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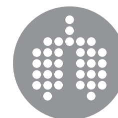

A role for 12R-lipoxygenase in MUC5AC expression by respiratory epithelial cells

Ignacio Garcia-Verdugo*,#,¶, Fatima BenMohamed*,#, Sonja Tattermusch*,#, Dominique Leduc*,#, Gilles Charpigny+, Michel Chignard*,#, Mario Ollero§ and Lhousseine Touqui*,#

ABSTRACT: **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 over-expressed in the airways of patients with these diseases. MUC5AC expression is mediated by an extracellular signal-regulated kinase (ERK)/Sp1 dependent mechanism.**

Our aim was to study the role of eicosanoids and their signalling pathways in MUC5AC expression.

Inhibitors of 12-LOX, but not those of COX, 5-LOX or 15-LOX, reduce MUC5AC expression induced by phorbol myristate acetate (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, lipopolysaccharide or transforming growth factor-α. 12R-LOX also participates in MUC2 and MUC5B expression, although to a lesser extent than for MUC5AC. Contrarily, 12R-LOX silencing does not modify interleukin-8 production. 12-LOX inhibitors reduce ERK activation and Sp1 translocation induced by PMA. Moreover, the 12R-LOX product 12(R)-hydroxyeicosatetraenoic acid, induces MUC5AC expression, ERK activation and Sp1 translocation.

12R-LOX is involved in MUC5AC expression. This occurs *via* ERK- and Sp1-signalling pathways.

KEYWORDS: **Eicosanoids, lipoxygenase, MUC5AC, mucins**

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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 polymerise 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 studied extensively, using the cell line NCI-H292 as a model [4, 7, 8]. Several studies from NADEL and co-workers [8, 9] have shown that phorbol myristate acetate (PMA) induces a matrix metalloprotease-mediated release of transforming growth factor-α (TGF-α), which subsequently binds and activates the epidermal growth factor receptor (EGFR) to induce MUC5AC synthesis and secretion. Phosphorylation of EGFR is followed by activation of the extracellular signal-regulated kinase (ERK)/Sp1 signalling pathway [4, 7]. EGFR participates in MUC5AC release induced by other pro-inflammatory stimuli like neutrophil elastase and bacterial lipopolysaccharide (LPS) [10].

Cytosolic phospholipase A_2 ($cPLA_2$) is a key enzyme controlling the release of arachidonic acid (AA). Once AA is released it is further metabolised by cyclooxygenases (COXs) and/or lipoxygenases (LOXs) 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 $cPLA_2$ in mucus hypersecretion in a mouse model of cystic fibrosis (CF) [13]. COX catalyses the biosynthesis of prostaglandins such as prostaglandin E_2 (PGE_2). Both inhibitory and stimulatory

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effects of PGE_2 on mucin secretion have been reported [12, 14]. More recently, two independent laboratories have shown a role for COX-2 in interleukin (IL)-1β-induced MUC5AC production [15, 16]. Conversely, LOXs are dioxygenases that catalyse the addition of oxygen to AA, yielding hydroperoxyl derivatives including hydroperoxyeicosatetraenoic acids (HpETEs) and the reduced form, HETE. Mammalian LOXs 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-LOXs, the platelet-type, also called 12S-LOX (gene *ALOX12*), and the epidermal-type termed 12R-LOX (gene *ALOX12B*). Although both LOXs generate 12-HETE, the 12S-LOX enzyme produces the 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]. OWENS *et al*. [19] have found an increased expression of 12-LOX in CF patients compared with controls in both the airways epithelium and submucosal glands, but the physiological relevance of 12-LOX overexpression has not been established. The 12R-LOX pathway plays an important role in the establishment of the epidermal barrier function [20]. Conversely, 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 overexpression in asthma [22].

In chronic inflammatory diseases of the airway, such as chronic obstructive pulmonary disease, asthma and CF, exacerbated production of mucus contributes to airway obstruction, favouring bacteria colonisation. Although therapies have been proposed to treat mucus hypersecretion, 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 hypersecretion. In this paper, using pharmacological and siRNA approaches, we demonstrate a role for 12R-LOX in mucin expression and, particularly, MUC5AC. Induction of MUC5AC expression by 12R-LOX occurs *via* ERK and Sp1 signalling 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 $\mu g \cdot mL^{-1}$) or TGF-α (20 $ng \cdot mL^{-1}$) in serum-free culture media, except for LPS, to which 1% of serum was added. 12-HETE was dried from ethanolic solutions and added to the cells. For inhibition studies cells were pretreated with the indicated drugs for 1 h before addition of PMA and inhibitor. Drugs used in the study were tested in a wide range of concentrations (0.1–50 μM) following other studies previously published [15, 16, 22]. Effects of drugs on MUC5AC expression and cell viability were measured in parallel. Concentrations presented in the study for 12-LOX inhibitors (baicalein, cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC)) showed an optimal inhibition of MUC5AC expression and no toxicity. Dose–response effects were also observed (data not shown). All control conditions included corresponding vehicles at the appropriate concentrations (ethanol for nordihydroguaiaretic acid (NDGA) and dimethylsulphoxide 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 (370 × *g* 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 subjects and cultured at the 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 bronchial epithelial cell basal medium (CC-3171; Lonza, Levallois-Perret, France) supplemented with antibiotics. The cells were then stimulated in the same medium with bacteria supernatants or an equivalent dose of bacterial growth medium (Luria–Bertani (LB)) and 24 h after RNA extractions were performed.

Bacteria supernatants of *Pseudomonas aeruginosa* (PAK strain) were harvested at exponential-phase growth in 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, USA) or control siRNAs were incubated with TransIT-siQUEST transfection reagent (Mirus Bio, Madison, WI, USA) (1 μL 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 were removed and new medium (serum-free) plus stimuli were added for a further 24 h. 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 previously described [13, 23] using anti-MUC5AC (clone 45M1, Neomarkers, Fremont, CA, USA) and horseradish peroxidise (HRP)-conjugated goat anti-mouse immunoglobulin (Ig)G 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 a -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.

IL-8 and PGE_2 concentrations were measured in supernatants using a human IL-8 Kit DuoSet sandwich ELISA (R&D Systems, Abingdon, UK) and specific PGE_2 EIA (Cayman Chemicals, Ann Arbor, MI, USA), respectively.

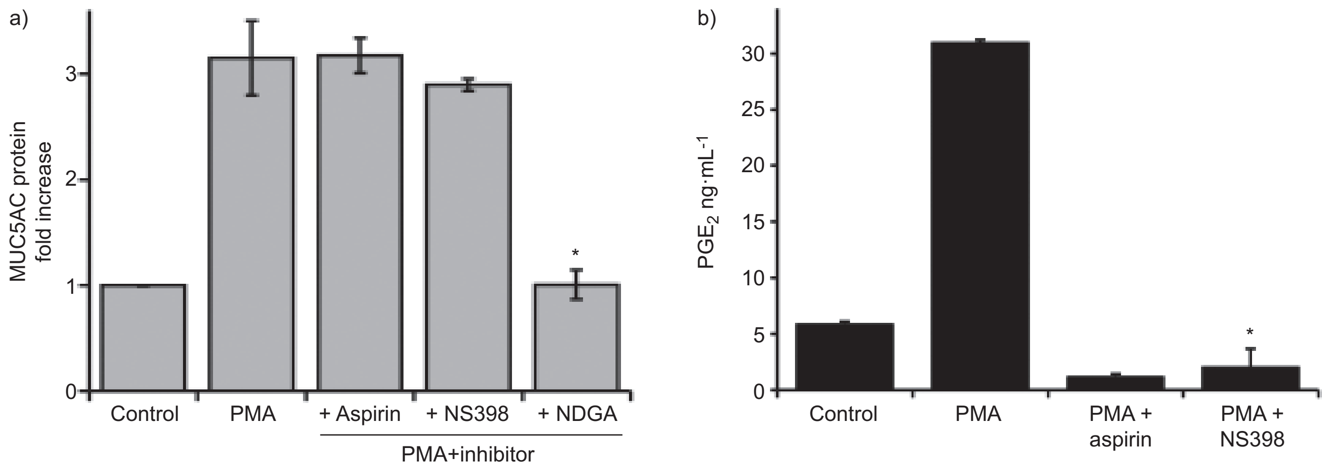

FIGURE 1. Expression of MUC5AC protein and prostaglandin E_2 (PGE_2) secretion induced by phorbol myristate acetate (PMA) in the presence of cyclooxygenase (COX) and lipoxygenase (LOX) inhibitors. a) MUC5AC protein was measured by ELISA in NCI-H292 cell lysates after 24 h of stimulation with PMA (20 nM) in the presence of aspirin (200 μM), NS398 (5 μM) or nordihydroguaiaretic acid (NDGA) (20 μM). Cells were pretreated with the indicated drugs for 1 h before addition of PMA. Data are represented as -fold increase referred to non-stimulated cells (control) (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. *: $p<0.05$ *versus* PMA treatment without inhibitors.

RT-PCR

Total RNA was extracted by RNeasy Mini Kit (Qiagen, Valencia, Spain). The corresponding cDNA was synthesised using random hexamers (Roche, Boulogne-Billancour, France) and M-MLV reverse transcriptase (Promega, Charbonnières-les-Bains, France). Real-time PCR was performed in 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using the SYBR Green Master Mix (Applied Biosystems). The primers for MUC5AC, MUC5B and MUC2 have been described previously [25]. Primers for 12R-LOX were as described by NIGAM *et al.* [26]. Other primers were designed using the Oligo Explorer 1.1.2 software. The primers were the following: human β-actin (Fw:5′-ggacttcgagcaagagatgg-3′; Rv:5′-gcagtgatctccttctgcatc-3′), platelet-type 12-lipoxygenase (Fw:5′-ccgaggagagaagcaatacc-3′; Rv:5′-tgagggcaggaacagtgt-3′), IL-8 (Fw:5′-agagacagcagagcacacaa-3′; Rw:5′-ttagcactccttggcaaaac-3′), 18sRNAr (Fw:5′- cttagagggacaagtggcg-3′; Rw:5′- acgctgagccagtcagtgta-3′). Triplicate Ct values were analysed in Microsoft Excel (Microsoft, Issy-les Moulineaux, France) using the comparative Ct (ΔΔCt) method as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta CT}$) was obtained as normalised to β-actin, using control cells as calibrator (arbitrary units=1) unless stated.

Western blotting

NCI-H292 cells in lysis buffer described above or radio-immunoprecipitation assay (RIPA) buffer supplemented with phosphatase-inhibitor and protease-inhibitor cocktail were electrophoresed and blotted in polyvinylidene fluoride (PVDF) membranes. Specific proteins were detected with rabbit anti-human phospho-p44/42 MAPK antibody (Cell Signaling, Danvers, MA, USA), mouse anti-human 12R-LOX antibody

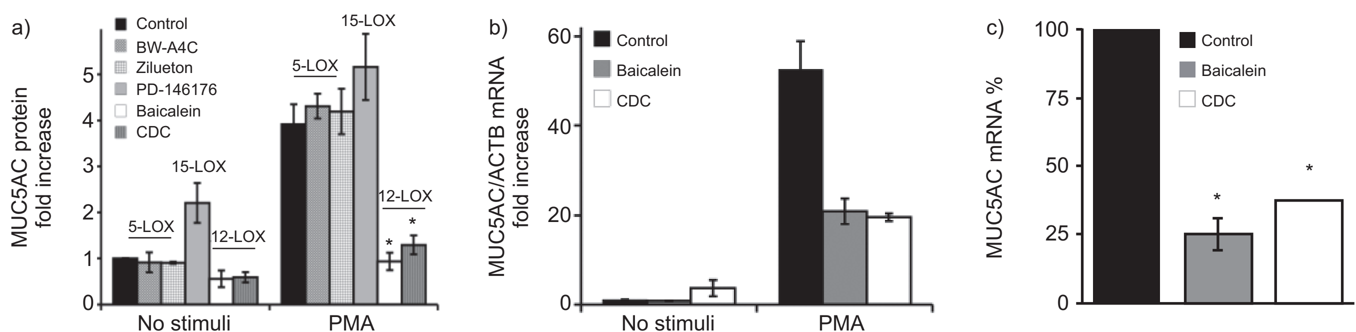

FIGURE 2. Expression of MUC5AC induced by phorbol myristate acetate (PMA) in the presence of lipoxygenase (LOX) inhibitors. a) MUC5AC protein or b) mRNA were measured in NCI-H292 cell lysates after 24 h of stimulation with PMA (20 nM) in the presence of BW-A4C, zileuton, PD-146176, baicalein (all drugs at 20 μM) or cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) at 10 μM. 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 (control) (arbitrary units=1). c) % MUC5AC expression in PMA-stimulated cells referred to cells stimulated only with PMA (100%). In a) and c) 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 is shown. *: $p<0.05$ *versus* PMA treatment without inhibitors.

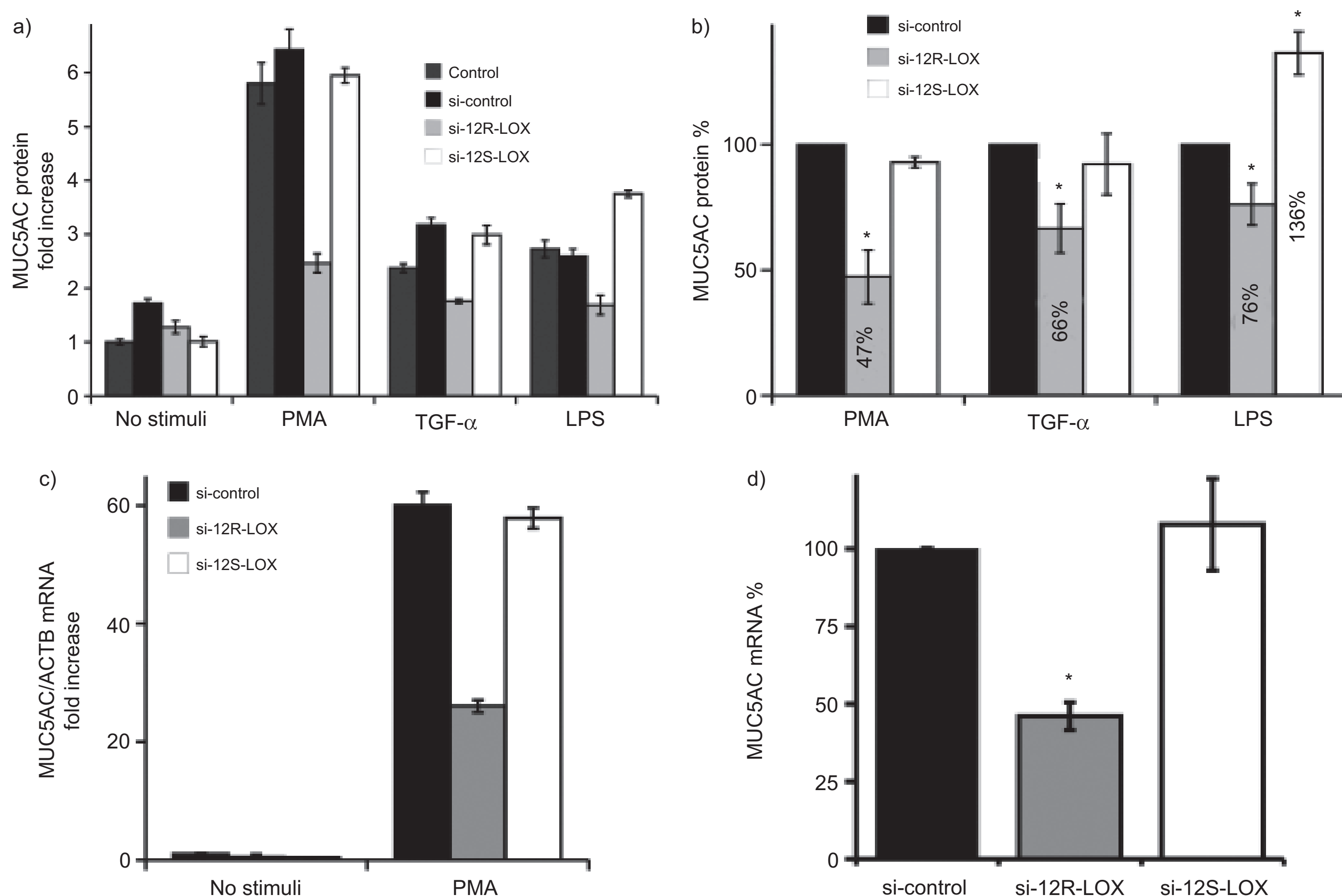

FIGURE 3. Expression of MUC5AC in 12-lipoxygenase (LOX) silenced NCI-H292 cells. Cells transfected with siRNA were stimulated with phorbol myristate acetate (PMA) (20 nM), transforming growth factor (TGF)-α (20 ng·mL^{-1}) or lipopolysaccharide (LPS) (10 μg·mL^{-1}) for 24 h. Then, MUC5AC protein was measured in cell lysates as fold increase referred to a) non-stimulated/non-transfected cells (control) (arbitrary units=1) or b) referred to stimulated/control si-RNA (si-control)-transfected cells (arbitrary value=100%). c) mRNA was measured and -fold increase expressed considering non-stimulated/si-control-transfected cells (arbitrary units=1). d) % MUC5AC expression in PMA-stimulated cells is referred to si-control transfected cells (100%). Mean±SD of triplicates are shown 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 are shown. *: $p<0.05$ *versus* cells transfected with si-control and stimulated with the corresponding stimulus.

(242-A01; Abnova, Taipei, Taiwan), mouse anti-human β-actin antibody (A5316; Sigma, Saint-Quentin Fallavier, France) 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.

Electrophoretic mobility shift assay
Nuclear proteins were extracted from 2×10^6 cells as described previously [27]. The double-stranded oligonucleotide containing a Sp1 binding site consensus sequence (5′-attcgatcggggcg-gggcgagc-3′) was labelled with [γ-^{32}P]dATP (3,000 Ci·mmol^{-1} at 10 mCi·mL^{-1}) and purified using a ProbeQuant G-50 micro column (GE Healthcare). The following steps were performed as described by Ignatov and Keath [28]. Binding reactions were performed in 40 mM hydroxyethyl piperazine ethane sulphonic acid (HEPES), pH 7.6, 10 mM NaCl, 1.5 mM $MgCl_2$, 2% glycerol, containing 1 μg of 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 (peroxisome proliferator-activated receptors (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 (Nikon, Champigny sur Marne, France).

Cell viability
Lactate dehydrogenase (LDH) activity released from cells was measured with the CytoTox 96 Nonradioactive Cytotoxicity assay (Promega).

Statistical analysis
Data are presented as mean±SD. ANOVA was used to determine statistically significant differences among groups followed by Student–Newman–Keuls multiple range test for paired comparisons.

When indicated, unpaired t-test was used to compare two groups. $p<0.05$ was considered significant.

RESULTS

Involvement of the LOX pathway in PMA-induced MUC5AC production

We aimed at identifying AA metabolites and signalling 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 (fig. 1a). We verified that COX inhibitors efficiently reduced PGE_2 release (fig. 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 (fig. 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 (fig. 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 (see the online supplementary figure), we examined the impact of transcriptional silencing of each 12-LOX form on MUC5AC expression. As fig. 3a shows, silencing of 12R-LOX expression decreased PMA-, TGF-α- and LPS-induced MUC5AC expression. MUC5AC inhibition was more pronounced after PMA treatment (53%) compared to TGF-α (34%) and LPS treatment (24%) (fig. 3b). By contrast, 12S-LOX silencing had no effect on either PMA- or TGF-α-induced MUC5AC (fig. 3a and b), though, surprisingly, it significantly enhanced LPS-induced MUC5AC production (fig. 3a and b). Next, we analysed 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 (fig. 3c and d). Interestingly, 12R-LOX siRNA did not modify PMA-induced IL-8 secretion (fig. 4a and b). 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 fig. 5a, CDC and baicalein reduced PMA-induced ERK phosphorylation. Moreover, electrophoretic mobility shift assay (EMSA) analyses showed an enhanced Sp1 translocation in PMA stimulated cells (fig. 5b). Conversely, baicalein reduced the Sp1-shifted band (fig. 5b).

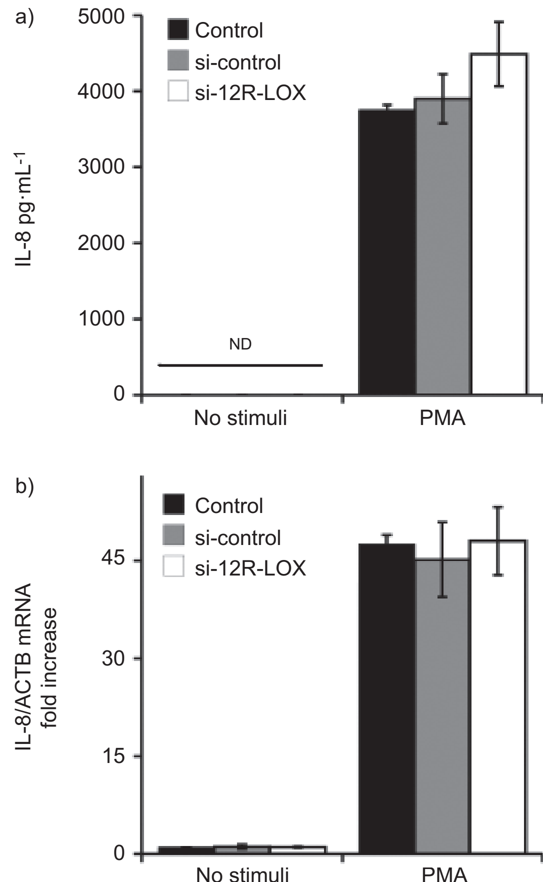

FIGURE 4. Expression of interleukin (IL)-8 induced by phorbol myristate acetate (PMA) in 12R-lipoxygenase (LOX) silenced NCI-H292. Cells transfected with 12R-LOX siRNAs (100 nM) were stimulated with PMA (20 nM) for 24 h. Then, IL-8 protein was measured in cell supernatants by a) sandwich ELISA and b) IL-8 phorbol myristate acetate mRNA expression by real time RT-PCR. Means±SD of triplicates are shown from one representative experiment of a total of three. ND: not detected; si-control: nontargeting si-RNA provided by manufacturer.

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 upregulates MUC5AC expression and activates ERK-Sp1 signalling pathways

12(R)-HETE, a 12R-LOX product, induced MUC5AC protein in a dose-dependent manner, with a maximal effect at 8 μM (fig. 6a). Furthermore, 12(R)-HETE was able to activate ERK (fig. 6b), whilst the MEK1/2 (ERK kinase) inhibitor (PD98059) blocked 12(R)-HETE-induced ERK phosphorylation (fig. 6b). In addition, 12(R)-HETE induced Sp1 translocation with a peak of activity at 2 h after stimulation (fig. 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 (fig. 7a). These effects were less pronounced than those observed on MUC5AC (53% inhibition) (fig. 7a). Finally, we

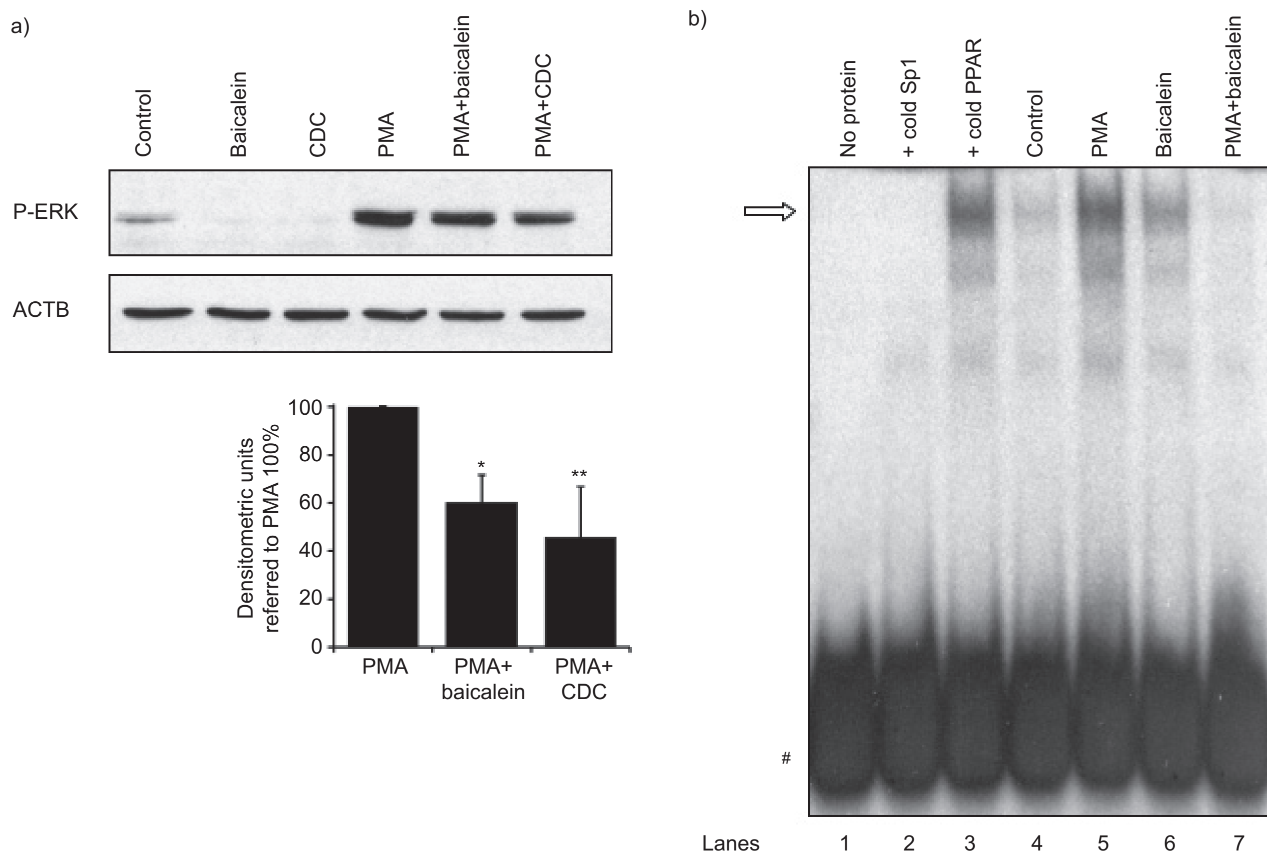

FIGURE 5. Activation of extracellular signal-regulated kinase (ERK) and Sp1 translocation induced by phorbol myristate acetate (PMA) in the presence of 12-lipoxygenase (LOX) inhibitors. a) Serum-deprived NCI-H292 cells were pre-incubated 2 h with either baicalein (20 μM) or cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) (10 μM) and stimulated for 20 min with PMA (20 nM) at 37°C. Afterwards cells lysates were collected in radioimmunoprecipitation assay (RIPA) buffer, electrophoresed (12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (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 normalised 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%. *: $p<0.05$; **: $p<0.01$ *versus* PMA treatment without inhibitors. b) Serum-deprived NCI-H292 cells pre-treated or not with baicalein (20 μM) were stimulated for 4 h with PMA (20 nM). After extraction of nuclear proteins, 5 μg were incubated with ^{32}P-labeled consensus Sp1 probe for 30 min at 4°C in binding buffer (40 mM hydroxyethyl piperazine ethane sulphonic acid, pH 7.6, 10 mM NaCl, 1.5 mM $MgCl_2$) in the presence of 50-fold molar excess of cold specific consensus Sp1 probe (+cold Sp1, line) or cold irrelevant peroxisome proliferator-activated receptors (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. Lane 1: probe only without nuclear proteins; lanes 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.

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. Increased AB staining was observed in cells stimulated with PMA compared to non-stimulated cells (fig. 7b), reflecting production of mucus induced by PMA. Baicalein strongly reduced this staining (fig. 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 *P. aeruginosa*, a pathophysiological stimulus in CF patients. *P. aeruginosa* supernatants induced MUC5AC and MUC2 (fig. 8a and 8b) but not MUC5B expression (fig. 8c). The presence of baicalein reduced 46% and 36 % of the *P. aeruginosa*-induced expression of MUC5AC and MUC2, respectively (fig. 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 a 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-neutralising antibodies or inhibitors of EGFR phosphorylation blocked PMA-induced MUC5AC expression [9]. In selected experiments more physiological stimuli (TGF-α, LPS, supernatants of *P. aeruginosa*) have been used to

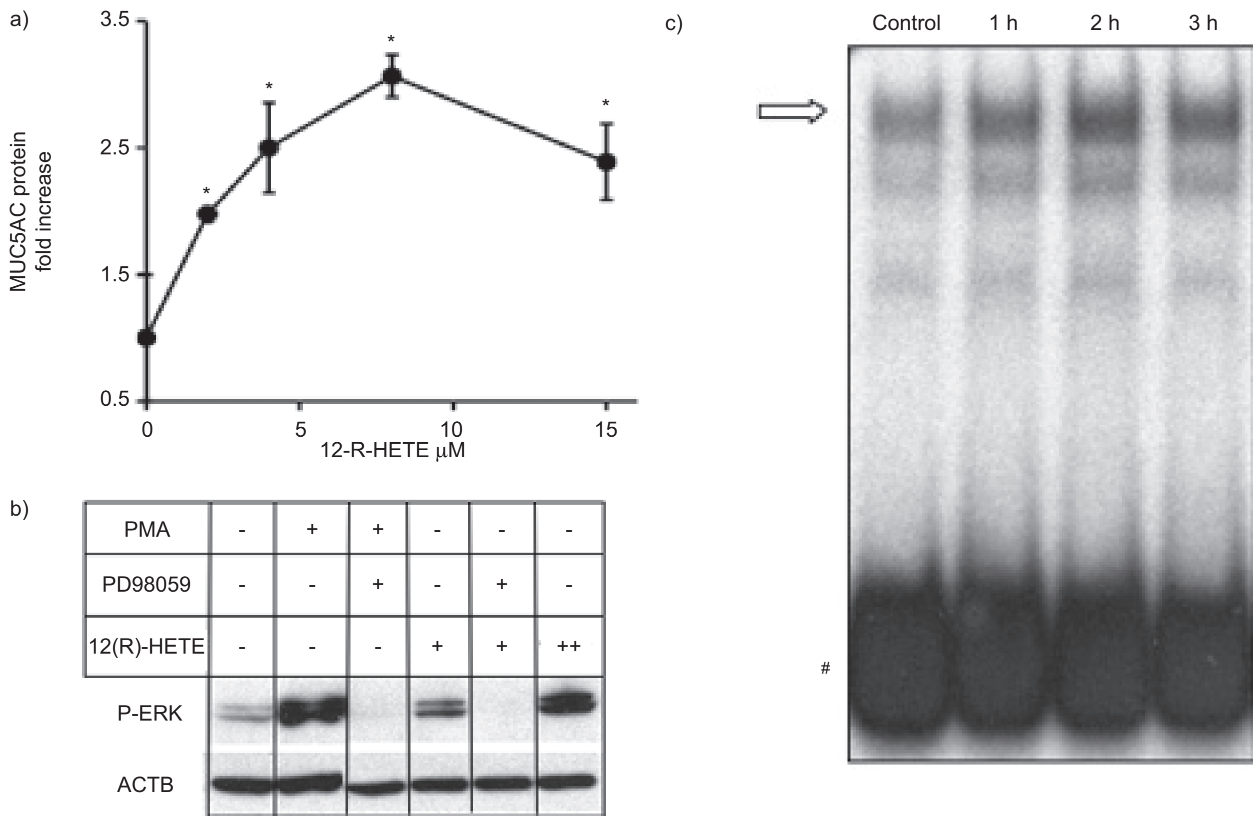

FIGURE 6. 12(R)-hydroxyeicosatetraenoic acid (HETE) induces MUC5AC expression, extracellular signal-regulated kinase (ERK) activation and Sp1 translocation in NCI-H292. a) 12(R)-HETE or vehicle were added to cells. After 24 h of culture, MUC5AC protein was measured in cell lysates by ELISA. Mean±SD is represented as -fold increase normalised to MUC5AC protein level in basal conditions from three independent experiments performed in triplicates. *: $p<0.05$ *versus* nonstimulated cells (t-test). b) ERK phosphorylation (P-ERK) was analysed as described in figure 5a after 20 min of incubation with phorbol myristate acetate (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 (control, t=0) before performing electrophoretic mobility shift assay as described in fig. 5b.

demonstrate the involvement of 12-LOX in mucin expression. Downstream signalling pathways after EGFR activation by PMA conducting to MUC5AC expression have been partially characterised [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 signalling pathways leading to mucin expression differ from those for PMA. PMA induces MUC5AC expression *via* matrix metalloprotease-mediated release of TGF-α, 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 TGF-α-induced MUC5AC expression (data not shown). Here again, differences in the signalling pathways activating MUC5AC expression (IL-13 receptor-dependent *versus* 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

FIGURE 7. Modulation of airway mucins and mucus by 12R-lipoxygenase (LOX). a) Real-time PCR from phorbol myristate acetate (PMA)-stimulated cells transfected with 12R-LOX siRNA or control siRNA, was performed with specific MUC5AC, MUC5B or MUC2 primers as described in the Materials and methods section. Levels of expression were normalised to PMA-stimulated cells transfected with control siRNA (100%). Mean±SD of triplicates from three independent experiments are represented. *: p<0.05 *versus* si-control transfected cells. b) Cells were first pre-incubated or not 1 h with baicalein (20 μM) and then stimulated or not for 24 h with PMA (20 nM). Cells were then fixed and stained with Alcian blue (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 cinnamyl-3,4-dihydroxy-α-cyanocinnamate (10 μM) showed the same results (data not shown).

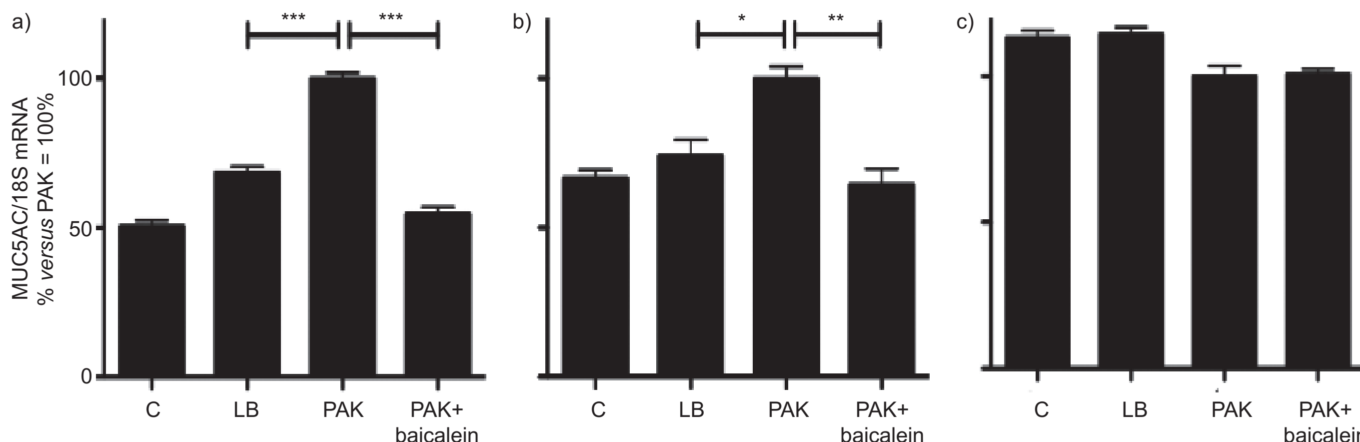

FIGURE 8. Effect of baicalein in *Pseudomonas aeruginosa* supernatant-induced mucins expression in human primary epithelial cells. The cells were cultured in air–liquid interface and pre-treated with baicalein (20 μM) 1 h and during treatment with bacteria (*P. aeruginosa*, strain PAK) supernatants diluted at 1:8 or bacteria culture medium, Luria–Bertani (LB, 1:8) for 24 h as indicated in the Materials and methods. Total mRNA was prepared and subjected to real-time quantitative PCR to measure a) MUC5AC, b) MUC2 and c) MUC5B levels. The results show the percentage of inhibition of mucins mRNA expression by baicalein compared to cells treated with PAK supernatant alone. Values represent mean±SD of three independent experiments from three different donors. *: p<0.05; **: p<0.01; ***: p<0.001.

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, TGF-α 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 TGF-α- 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. aeruginosa* supernatants, a pathogen related to lung dysfunction in CF, was reduced by a 12-LOX inhibitor in primary bronchial epithelial cells. Altogether, 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 TGF-α-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 signalling 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 thus: 1) 12R-LOX independent pathways are involved in PMA-induced MUC5AC expression; 2) other metabolites derived (directly or indirectly) from 12R-LOX activity might also participate in the process. Regarding the mechanism through which 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 explained 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 overexpression 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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STATEMENT OF INTEREST
None declared.

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