM) alone or in combi-
nation with RNO (1 μM) for 2 h before the addition of NE. Cell lysates (500 μg of total protein per array) were applied to the phosphoprotein array (the Proteome Profiler Human Phosphokinase Array Kit, R&D Sys-
tems) according to the manufacturer’s instructions.

2.7. The MITT cell viability assay

Neutrophil elastase’s cytotoxicity for A549 cells was checked with the MITT cell viability assay. Cell cultures were incubated with MITT (final concentration: 0.5 mg/mL) at 37 °C for 2 h. The formazan reaction product was extracted in dimethylsulfoxide (DMSO) and the OD was measured spectrophotometrically at 570 nm (with DMSO as a blank). Cell viability was expressed as a percentage of the value observed with untreated cells.
2.8. Statistical analysis

The results are expressed below as the mean ± S.E.M. A one-way analysis of variance (ANOVA) was used to assess between-group treatment effects. Treatment interactions were compared in a Tukey post-test. In all analyses, the threshold for statistical significance was set to P < 0.05.

3. Results

3.1. The effect of NE on the production of IL-8/CXCL8, Gro-α/CXCL1 and MCP-1/CCL2 by A549 cells

Incubation of A549 epithelial cells with NE (10 nM) was associated with significantly greater release of IL-8/CXCL8, MCP1/CCL2 and Gro-α/CXCL1 (Fig. 1A, B and C). Lower (1 nM) or higher (50 or 100 nM) concentrations of NE were not able to increase the levels of chemokine. Indeed, the highest tested concentration of NE (100 nM) was cytotoxic (Fig. 1D). Hence, a concentration of 10 nM was selected for investigation of NE’s effects on chemokine mRNA expression; NE was associated with significantly greater mRNA expression for IL-8/CXCL8, MCP1/CCL2 and Gro-α/CXCL1 at 2 h (Fig. 2A, B and C).

3.2. Effects of the EGFR inhibitor AG-1478 on NE-induced release of chemokine by A549 cells

Given that the EGFR is known to be activated by NE, we next sought to determine whether EGFR inhibitors could inhibit chemokine production from A549 cells. The incubation of epithelial cells with AG-1478 (at 0.1 μM, 0.3 μM and 1 μM) was associated with concentration-dependent inhibition of the release of IL-8/CXCL8 (Fig. 3A). Moreover, AG-1478 at 0.3 μM or 1 μM inhibited the NE-induced release of Gro-α/CXCL1 (Fig. 3C) but not the release of MCP1/CCL2 (Fig. 3B).

3.3. Effects of a combination of RNO and PGE₂ on NE-induced chemokine release by A549 epithelial cells

Roflumilast N-oxide alone had no effect on NE-induced chemokine release (data not shown). In contrast, the combination of RNO with 10 nM PGE₂ decreases IL-8/CXCL8, MCP1/CCL2 and Gro-α/CXCL1 release. The incubation of epithelial cells with PGE₂ alone is able to decrease of NE-induced IL-8/CXCL8, MCP1/CCL2 and Gro-α/CXCL1 release (Fig. 4A, B and C), however the combination of RNO with 10 nM PGE₂ further reduced the release of MCP1/CCL2 and Gro-α/CXCL1 (Fig. 4B and C) with respect to PGE₂.

3.4. Effects of NE on the activation of signaling pathways

Mitogen-activated protein kinases are involved in a number of processes (such as oxidative stress and inflammation), and p38α is reportedly induced by NE. The time-dependent phosphorylation of ERK, JNK and p38α mitogen-activated protein (MAPK) kinases was monitored following NE stimulation. Significantly greater p38α MAPK activation was observed 5 min after NE treatment but not after 15 or 30 min (Fig. 5A and E). In contrast, NE did not activate the JNK, ERK 1/2 and STAT3 pathways (Fig. 5B, C, and D). Total levels of p38α, JNK, ERK 1/2 and STAT3 were not altered by NE treatment, relative to control experiments (Fig. 5A, B, C, and D).

3.5. Activation of the p38α kinase pathway by NE is less intense after treatment with RNO

The treatment of epithelial cells with 10 nM PGE₂ (whether alone or in combination with RNO) markedly reduced the NE-induced activation of p38α kinase after 5 min (Fig. 6A). However, only the combination of RNO and PGE₂ was associated with significantly lower p38α activation (Fig. 6A). This finding was confirmed by the results of a specific ELISA.

Fig. 1. Effects of NE on IL-8/CXCL8, Gro-α/CXCL1 and MCP-1/CCL2 release and cell viability (in an MTT assay). Serum-starved A549 cells were incubated with medium alone (control), PBS or different concentrations of NE (1 nM-100 nM) for 30 min and then with medium alone for 24 h. After collection of the culture supernatants, the concentrations of chemokines were measured by ELISA (A, B, and C) and cell viability was determined with an MTT assay (D). The data represent the mean ± S.E.M of 6–10 independent experiments. *P < 0.05, compared with the control.
for phosphorylated p38α (Fig. 6B). Whether alone or in combination with RNO, PGE2 did not alter the activation of ERK 1/2 and JNK pathways or the total amount of protein (Supplementary Fig. 1).

3.6. Effects of RNO on NE-induced protein phosphorylation in A549 cells

We first used a human phosphoprotein array kit to establish whether or not other signaling pathways were activated by NE 10 nM in A549 cells pre-treated with PGE2. We then analyzed the effects of a combination of RNO and PGE2, relative to PGE2 alone. After the pre-treatment of A549 cells with PGE2, incubation with NE was associated with activation (an increase of 30% compared to the control was considered) of 12 kinases (gray bars; RSK 1/2/3, c-Jun, Akt T308, Hck, Fak, STAT5 a/b, p53 S46, p70 S6, PLCγ1, Chk2, Pyk2, and PRAS 40 t) of the 45 tested (Fig. 7A). Of the 12 kinases activated by NE in the presence of PGE2, five (blanc bars; Hck, FAK, STAT5 a/b, P70s6 and CHK-2) appeared to be less strongly activated when PGE2 was combined with 1 μM RNO (Fig. 7Aa and B).

4. Discussion

In the present study, we investigated the anti-inflammatory effects of RNO (a PDE type 4 inhibitor) on NE-induced changes in the A549 alveolar epithelial cell line. We found that NE raises the production of three chemokines by these epithelial cells and that this effect is mediated by the p38α signaling pathway. An EGFR inhibitor lowered the NE-induced release of IL-8/CXCL8 and Gro-α/CXCL1 (but not of MCP-1/CCL2). When combined with PGE2, RNO had a more marked inhibitory effect on chemokine release and p38α kinase activation. RNO also inhibited five other NE-induced kinases.

Previous studies have found that smokers (particularly those suffering from COPD) have high neutrophil counts in the airways [5]. Other literature data have shown that the NE produced by neutrophils causes the release of cytokines and chemokines from airway epithelial cells [6, 9, 20]. In the present study, NE induced the release of IL-8/CXCL8, MCP-1/CCL2 and Gro-α/CXCL1 and greater chemokine mRNA expression by A549 cells. Elevated levels of these chemokines are found in the BAL fluid from COPD patients, and are involved in the physiopathology of COPD [21]. We also found that chemokine release was greatest at 10 nM NE, with less release at higher concentrations of the enzyme. This paradoxical effect may be due to proteolysis of the chemokines by NE [20]. Indeed, we found that 50 nM NE was associated with detachment of about 10% of the cells (data not shown). In vivo, the activity of NE is controlled by endogenous inhibitors (such as α1-proteinase, secretory leukoprotease inhibitor and elastin). The absence of these inhibitors in vitro may explain why NE is active at low concentrations [5].

It has been reported that NE-induced release of IL-8/CXCL8 from bronchial epithelial cells can be prevented by specific inhibitors, suggesting that chemokine release involves NE’s proteolytic activity (via the activation of surface receptors such as TLR-4 and EGFR) [6, 7]. Indeed, the NE protease cleaves the cytoplasmic membrane protein meprin-α, which in turn activates TLR-4 and EGFR and leads to the production of CXCL8/IL8 via the NFκB and MAP kinase pathways. Bergin and colleagues (2008) showed that TLR-4 inhibitors can inhibit NE-

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**Fig. 2.** Effects of NE on mRNA expression for IL-8/CXCL8, Gro-α/CXCL1 and MCP-1/CCL2. Serum-starved A549 cells were incubated with medium alone (control), PBS or NE (10 nM) for 30 min and then with medium alone for 2 h. mRNA expression was determined by real-time quantitative RT-PCR. The gene expression results are normalized against the values for GAPDH. The data represent the mean ± S.E.M of four independent experiments. *P < 0.05, compared with the control.

**Fig. 3.** Effects of AG-1478 on the NE-induced release of chemokines by A549 cells. Serum-starved A549 cells were incubated with AG-1478 or vehicle 30 min prior to stimulation with NE (10 nM) for 30 min, and were then incubated with medium alone for 24 h. The culture supernatants were collected and the concentrations of chemokines were measured by ELISA. The data represent the mean ± S.E.M of three independent experiments. #P < 0.05, compared with vehicle.
Fig. 4. Effects of PGE2 in the presence and absence of RNO on NE-induced IL-8/CXCL8, Gro-α/CXCL1 and MCP-1/CCL2 release by A549 cells. Cells were pre-incubated with 10 nM PGE2 alone or in combination with RNO (0.1 μM; 0.3 μM or 1 μM) or vehicle for 2 h and were then stimulated with NE for 30 min. After 24 h, cell culture supernatants were collected and the levels of chemokines were quantified by ELISA. Results are expressed as the mean ± S.E.M of four independent experiments. *P < 0.05, compared with NE (10 nM), #P < 0.05, compared with PGE2.

Fig. 5. Effects of NE on ERK1/2, p38α and JNK kinase in A549 cells. Serum-starved A549 cells were incubated with serum free medium alone (the control), PBS or NE 10 nM for 5, 15 or 30 min. Total cell lysates were immunoblotted with antibodies specific for phospho-p38α kinase and total p38α kinase (A), phospho-JNK and total JNK (B), phospho ERK1/2 and total ERK1/2 (C) and phospho-STAT3 and total STAT3 (D). Densitometry of the phospho-p38α kinase (E), phospho-JNK (F), phospho ERK1/2 (G), phospho STAT3 (H) blots. Results are expressed as the mean ± S.E.M of three independent experiments, *P < 0.05, compared with the control.
Fig. 6. Effects of PGE2 in the presence and absence of RNO on NE-induced activation of the p38α pathway. Serum-starved A549 cells were incubated with PGE2 alone or in the presence of RNO and were then stimulated with PBS or 10 nM NE for 5 min. Total cell lysates were immunoblotted with antibodies specific for phospho-p38α kinase and total p38α kinase (A). The amount of phosphorylated p38α in the cell lysate was determined by ELISA (B). Results are expressed as the mean ± S.E.M of three independent experiments. Statistically significant p38α phosphorylation was detected using the Mann–Whitney U-test. *P < 0.05, compared with the PBS. **P < 0.05, compared with NE (10 nM).

Fig. 7. Effects of RNO, in the presence PGE2, on NE-induced protein phosphorylation in A549 cells. Cells were pre-incubated for 2 h with PGE2 (10 nM) alone or in the presence of RNO (1 μM) and were then stimulated 5 min with 10 nM NE. Cell extracts were probed on human phosphoprotein arrays. Results representing the signal intensity (in arbitrary units) were expressed as a percentage of the control value (serum-free medium alone).
5. Conclusions

We found that NE is able to increase the release of chemokines from epithelial cells via the activation of p38α MAP kinase. Moreover, we showed that an EGFR receptor inhibitor is able to inhibit NE-induced production of IL-8/CXCL8 and Gro-α/CXCL1 but not of MCP-1/CCL2. In contrast, treatment of the cells with a combination of RNO and PGE2 was associated with lower IL-8/CXCL8, MCP-1/CCL2 and Gro-α/CXCL1 release and a lower degree of activation of several kinases. Our results confirm RNO’s anti-inflammatory effect in this in vitro elastolytic model and help to explain the compound’s mechanism of action. Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.intimp.2015.11.019.

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References


It was recently confirmed that roflumilast has therapeutic value in treating exacerbations of COPD [13,28]. The effect of roflumilast on the NE-induced release of chemokines and kinases activation in has not previously been described. We therefore decided to investigate the effect of RNO (the active metabolite of roflumilast) on the NE-induced activation of A549 cells. Our results show that RNO is able to reinforce PGE2’s inhibitory effect on NE-induced chemokine production. In this in vitro model, PGE2 was needed to activate adenylate cyclase because the A549 epithelial cell line does not produce endogenous cAMP [27]. By increasing levels of cAMP, PGE2 reduces chemokine release and p38α activation. However, a combination of RNO and PGE2 exerted a more marked inhibitory effect than PGE2 alone. We next looked at whether RNO exerted inhibitory effects on other kinases (i.e. those not affected by PGE2 alone). In the presence of PGE2, RNO prevented the phosphorylation of five protein kinases (at least 50% after treatment with Roflumilast n-oxide in relation to the untreated group): Hck, Fak, STAT 5a/b, p70 S6 and CHK2 (Fig. 7). Our results are in line with the literature data on RNO effects on kinase phosphorylation and cytokine release by bronchial epithelial cells and macrophages [18,28].

Hck is a member of the Src family of tyrosine kinases. Several studies have shown that Hck is involved in inflammatory signaling pathways. Indeed, Ziegel and colleagues (1988) observed that LPS-mediated macrophage activation was associated with elevated expression of Hck [29]. STAT5 activation may be involved in cytokine production by A549 cells after EGF stimulation [30]. We showed that NE-induced several different kinases, that can be involved in cytokines release. Then, the effects of RNO in others kinases (Hck, Fak, STAT 5a/b, p70 CHK-2) activation needs to be further investigated, as perhaps its inhibition may be involved in the release of cytokines in COPD context. Taken as a whole, our findings suggest that RNO’s anti-inflammatory effect is complex and involves several kinases and pathways.


