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Azithromycin fails to reduce inflammation in cystic fibrosis airway epithelial cells

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ABSTRACT

Cystic fibrosis is a hereditary disease caused by a mutation in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene that encodes a chloride (Cl^-) channel. Cystic fibrosis pulmonary pathophysiology is characterised by chronic inflammation and bacterial infections. Azithromycin, a macrolide antibiotic, has shown promising anti-inflammatory properties in some inflammatory pulmonary diseases. Moreover, all clinical studies have presented an improvement of the respiratory condition of cystic fibrosis patients, but the molecular and cellular mechanisms remain unknown. The aim of this study was to investigate, in bronchial epithelial cells, the effects of azithromycin on inflammatory pathways involved in cystic fibrosis. We have analysed the effects of azithromycin on cystic fibrosis and non-cystic fibrosis bronchial epithelial cell lines but also in non-immortalized non-cystic fibrosis human glandular cells. To create an inflammatory context, cells were treated with Tumor Necrosis Factor (TNF)-α or Interleukin (IL)1-β. Activation of the NF-κB pathway was investigated by luciferase assay, western blotting, and by Förster Resonance Energy Transfer imaging, allowing the detection of the interaction between the transcription factor and its inhibitor in live cells. In all conditions tested, azithromycin did not have an anti-inflammatory effect on the cystic fibrosis human bronchial epithelial cells and on CFTR-inhibited primary human bronchial glandular cells. More, our data showed no effect of azithromycin on IL-1β– or TNF-α–induced IL-8 secretion and NF-κB pathway activation. Taken together, these data show that azithromycin is unable to decrease *in vitro* inflammation in cystic fibrosis cells from airways.

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1. Introduction

Cystic fibrosis is the most common genetic disorder in Caucasian populations. Cystic Fibrosis Transmembrane conductance Regulator (*CFTR*) gene is a member of the "ATP-binding cassette" superfamily of transporters, that encodes a chloride (Cl^-) channel (O'Sullivan and Freedman, 2009). Lung disease is the foremost cause of morbidity and mortality in cystic fibrosis patients due to an exaggerated inflammation and to abnormally thick mucus resulting in chronic infections. In cystic fibrosis airways, the classical origin of inflammation supports the concept of an increase in the transcription Nuclear Factor-κB (NF-κB) and the Mitogen-Activated Protein kinases (MAP kinases)/Activator Protein (AP)-1 activities that leads to an increase in interleukin (IL)-8 production and neutrophil recruitment (Muselet-Charlier et al., 2007; Perez et al., 2007; Raia et al., 2005; Srivastava et al., 2006; Tabary et al., 2006).

To treat this inflammation many therapeutic strategies have been tested like corticosteroids and macrolides. Macrolides are antibiotics that have shown anti-inflammatory effects in some pulmonary diseases and on bronchial epithelial cells through the inhibition of NF-κB and/or MAP kinases pathways (Cigana et al., 2006, 2007; Shinkai et al., 2006). Macrolides, such as erythromycin and azithromycin, have been used to successfully treat patients with diffuse panbronchiolitis (DPB), which exhibits some similarities with cystic fibrosis (Brugiere et al., 1996; Sakito et al., 1996). Because macrolides can increase survival among DPB patients, they have been tested in cystic fibrosis patients. These studies have reported improvement of the clinical parameters in cystic fibrosis and summarized in a meta-analysis (Florescu et al., 2009). Thus, the authors demonstrated that azithromycin is clearly associated with improvement of lung parameters with a reduced rate of decline of lung function by an unknown mechanism.

This study investigates the effects of macrolides on inflammatory pathways in CFTR-sufficient and CFTR-deficient *in vitro* bronchial epithelial cells.

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2. Materials and methods

2.1. Materials

CFTR inhibitor, CFTR inhibitor (inh-172, 10 μM), clarithromycin and erythromycin were purchased from Sigma-Aldrich (both macrolides were used at a 10 μg/ml-concentration). Azithromycin (10 μg/ml) was a kind gift from Pfizer. This concentration is physiologically relevant and concordant with data obtained by Di Paolo *et al.* who have shown that a 9,17 μg/ml concentration was found in the lungs of non-cystic fibrosis patients treated with a 500 mg/day oral dose of azithromycin (Di Paolo et al., 2002). TNF-α and IL-1β were purchased from R&D Systems (R&D Systems, Lille, France) and used at the final concentration of 10 ng/ml as previously described (Muselet-Charlier et al., 2007).

2.2. Cell culture

IB3-1 is a bronchial epithelial cell line derived from a cystic fibrosis patient with a F508del/W1282X CFTR genotype. It was used in conjunction with the S9 cell line that was derived from IB3-1 cells after stable transduction with wild-type *CFTR* (CFTR-corrected cells) (Zeitlin et al., 1991). Both cell lines were purchased from the American Type Culture Collection (LGC Promochem SARL, Strasbourg, France) and cultured as previously described (Roque et al., 2008). It has been shown that CFTR Cl^- channel dysfunction in cystic fibrosis tracheal submucosal gland cells leads to abnormal transepithelial salt and fluid secretion. Based on these observations it is reasonable to postulate that intrinsic abnormalities in these cells related to CFTR deficiency could lead early inflammation in cystic fibrosis airways. More, a recent study comparing the genome-wide expression profile of tracheal and bronchial human airway epithelia *in vivo* to the expression profile of primary cultures of human airway epithelia has shown that the use of primary cultures is important to recapitulate airway epithelia biology (Pezzulo et al., 2011). Therefore, non-immortalized Human Bronchial Glandular (HBG) cells from non-cystic fibrosis adult patients were also used, and exhibited *in vitro* characteristics of bronchial secretory glandular epithelial cells as previously described (Tabary et al., 1998). The protocol was approved by the local ethics committee of Broussais Hospital (Paris, France). Briefly, cells isolation were obtained by enzymatic digestion from bronchial submucosal and grown onto type I collagen coated culture flasks in a DMEM/Ham's F12 mixture (50/50%, v/v) supplemented with 1% Ultroser G (Pall Corporation, Saint Germain en Laye, France) (Kammouni et al., 1997). After 4 weeks, third-passage HBG cells has proliferated and exhibited characteristics of homogeneous submucosal epithelial and secretory cells (Tabary et al., 1998). For this study, cells were treated for 72 h with a CFTR inhibitor (Inh-172) or with vehicle alone (dimethyl sulfoxide) before macrolide treatment.

2.3. CFTR activity

The activity of CFTR protein was assessed by I^- quenching of halide-sensitive YFP using Premo Halide sensor technology (Invitrogen, Villebon sur Yvette, France). Assays were performed using a FluoStar fluorescence plate readers (BMG Labtechnologies, Champigny sur Marne, France). As previously described, each well of a 96-well plate, containing the cells, was washed three times with PBS (200 μl/wash), leaving 50 μl of PBS. CFTR conductance was stimulated by an agonist mixture (forskolin, 3-isobutyl-1methylxanthine, apigenin). After 15 min, the 96-well plates were transferred to a plate reader for fluorescence assay. Each well was assayed individually for CFTR-mediated I^- efflux by recording fluorescence continuously (400 ms/point) for 2 s (base line), then 50 μl of a 140 mM I^- solution. Iodide rather than chloride is used because of strong YFP-H148Q/I152L quenching by iodide, and because CFTR is permeable to iodide (Verkman and Galietta, 2009).

2.4. ELISA IL-8

Cells were pre-treated with the macrolides or the vehicle for 30 and TNF-α, IL-1β, or LPS solutions were added for 16 h before assays. Culture supernatants were collected, centrifuged to remove the debris and stored at −80 °C until IL-8 protein concentration was determined using the human IL-8 Duo Set kit (R&D Systems, France), following the manufacturer's instructions.

2.5. 7-plex phosphoprotein assay

After treatment, proteins were extracted using Bio-Plex cell lysis kit (Bio-Rad, Marnes-la-Coquette, France) following the manufacturer's instructions and proteins were quantified. To detect the chosen phosphoproteins (P-p65-NF-κB, P-p38 MAP kinase, P-JNK, P-ERK, P-STAT3, P-STAT6, P-CREB), the Phosphoprotein Detection Kit was used (Bio-Rad, France) and fluorescently labelled streptavidin reporter was added to wells before the phosphoproteins were quantified with the Bio-Plex Array Reader.

2.6. NF-κB and AP-1 luciferase reporter gene assay

Cells were grown to 70% confluence on 12-well plates and were transiently transfected with 0.5 μg of one of the luciferase expression vector (NF-κB-Luc or AP-1-Luc; Stratagene, Montigny-le-Bretonneux, France), along with 0.5 μg of pRL-TK vector (Promega, Lyon, France) as an internal control, using the Exgen 500 reagent (Euromedex, Souffelweyersheim, France) following the protocol previously published (Roque et al., 2008).

2.7. SDS-PAGE and immunoblotting

Equal amounts of total protein (20 μg) from cultured cystic fibrosis and non-cystic fibrosis airway epithelial cells were loaded on 10% SDS-polyacrylamide gels. Specific antibody against IκB-α (Abcam, Paris, France) and β-actin (Sigma, St. Quentin Fallavier, France) was performed and quantified on a LAS 1000 densitometer; the intensities of the bands were compared on a basis of adjusted volume (mean optical density x area).

2.8. Förster Resonance Energy Transfer (FRET) experiments

FRET experiments were performed with YFP-p65/NF-κB and IκBα-CFP plasmids as previously described (Tabary et al., 2006).

2.9. Data analysis

The data are expressed as mean ± S.E.M. of at least five different experiments in duplicate. The n in the figure legend indicates the number of experiments. Statistical differences were determined using either a paired or an unpaired, one-tailed *t* test. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. IL-1β and TNF-α, but not LPS from P. aeruginosa, induce IL-8 secretion by cystic fibrosis and non-cystic fibrosis cell lines

To determine CFTR activity in our cells, we have performed experiments using the iodide-sensitive fluorescent protein method (Verkman and Galietta, 2009). Fig. 1A illustrates screening in a 96-well format with normal or corrected cells (HBG and S9 cells) and CFTR deficient cells (IB3-1, S9) and HBG treated with CFTR inhibitor, CFTR (Inh-172). In S9 and HBG cells, we observed a strong decrease of YFP fluorescence that was inhibited in IB3-1 and in S9 or HBG cells cultured with the CFTR inhibitor.

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