Lipoxin A4 stimulates calcium-activated chloride secretion and increases airway surface liquid height in normal and cystic fibrosis airway epithelia

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LIPOXIN A₄ STIMULATES CALCIUM-ACTIVATED CHLORIDE SECRETION AND INCREASES AIRWAY SURFACE LIQUID HEIGHT IN NORMAL AND CYSTIC FIBROSIS AIRWAY EPITHElia

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Running head: LXA₄ increases chloride secretion and ASL height in CF airways

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Cystic Fibrosis (CF) is a genetic disease characterised by a deficit in epithelial Cl⁻ secretion leading to airway dehydration and a reduced Airway Surface Liquid (ASL) height. The endogenous lipoxin LXA₄ is a member of the newly identified eicosanoids playing a key role in ending the inflammatory process. Levels of LXA₄ are decreased in the airways of patients with CF. We have previously shown that in normal human bronchial epithelial cells, LXA₄ produced a rapid and transient intracellular Ca²⁺ increase. We have investigated here, the effect of LXA₄ on Cl⁻ secretion and the functional consequences on ASL height in bronchial epithelial cells obtained from CF and non-CF patient biopsies and in bronchial epithelial cell lines. We found that LXA₄ stimulated a rapid intracellular Ca²⁺ increase in all of the different CF bronchial epithelial cells tested. In non-CF and CF bronchial epithelia, LXA₄ stimulated whole-cell Cl⁻ currents which were inhibited by NPPB (calcium-activated Cl⁻ channel inhibitor), BAPTA-AM (chelator of intracellular Ca²⁺) but not by CFTRinh-172 (CFTR inhibitor). We found, using confocal imaging, that LXA₄ increased the ASL height in non-CF and in CF airway bronchial epithelia. The LXA₄ effect on ASL height was sensitive to bumetanide an inhibitor of transepithelial Cl⁻ secretion. The effects of LXA₄ on intracellular Ca²⁺, whole-cell Cl⁻ currents, conductances and ASL height were inhibited by Boc-2 the antagonist of the ALX/FPR2 receptor. Our results provide, for the first time, evidence for a novel role of LXA₄ in the stimulation of Ca²⁺ signalling, Ca²⁺-activated Cl⁻ secretion and ASL height in non-CF and CF bronchial epithelia.

We have investigated the role of lipoxin A₄ on cystic fibrosis airway epithelium secretory function. Cystic fibrosis is caused by the mutation of the gene coding for the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), a cyclic AMP-dependent Cl⁻ channel. The major clinical features of CF are chronic pulmonary disease, exocrine pancreatic insufficiency and male infertility (1, 2). The lung disease is the main cause of morbidity and mortality in CF. The airway epithelium of patients with CF fails to transport Cl⁻ and water, resulting in a reduced ASL height and impaired mucociliary clearance. The hyper-absorption of Na⁺ observed in the CF bronchial epithelium may further exacerbate the dehydration of the ASL. It is generally accepted that the dehydration of the airway lumen favours chronic infection and inflammation leading to progressive destruction of the lung (3). Identification of agents, particularly natural endogenous biologicals, which stimulate alternative non-CFTR Cl⁻ secretory pathways and promote ASL hydration and optimal ASL height recovery are likely to be of therapeutic benefit in
improving mucociliary clearance in patients with CF.

The levels of LXA₄ have been reported to be decreased in the airways of patients with CF (4). Lipoxins are bioactive lipids derived from omega-6 polyunsaturated fatty acids playing important roles in various biological functions (5). The endogenous lipoxin A₄ (LXA₄; 5S,6R,15S-trihydroxy-7,9,13-trans-11-eicosatetraenoic acid) is produced at inflammatory sites from the interaction of lipooxygenase activities of several cell types including leukocytes, platelets and epithelial cells. This lipid mediator is one member of the newly identified molecules playing a role in ending/resolving the inflammatory process by modulating neutrophil inflammation, clearing apoptotic PMN and inhibiting the production of pro-inflammatory cytokines (6). The deficit in LXA₄ in CF could be a contributing factor in chronic airway inflammation which characterises these patients.

Very little is known about the role of LXA₄ in the lung beyond its anti-inflammatory effects. We have previously shown that normal human bronchial epithelial cells are a biological target for LXA₄. The receptor for LXA₄ (ALX/FPR2) is expressed in the bronchial epithelial cell line 16HBE14o- and LXA₄ stimulates an intracellular Ca²⁺ mobilisation in these cells (7). Intracellular Ca²⁺ is a major regulator of Cl⁻ transport and the stimulation of epithelial Cl⁻ secretion would be of major therapeutic benefit in CF to restore efficient airway clearance, therefore we have investigated the effect of LXA₄ on epithelial Cl⁻ secretion and its functional consequences on ASL height using bronchial epithelial cells obtained from CF and non-CF patient biopsies and in a variety of bronchial epithelial cell lines commonly used as models for CF ion transport and immunological studies.

**Experimental procedures**

**Cell Culture.** For the primary culture of human bronchial epithelium, the cells were obtained from endobronchial biopsies in non-CF and CF patients (Local Ethics Committee approval and patient consent). The bronchial epithelial cell samples were taken from a normal area of bronchi in 4 patients with a normal lung function and from 3 CF patients homozygote for the Phe508del mutation. Bronchial epithelium biopsies were washed and incubated for two hours at room temperature with 250 µg/ml amphotericin B in Phosphate Buffer Saline (PBS) without calcium and magnesium. After centrifugation, the explants were collected and re-suspended in 500 µl of Bronchial Epithelium Basal Medium (BEBM, Clonetics, BioWhittaker, San Diego, USA) supplemented with 0.5µg/ml human recombinant epidermal growth factor, 7.5 mg/ml bovine pituitary extract, 0.5 mg/ml epinephrine, 10 mg/ml transferrin, 5 mg/ml insulin, 0.1 µg/ml retinoic acid, 6.5 µg/ml triiodothyronine, and 50 mg/ml gentamicin (BD, Erembodegem, Belgium) and 250 µg/ml amphotericin B (BD, Erembodegem, Belgium). The explants were plated in a 24 well plate (Nunc, Roskilde, Denmark) previously coated with a fibronectin/collagen solution and incubated at 37°C in a humidified 5% CO₂ atmosphere. Twenty four hours after seeding, the volume of media was adjusted to 400µl. The cells were cultured under these conditions for six to nine days (confluence close to 70 %) before splitting. Fibroblasts were removed by 1 minute treatment with trypsin EDTA (Gibco, Invitrogen, Paisley, UK). Epithelial cells were then trypsinised and re-suspended after centrifugation, in supplemented BEBM. The cells were seeded at 2500-4000 cells/cm² in flasks (BD, Erembodegem, Belgium).

NuLi-1, CuFi-1, CuFi-3, and CuFi-4 cells were kindly donated by Prof Zabner, University of Iowa, USA. The NuLi-1 cell line was derived from human airway epithelium of normal genotype, whereas CuFi-1, Cufi-3 and CuFi-4 cell lines were derived from CF patients with Δ508/Δ508, R553X /Δ508, and G551D/Δ508 genotypes respectively. The cell lines were transformed with a RT component of telomerase and human papillomavirus type 16 E6 and E7 genes (8). Cells were initially grown to confluence in flasks using BEBM with EGF, hydrocortisone, bovine pituitary extract, transferrin, bovine insulin, triiodothyronine, epinephrine, retinoic acid, penicillin-streptomycin (0.025 µg/ml), gentamicin (0.05 µg/ml), and amphotericin (25 µg/ml).

Airway epithelial cells were plated at 2 x 10⁶ cells/cm² on Millicell hanging cell culture inserts (Millipore, Billerica, USA) for ASL height measurements. All inserts were pre-coated with collagen type VI and grown in BEGM medium until confluence was achieved.
Once cell confluence was confirmed under visual inspection, the medium was switched to DMEM/F-12 (Invitrogen, Auckland, New Zealand) to aid cell differentiation. This medium was supplemented with Ultraser G (2%, Pall Biospera, Cergy-Saint-Christophe, France), which enhances ion transport (8), and penicillin-streptomycin (0.025 µg/ml), gentamicin (0.05 ng/ml), and amphotericin (25 µg/ml). Medium at the apical aspect was aspirated every 3-4 days until the establishment of an air-liquid interface. The basolateral culture medium was replaced every 2-3 days. After 4-6 weeks growth, the cells formed a polarised confluent monolayer with a high transepithelial electrical resistance (TER) of > 700 Ω/cm².

**Intracellular calcium imaging.** Intracellular Ca²⁺ was measured by epifluorescence microscopy as previously described (9). The human airway epithelial cells were cultured on fibronectin-collagen coated (for primary culture cells) and on collagen VI coated (for NuLi-1 and CuFi-1 cells) glass bottom dishes (WPI, Stevenage, UK) for 6 days until 70% of confluence was reached. Cells were loaded with 5 µM of the Ca²⁺-sensitive fluorescent probe fura-2-acetoxy-methyl ester (fura 2-AM, Invitrogen, Auckland, New Zealand) for 30 min, in the dark, at room temperature (22°C) and were then washed twice in HEPES-buffered Krebs-Henseleit solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.4, 280-290 mOsmol). The glass bottom dishes covered with the fura 2-AM loaded epithelial cell monolayer were mounted on the stage of an inverted microscope equipped for epifluorescence (TE-300, Nikon, Badhoeve Dorp, Netherlands). Intracellular Ca²⁺ imaging was performed using the Metafluor Imaging System (Universal Imaging Corporation). The cell preparation was excited alternatively with monochromatic light at 340 and 380 nm using an Optoscan monochromator (Cairn Research Ltd, Kent, UK). The emission fluorescence produced after fura 2-AM excitation was filtered at 512nm. The emitted light image was detected using a Photometrics CoolSNAP-fx video camera (Roper Scientific, Ery, France) coupled to the microscope. The fluorescence obtained at each excitation wavelength (F340 and F380) depended upon the level of Ca²⁺ binding to fura 2-AM. The results are given as ratiometric data (F340/F380), or as amplitude of variation compared to the basal ratio level (ΔF340/F380).

**Whole-cell patch-clamp recording.** Freshly isolated epithelial cells obtained from 4 non-CF patients and from 3 CF patients (genotype: ΔF508/ΔF508) and from the NuLi-1 and CuFi-3 cell lines were used for patch-clamp experiments. The CuFi-1 cells were not used for patch-clamp experiments since we could not reach a Giga ohm seal with these cells. Cells were patch-clamped at room temperature (25°C) on an inverted microscope (TE-300, Nikon, Badhoeve Dorp, Netherlands). Patch-pipettes were prepared from soda glass (Vitrex, Modulhom, Herlev, Denmark), pulled on a programmable puller (P80/PC, Sutter Instrument Company, USA). The whole-cell configuration was obtained from cell-attached mode after breaking the patch membrane by applying a brief negative pressure in the patch pipette. Whole-cell currents were amplified (Axopatch 200B, Axon instrument, CA) and digitized using a 16-bit data converter (Digidata 1322A, Axon instrument, CA) following low pass filtering at 5KHz and sampled in real-time. Whole-cell current voltage (IV) relationships were analysed using Clampfit software (Axon instrument, CA).

The patch pipette was filled with a “high K” solution” at pH=7.2, 290mosm: 110mM K-gluconate, 20mM NaCl, 1.2mM KH₂PO₄, 3.46mM, 3mM KH₂PO₄, 5mM EGTA, 6mM HEPES, 2.78mM CaCl₂, pH= 7.2 adjusted with KOH. The bathing solution had the following composition: 140mM NaCl, 5mM KCl, 6mM Hepes, 2mM CaCl₂, 1.2mM KH₂PO₄, 1.2mM MgSO₄ and pH= 7.4. The Nernst potentials between the patch pipette and bath for K⁺ and for Cl⁻ were -77mV and -39mV, respectively. The access resistance (Ra) was determined by fitting the current transients produced by a 5mV voltage pulse with a single exponential. The measured Ra was 5.43±0.08Ω (n=40).

**Airway Surface Liquid (ASL) height measurements.** ASL height was measured using a protocol adapted from Tarran et al. (10), using live-cell confocal fluorescence microscopy. To label the ASL, 8µl PBS containing 1 mg/ml Texas red®-dextran (10kD; Invitrogen, Auckland, New Zealand) was added to the apical surface of the well-differentiated airway epithelium. The epithelial cells were stained using Calcein-AM.
(5μM, Invitrogen, Auckland, New Zealand) dissolved in medium culture for 30 minutes and introduced to the basolateral compartment of the insert. The Fluorinet™ electronic fluid Perfluorocarbon-72 (FC-72, 3M, St Paul, USA) was added to the apical compartment of the insert at a volume of 0.5ml. Perfluorocarbon-72 is immiscible with the ASL and was used to prevent ASL evaporation on transferring the inserts from the incubator to the microscope stage and during the confocal scanning experiments. Epithelia were Z-scanned using a Zeiss LSM 510 Meta using a 40X objective. To evaluate the average ASL height, for each preparation, 9 separate regions of interest were determined within the microscope field and were XZ scanned. Images were analysed using the Zeiss LSM Image analyser software (Carl Zeiss Microlmaging GmbH, Germany).

**Drugs.** The lipoxin LXA₄ was purchased from Calbiochem. Aliquots of LXA₄ solution (100μM) in ethanol were stored at -80 °C to avoid degradation of the molecule. The peptide Boc-Phe-Leu-Phe-Leu-Phe (Boc-2) (Phoenyx pharmaceutical, Belmont, USA) was used as specific inhibitor of the ALX/FPR2 receptor (11). For these latter experiments, cells were pre-incubated with 10⁻⁵ M Boc-2 for 24 hours at 37°C. BAPTA-AM (10μM, Molecular probes, Leiden, Netherlands) was used to chelate intracellular Ca²⁺ (12). The 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB, Sigma, USA) used at 1μM is an inhibitor of Ca²⁺-activated Cl⁻ channels (13). CFTRinh-172 an antagonist of the CFTR channel and bumetanide an inhibitor of the NKCC1 cotransporter were supplied by Sigma (14).

**Data Analysis.** The intracellular Ca²⁺ variations were measured as the difference between the mean F340/F380 ratio during the 2 min prior to exposure to LXA₄ and the ratio measured at the peak of the Ca²⁺ response and 2 and 5 min after the peak. In each experiment, the mean ratio was obtained from all cells in the microscopic field. In whole-cell patch-clamp experiments, conductances were determined by linear regression of the current-voltage relationship obtained in n cells. For the ASL height measurements, three confocal image acquisitions were performed on each epithelial cell monolayer and nine regions of interest were analysed in the acquisition field using the LSM image browser (Zeiss). Values were loaded in an Excel spreadsheet and averaged. Mean value were obtained from n independent experiments. The experiments were repeated under the same conditions on a minimum of three different cell passages. Data are presented as the mean ± S.E.M. of n experiments. Measures of statistical significance were obtained using the Student’s t test for paired data. A p value <0.05 was deemed to be significant. All statistical operations were performed using Excel software (Microsoft).

**RESULTS**

**LXA₄ effects on intracellular Ca²⁺ in normal and CF bronchial epithelial cells.** LXA₄ induced a rapid increase of intracellular Ca²⁺ in all human airway epithelial cell types tested. There was no significant difference in the maximum increase induced by LXA₄ (100nM) between non-CF and CF bronchial epithelial cell lines and between non-CF and CF epithelial primary cultures, although the absolute peak values were higher in the cell lines than in primary cultures. However, as shown on figure 1A and 1B, the kinetics of the Ca²⁺ responses were not identical from one cell type to another. In non-CF bronchial epithelial cell lines (Nuli-1 cell line and NHBE primary cultures), the intracellular Ca²⁺ rise induced by LXA₄ was fast and transient with a Ca²⁺ recovery to basal values within 2 to 5 min. In contrast, in CF bronchial epithelial cells (CuFi cell lines and CFE primary cultures), LXA₄ induced a slower Ca²⁺ increase and a delayed (or absent) recovery toward basal values (Figure 1B). Thus the total amount of Ca²⁺ mobilised in the cytosol upon LXA₄ exposure was higher in CF than in non-CF bronchial epithelial cells.

In order to investigate the origin of the calcium signal induced by LXA₄, we tested the effect of LXA₄ on intracellular Ca²⁺ in the absence of extracellular Ca²⁺ in normal and CF airway epithelial cells. The results presented in figure 1A showed that in Nuli-1 cells bathed in external Ca²⁺-free solution (grey dotted line), the response to LXA₄ was not different from the response obtained under control conditions (plain line). In Nuli-1 cells, there was no significant difference in the maximum Ca²⁺ increase obtained after LXA₄ exposure with or without external Ca²⁺ (F340/F380 : control 8.49±0.48 and external Ca²⁺-
Role of the ALX/FPR2 receptor in the calcium response to LXA₄. The role of the ALX/FPR2 receptor in the Ca²⁺ response to LXA₄ was investigated using the specific inhibitor, Boc-2, in NuLi-1 and CuFi-3 cells (figure 2). The effect of LXA₄ (100nM) on intracellular Ca²⁺ was completely abolished after treatment with Boc-2 (10µM) (figure 2A and 2B). However, ATP (100µM), a known stimulator of intracellular Ca²⁺ mobilisation via purinergic receptor stimulation, produced a Ca²⁺ signal in cells treated with Boc-2 (figure 2A). These results indicate the involvement of the ALX/FPR2 receptor in the Ca²⁺ signalling response to LXA₄.

LXA₄ effects on whole-cell currents in non-CF and CF bronchial epithelial cells. The current-voltage relationship obtained from whole-cell patch-clamp recordings in non-CF (NHBE) and CF (CFBE) primary cultures of human bronchial epithelial cells. The time-dependence and dose-dependence of the LXA₄ effects on whole cell current and conductance are presented in figures 3 and 4, respectively. Under control conditions, the whole-cell currents were outwardly rectified in non-CF (gₒᵤₐₐ=612.2±165.2pS and gₑᵤₐ=227.7±99.3pS, n=16) and CF (gₒᵤₐ=855.4±192.9pS and gₑᵤₐ=302.9±100.4pS, n=8) primary airway epithelial cells. The reversal potentials were Eᵣᵢᵥᵦ=-22.4±3.9mV and Eᵣᵢᵥᵦ=-22.4±1.9mV in the non-CF and CF cells, respectively, indicating that Cl⁻ ions (Eᵣᵢᵥᵦ=-39mV) were the main charge carrier under these conditions (see table 1).

LXA₄ (100nM) exposure stimulated the whole-cell currents in NHBE and CFBE cells, in a time-dependent manner, as illustrated in figure 3. In the non-CF NHBE primary cultures, LXA₄ enhanced the basal outward conductance by 4.12±0.17 fold (n=13), and the inward conductance by 2.98±0.15 fold (n=13), without producing a significant change in the reversal potential (Eᵣᵢᵥᵦ=-21.4±5.8 mV). LXA₄ also stimulated the whole-cell current in CF bronchial cells (figure 3B). LXA₄ increased the outward conductance in CFBE cells by 3.97±0.19 fold (n=8) and the inward conductance by 3.57±0.19 fold (n=8) without affecting the reversal potential (Eᵣᵢᵥᵦ=-23.8±3.1mV). The maximum stimulatory effect on membrane conductance in NHBE cells was obtained after 10 min exposure to LXA₄ and declined thereafter to control levels (figure 3A). In CF airway epithelial cells, the increased membrane current and conductance changes induced by LXA₄ were sustained without recovery to basal values over the 15 min period of observation (figure 3B). The inward conductance (for outward flux of Cl⁻ from the cell) was significantly increased by LXA₄ in all cell types studied including primary cultures of CF and non-CF bronchial epithelia and in the CF and non-CF cell lines (figure 3C).

The stimulatory effect of LXA₄ on membrane current and conductance in non-CF NuLi-1 cells (figure 4A) and CF CuFi-3 cells (figure 4B) was found to be dose-dependent with significant responses observed at concentrations as low as 1pM and the maximum response achieved at 10nM lipoxin (figure 4C).

Role of the ALX/FPR2 receptor in the membrane current and conductance responses to LXA₄. We have tested the effect of the ALX/FPR2 receptor antagonist Boc-2 on the whole-cell current and conductance response to 100nM LXA₄ treatment in NuLi-1 cells (figure 5A) and CuFi-3 cells (figure 5B). Treatment of these cells with Boc-2 (10µM) for 1h completely abolished the effect of LXA₄ on the whole-cell current (figure 5A, 5B) and inward conductance (Figure 5C).
**LXA₄ effect on Cl secretion.** To investigate the contribution of Cl currents to the LXA₄ induced current, we tested the effects of NPPB (calcium-activated Cl channel inhibitor) and CFTR-inh172 (CFTR Cl channel inhibitor), on membrane current and conductance responses to LXA₄ in CF and non-CF cells (figure 6). The addition of NPPB (1µM) cells 10 min after exposure to LXA₄ (100nM), immediately inhibited the whole-cell current by 70.19±1.42% in non- CF primary cultures (n=4) and by 76.19±4.12% in CF primary cultures (n=3). A typical experiment in NHBE cells is shown in figure 6A. Pre-treatment of CFE primary cultured cells for 2 min with NPPB (1µM) prior to LXA₄ exposure, completely inhibited the stimulatory effect of LXA₄ on whole-cell current (p>0.5, n=3). In addition, when equimolar Cl⁻ was used in the bath and patch pipette, LXA₄ did not stimulate whole-cell current in any of the cell types tested (data not shown). NBBP also inhibited the LXA₄-stimulated membrane inward conductance in CF and non-CF cells. A summary of the inhibitory effects of NPPB on inward conductance in the CFE and non-CF primary cultured cells, and the CuFi-3 cell line are presented in figure 6A.

The specific CFTR inhibitor, CFTR-inh172, did not affect the stimulation of the whole-cell current by LXA₄ in non-CF NuLi-1 cells (figure 6B). There was no effect of CFTR-inh172 on basal or LXA₄-stimulated membrane current and conductance in CFE and CuFi-3 cells (data not shown), consistent with the absence of functional CFTR. When Nuli-1 cells were pre-treated with CFTR-inh172 (5mM), subsequent LXA₄ exposure stimulated the outward and inward conductances by 2.1±0.15 fold and 2.5±0.14 fold (n=3), respectively (figure 6B). These results indicate that the stimulatory effect of LXA₄ on whole-cell current and conductance in non-CF and CF bronchial cells is mainly due to activation of NPPB-sensitive Cl⁻ channels and does not involve CFTR channels.

**Role of intracellular Ca²⁺ in the whole-cell current responses to LXA₄.** In order to evaluate the role of intracellular Ca²⁺ in the LXA₄ induced whole-cell currents, we used BAPTA-AM as a chelator of intracellular Ca²⁺. Cells were bathed in Kreb’s solution and the patch pipette contained 100nM Ca²⁺ with 5mM EGTA and 10µM BAPTA-AM. Under these conditions of low intracellular Ca²⁺ (~1pM, estimated from free software WEBMAXC http://www.stanford.edu/~cpatton/webmaxc/webmaxS.htm), the stimulatory effect of LXA₄ on whole-current was abolished in NuLi-1 cells (figure 7A) and in CuFi-3 cells (figure 7B). Furthermore, BAPTA-AM pre-treatment completely abolished the effect of LXA₄ on the membrane inward conductance in these cells (Figure 7C).

**LXA₄ effects on Airway Surface Liquid Height.** The consequence of LXA₄ stimulation of whole-cell Cl currents and transepithelial Cl⁻ secretion on ASL height was investigated in NuLi-1, CuFi-1 and CuFi-3 epithelial monolayers. The ASL measurements were carried out on the monolayers after a period of 24 hours exposure to an air-liquid interface to allow fluid absorption/secretion and ASL height to reach a steady state. LXA₄ (100nM) was applied to the epithelium from the basolateral compartment of the chamber 15 minutes prior to ASL measurements. Under control conditions (without LXA₄ and 24h after a steady-state ASL height was established), the non-CF NuLi-1 cell monolayers showed a continuous unbroken ASL layer (figure 8A) and a mean ASL height of 7.2±0.1µm (n=3), whereas CF cell monolayers presented a disrupted and thinner ASL layer of 4.6±0.1µm in CuFi-1 cells (n=6, p<0.005) and of 6.2±0.1µm in CuFi-3 cells (n=4, p<0.005) (figure 8A).

Exposure to LXA₄ (100nM) significantly increased the ASL height in both non-CF and CF cell monolayers (figure 8A,B). LXA₄ treatment produced a maximum ASL height increase from 7.2±0.1µm to 10.0±0.2µm in NuLi-1 epithelia (n=3), and from 4.6±0.1µm to 11.1±0.2µm in CuFi-1 epithelia (n=6), and from 6.2±0.1µm to 9.8±0.2µm in CuFi-3 epithelia (n=4) (p<0.001). In CF epithelium, the disrupted appearance of the ASL layer largely disappeared after LXA₄ treatment (figure 8A).

**Role of Cl transport in the ASL height responses to LXA₄.** In order to investigate the contribution of Cl⁻ secretion in the generation of the ASL, we used bumetanide to inhibit the basolateral Na/K/2Cl co-transporter. Bumetanide (1µM) treatment significantly decreased the basal ASL height from 7.2±0.1µm to 5.9±0.1µm (p<0.001, n=6) in NuLi-1 monolayers but had no
significant effect on ASL height in CuFi-1 epithelia (4.6±0.1µm in control and 4.82±0.1µm (p >0.05, n=6) after bumetanide) and in CuFi-3 epithelia (6.2±0.1µm in control and 6.1±0.1µm (p >0.05, n=4) after bumetanide). These data indicate that Cl⁻ channels contribute to the generation of the basal ASL height in the non-CF epithelium. Furthermore, bumetanide (1µM) significantly abolished the ASL height increase induced by LXA₄ in NuLi-1 and CuFi-3 monolayers (figure 8B). The ASL height measured after LXA₄ exposure in the presence of bumetanide was significantly decreased in NuLi-1 (5.7±0.1µm, n=3, p<0.05) and in CuFi-1 (4.86±0.1µm, n=6, p<0.001) and in CuFi-3 (6.2±0.1µm, n=6, p<0.001) compared to LXA₄ alone (figure 8B). Taken together, these results indicate that the stimulatory effect of LXA₄ on ASL height mainly involves Ca²⁺-dependent Cl⁻ secretion via NPPB-sensitive channels in both CF and non-CF epithelia.

**Role of the ALX/FPR2 receptor in the ASL height responses to LXA₄.** We tested the effect of the ALX/FPR2 receptor antagonist Boc-2 on the ASL height response to LXA₄. Boc-2 significantly reduced the effect of LXA₄ on ASL height in Nuli-1 and CuFi-3 epithelial monolayers without affecting the basal ASL height (figure 8B). These data, taken together, support the conclusion that the ALX/FPR2 receptor mediates the effect of LXA₄ on ASL height as well as on calcium mobilization and Cl⁻ secretion.

**DISCUSSION**

In this study, we report the first evidence for a novel effect of the endogenous lipoxin LXA₄ to stimulate an increase in Airway Surface Liquid height, by enhancing Ca²⁺ activated Cl⁻ transport in bronchial epithelial cells obtained from patients with CF and non-CF patients and in airway cell lines.

In the healthy lung, the ASL, a thin layer of fluid covering the surface of the bronchial epithelium allows cilia to beat effectively (15). Maintenance of an optimal height of the ASL for ciliary beat is crucial for the quality of mucociliary clearance (16-19). Epithelial ion transport regulates the ASL height, mainly by generating osmotic gradients that provide a driving force for transepithelial ion and water movement (20). In CF, the lack of functional CFTR leads to a reduced ASL height, resulting in an impaired mucociliary clearance that promotes chronic bacterial infection of the airways (3). In a previous study, we reported that LXA₄ stimulated an intracellular Ca²⁺ mobilization in a normal human airway epithelial cell line 16HBE140- (7). Here, we tested the hypothesis that LXA₄, which is reduced in CF airways (4), could induce a Ca²⁺ signal coupled to an increased Cl⁻ secretion in CF epithelium thus providing a compensatory mechanism for the absent cAMP-mediated Cl⁻ transport via mutated CFTR and enhancing airway lumen hydration and ASL height.

Other studies have shown that LXA₄ exerts biological actions on human airway epithelial cells, with a maximal effect observed at 100nM. LXA₄ (100nM) inhibited IL-8 production by airway epithelial cells (21), stimulated an intracellular Ca²⁺ increase (7), increased ZO-1 expression at the plasma-membrane and transepithelial electrical resistance (22), and enhanced epithelial repair after an acid injury (23). We report here, novel effects of LXA₄ on ion and fluid transport in normal and CF bronchial epithelia.

Our studies show that LXA₄ induces an intracellular Ca²⁺ mobilization in normal and CF epithelium. This Ca²⁺ response to LXA₄ involves the ALX/FPR2 receptor, since the FPR2 receptor antagonist Boc-2 inhibited the effect. This result supports our previous study which suggested that the Ca²⁺ signal induced by LXA₄ was mediated by the ALX/FPR2 receptor since the Ca²⁺ response to LXA₄ was only obtained in the 16HBE140-airway epithelial cell line that express the receptor whereas LXA₄ did not produce any Ca²⁺ response in the A549 cell line which does not express ALX/FPR2 (7).

Our results indicate that in non-CF airway epithelial cells, LXA₄ generates a rapid and transient calcium signal mainly due to the release of Ca²⁺ from intracellular stores and not by Ca²⁺ entry since the calcium signal was not affected by the removal of external Ca²⁺. This is in accordance with our previous report showing that most of the Ca²⁺ mobilization induced by LXA₄ was generated from thapsigargin sensitive stores (7). In contrast, in CF airway epithelial cells, the duration of the Ca²⁺ signal induced by LXA₄ was greater than in non-CF cells. Although, in CF cells, the removal of external calcium did not affect the maximum peak calcium increase, the calcium response to LXA₄ became more transient.
These results suggest that, in CF airway epithelial cells, in addition to the calcium flux from intracellular calcium stores, LXA₄ also stimulates a calcium entry which leads to an overall larger calcium mobilisation than in normal airway epithelial cells. The observed differences reported in the literature between the Ca²⁺ signal obtained upon agonist exposure in CF and non-CF airway epithelial cells are controversial. Some authors reported that expression of either CFTR or ΔF508CFTR in airway epithelial cells had no effect on intracellular Ca²⁺ (24). However, our results are in accordance with the demonstration that Ca²⁺ signaling is abnormal in CF airway epithelial cells and that correction of the abnormal trafficking of ΔF508CFTR protein restored intracellular Ca²⁺ homeostasis (25). Recent reports also indicate that intracellular Ca²⁺ signals induced by pro-inflammatory mediators are increased in CF airway epithelia compared to non-CF due to an expansion of the apical ER Ca²⁺ stores in CF airway epithelial cells (26). This finding is coherent with several studies showing that the nasal transepithelial electrical potential responses to agents that promote an intracellular calcium mobilisation than Ca²⁺-dependent Cl⁻ conductance were higher in CF patients than in normal subjects (27-30).

Intracellular Ca²⁺ regulates several epithelial functions including ion transport, mucin secretion, and ciliary beat frequency which constitute a primary mode of a non-specific cleansing process and lung protection. Our results indicate that the Ca²⁺ signal induced by LXA₄ is coupled to an increased Cl⁻ secretion in CF epithelium. LXA₄ stimulated the whole-cell current and conductance in non-CF and CF epithelial cells. The inhibitory effect of BAPTA used as a chelator of intracellular Ca²⁺ demonstrated the role of Ca²⁺ in the stimulation of the whole currents by LXA₄. The sensitivity of basal and stimulated whole-cell currents to NPPB or Cl⁻ substitution, underscores the major contribution of Cl⁻ secretion to the generation of the whole-cell current. These results agree with our previous report indicating that LXA₄ stimulated a Ca²⁺-activated transepithelial Cl⁻ secretion in non-CF bronchial epithelial cells (7). Since we found that LXA₄ stimulation of the whole-cell currents was present in CF airway epithelia (in which CFTR is not functionally expressed), LXA₄ most probably affects Cl⁻ channels other than CFTR. In addition, we found that the time course of the LXA₄ effect on whole-cell currents was different between non-CF and CF cells, with a transient current increase in non-CF cells compared to CF cells where the current increase was more sustained. One explanation may be that the time course of the effect of LXA₄ on the Cl⁻ currents is directly related to the time course of the intracellular calcium change induced by LXA₄. Therefore, the greater and sustained effect of LXA₄ on whole-cell currents in CF cells could be related to the long lasting Ca²⁺ signal obtained in CF airway epithelial cells. Finally, the ineffectiveness of CFTR inh-172 on the LXA₄ stimulation of whole-cell currents indicates that the effect of LXA₄ on Cl⁻ secretion is not mediated by CFTR activation. This conclusion is strengthened by the observation that bumetanide reduces further the ASL height compared to Boc-2 treatment in Nuli-1 cells but not in CuFi-3 cells where functional CFTR is absent. If LXA₄ had stimulated CFTR and Ca²⁺-dependent Cl⁻ channels we would expect equivalent inhibition of ASL height by Boc-2 and bumetanide in Nuli-1 cells.

Our results show that the stimulatory effect of LXA₄ on Cl⁻ secretion induces a subsequent increase ASL height in both normal and CF epithelia. The CF monolayers generate a thinner ASL layer than non-CF airway epithelial monolayers which correlates with the diminished ASL in CF airways reported in the literature (19). In addition, we observed that in control conditions, the non-CF cell monolayers showed a continuous ASL layer whereas in CuFi-1 monolayers this liquid layer was disrupted. The ASL gaps are localised over mucin secreting goblet cells and could result from localised dehydration and mucus plugging of the ASL. After LXA₄ exposure in CF cells, the ASL height significantly increased with no disruption of the ASL layer, suggesting that LXA₄ induces hydration of the ASL and possibly reduced mucin secretion at the apical surface of the epithelium. The inhibitory effect of bumetanide indicates that the effect of LXA₄ on ASL height is mainly dependent on stimulation of transepithelial Cl⁻ transport. However, we cannot exclude the possibility that LXA₄ can also exert its action to increase ASL height through the inhibition of ENaC activity which is known to be downregulated by increased intracellular Ca²⁺. Taken together, our results provide evidence for a novel role of LXA₄ in stimulating Ca²⁺ activated Cl⁻ secretion and ASL generation in CF and non-CF
Thus LXA₄ or its stable analogues may provide a novel therapeutic strategy to rehydrate the CF airway by modulating ion transport and airway surface liquid volume via pathways which bypass defective CFTR. Our results also indicate that the reduced levels of LXA₄ observed in CF patients may be an additional contributory mechanism by which mucociliary clearance is altered in CF airways.

REFERENCES

ACKNOWLEDGEMENTS

This work was funded by the French National Institute of Health (INSERM), the CHU of Montpellier, the French Cystic Fibrosis Association (Vaincre La Mucoviscidose), a Career Enhancement and Mobility Programme Marie Curie Fellowship, the Higher Education Authority of Ireland under the Programme for Research in Third Level Institutions (PRTLI) Cycle 4 and the National Biophotonics and Imaging Platform, Ireland.
FIGURE LEGENDS

Figure 1. LXA₄ effect on intracellular Ca²⁺ activity in non-CF and CF bronchial epithelial cells. 
(A) Typical effect of LXA₄ (100nM) on the cytosolic Ca²⁺ (ratio F340/F380) measured in Nuli-1 and CuFi-3 cell lines and in normal and CF primary cultures of bronchial epithelial cells (NHBE and CFBE) in control (plain line) and in external Ca²⁺-free conditions (dotted line). (B) Mean values of the maximum increase in Ca²⁺ in non-CF (Nuli-1 and NHBE) and in CF (CuFi-1, CuFi-3, CuFi-4 cell lines and CFBE) bronchial epithelial cells. (* denotes: p<0.05, ** denotes: p<0.01).

Figure 2. Effect of Boc-2 on the intracellular Ca²⁺ signal induced by LXA₄. (A)Typical effect of LXA₄ (100nM) and ATP (100μM) on cytosolic Ca²⁺ (ratio F340/F380) in NuLi-1 cells in control conditions (upper panel) and after 24 hours of pre-treatment with Boc-2 (10μM) a specific inhibitor of ALX/FPR2 (lower panel). (B) Mean values corresponding (* denotes: p<0.05, ** denotes: p<0.01).

Figure 3. Time dependency of the effect of LXA₄ on the whole-cell currents in normal (NHBE) and CF (CFHBE) bronchial epithelial cells in primary culture. 
Typical I-V relationships and corresponding current records obtained before and after 5, 10 and 15 min exposure to LXA₄ (100nM) in NHBE (A) and CFHBE (B) isolated bronchial epithelial cells in primary culture. (C) Bar charts representing averages of the inward conductances (pS) in NHBE and in CFHBE bronchial epithelial cells. Mean Mean inward conductance changes normalized to control values (gi/gic) obtained in control conditions (open bars) and 10 min after LXA₄ (100nM) exposure (black bars).

Figure 4. Dose dependency of the effect of LXA₄ on the whole-cell currents of normal (Nuli-1) and CF (CuFi-3) bronchial epithelial cell lines. 
Typical I-V relationships and corresponding current records obtained before and after 10 min exposure to 1pM, 1nM, 10nM and 100nM LXA₄ in Nuli-1 (A) and CuFi-3 (B) cell lines. (C) Mean inward conductance changes normalized to control values(gi / gic obtained without LXA₄) as a function of LXA₄ concentration in Nuli-1 (open bars) and CuFi-3 (black bars) cells.

Figure 5. Effect of Boc-2 on the whole-cell currents stimulated by LXA₄ in normal (Nuli-1) and CF (CuFi-3) bronchial epithelial cell lines. 
Typical I-V relationships and corresponding current records obtained in control condition, with Boc-2 (10μM) and Boc-2 with LXA₄ (10min, 100nM) in Nuli-1 (A) and CuFi-3 (B) isolated cells. (C) Mean inward conductance changes normalized to control values(gi/gic obtained upon exposure to LXA₄ alone (10min, 100nM) and after Boc-2 pretreatment in Nuli1 (open bars) and CuFi-3 (black bars) cells.

Figure 6. Effects of Cl⁻ channel inhibitors on the whole-cell currents stimulated by LXA₄. 
(A) Typical I-V relationship of the effect of NPPB (1μM) after the stimulation of primary cultures of bronchial epithelial cells with LXA₄ (100nM). Histogram of the inhibitory effect of NPPB on the inward currents obtained in non-CF primary NHBE cells, CuFi-3 cells and CF primaryCFHBE cells after stimulation with LXA₄ (100nM). (B) Typical I-V current relationships of the effect of LXA₄ (100nM) obtained in NuLi-1 cells after treatment with the CFTR channel inhibition CFTR-inh172 (5mM) and histogram showing the absence of inhibitory effect in Nuli-1 cells.

Figure 7. Effect of BAPTA-AM on the whole-cell currents induced by LXA₄. 
Typical I-V current relationships obtained upon exposure to LXA₄ (100nM, 10min) with or without BAPTA (10μM) treatment 1h prior to LXA₄ exposure in NuLi-1 (A) and CuFi-3 (B) cells. (C) Mean inward conductance changes normalized to control values (gi/gic) obtained upon exposure to LXA₄
alone (10min, 100nM) and after BAPTA-AM pre-treatment in Nuli1 (open bars) and CuFi-3 (black bars) cells.

**Figure 8.** LXA₄ (100nM) effects on airway surface liquid height in NuLi-1 and CuFi-3 epithelia. (A) Typical z-plane confocal sections of showing ASL responses to LXA4 in NuLi-1 and CuFi-3 epithelial cell monolayers labelled with calcein green, and ASL labelled with dextran-conjugated Texas red™ fluorochrome. (B) Effect of bumetanide (10µM) and Boc-2 (10µM) on the ASL height responses to LXA₄ treatment in NuLi-1 and CuFi-3 epithelial monolayers
Figure 1
Figure 3
Figure 4
Figure 6
Figure 7
A  

NuLi-1  CuFi-3  

control  

LXA₄  

B  

Nuli-1  CuFi-3  

- without inhibitor  
- Boc-2  
- bumetanide  

ASL height (μm)  

control  LXA₄  

Figure 8
Table 1

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Mean outward ($G_{out}$) and inward ($G_{in}$) conductances and reversal potentials ($V_r$) measured in non CF and CF airway epithelial cells in primary culture and NuLi-1 and CuFi-3 cell lines in control conditions and stimulated by LXA$_4$ (100nM, 10 min).