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Fluticasone reduces IL-6 and IL-8 production of cystic fibrosis bronchial epithelial cells *via* IKK-β kinase pathway

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ABSTRACT: Inhaled fluticasone propionate (FP) is widely used to reduce pulmonary inflammation in chronic obstructive pulmonary disease, but the potential effects of FP on airway epithelial cells from patients with cystic fibrosis (CF) are unknown. In CF disease, a nonregulated inflammatory lung response occurs through exaggerated nuclear factor (NF)-κB activation and elevated pro-inflammatory cytokines production by airway epithelial cells.

To determine whether FP reduces cytokine production in bronchial epithelial cells *via* NF-κB, the authors investigated the nonstimulated and the *Pseudomonas aeruginosa* lipopolysaccharide (LPS) stimulated production of NF-κB-dependent interleukin (IL)-6, IL-8 and RANTES (regulated on activation, T-cell expressed and secreted) along with the activation of NF-κB in non-CF and CF human bronchial gland epithelial cells.

It was demonstrated that a relevant concentration of FP (10^{-8} M) inhibited constitutive and *P. aeruginosa* LPS-induced IL-6 and IL-8 production of non-CF and CF bronchial epithelial cells. Interestingly, the expression of two IκB kinases (IKK)-α/β, the degradation of cytosolic IκB-β inhibitor and the NF-κB deoxyribonucleic acid binding activity were markedly reduced after FP treatment in both CF and non-CF bronchial epithelial cells.

It was shown by the authors that fluticasone propionate exerts an anti-inflammatory effect by blocking a signal transduction leading to a reduced level of IκB-α/β kinases in bronchial epithelial cells. In particular the strong effect on the IκB-β kinase, which is known to be elevated in bronchial epithelial cells in cystic fibrosis patients, was observed.

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Cystic fibrosis (CF) is a lethal inherited disorder caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) in airway epithelial cells [1]. One of the major clinical manifestations of CFTR mutations is neutrophil dominated inflammation [2, 3], implying that defective CFTR affects the immune function of airway epithelial cells, in particular the production of nuclear factor (NF)-κB-dependent chemokines. Increasing evidence suggests that respiratory epithelial cells with dysfunctional CFTR have exaggerated activation of NF-κB/inhibitor of NF-κB complex associated with upregulated expression of chemokines [4, 5]. The present authors and previous studies, have previously demonstrated that epithelial expression of interleukin (IL)-8 is excessively elevated in the bronchial epithelial cells of human CF adults [6]. This was observed in both matched CF and corrected respiratory epithelial cell lines [7, 8] and in CF transmembrane conductance regulator homozygous deficient mice (CFTR-/-) compared to normal mice after *Pseudomonas aeruginosa* stimulation [9]. How CFTR dysfunction in CF airway cells contributes to the activation and nuclear localisation of NF-κB is still unclear. Recently, an *in vitro* study on the expression of ΔF508 CFTR (CFTR which contains a deletion of phenylalanine at position 508) in the ovary cells of Chinese hamsters, which do not express CFTR, revealed a seven-fold increase in the activation of NF-κB compared to the wild-type CFTR or the G551D mutant. This effect was also paralleled with elevated IL-8 expression [5].

Therapeutic strategies include the blocking of transcription factors such as NF-κB that lead to inflammatory gene activation and the inhibition of signaling pathways that are stimulated in lung diseases. The effects of corticosteroids are predominantly mediated by the inhibition of NF-κB deoxyribonucleic acid (DNA)-binding activity, which controls genes encoding inflammatory cytokines, chemokines and adhesion molecules in respiratory epithelial cells [10–13]. The inhaled steroid fluticasone propionate (FP) is widely used clinically as an anti-inflammatory and immunosuppressive agent, especially in the treatment of asthma [14] and allergic rhinitis [15]. Recent reports suggest that *in vivo* administration of FP attenuates pulmonary inflammation through its ability to reduce the number of eosinophils in airway biopsies [16] and inhibit neutrophil chemotaxis [17]. FP induces apoptosis of eosinophils [18] and also reduces the production of several cytokines such as IL-1β, IL-6, IL-8 and RANTES (regulated on activation, T-cell expressed and secreted) by alveolar macrophages [19] and lymphocytes [20, 21]. Despite all of these anti-inflammatory effects, molecular targets of FP in bronchial epithelial cells are unknown. Understanding such mechanisms is of great interest and may lead to the development of new therapeutic approaches capable of reducing airway inflammation, which is early, excessive and sustained in CF patients.

The aims of the present study were to: 1) evaluate the ability of FP to reduce the constitutive and *P. aeruginosa* lipopolysaccharide (LPS)-induced production of pro-inflammatory

cytokines IL-6, IL-8 and RANTES in cultured bronchial gland epithelial cells from CF and non-CF patients; and 2) to examine the FP action on the molecular targets of the NF-κB/IκB-α pathway.

Materials and methods

Cell culture

Cell isolation and culture procedures of human bronchial submucosal gland epithelial cells were performed on bronchial tissues collected from adult CF patients (all ΔF508 homozygous for CFTR mutation) and non-CF control patients undergoing lung transplantation, as previously described [22]. Bronchial tissues for non-CF control experiments were obtained from patients with primary pulmonary hypertension and pulmonary idiopathic fibrosis. Bronchial gland epithelial cells were isolated by enzymatic digestion from bronchial submucosa and grown on type I, collagen coated, 25 cm^2 tissue-culture flasks in Dulbecco modified Eagle's medium (DMEM) with Ham's F12 (50/50%, v/v), supplemented with 1% Ultroser G (a serum substitute; Sepracor, Villeneuve-la-Garenne, France), glucose (10 $g \cdot L^{-1}$), sodium pyruvate (0.33 $g \cdot L^{-1}$). Penicillin G (100 $U \cdot mL^{-1}$) and streptomycin (100 $\mu g \cdot mL^{-1}$) were also added as antibiotics.

Exposure of cystic fibrosis and noncystic fibrosis bronchial epithelial cells to fluticasone propionate

CF and non-CF bronchial epithelial cells (1×10^4 cells) were grown in 24-well culture plates. Prior to their exposure to FP, CF and non-CF bronchial epithelial cells were incubated for 16 h in an Ultroser G-free DMEM/Ham's F12 medium in 95% air and 5% CO_2. At the end of the 16-h period, CF and non-CF bronchial epithelial cells were pretreated with either DMEM/Ham's F12 medium alone or the medium with various concentrations of FP (10^{-9}–10^{-6} M) for 2 h. Afterwards the cells were activated for 4 h with DMEM/Ham's F12 containing 1.0 $\mu g \cdot mL^{-1}$ *P. aeruginosa* LPS (serotype 10; Calbiochem, San Diego, CA, USA). A stock solution of FP (10^{-3} M) generously provided by GlaxoSmithKline was prepared in 99.5% ethanol (Merck Eurolab, Darmstadt, Germany). FP was further diluted with DMEM/Ham's F12 medium to final FP concentrations (10^{-9}–10^{-6} M) and used throughout the experiments. Immediately after each period of cell exposure, cells and culture supernatants were collected and stored at -80°C.

Enzyme-linked immunoabsorbent assay for interleukin-6 and intereukin-8 and regulated on activation, T-cell expressed and secreted

The enzyme-linked immunoabsorbent assay (ELISAs) for IL-6, IL-8 and RANTES cytokine detection, were sensitive at 2 $pg \cdot mL^{-1}$, 5 $pg \cdot mL^{-1}$ and 2 $pg \cdot mL^{-1}$ respectively, were performed by following the manufacturer's instructions (Biosource International, Camarillo, CA, USA). All reagents were molecular biology grade and all buffers and solutions were prepared using pyrogen-free grade water. In all culture supernatants, undetectable levels of endotoxin (detection limit $\geqslant 5$ $pg \cdot mL^{-1}$) were found using a quantitative chromogenic Limulus Amoebocyte Lysate assay (LAL; Bio Whittaker, Emerainville, France). Cell viability of CF and non-CF bronchial epithelial cells always exceeded 97%, as determined by trypan blue exclusion, after all experimental procedures. Data were expressed either as $ng \cdot mL^{-1}$ per 10^6 viable cells or as released cytokine percentage compared to control.

Immunofluorescence for IκB-α kinase expression

CF and non-CF bronchial epithelial cells cultured in the absence or presence of FP (10^{-8} M, 16 h) were fixed *in situ* in cold methanol at -20°C, air dried, and rehydrated in phosphate-buffered saline (PBS) at pH 7.2. Cells were stained for IκB-α expression using sheep antiserum to human IκB-α (Serotec Ltd, Oxford, UK) for 1 h at room temperature and a donkey anti-sheep fluorescein isothiocyanate (FITC) conjugated antibody (Ab) for 45 min at room temperature as previously described [23]. Negative controls were obtained by using either nonspecific immunoglobulin (Ig)G as the primary Ab (M7769; Sigma Aldrich Chimie, Lyon, France) or FITC conjugated Ab alone. Representative fields of CF and non-CF bronchial gland cells cultured in different conditions were recorded using a Zeiss Axiophot microscope (Zeiss, Le Pecq, France).

Cell extracts and Western blot analysis

CF and non-CF bronchial epithelial cells cultured in the absence or presence of FP (10^{-8} M, 16 h) were washed in PBS (pH 7.2) and cell proteins were extracted (15 min, 4°C) in radioimmunoprecipitation buffer (50×10^{-3} M Tris, pH 7.4, 15×10^{-4} M NaCl, 1% Triton X100, 0.1% sodium doodecylsulphate, 5×10^{-3} M iodoacetamide, sodium deoxycholate and 1% Nonidet P-40), which was supplemented with 1% phenylmethylsulfonyl fluoride. Cells were then harvested and the protein extracts were centrifuged (12,500×*g*, 10 min, 4°C). Supernatants were collected and used to assay IκB-α inhibitor and the two IκB kinases (IKK-α/β) as previously described [22]. An equal amount of protein (10 $\mu g \cdot mL^{-1}$) from each protein extraction was submitted to electrophoresis under denaturing conditions using 4–15% polyacrylamide gels (Pharmacia Biotech., Paris, France). The gels were then transferred onto a nitrocellulose membrane (Millipore, Bedford, MA, USA) by electroblotting. After successive washes, the membranes were analysed by using the Western blot technique with rabbit polyclonal antihuman IκB-α, antihuman IKK-α and antihuman IKK-β antibodies (Santa Cruz Biotechnology, CA, USA) and developed with an enhanced chemiluminescence kit (Amersham Life Science, Freiburg, Germany). Densitometric analysis of Western blots was performed on a Bio-Rad model GS-690 imaging densitometer. The gels were scanned in the transmittance mode at a resolution setting of 800 dots per inch. The intensity of each band was compared with the adjusted volume (mean optical density×area in mm^2).

Nuclear protein extraction and electrophoretic mobility shift assay

Nuclear extracts were prepared and analysed after the CF and non-CF bronchial epithelial cells were incubated in different conditions, as previously described [22]. Nuclear extracts of CF and non-CF bronchial epithelial cells were incubated with a ^{32}phosphate (P)-labelled NF-κB oligonucleotide. The consensus κB DNA sequence used for the electophoretic mobility shift assay (EMSA) was 5′AGTTGAGGGGACTTTCCCAGGC3′ (Promega Corp., Madison, WI, USA). The oligonucleotide was radiolabelled with [α^{32}P] using T4 polynucleotide kinase (Pharmacia Biotech.). The protein

DNA complexes were electrophoresed on a nondenaturing 5% polyacrylamide gel, then dried under vacuum and exposed at -80°C with autoradiographic film. In competition studies and supershift assay, a 100-fold molar excess of unlabelled oligonucleotide or 1 μg Ab was added to the binding reaction mixture, prior to the addition of the labelled κB probe. Identification of the different NF-κB heterodimeric proteins was carried out by incubating the nuclear extracts with polyclonal antibodies against the NF-κB proteins NF-κB1 (p50) and (p65) RelA (Santa Cruz Biotechnology), prior to the addition of the labelled κB probe.

Statistical analysis

Results were expressed as mean±SD. Each data point was confirmed in triplicate and each cell culture experiment was performed $\geqslant 3$ times. The statistical significance of differences in cytokine levels was determined by analysis of variance.

Results

Effects of fluticasone propionate on the secretion of interleukin-6, interleukin-8 and regulated on activation, T-cell expressed and secreted from cystic fibrosis and non-cystic fibrosis bronchial epithelial cells

Under resting culture conditions (*i.e.* over a 4-h period in the unstimulated state), the spontaneous secretions of IL-8, IL-6 and RANTES were 3.5-, 8.0- and 10.0-fold higher in CF bronchial epithelial cells compared to non-CF bronchial epithelial cells, respectively (fig. 1). Exposure of both CF and non-CF bronchial epithelial cells to FP (10^{-9}–10^{-6} M, 4 h) significantly ($p<0.05$) reduced the IL-6 and IL-8 secretion in a dose-dependent manner (fig. 2a–d). A significant reduction ($p<0.05$) of RANTES was observed only in non-CF bronchial epithelial cells with FP at 10^{-7} M and 10^{-8} M (fig. 2c and d). Interestingly, pretreatment with FP (10^{-8} M) significantly ($p<0.05$) reduced the *P. aeruginosa* LPS induced IL-8 and IL-6 production in CF and non-CF bronchial cells. RANTES production was significantly ($p<0.01$) reduced in *P. aeruginosa* LPS stimulated non-CF bronchial epithelial cells with FP at 10^{-8} M. There was no significant effect of FP on unstimulated and *P. aeruginosa* LPS stimulated RANTES secretion in CF bronchial epithelial cells (fig. 2c and d).

Exposure of CF and non-CF bronchial epithelial cells to FP at 10^{-8} M for a 16-h incubation period resulted in a significant decrease of IL-6 and IL-8 production (fig. 3a and b). In contrast to non-CF bronchial epithelial cells, no significant decrease of RANTES secretion was observed in CF bronchial epithelial cells (fig. 3c).

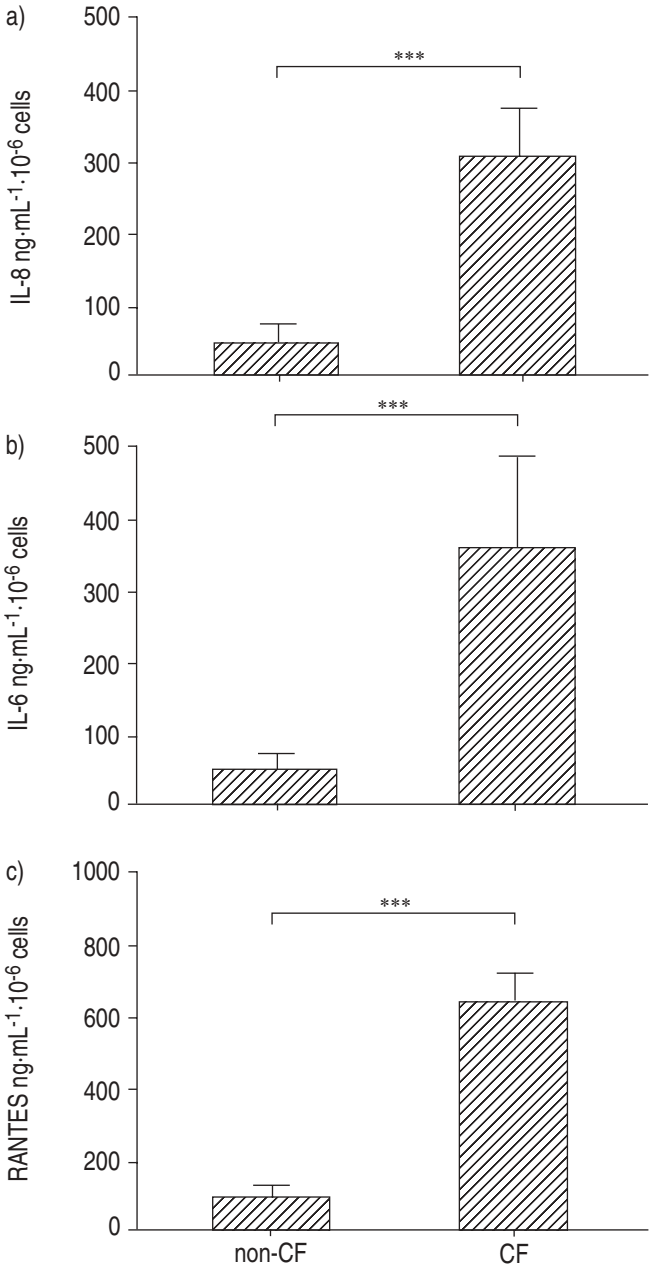

Fig. 1.–Spontaneous levels of cytokines interleukin (IL)-8, IL-6 and regulated upon activation, normal T-cell expressed and secreted (RANTES) released by cultured non-cystic fibrosis (non-CF) and ΔF508 homozygous cystic fibrosis (CF) bronchial epithelial cells in the unstimulated (4 h-period) state. Data are presented as mean±SD of three separate experiments. ***: $p<0.001$.

Fluticasone propionate, a potent inhibitor of constitutive nuclear factor-κB activation in cystic fibrosis bronchial epithelial cells

Because most pro-inflammatory cytokine genes including IL-6 and IL-8 contain κB-binding motifs in their promoter regions, the authors investigated whether the inhibition of cytokine expression by FP (10^{-8} M, 16 h) was due to the reduction of NF-κB activation.

The electrophoretic mobility shift assays (EMSAs) of nuclear extracts harvested from unstimulated-CF bronchial epithelial cells demonstrated high constitutive level of activated NF-κB (fig. 4a, lane 3). In *P. aeruginosa* LPS stimulated non-CF bronchial epithelial cells in which the activation of NF-κB was observed (fig. 4a, lane 1), FP treatment markedly reduced *P. aeruginosa* LPS induced NF-κB binding activity (fig. 4a, lane 2 compared to lane 1). No endogenous NF-κB binding activity was found in unstimulated-non-CF bronchial epithelial cells, as previously reported [24] (data not shown). Exposure of CF bronchial epithelial cells to FP led to a strong downregulation of NF-κB (fig. 4a, lane 4). The specificity of NF-κB DNA-binding was confirmed in competition experiments. Incubation of nuclear extracts from resting CF bronchial cells with a 100-fold excess of unlabelled (cold κB) NF-κB oligonucleotide led to a complete inhibition of binding activity (fig. 4b, lane 1). The components of the NF-κB DNA-binding protein complex was determined by performing supershift assays with antibodies to the p65

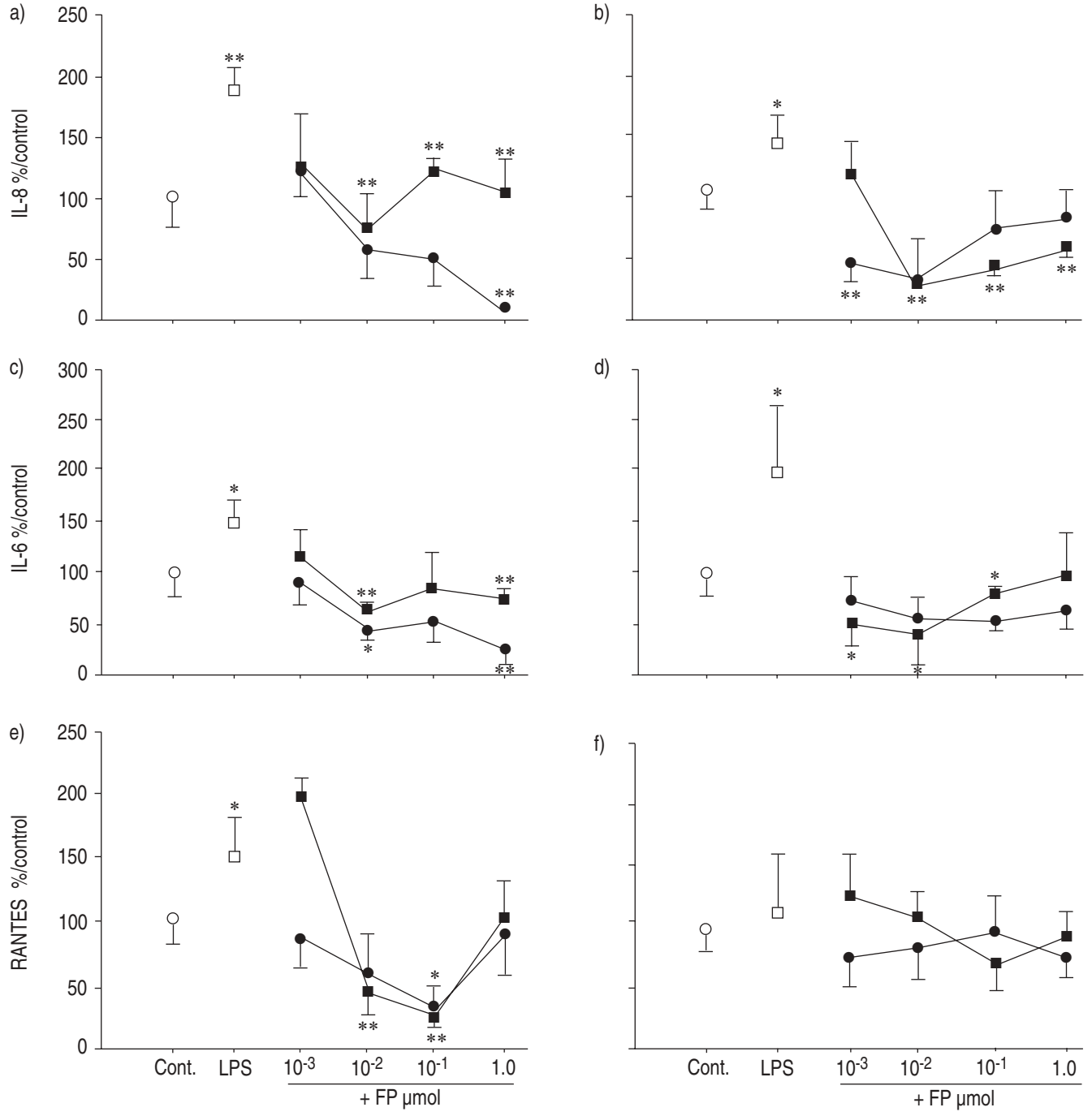

Fig. 2. – Concentration effect of fluticasone propionate (FP) on the release of interleukin (IL)-8 (a, b), IL-6 (c, d) and regulated upon activation, normal T-cell expressed and secreted (RANTES) (e, f) from cultured human non-cystic fibrosis (non-CF) (a, c and e) *versus* ΔF508 homozygous cystic fibrosis (CF) bronchial epithelial cells incubated for 2 h (b, d, f). Control (Cont.) non-CF and CF bronchial epithelial cells were maintained either in basal growth medium alone (○) or with *Pseudomonas aeruginosa* lipopolysaccharide (LPS) added (1.0 µg·mL^{-1}; □). Non-CF and CF bronchial cells were pretreated with FP for 2 h with (●) or without (■) the addition of *P. aeruginosa* LPS (1.0 µg·mL^{-1}) for 4 h. Data are presented as percentage over control CF and non-CF bronchial epithelial cells, respectively. Data represent mean±SD of three separate experiments each condition carried out in duplicate. *: $p<0.05$; **: $p<0.01$ compared with basal growth medium alone or with *P. aeruginosa* LPS added.

subunit of NF-κB. As shown in figure 4b, the addition of the antibodies to p65 caused a supershift (fig. 4b, lane 2).

Fluticasone propionate induces the nuclear factor inhibitor-κB-α expression

The expression of IκB-α in the absence or presence of FP (10^{-8} M, 16 h) was analysed in both CF and non-CF bronchial epithelial cells. It was shown that an increase in IκB-α occurs in the cytoplasm of non-CF bronchial epithelial cells after FP treatment (fig. 5). Compared with untreated CF bronchial epithelial cells, in which no detectable immunoreactivity for IκB-α was observed, FP treatment permitted the induction and maintenance of IκB-α expression in CF bronchial epithelial cells (fig. 5a). These results were further confirmed by Western blot showing that the IκB-α level in FP-treated CF bronchial epithelial cells, evaluated by

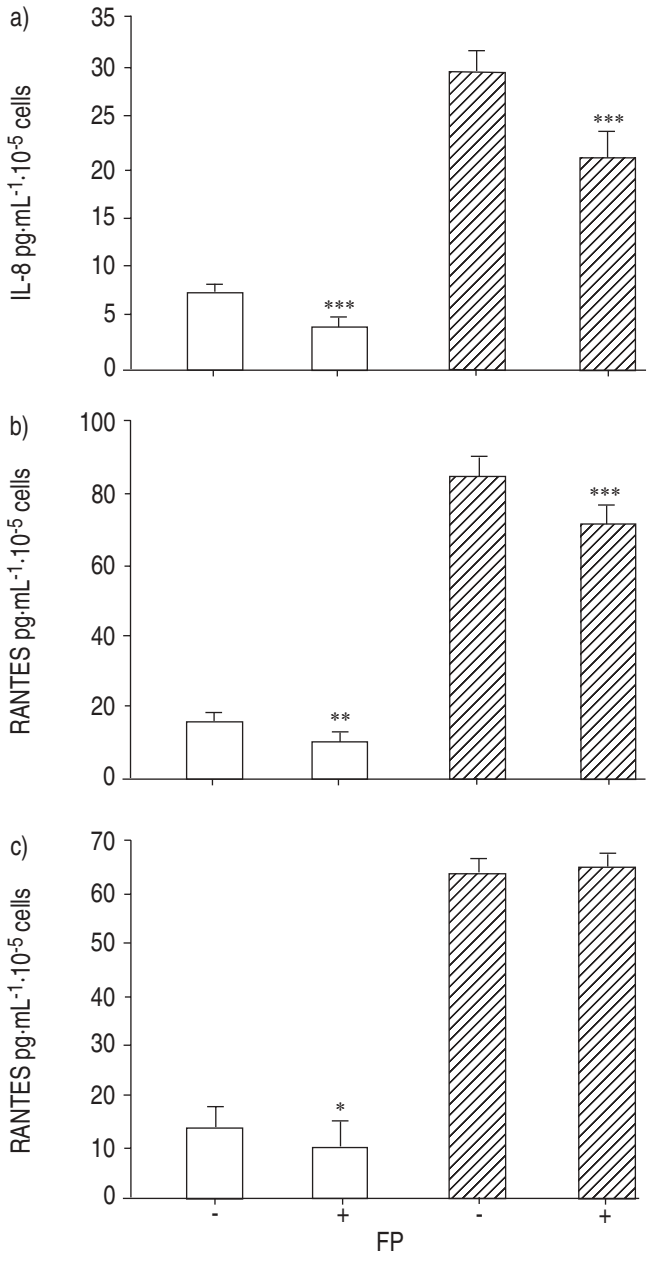

Fig. 3. – Levels of cytokines interleukin (IL)-8, IL-6 and regulated upon activation, normal T-cell expressed and secreted (RANTES) released by non-cystic fibrosis (non-CF: □) and ΔF508 homozygous cystic fibrosis (CF: ▨) bronchial epithelial cells after their exposure to fluticasone propionate (FP) (10^{-8} M) for 16 h (+). Data are presented as mean±SD of three separate experiments in which each condition was carried out in duplicate. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$, compared with the medium without FP (-).

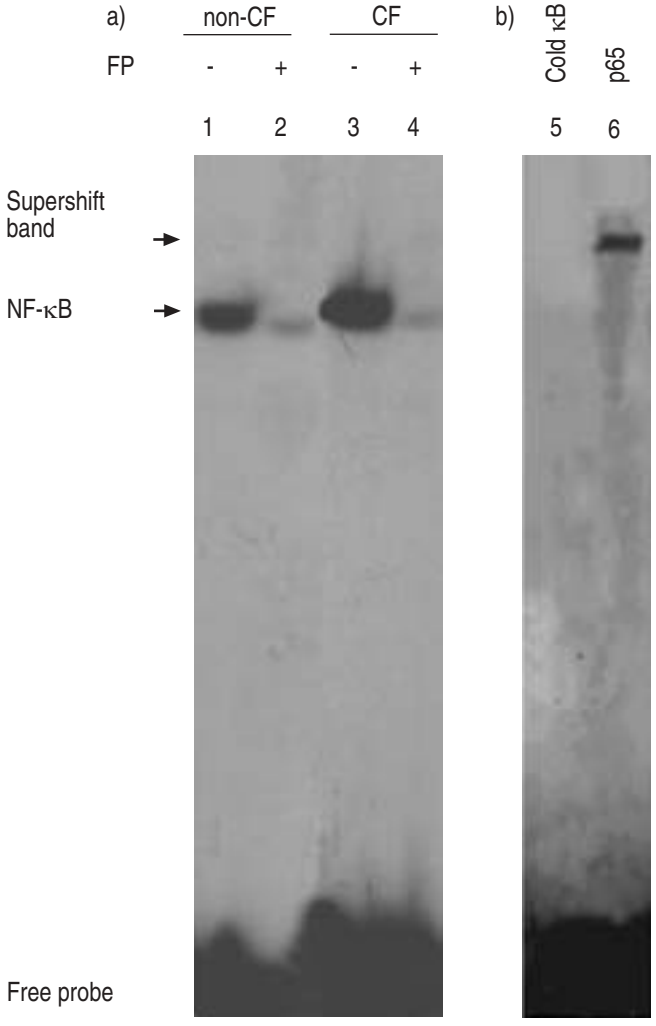

Fig. 4. – Nuclear factor (NF)-κB binding activity in *Pseudomonas aeruginosa* lipopolysacharide (LPS) stimulated non-cystic fibrosis (non-CF) bronchial cells (a, lane 1) and unstimulated ΔF508 homozygous cystic fibrosis (CF) bronchial epithelial cells (a, lane 3) incubated with fluticasone propionate (FP) (10^{-8} M) for 16 h (a, lanes 2 and 4, respectively). To demonstrate the specificity of binding of the NF-κB oligonucleotide, a 100-fold M excess of unlabelled NF-κB (b, lane 5, cold κB) was used to compete with the labelled NF-κB probe. The addition of antibody to RelA (p65 subunit) component (b, lane 6, p65) caused a supershift, as indicated. This figure is representative of three experiments.

densitometric analyses raised to 60% of the IκB-α level found in untreated non-CF bronchial epithelial cells (fig. 5e). These results, which agree with the EMSA data (fig. 4), indicate that constitutive NF-κB activation detected in CF bronchial epithelial cells is reduced with FP treatment *via* the induction and the maintenance of cytosolic IκB-α.

Fluticasone propionate reduces the IκB-α/β kinase protein expressions

To determine whether the level of IκB-α in CF and non-CF bronchial gland cells before and after FP treatment was related to the level of IKK-α and IKK-β expressed, Western blots were performed on cell extracts from CF and non-CF bronchial gland cells cultured in the absence or presence of FP (10^{-8} M, 16 h). In contrast to the level of IKK-α, an elevated basal level of IKK-β was found in CF bronchial epithelial cells (a 4.5-fold increase) compared to that observed in non-CF bronchial epithelial cells. Consistent with the increased level of IκB-α inhibitor after FP treatment (fig. 6), FP induced a significant reduction of both the IKK-α and IKK-β kinases in CF and non-CF bronchial epithelial cells (fig. 6a and b). Interestingly, image analysis of digitised Western blots of IKK-β level in CF bronchial epithelial cells after FP treatment revealed a marked decrease of IKK-β (an 80% reduction) to a level similar to that found in untreated non-CF bronchial epithelial cells (fig. 6b, lane 4 compared to lane 1).

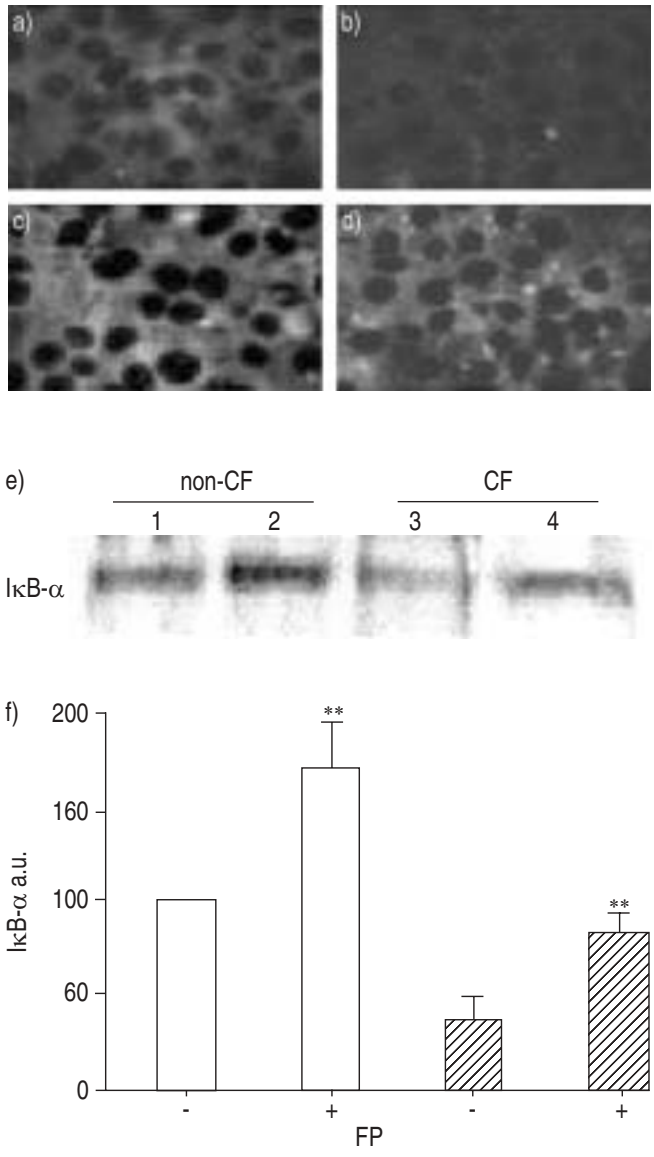

Fig. 5. – Expression of cytosolic inhibitor of nuclear factor-κB (IκB-α) protein in non-cystic fibrosis (non-CF: □) and ΔF508 homozygous cystic fibrosis (CF: ▨) bronchial epithelial cells before (a, b) and after their exposure to fluticasone propionate (FP) (10^{-8} M) for 16 h (c, d), as demonstrated by immunofluorescence. Equal amounts of protein extract from non-CF and ΔF508 homozygous CF cells treated with FP or not were analysed for levels of IκB-α by Western blotting using specific antibodies to IκB-α (e). Densitometric analyses of the data combined with three similar studies (f), are expressed in arbitrary units (a.u.). Densitometric results are reported as percentage of IκB-α levels compared with those obtained from untreated non-CF bronchial gland cells (e, lane 1). One representative experiment of three independent experiments is shown. **: $p<0.01$.

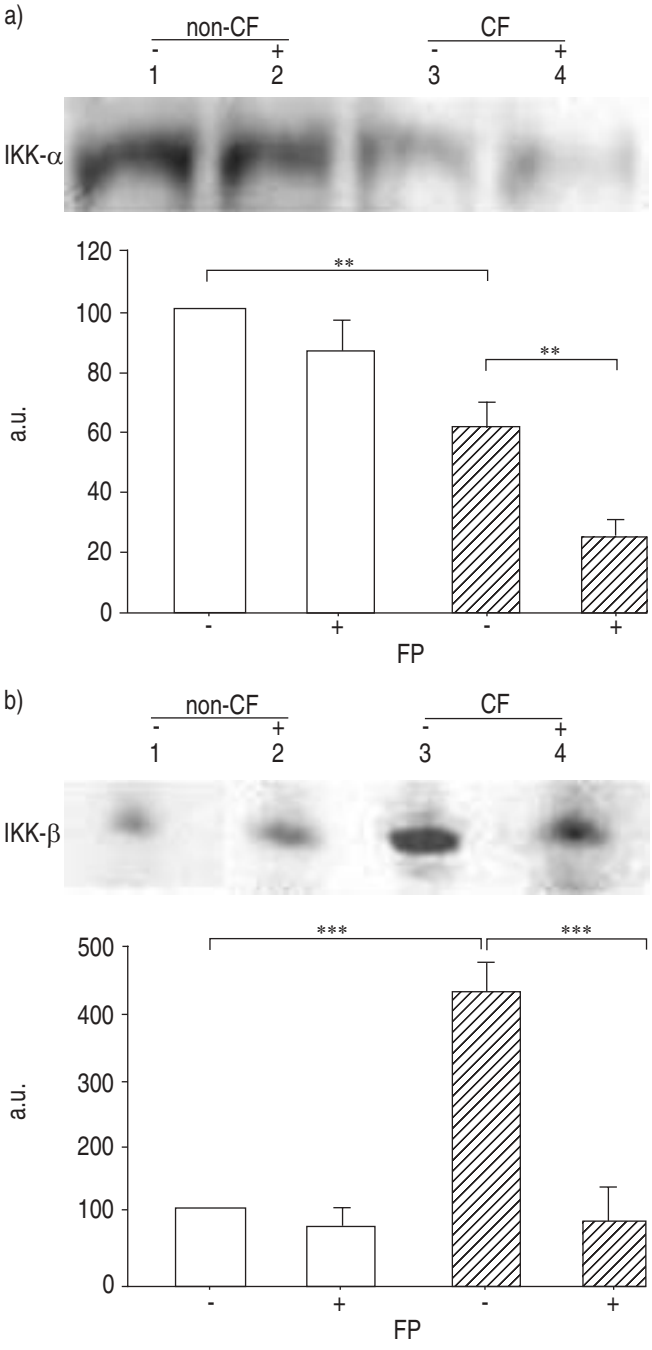

Fig. 6. – Expression of inhibitor of nuclear factor-κB kinase (IKK)-α and IKK-β protein levels in non-cystic fibrosis (non-CF: □) and ΔF508 homozygous cystic fibrosis (CF: ▨) bronchial epithelial cells after their exposure to 10^{-8} M fluticasone propionate (FP) for 16 h, as demonstrated by Western blotting (a and b). Equal amounts of protein extract from non-CF and ΔF508 homozygous CF cells treated with or without FP were analysed for the levels of IKK-α and IKK-β kinases. Densitometric analyses of the data combined with three similar studies, are expressed in arbitrary units (a.u.). Densitometric results are reported as percentage of IKK-α and IKK-β protein levels compared with those obtained from untreated non-CF bronchial epithelial cells (a and b, lane 1), respectively. The figure shows one representative experiment of three independent experiments. **: $p<0.01$; ***: $p<0.001$.

Discussion

The present findings show that FP reduces the IL-6 and IL-8 production of both unstimulated and *P. aeruginosa* LPS stimulated bronchial epithelial cells from non-CF and CF patients. In CF bronchial epithelial cells, this reduction of IL-6 and IL-8 production by FP was associated with a strong reduction of constitutive NF-κB activation, an increased accumulation of cytosolic IκB-α inhibitor and a reduction of both IKK-α and IKK-β kinase expression, particularly marked for IKK-β.

In this study *P. aeruginosa* LPS was used as an inflammatory stimulus in order to increase the pro-inflammatory cytokine release and to mimic the airway epithelium inflammatory response. Recently, ESCOTTE *et al.* [25] have described

an *in vivo* xenograft model of mild inflammation and showed that FP decreases the LPS-induced IL-8 release. Few studies have focused on the regulation of IκB proteins in the lungs after LPS stimulation. MIZGERD *et al.* [26] have recently reported that LPS induces a significant loss of IκB-α and IκB-β in a time-dependent manner, but they did not study the effect of an anti-inflammatory drug.

In contrast to the decrease of IL-6 and IL-8 after FP in non-CF and CF bronchial epithelial cells, a weak reduction of RANTES production was observed in CF bronchial epithelial cells after FP treatment. Some authors have shown that induction of RANTES expression requires activation of NF-κB in primary epithelial cells [27], whereas others have shown that RANTES and IL-8 gene expression are differently regulated by NF-κB [28]. In the present study, the lack of FP effect on RANTES expression in CF epithelial cells suggests that signalling pathways other than NF-κB may be implicated. DHAWAN *et al.* [29] recently identified a novel NF-κB inducing mitogen-activated protein kinase (MAPK) signaling pathway which is involved in the activation of IKKs and NF-κB. In the present study, MAPK signaling might regulate RANTES expression in a NF-κB independent manner in CF bronchial epithelial cells at resting state and after FP treatment. Further investigations are necessary to confirm this hypothesis.

In CF patients, bronchial epithelial cells produce large amounts of chemokine IL-8, even in the absence of bacterial infection [6, 24] and lower levels of anti-inflammatory cytokine IL-10 production [30], which play a central role in the onset and sustained airway inflammation in CF. Consequently, bronchial epithelial cells represent an important target of anti-inflammatory molecules and particularly of inhaled steroids. As previously reported, FP is a steroid with anti-inflammatory activity characterised by a high affinity for the steroid-binding site [31]. It has been described as more potent than other glucocorticoids, such as budenoside and beclomethasone. at repressing NF-κB activation in alveolar epithelial cells [32, 33]. In the present study, it was demonstrated that FP exerts maximal anti-inflammatory effects with a relevant concentration of FP (10^{-8} M) in both non-CF and CF bronchial epithelial cells. Previously the authors had reported that dexamethasone (DEX) used at higher concentrations (10^{-7}–10^{-5} M) was unable to significantly reduce the IL-8 production in CF but not in non-CF bronchial epithelial cells [24]. The reason why FP is more potent than DEX may be related to the lipophilic nature of FP and to a higher affinity for its glucocorticoid receptor (GR).

To further identify the potential molecular targets by which FP exerts its anti-inflammatory effect, the NF-κB/IκB-α pathway, known to be the major signaling pathway for the expression of most pro-inflammatory cytokine genes, was investigated. It was demonstrated, for the first time in the present study, that within CF bronchial epithelial cells, FP strongly reduces NF-κB activation which is paralleled with an increased level of cytosolic IκB-α inhibitor. In addition, it was also shown that FP decreases the level of both IKK-α and IKK-β expression, particularly for IKK-β (by a 4.5-fold reduction) in CF but not in non-CF bronchial epithelial cells. Further studies will be required to characterise the potential changes of IKK-α/β kinases and IκB-α at the gene expression level to improve the understanding of the molecular mechanisms (pre- and/or post-transcriptional mechanisms) of FP action on CF and normal bronchial epithelial cells.

In this study it was shown that the basal level of IKK-β is excessively elevated in CF bronchial epithelial cells, supporting the hypothesis that mechanisms leading to the regulation of IKK-β expression are altered in CF, a phenomenon that is probably associated with the sustained inflammatory process characterising the CF airway disease. The lack of cytosolic IκB-β inhibitor that had previously been reported by the authors in CF bronchial epithelial cells *in vivo* and *in vitro* [24] might be mediated in part by the elevated endogenous level of IKK-β. It is well known that one of the critical steps in the activation of NF-κB is the phosphorylation of the IκB-α inhibitor by two IκB kinases, IKK-α and IKK-β, which leads to its degradation [34]. The mechanism of IKK activation by pro-inflammatory stimuli is still poorly defined [35]. The identity of physiological IKK kinases that mediate its activation in response to different stimuli is the subject of intense investigation [36]. It has been well recognised that only *in vivo* IKK-β and not IKK-α is required for activation of NF-κB [35]. To date, it is unknown why CF bronchial epithelial cells exhibit elevated endogenous IKK-β expression and it remains to be determined how FP reduces IKK-β expression in CF bronchial epithelial cells. IKK kinases may be activated by receptor activation at an upstream level, resulting in IKK phosphorylation. Several potential upstream IKK kinases could be involved in this activation [35]. Further investigations are necessary to clarify whether upstream of IKKs, *i.e.* NF-κB-inducing kinase and MAP kinases may be the molecular targets of FP action.

The present data shows, that fluticasone propionate acts as a negative regulator of nuclear factor-κB activation in both noncystic fibrosis and cystic fibrosis bronchial epithelial cells. The data also suggests that elevated endogenous nuclear factor inhibitor-κB-β kinase expression found in cystic fibrosis bronchial epithelial cells may be involved in this regulation. The authors hypothesised that altered nuclear factor inhibitor-κB-β kinase expression in bronchial epithelium cells may represent a possible mechanism responsible for aberrant inflammatory responses, characteristic of cystic fibrosis. Possibly as a result of partially reduced negative regulation of the nuclear factor-κB activity and the subsequent dysregulation of interleukin-6 and -8 production. In patients with bronchectasis, it has been shown that inhaled fluticasone propionate reduced the level of inflammatory mediators in sputum [37]. In cystic fibrosis patients, fluticasone propionate did not induce significant changes in most of the parameters of pulmonary inflammation (sputum leukocyte count, myeloperoxydase activity and superoxide anion release). This may be related to the failure of fluticasone propionate to penetrate the viscous mucus lining cystic fibrosis airways [38]. *In vivo*, concentrations of inhaled fluticasone propionate (1 $mg \cdot day^{-1}$) are estimated to be in the nanomolar range in human lung tissues for >17 h [39]. In the present study it was shown that a relevant concentration of fluticasone propionate (10^{-8} M for 16 h) reduced the negative regulator of the nuclear factor-κB activation and subsequent interleukin-6 and -8 production, by bronchial epithelial cells of cystic fibrosis patients. Further work is now required to determine whether the *in vitro* observations found in this study can be translated into a clinical meaningful effect in cystic fibrosis patients.

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