A ROLE FOR 12R-LIPOXYGENASE IN MUC5AC EXPRESSION BY RESPIRATORY EPITHELIAL CELLS

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Running title: 12R-lipoxygenase induces mucin expression
ABSTRACT

Background: Eicosanoids are metabolites of arachidonic acid produced by cyclooxygenases (COXs) or lipoxygenases (LOXs). They mediate inflammation and mucus secretion in chronic pulmonary inflammatory diseases. The gel-forming mucin MUC5AC is overexpressed in the airways of these patients. MUC5AC expression is mediated by extracellular-signal-regulated kinase (ERK)/Sp1 dependent mechanism.

Aim: Study the role of eicosanoids and their signaling pathways in MUC5AC expression.

Results and Methods: Inhibitors of 12-LOX, but not those of COX, 5-LOX or 15-LOX, reduce MUC5AC expression induced by PMA in the bronchial epithelial cell line NCI-H292. These inhibitors also abrogate the production of whole mucus by cell monolayers. Two forms of 12-LOX (R and S) exist in mammals. Using siRNAs we show that 12R-LOX but not 12S-LOX is involved in MUC5AC expression induced by PMA, LPS or TGFα. 12R-LOX also participates in MUC2 and MUC5B expression although to a lesser extent than for MUC5AC. Contrarily, 12R-LOX silencing does not modify IL-8 production. 12-LOX inhibitors reduce ERK activation and Sp1 translocation induced by PMA. Moreover, the 12R-LOX product 12(R)-hydroxyeicosatetraenoic (12(R)-HETE), induces MUC5AC expression, ERK activation and Sp1 translocation.

Conclusion: 12R-LOX is involved in MUC5AC expression. This occurs via ERK- and Sp1 signaling pathways.
KEYWORDS

Eicosanoids, lipoxygenase, MUC5AC, mucins
INTRODUCTION

Mucins, the major proteins within the mucus, are highly glycosylated and consist of a protein backbone with a central domain (mucin domain) susceptible to O-glycosylation [1]. Gel-forming mucins polymerize through their cysteine-rich terminal domains and are secreted in the extracellular media. In the airways of adults, the most representative gel-forming mucins are MUC5AC, MUC5B and to a lesser extent MUC2 [2]. These mucins are clustered on the p15 arm of chromosome 11 [3] and they display common and different regulatory mechanisms of expression [4, 5]. MUC5AC is the most abundant gel-forming mucin present at the airway surface [6]. Its mechanisms of expression have been extensively studied, using the cell line NCI-H292 as a model [4, 7, 8]. Several studies from Nadel and co-workers have shown that PMA induces a matrix metalloprotease-mediated release of transforming growth factor α (TGFα), which subsequently binds and activates the EGF receptor (EGFR) to induce MUC5AC synthesis and secretion [8, 9]. Phosphorylation of EGFR is followed by activation of the extracellular-signal-regulated kinase (ERK)/Sp1 signaling pathway [4, 7]. EGFR participates in MUC5AC release induced by other pro-inflammatory stimuli like neutrophil elastase and bacterial lipopolysaccharide (LPS) [10].

Cytosolic phospholipase A2 (cPLA2) is a key enzyme controlling the release of arachidonic acid (AA). Once AA is released it is further metabolized by cyclooxygenases (COX) and/or lipoxygenases (LOX) to generate eicosanoids. Although early studies showed that eicosanoids regulate mucin production [11, 12], the underlying mechanisms remain unknown. Recently, we have shown a role of cPLA2 in mucus hypersecretion in a mouse model of cystic fibrosis (CF) [13]. COX catalyzes the biosynthesis of prostaglandins like PGE2. Both inhibitory and stimulatory effects of PGE2 on mucin secretion have been reported [12, 14]. More recently, two independent laboratories have shown a role for COX-2 in IL-1β-induced MUC5AC production [15, 16]. Conversely, LOX are dioxygenases that catalyze the
addition of oxygen to AA yielding hydroperoxyl derivatives including hydroperoxyeicosatetraenoic acids (HpETEs) and its reduced form HETE. Mammalian LOX are traditionally classified as 5-, 8-, 12-, or 15-LOX, according to the site of oxygen insertion within AA. In humans there are two different 12-LOX, the platelet-type, also called 12S-LOX (gene \textit{ALOX12}), and the epidermal-type termed 12R-LOX (gene \textit{ALOX12B}). Although both LOX generate 12-HETE, the 12S-LOX enzyme produces 12(S)-HETE enantiomer whereas the 12R-LOX enzyme, produces almost exclusively the 12(R)-HETE enantiomer, representing the only mammalian LOX that directs molecular oxygen into the R position of AA [17].

12S-LOX mediates a major pathway in the metabolism of AA on platelet activation [18]. Owen et al. have found an increased expression of 12-LOX in CF patients compared with controls in both the airways epithelium and submucosal glands [19], but physiological relevance of 12-LOX over-expression has not been established. The 12R-LOX pathway plays an important role in the establishment of the epidermal barrier function [20]. On the other hand, 15-LOX-1 is involved in inflammatory diseases, including atherosclerosis, cancer, osteoporosis, angiotensin II-dependent hypertension and diabetes [21]. Recently, 15-LOX-1 has been implicated in MUC5AC over-expression in asthma [22].

In chronic inflammatory diseases of the airway, such as chronic obstructive pulmonary disease (COPD), asthma and CF, exacerbated production of mucus contributes to airways obstruction favoring bacteria colonization. Although therapies have been proposed to treat mucus hyper-secretion, effective treatments are still lacking. Improving the understanding of the mechanisms of mucus production and secretion will help in the design of new pharmacological approaches in the treatment of mucus hyper-secretion. In this paper, using pharmacological and siRNA approaches, we demonstrated a role for 12R-LOX in mucin expression and particularly MUC5AC. Induction of MUC5AC expression by 12R-LOX
occurs via ERK and Sp1 signaling pathways. We propose for the first time a role of 12R-LOX in chronic pulmonary inflammatory diseases.

MATERIALS AND METHODS

Cell culture and stimulation of NCI-H292

NCI-H292 cells obtained from ATCC (Molsheim, France), were cultured as described [13]. After 24 h of culture under serum free conditions, cells were stimulated with PMA (20 nM), LPS (10 µg/ml) or TGFα (20 ng/ml) in serum free culture media except for LPS where 1% of serum was added. 12-HETE was dried from ethanolic solutions and added to the cells. For inhibition studies cells were pre-treated with the indicated drugs for 1 h before addition of PMA + inhibitor. Drugs used in the study were tested in a wide range of concentrations (0.1-50 µM) following other studies previously published. Effects of drugs on MUC5AC expression and cell viability were measured in parallel. Concentrations presented in the study for 12-LOX inhibitors (baicalein, CDC) showed an optimal inhibition of MUC5AC expression and no toxicity. Dose-respond effects were also observed (data not shown). All control conditions included corresponding vehicles at the appropriate concentrations (ethanol for NDGA and DMSO for PMA and other inhibitors). After 24 h in the presence of stimuli/inhibitors, supernatants were collected and the cells lysed in lysis buffer (150 mM sodium phosphate, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) deoxycholate, pH 7.2 supplemented with protease inhibitor cocktail). Pre-cleared samples (370xg for 10 min at 4°C) were stored at -80°C until analysis.

Cell culture and stimulation of primary human bronchial epithelial cells

Differentiated human primary bronchial epithelial cells, MucilAir, were purchased from Epithelix (Epithelix Sarl, Geneve, Switzerland). These cells were isolated from the
bronchi of healthy subject and cultured at air-liquid interface for 3 weeks in mucilAir culture medium (Epithelix) until differentiation. To reduce basal levels of mucin expression, cells were cultured for 48 h in BEBM basal medium (Lonza, CC-3171) supplemented with antibiotics. The cells were then stimulated in the same medium with bacteria supernatants or a equivalent dose of bacterial growth medium (LB) and 24 h after RNA extractions were performed.

Bacteria supernatants of *Pseudomonas aeruginosa* (PAK strain) were harvested at exponential-phase growth in Luria-Bertani (LB) medium. After centrifugation to eliminate bacteria, supernatants and LB medium (used as control) were filtered (0,2 µm) and stored at -80°C until use.

**siRNA experiments**

Pre-designed siRNAs from Santa Cruz Biotechnologies (Santa Cruz, CA) or control siRNAs were incubated with TransIT-siQUEST transfection reagent (Mirus Bio, Madison, WI) (1µl per well) for 20 min at room temperature in serum-free culture media (50 µl final volume). NCI-H292 were grown until sub-confluent (80 %) and cultured in complete cell culture media (350 µL) containing the appropriate siRNA (final concentration 100 nM). After 24 h of incubation, media was removed and new medium (serum free) plus stimuli were added for 24 h more. Real time RT-PCR and Western blotting were performed to follow-up silencing at 24 h or 48 h after transfection.

**ELISA**

MUC5AC ELISA was performed as described in [13, 23] using anti-MUC5AC (clone 45M1, Neomarkers, Fremont, CA) and HRP-conjugated goat anti-mouse IgG antibodies. Because expression of MUC5AC in NCI-H292 changes with cell passage [23] and there is no commercial standard available for human MUC5AC, its expression is represented as -fold
increase referred to basal production (control cells). Specificity of the 45M1 clone has been
demonstrated [24]. Total protein from cell lysates was quantified in order to eliminate effects
of the stimuli in cell proliferation.

Interleukin-8 (IL-8) and PGE2 concentrations were measured in supernatants using a human
IL-8 Kit DuoSet sandwich ELISA (R&D Systems, Abingdon, UK) and specific PGE2 EIA
(Cayman Chemicals), respectively.

**Real time RT-PCR**

Total RNA was extracted by RNeasy Mini Kit (Qiagen, Valencia, Spain). The corresponding
cDNA was synthesized using random hexamers (Roche) and M-MLV reverse transcriptase
(Promega). Real-time polymerase chain reaction (PCR) was performed in 7500 Fast Real-
Time PCR System (Applied Biosystems, Carlsbad, CA) using the SYBR Green Master Mix
(Applied Biosystems). The primers for MUC5AC, MUC5B and MUC2 have been described
in [25]. Primers for 12R-LOX were from [26]. Other primers were designed using the Oligo
Explorer 1.1.2 software. The primers were the following: human β-actin (Fw:5’-
ggacctcgagaagagatgg-3’; Rv:5’-gcagtgatctctcttgcatc-3’), platelet-type 12-lipoxygenase
(Fw:5’-ccgaggagaagcaatacc-3’; Rv:5’-tgagggcaggaacagtgt-3’), IL-8 (Fw:5’-
agagacagcagcaaccaac-3’; Rw:5’-ttagcactctttggeaaaaac-3’), 18sRNA (Fw:5’-
cttagagggacaagtgg -3’; Rw:5’- acgctgagccagtctgta-3’). Triplicate Ct values were
analyzed in Microsoft Excel using the comparative Ct (ΔΔCt) method as described by the
manufacturer (Applied Biosystems). The amount of target (2^ΔΔCt) was obtained as
normalized to β-actin, using control cells as calibrator (arbitrary units=1) unless stated.

**Western Blotting**

NCI-H292 cells in lysis buffer described above or RIPA buffer supplemented with
phosphatase-inhibitor and protease-inhibitor cocktail were electrophoresed and blotted in
PVDF membranes. Specific proteins were detected with rabbit anti- human phospho-p44/42
MAPK antibody (Cell Signaling), mouse anti-human 12R-LOX antibody (Abnova, 242-A01,
Taipei, Taiwan), mouse anti-human β-actin antibody (Sigma, A5316) and the corresponding secondary antibodies (anti-mouse IgG for 12R-LOX and β-actin primary antibodies or anti-rabbit IgG for anti-phospho-p44/42 primary antibody) HRP-labeled. ECL (GE Healthcare, Saclay-Orsay, France) was used as chemiluminescence detection kit.

**EMSA**

Nuclear proteins were extracted from 2x10^6 cells as described previously [27]. The double-stranded oligonucleotide containing a Sp1 binding site consensus sequence (5’-attcgatcggggcgggccgacg-3’) was labelled with [gamma-^32^P]dATP (3.000 Ci/mmol at 10 mCi/ml) and purified using a ProbeQuant G-50 micro column (GE Healthcare). The following steps were performed as described in [28]. Binding reactions were performed in 40 mM HEPES, pH 7.6, 10 mM NaCl, 1.5mM MgCl2, 2 % glycerol, containing 1 µg of poly(dI-dC) poly(dI-dC), 0.8 µCi of labeled oligonucleotide (1 ng) and 5 µg of nuclear extract. The binding specificity was assessed with 50-fold excess cold specific (Sp1 consensus) or nonspecific oligonucleotide (PPAR consensus).

**Alcian Blue Staining**

NCI-H292 cells were fixed (formalin 10 %, 10 min) and stained with alcian blue colorant in acetic acid (pH 2.5) for 30 min at room temperature. Cells were counterstained with saphranine for nuclei observation, mounted and observed in a Nikon E 800 microscope. Pictures were acquired using a Nikon digital still DXM 1200 camera controlled by ACT-1 software version 2.

**Cell viability**

LDH activity released from cells was measured with the CytoTox 96 Nonradioactive Cytotoxicity assay (Promega, Charbonnières-les-Bains, France)

**Statistical analysis**
Data are presented as means ± SD. ANOVA was used to determine statistically significant differences among groups followed by Student Newman-Keuls multiple range test for paired comparisons. When indicated, Students' t-test was used to compare two groups. P<0.05 was considered significant.

RESULTS

Involvement of LOX pathway in PMA-induced MUC5AC production

We aimed at identifying AA metabolites and signaling pathways involved in MUC5AC expression. For this purpose, we examined MUC5AC levels in supernatants and cell lysates of PMA-stimulated NCI-H292 cells, in the presence of specific COX or LOX inhibitors. Only results from cell lysates are presented but similar findings were observed on supernatants. We examined the effect of two COX inhibitors (Aspirin, NS398) and a general LOX inhibitor (NDGA) on MUC5AC expression. NDGA significantly reduced PMA-induced MUC5AC expression, whereas COX inhibitors had no effect (Figure 1A). We verified that COX inhibitors efficiently reduced PGE₂ release (Figure 1B). These data indicate that the LOX but not the COX pathway is involved in MUC5AC expression in PMA stimulated cells.

Role of 12-LOX in MUC5AC production

Using more selective LOX inhibitors, we observed that only those targeting 12-LOX (CDC, baicalein) but not those targeting 5-LOX (BW-A4C, Zileuton) or 15-LOX inhibitors (PD-146176), reduced PMA-induced MUC5AC expression (Figure 2A). In contrast, neither CDC nor baicalein reduced IL-8 secretion induced by PMA (data not shown). Furthermore, both baicalein and CDC decreased significantly PMA-induced MUC5AC mRNA expression (Figure 2B). Effects of these inhibitors were not due to cell cytotoxicity as measured by LDH
release (materials and methods section). Therefore, these results indicate that 12-LOX modulates MUC5AC expression at a transcriptional level.

**12R-LOX is involved in MUC5AC expression**

Because NCI-H292 cells express both 12S-LOX and 12R-LOX (Figure Suppl-1), we examined the impact of transcriptional silencing of each 12-LOX form on MUC5AC expression. As Figure 3A shows, silencing of 12R-LOX expression decreased PMA-, TGFα- and LPS-induced MUC5AC expression. MUC5AC inhibition was more pronounced after PMA treatment (53%) compared to TGFα (34%) and LPS treatment (24%) (Figure 3B). By contrast, 12S-LOX silencing had no effect on either PMA- or TGFα-induced MUC5AC (Figure 3A and 3B), though, surprisingly; it significantly enhanced LPS-induced MUC5AC production (Figure 3A and 3B). Next, we analyzed the effect of 12-LOX silencing on MUC5AC mRNA expression in PMA-stimulated cells. Silencing of 12R-LOX, but not that of 12S-LOX, reduced MUC5AC mRNA expression (Figure 3C and 3D). Interestingly, 12R-LOX siRNA did not modify PMA-induced IL-8 secretion (Figure 4A and 4B). Taken together these data show a role for 12R-LOX in the induction of MUC5AC expression.

**12-LOX inhibitors reduce PMA-induced ERK and Sp1 activation**

In NCI-H292 cells, activation of PKC by PMA, activates ERK and translocates Sp1 which binds to MUC5AC promoter to initiate transcription [7, 9]. As shown in Figure 5A, CDC and baicalein reduced PMA-induced ERK phosphorylation. Moreover, EMSA analyses showed an enhanced Sp1 translocation in PMA stimulated cells (Figure 5B). Conversely, baicalein reduced the Sp1 shifted band (Figure 5B). Similar results were obtained with the other 12-LOX inhibitor, CDC (data not shown). Taken together, these data suggest that 12-LOX plays a role in PMA-induced ERK-Sp1 activation.
12(R)-HETE up-regulates MUC5AC expression and activates ERK-Sp1 signaling pathways

12(R)-HETE, a 12R-LOX product, induced MUC5AC protein in a dose dependent-manner, with a maximal effect at 8 μM (Figure 6A). Furthermore, 12(R)-HETE was able to activate ERK (Figure 6B), whilst the MEK1/2 (ERK kinase) inhibitor (PD98059) blocked 12(R)-HETE-induced ERK phosphorylation (Figure 6B). In addition, 12(R)-HETE induced Sp1 translocation with a peak of activity at 2 h after stimulation (Figure 6C). These findings indicate that 12(R)-HETE stimulates the ERK-Sp1-signalling pathway.

12R-LOX also regulates other respiratory mucins

We examined whether 12R-LOX also modulates PMA-induced MUC5B and MUC2 expression. Silencing of 12R-LOX reduced MUC5B and MUC2 expression by 28 % and 35 %, respectively (Figure 7A). These effects were less pronounced than those observed on MUC5AC (53% inhibition) (Figure 7A). Finally, we investigated the role of 12-LOX on the production of whole mucus by NCI-H292 cells. This was performed using Alcian Blue (AB) staining on cell monolayers in the presence or absence of baicalein. An increased AB staining was observed in cells stimulated with PMA compared to non-stimulated cells (Figure 7B), reflecting production of mucus induced by PMA. Baicalein strongly reduced this staining (Figure 7B), a finding confirmed when cells were treated with CDC (data not shown).

12-LOX regulates respiratory mucins in primary human bronchial epithelial cells

To examine the role of 12-LOX in the expression of respiratory mucins in primary cells, human bronchial epithelial cells were growth in air-liquid interface and stimulated with supernatants of Pseudomonas aeruginosa (P.a), a pathophysiological stimuli in CF patients.
P. a supernatants induced MUC5AC and MUC2 (Figure 8A and 8B) but not MUC5B expression (Figure 8C). The presence of baicalein reduced 46 % and 36 % of the P. a-induced expression of MUC5AC and MUC2, respectively (Figure 8A and 8B).

DISCUSSION

In the present work, performed on the NCI-H292 cell line and primary bronchial epithelial cells, we demonstrate a key role for 12-LOX in mucin MUC5AC expression. We have used PMA as mucin inducer because previous studies have shown that expression of MUC5AC induced by this drug is dependent of EGFR activation, a key receptor controlling mucin production in the airways [10]. EGFR neutralizing antibodies or inhibitors of EGFR phosphorylation blocked PMA-induced MUC5AC expression [9]. In selected experiments more physiological stimuli (TGFα, LPS, supernatants of Pseudomonas aeruginosa) have been used to demonstrate the involvement of 12-LOX in mucin expression. Downstream signaling pathways after EGFR activation by PMA conducting to MUC5AC expression have been partially characterized [4, 7] but the role of 12-LOX in these pathways has not been explored.

Although active COX enzymes are present in NCI-H292 cells, COX inhibitors had no effect on PMA-induced MUC5AC expression. Previous works have shown a role of COX in MUC5AC expression [15, 16]. However, it should be noted that in these reports the authors used IL-1β as MUC5AC inducer instead of PMA. IL-1β signaling pathways leading to mucin expression differ from those for PMA. PMA induces MUC5AC expression via matrix metalloprotease-mediated release of TGFα, which subsequently activates EGFR [29], a mechanism different from that for IL-1β [15]. Moreover, Gray et al. [15] used a model of tracheobronchial epithelial cells, in which different cell types are present (e.g. ciliated, basal, mucus cells). Therefore, in their model, one possibility is that COX metabolites modulate MUC5AC expression by mucus producing cells via a paracrine mechanism. In contrast, our
study was performed on a single cell type, which involves the study of the autocrine regulation of mucin expression.

LOX represents a family of enzymes that play diverse biological roles. Using pharmacological and transcriptional inhibition we explored which LOX is involved in MUC5AC expression. We first examined whether 5-LOX, which leads to leukotriene production, is involved in MUC5AC expression. Indeed, leukotrienes have been shown to induce mucus expression in the lung [11], and human bronchial epithelial cells express an active 5-LOX [30]. Our results showed that this LOX is not involved in MUC5AC expression in our cell system.

A recent study has shown that a 15-LOX-1 inhibitor abrogates MUC5AC expression induced by long-term IL-13 stimulation of bronchial epithelial cells [22]. However, in the present study, using the same 15-LOX-1 inhibitor reported in the study by Zhao et al. [22], we found that this enzyme is not involved in PMA-induced MUC5AC expression. We also observed that the 15-LOX-1 inhibitor did not reduce TGFα-induced MUC5AC expression (data not shown). Here again, differences in the signaling pathways activating MUC5AC expression (IL-13 receptor-dependent vs. EGFR-dependent pathways) and duration of the stimulation, might explain these discrepancies between our findings and those of Zhao et al. [22].

Our pharmacological approach showed that 12-LOX is the only LOX that plays a role in MUC5AC expression in NCI-H292 cells. Because two 12-LOX (12R-LOX and 12S-LOX) encoded by distinct genes are present in mammalian cells, we used a siRNA strategy to identify which form is involved in mucus production in our cell system. Our results showed that PMA-induced MUC5AC expression is reduced by 12R-LOX but not by 12S-LOX siRNA. Interestingly, using more physiological stimuli, TGFα and LPS (ligands for EGFR and TLR-4 receptor respectively, two key receptors in lung pathophysiology), we demonstrate
a stimulating role of 12R-LOX in TGFα and LPS-induced MUC5AC expression. We also showed that 12R-LOX plays a role in the modulation of MUC5B and MUC2 expression and in the production of whole mucus by NCI-H292 monolayers induced by PMA. Moreover, induction of mucins by P.a supernatants, a pathogen related to lung dysfunction in CF, was reduced by a 12-LOX inhibitor in primary bronchial epithelial cells. All together, our data suggest a role of 12-LOX in the expression of mucins relevant to the pathogenesis of lung obstructive diseases.

Trans-activation of EGFR by PMA [9] is known to induce ERK activation and binding of the transcription factor Sp1 to the MUC5AC promoter [4, 7]. Here, we show that 12R-LOX siRNA inhibits TGFα-induced MUC5AC expression and that 12-LOX inhibitors reduce ERK activation and Sp1 translocation induced by PMA. Altogether, these results suggest that 12R-LOX, but not 12S-LOX, mediates PMA-induced MUC5AC expression via an EGFR-related signaling pathway involving ERK- and Sp1 activation.

We next examined whether 12(R)-HETE, one product of 12R-LOX, mediates MUC5AC expression in NCI-H292 cells. We show that addition of 12R-HETE stimulates MUC5AC expression, ERK activation and Sp1 translocation. However, the effects of 12R-HETE were less pronounced than those of PMA in terms of MUC5AC production. This could be explained by: i) 12R-LOX independent pathways are involved in PMA-induced MUC5AC expression; ii) other metabolites derived (directly or indirectly) from 12R-LOX activity might also participate in the process. Regarding the mechanism through 12(R)-HETE induces MUC5AC, 12(R)-HETE is a potent activator of the arylhydrocarbon receptor (AhR) [31], a ligand-regulated transcription factor related to environmental insults. Activation of AhR receptor results in upregulation of MUC5AC expression in mouse lungs and in NCI-H292 cells [32, 33]. Therefore, 12R-LOX stimulating action could be explain by the capacity of 12R-LOX products (e.g. 12(R)-HETE) to activate AhR.
In conclusion, this study demonstrates that 12R-LOX, an enzyme that produces AA metabolites with R-chirality, mediates mucus production by mucus producing cells, a main feature of pulmonary obstructive diseases. Therefore, 12R-LOX represents a suitable new target for therapeutic intervention to reduce mucus production in these pathologies. Moreover, bronchioloalveolar carcinoma occurs with excessive production of sputum associated to over-expression of respiratory gel forming mucins. Therapies preventing EGFR activation have shown beneficial effects in these patients [34]. The role of 12-LOX in EGFR-dependent mucin production suggests 12-LOX inhibitors as potential agents for the treatment of bronchioloalveolar carcinoma.

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REFERENCES


**FOOTNOTES**
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FIGURE LEGENDS
Figure 1. Expression of MUC5AC protein and PGE\(_2\) secretion induced by PMA in the presence of COX and LOX inhibitors. A) MUC5AC protein was measured by ELISA in NCI-H292 cell lysates after 24 h of stimulation with PMA (20 nM) in the presence of aspirin (200 \(\mu\)M), NS398 (5 \(\mu\)M) or NDGA (20 \(\mu\)M). Cells were pre-treated with the indicated drugs for 1 h before addition of PMA. Data are represented as fold increase referred to non-stimulated cells (CTRL) (arbitrary units=1). Inhibitors alone did not modify MUC5AC basal secretion levels (data not shown). B) Concentration of PGE\(_2\) measured by EIA in supernatants of NCI-H292 cells stimulated as described in A). Mean ± SD of triplicates from three independent experiments is represented. * p<0.05 vs. PMA treatment without inhibitors.

![Figure 1](image1.png)

Figure 2. Expression of MUC5AC induced by PMA in the presence of LOX inhibitors. MUC5AC protein (A) or mRNA (B) were measured in NCI-H292 cell lysates after 24 h of stimulation with PMA (20 nM) in the presence of BW-A4C, Zilueton, PD-146176, Baicalein (all drugs at 20 \(\mu\)M) or CDC at 10 \(\mu\)M. Cells were pre-treated with the indicated drugs for 1 h before addition of PMA. Data are represented as fold increase referred to non-stimulated cells (CTRL) (arbitrary units=1). Panel B inset, % of MUC5AC expression in PMA-stimulated

![Figure 2](image2.png)
cells is referred to cells stimulated only with PMA (100%). In A) and inset B), the Mean ± SD of triplicates from three independent experiments is represented. In B), Mean ± SD of triplicates from one representative experiment of a total of three. * p<0.05 vs. PMA treatment without inhibitors.

Figure 3. Expression of MUC5AC in 12-LOX silenced NCI-H292 cells. Cells transfected with siRNA were stimulated with PMA (20 nM), TGFα (20 ng/ml) or LPS (10 μg/ml) for 24 h. Then, MUC5AC protein was measured in cell lysates as fold increase referred to non-stimulated/non-transfected cells (CTRL) (arbitrary units=1) in A), or referred to stimulated/control si-RNA(si-ctrl)-transfected cells (arbitrary value=100 %), in B). In Panel C, mRNA was measured and fold increase expressed considering non-stimulated/si-ctrl-transfected cells =1. In D: percent of MUC5AC expression in PMA-stimulated cells is referred to si-ctrl transfected cells (100 %). Mean ± SD of triplicates are showed from one representative experiment of a total of three in A) and C). In B) and D) Mean ± SD of triplicates from three independent experiments. * p<0.05 vs. cells transfected with si-ctrl and stimulated with the corresponding stimulus.
**Figure 4.** Expression of IL-8 induced by PMA in 12R-LOX silenced NCI-H292. Cells transfected with 12R-LOX siRNAs (100 nM) were stimulated with PMA (20 nM) for 24 h. Then, IL-8 protein was measured in cell supernatants by sandwich ELISA (A) and IL-8 mRNA expression by real time RT-PCR (B). Means ± SD of triplicates are shown from one representative experiment of a total of three.
Figure 5. Activation of ERK and Sp1 translocation induced by PMA in the presence of 12-LOX inhibitors. A) Serum deprived NCI-H292 cells were pre-incubated 2 h with either baicalein (20 μM) or CDC (10 μM) and stimulated for 20 min with PMA (20 nM) at 37°C. Afterwards cells lysates were collected in RIPA buffer, electrophoresed (12 % SDS-PAGE) and blotted. Activated ERK (P-ERK) was detected using an anti-phospho-p44/42 MAPK antibody. Membranes were stripped and re-probed with anti-β-actin (ACTB) antibodies. The P-ERK reactive band was normalized to ACTB baseline value. One representative blot is shown of a total of three independent experiments. Densitometric units are from three independent experiments considering P-ERK/ACTB from PMA stimulated cells as 100% * p<0.05, ** P<0.01 vs. PMA treatment without inhibitors. B) Serum deprived NCI-H292 cells pretreated or not with baicalein (20 μM) were stimulated for 4h with PMA (20 nM). After extraction of nuclear proteins, 5 μg were incubated with 32P-labeled consensus Sp1 probe for 30 min at 4°C in binding buffer (40 mM HEPES, pH 7.6, 10 mM NaCl, 1.5 mM MgCl2) in the presence of 50-fold molar excess of cold specific consensus Sp1 probe (+cold Sp1, line) or cold irrelevant PPAR consensus probe (+cold PPAR, line). Sp1-probe complexes (arrow) were separated from free probe (*) in 5 % polyacrylamide native gel pre-chilled at 4°C. In lane-1: only probe without nuclear proteins; lane-2,3,5: nuclear proteins from PMA stimulated cells ; lane-4: nuclear proteins from control cells; lane-6: nuclear proteins from baicalein treated cells; lane-7: nuclear proteins from baicalein and PMA treated cells. One representative gel from three independent experiments is shown.
**Figure 6.** 12(R)-HETE induces MUC5AC expression, ERK activation and Sp1 translocation in NCI-H292. A) 12(R)-HETE or vehicle were added to cells. After 24 h of culture, MUC5AC protein was measured in cell lysates by ELISA (A). Mean ± SD is represented as fold increase normalized to MUC5AC protein level in basal conditions from three independent experiments performed in triplicates. * p<0.05 vs. non stimulated cells (t-Student). B) ERK phosphorylation (P-ERK) was analyzed as described in Figure 5A after 20 min of incubation with PMA (20 nM), PD98059 (20 μM), 12(R)-HETE (2 μM(+) or 8 μM (++)) or the combinations indicated in the table. PD98059 was added also 30 min before either PMA or 12(R)-HETE. C) Cells were treated with 12(R)-HETE (8 μM) at indicated times (Ctrl, t=0) before perform EMSA as described in Figure 5B.
Figure 7. Modulation of airway mucins and mucus by 12R-LOX. A) Real time RT-PCR from PMA-stimulated cells transfected with 12R-LOX siRNA (closed bars) or control siRNA (si-ctrl, grey bars), was performed with specific MUC5AC, MUC5B or MUC2 primers as described in Methods section. Levels of expression were normalized to PMA stimulated cells transfected with control siRNA (100%). Means ± SD of triplicates from three independent experiments are represented. * p<0.05 vs. si-ctrl transfected cells. B) Cells were first pre-incubated or not 1 h with baicalein (20 mM) and then stimulated or not for 24 h with PMA (20 nM). Cells were then fixed and stained with AB (acidic mucins in blue) and saphranine (nuclei in red). Scale bar = 100 μM. Representative images from one out of two independent experiments are shown. Treatment with CDC (10 μM) showed the same results (data not shown).
Figure 8. Effect of Baicalein in *P. aeruginosa* supernatant-induced mucins expression in human primary epithelial cells. The cells were cultured in air-liquid interface and pretreated with baicalein (Baic, 20 µM) one hour and during treatment with bacteria (*Pseudomonas aeruginosa*, strain PAK) supernatants diluted at 1:8 or bacteria culture medium, Luria-Bertani (LB, 1:8) for 24 h as indicated in Material and Methods. Total mRNA was prepared and subjected to RT-qPCR to measure MUC5AC (A), MUC2 (B) and MUC5B (C) levels. The results show the percentage of inhibition of mucins mRNA expression by Baicalein compared to cells treated with PAK supernatant alone. Values represent means ± SD of 3 independent experiments from three different donors. * $P<0.05$; ** $P<0.01$ and *** $P<0.001$. 
Supplementary data

**Figure Suppl-1.** Expression of 12-LOX in NCI-H292. Cells transfected with 12R-LOX siRNAs (si-12R-LOX), 12S-LOX siRNAs (si-12S-LOX) or control si-RNAs (si-ctrl) (100 nM, final concentration) were stimulated with PMA (20 nM) for 24 h. Then, total RNA or cell lysates were obtained. Real time RT-PCR was performed using specific primers for 12S-LOX (panel A) or 12R-LOX (panel B). In C) Western blots from non-stimulated NCI-H292 cells transfected with si-ctrl or si-12R-LOX were performed using anti-12R-LOX antibodies, followed by stripping and re-probing of membranes with anti-β-actin (ACTB) antibodies. *Jurkat* lane, corresponds to a cell extract from Jurkat clone E6-1 cell line (positive control for the antibody). The *arrow* indicates 12R-LOX specific band. *Asterisk* points non-specific band detected by the antibody. Control siRNAs did not modify 12-LOX expression neither in basal or PMA-induced conditions (data not shown). D) Quantification of 12R-LOX band intensity
normalized to ACTB from C). In A) and B), Mean ± SD of triplicates from three independent experiment is shown. * p<0.05 vs. cells transfected with control siRNAs (si-ctrl).