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### High Susceptibility for Cystic Fibrosis Human Airway Gland Cells to Produce IL-8 Through the I $\kappa$ B Kinase $\alpha$ Pathway in Response to Extracellular NaCl Content<sup>1</sup> ✓

Olivier Tabary; ... et. al

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# High Susceptibility for Cystic Fibrosis Human Airway Gland Cells to Produce IL-8 Through the I $\kappa$ B Kinase $\alpha$ Pathway in Response to Extracellular NaCl Content<sup>1</sup>

Olivier Tabary,\* Sandie Escotte,\* Jean Paul Couetil,<sup>†</sup> Dominique Hubert,<sup>‡</sup> Daniel Dusser,<sup>‡</sup> Edith Puchelle,\* and Jacky Jacquot<sup>2\*</sup>

Increasing evidence suggests that in airways from cystic fibrosis (CF) patients, inflammation may precede bacterial infection and be related to an endogenous dysregulation of proinflammatory cytokines in airway epithelial cells. Several investigators have reported that, in CF airway fluids, elevated NaCl concentrations may also contribute to the diseased state by inhibiting the bactericidal properties of airway fluid. Because many proinflammatory cytokines are transcriptionally regulated by the NF- $\kappa$ B, we investigated whether an elevated extracellular NaCl content in airway fluids significantly impaired the regulation of the NF- $\kappa$ B/I $\kappa$ B $\alpha$  complex and the chemokine IL-8 production in primary non-CF and CF human bronchial gland epithelial cells. Exposure of non-CF gland cells to hypotonic (85 mM) NaCl solution, compared with isotonic (115 mM) NaCl and hypertonic (170 mM) NaCl solutions, resulted in a significant decrease in IL-8 production that was paralleled by a strong inhibition of activated NF- $\kappa$ B associated with an increased cytosolic expression of I $\kappa$ B $\alpha$  and a decrease in the I $\kappa$ B kinase  $\alpha$  protein level. In CF gland cells, we demonstrated that, compared with the high IL-8 in an hypertonic solution, the release of IL-8 was significantly reduced 2-fold in an isotonic solution and 5-fold in a hypotonic solution. Strikingly, exposure of CF bronchial gland cells to either hypotonic or isotonic milieu did not result in a marked inhibition of the activated NF- $\kappa$ B/I $\kappa$ B $\alpha$  system. This is the first demonstration that primary human CF bronchial gland cells exhibit abnormally high IL-8 production through constitutively activated NF- $\kappa$ B and high I $\kappa$ B kinase  $\alpha$  level, whatever the hypo-, iso-, and hypertonic NaCl milieu. *The Journal of Immunology*, 2000, 164: 3377–3384.

Cystic fibrosis (CF)<sup>3</sup> is a lethal inherited disorder caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) in airway epithelial cells. The CFTR protein provides cAMP-regulated chloride conductance (1) and acts as a regulator of apical Na<sup>+</sup> absorption (2, 3). Lung disease is the main cause of morbidity and mortality in CF patients. Despite recent and considerable progress in our understanding of the structure and functions of the CFTR protein in airways, the mechanisms by which the absence or dysfunction of CFTR causes numerous pathological manifestations, including early chronic airway inflammation, remain unexplained in human CF lung disease (4, 5). We and other investigators recently

favor the hypothesis that an endogenous pathway for airway inflammation may exist in CF airway epithelial cells before the manifestation of a bacteria-related infection (6–8). We previously reported that CF mice raised in pathogen-free conditions exhibited a greater number of lymphocytes in the airway submucosa compared with wild-type littermates (6). Recently, we demonstrated both in vivo and in vitro that  $\Delta$ F508 CF human bronchial gland (HBG) cells exhibited a selective up-regulation in chemokine IL-8 production (7) associated with constitutively activated NF- $\kappa$ B and a lack of cytosolic I $\kappa$ B $\alpha$  protein (9). The constitutive and/or excessive production of IL-8 by CF bronchial epithelial cells could implicate these cells in facilitating the neutrophil, T lymphocyte, and monocyte migration associated with airway inflammation in CF patients.

In CF HBG, the mechanism(s) directly responsible for constitutive NF- $\kappa$ B activation remains unknown. NF- $\kappa$ B dimers exist as inactive complexes in the cytoplasm of unstimulated cells due to their interaction with a family of inhibitory proteins collectively designated I $\kappa$ Bs. These I $\kappa$ Bs are phosphorylated by cellular kinase complexes known as I $\kappa$ B kinases (IKK)- $\alpha$  and - $\beta$  (10, 11). The prototypic and best-studied member of the I $\kappa$ B family, I $\kappa$ B $\alpha$ , binds to the nuclear translocation sequence of p65 and sequesters NF- $\kappa$ B in the cytoplasm. The key regulatory steps in the signal-induced control of NF- $\kappa$ B include the phosphorylation and degradation rates of I $\kappa$ B $\alpha$ . Cytosolic inhibitor factor I $\kappa$ B $\alpha$  appears to be a major regulator of NF- $\kappa$ B, as indicated by the degradation of I $\kappa$ B $\alpha$  matching the translocation of NF- $\kappa$ B to the nucleus (11, 12). Elevated NaCl concentrations in CF airway fluids have been noted by several investigators in both in vivo and in vitro studies. For example, Gilljam et al. found a Cl<sup>-</sup> concentration of 170  $\pm$  80 mM in bronchial fluid from CF patients vs 85  $\pm$  54 mM in control subjects (13). Two more recent publications have demonstrated

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<sup>3</sup> Abbreviations used in this paper: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; HBG, human bronchial gland;  $\Delta$ F508 homozygous CF HBG cells, CF human bronchial submucosal gland cells expressing the cystic fibrosis transmembrane conductance regulator that contains a deletion of phenylalanine at position 508; IKK, I $\kappa$ B kinase; NIK, NF- $\kappa$ B-inducing kinase; MAPK, mitogen-activated protein kinase.

abnormally high Na<sup>+</sup> and Cl<sup>-</sup> concentrations (~180 mM) in airway surface fluid recovered from CF-cultured epithelial cells and CF bronchial xenografts and a significantly reduced ability of airway fluid to kill bacteria, unless these fluids were diluted in water (14, 15). To date, it is not known whether the NF- $\kappa$ B/I $\kappa$ B $\alpha$  complex in airway epithelial cells plays a determinant role in airway inflammatory processes in patients with CF or whether the NF- $\kappa$ B activation in airway epithelial cells is sensitive or not to variations of extracellular NaCl concentration. Nor is it known whether a loss of CFTR protein activity, i.e., loss of chloride transport in the human CF airway gland serous cells, which is well recognized to express high amount of CFTR protein (16, 17), alters the activity of transcription factors such as NF- $\kappa$ B and the subsequent induction of IL-8 gene expression. Therefore, we address the question of whether different extracellular NaCl concentrations would impair the regulation of NF- $\kappa$ B/I $\kappa$ B $\alpha$  complex and subsequent IL-8 production in human CF compared with non-CF airway epithelial cells. Understanding such mechanisms is of great interest, as it may lead to the development of novel therapeutic strategies in CF airway disease.

In this report, we investigated the effects of hypotonic (85 mM), isotonic (115 mM), and hypertonic (170 mM) NaCl solutions on the activation of the NF- $\kappa$ B/I $\kappa$ B $\alpha$  system and subsequent IL-8 production in non-CF vs  $\Delta$ F508 homozygous CF bronchial gland epithelial cells. We also focused our study on the phosphorylation and degradation status of I $\kappa$ B $\alpha$  to gain further insight into the NF- $\kappa$ B-inducing kinase (NIK) and IKK- $\alpha$  and - $\beta$  pathways in the salt-dependent IL-8 production in human non-CF and CF bronchial gland cells.

Our data demonstrate that extracellular fluid NaCl content modulates the NF- $\kappa$ B activity and subsequent IL-8 production in human non-CF gland bronchial epithelial cells but not in CF bronchial gland cells. On exposure to isotonic (115 mM) and hypertonic (170 mM) NaCl milieu, primary human CF bronchial gland epithelial cells exhibit abnormally high IL-8 production through constitutive NF- $\kappa$ B binding activity and high levels of IKK- $\alpha$ , thus contributing to the heightened inflammatory response seen in airways of CF patients (4–9).

## Materials and Methods

### Cell culture

Cell isolation and culture procedures of HBG cells were performed on bronchial tissues collected from eight  $\Delta$ F508 homozygous CF patients (four females and four males; mean age 17.3 years, range 9–27 years) and four non-CF patients (two males with primary pulmonary hypertension, aged 28 and 29 years, respectively, and two males with pulmonary idiopathic fibrosis, aged 40 and 61 years, respectively), as described previously (7). Briefly, HBG cells were isolated by enzymatic digestion from bronchial submucosa and grown onto type I collagen-coated 25-cm<sup>2</sup> tissue culture flasks in a DMEM/Ham's F12 mixture (50/50%, v/v) supplemented with 1% Ultrosor G (a serum substitute from Sepracor, Villeneuve-la-Garenne, France), glucose (10 g/L), and sodium pyruvate (0.33 g/L). Penicillin G (100 U/ml) and streptomycin (100 U/ml) were also added. After 4 wk in primary culture, second- and third-passage CF-HBG and non-CF HBG cells had proliferated and exhibited characteristics of homogenous submucosal epithelial and secretory gland cells, as previously described (18, 19). Using the halide-sensitive fluorescent dye 6-methoxy-N-(3-sulfo-propyl)-quinolinium, we have previously shown a significant increase in Cl<sup>-</sup> channel activity via the CFTR protein in non-CF HBG cells in response to forskolin treatment (demonstrating a cAMP-dependent activation of a Cl<sup>-</sup> efflux), which is not preserved in cultured CF HBG cells (7).

### Exposure of bronchial epithelial cells to different extracellular NaCl solutions

Before the exposure of bronchial epithelial cells to either low (hypotonic, 85 mM NaCl), intermediate (isotonic, 115 mM NaCl), or high (hypertonic, 170 mM NaCl) saline solutions, confluent cultures of  $\Delta$ F508 homozygous CF and non-CF HBG cells were incubated for 16 h in an Ultrosor G-free

RPMI 1640 medium in 95% air/5% CO<sub>2</sub> to ensure that cells were in a quiescent state. At the end of the 16-h period, individual monolayers of primary CF and non-CF HBG cells were exposed for a further 6-h period to Cl<sup>-</sup> solutions containing either 85 mM, 115 mM, or 170 mM Cl<sup>-</sup>, respectively. The three chloride-containing solutions used in this study (85 mM Cl<sup>-</sup>, 115 mM Cl<sup>-</sup>, and 170 mM Cl<sup>-</sup>) contained 1 mM CaCl<sub>2</sub>, 20 mM KCl, and either 60 mM, 85 mM, or 148 mM NaCl, pH 7.4, respectively, as previously reported (7, 14). Immediately after each period of CF and non-CF cell exposure, supernatants were collected and stored at -80°C until testing for the presence of IL-8. 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,  $\geq$ 5 pg/ml) were found using a quantitative chromogenic *limulus* amoebocyte lysate assay (BioWhittaker, Emerainville, France).

### ELISA for IL-8

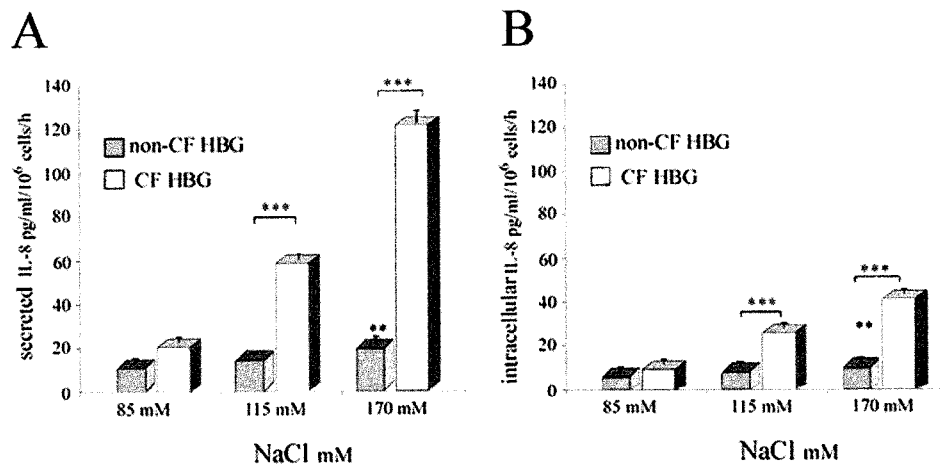
IL-8 ELISA in the culture supernatants and cell lysates of non-CF and CF HBG cells were conducted according to the manufacturer's instructions in commercially available ELISA kits (Biosource International, Camarillo, CA). Cell lysates were prepared as follows. After each period of different saline treatments, CF and non-CF HBG cell monolayers were washed in PBS, pH 7.2, harvested by scraping, centrifuged (300  $\times$  g, 5 min, 4°C), and total protein was extracted (30 min, 4°C) in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl<sub>2</sub>, 1% Nonidet P-40 (Sigma), 0.5% deoxycholate (Sigma), and 0.1% SDS). Cell debris were centrifuged (12,000  $\times$  g, 30 min, 4°C), and supernatants were retained and stored at -80°C. The ELISAs for IL-8 were sensitive down to a level of 5 pg/ml. The uniformity of the CF and non-CF HBG cells in monolayer culture was determined by quantifying the cell number/well. Cell viability of CF and non-CF HBG cells exceeded 97%, as determined by trypan blue exclusion after all experimental interventions. All results were expressed as pg/ml/viable 10<sup>6</sup> cells/h.

### Immunofluorescence

After each period of the different saline treatments described above, CF and non-CF HBG cell monolayers were fixed in situ in cold methanol for 10 min at -20°C, air dried, and rehydrated in 0.1 M PBS at pH 7.4 before immunofluorescence detection. Cells were stained for I $\kappa$ B $\alpha$  expression using rabbit antiserum to human I $\kappa$ B $\alpha$  (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature and a donkey anti-rabbit FITC-conjugated Ab for 45 min at room temperature, as previously described (9). Negative controls were obtained by using either nonspecific IgG as the primary Ab (M7769; Sigma) or with FITC-conjugated Ab alone. Representative fields of CF and non-CF HBG cells in different saline conditions were recorded with a Zeiss Axiophot microscope (Zeiss, Le Pecq, France) and employing epifluorescence and Nomarski differential interference illumination.

### Cell extracts and Western blot analysis

After each period of different saline treatments, CF and non-CF HBG cell monolayers were washed in PBS, pH 7.2, harvested by scraping, centrifuged (300  $\times$  g, 5 min, 4°C), and total protein was extracted (30 min, 4°C) in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl<sub>2</sub>, 1% Nonidet P-40 (Sigma), 0.5% deoxycholate (Sigma), and 0.1% SDS). Protein extracts were centrifuged (12,000  $\times$  g, 30 min, 4°C), and protein concentrations were measured using the Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of protein were boiled for 4 min in Laemmli buffer, and electrophoresis was conducted under denaturing conditions using 4–15% polyacrylamide gels (Pharmacia Biotech, Orsay, France), which were then transferred onto a nitrocellulose membrane (Millipore, Bedford, MA) by electroblotting. Membranes were blocked with TBS containing Tween 20 (40 mM Tris, pH 7.6, 300 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk for 4 h at room temperature before exposure to rabbit polyclonal anti-human I $\kappa$ B $\alpha$  and anti-I $\kappa$ B $\beta$  (Santa Cruz Biotechnology). For analysis of I $\kappa$ B kinases  $\alpha$  and  $\beta$  (IKK- $\alpha$  and IKK- $\beta$ ), membranes were exposed to rabbit polyclonal anti-IKK- $\alpha$  and IKK- $\beta$  Abs, respectively (Santa Cruz Biotechnology). The level of phosphorylated I $\kappa$ B $\alpha$  was analyzed by Western blot using a polyclonal phospho-specific anti-I $\kappa$ B $\alpha$  (New England Biolabs, Beverly, MA) Ab, which detects I $\kappa$ B $\alpha$  only when activated by phosphorylation at Ser<sup>32</sup>. Proteins were visualized using HRP-conjugated donkey anti-rabbit IgG (Boehringer Mannheim, Mannheim, Germany) and the enhanced chemiluminescence detection kit (Amersham Life Science, Arlington Heights, IL). Prestained m.w. markers (Bio-Rad, Hercules, CA) were loaded on each gel to verify the effective transfer of proteins to membranes and to determine the m.w. of proteins. Densitometric analyses of Western blots were performed on a Bio-Rad model GS-690 imaging densitometer using Molecular Analyst software, version 1.4.1.



**FIGURE 1.** Levels of IL-8 production in cultured human non-CF vs  $\Delta$ F508 homozygous CF bronchial gland epithelial cells after their exposure to increasing extracellular NaCl content. Confluent monolayers of non-CF and CF HBG cells were covered by 2 ml of a solution containing 1 mM  $\text{CaCl}_2$ , 20 mM KCl, and either 60 mM, 85 mM, and 148 mM NaCl (total  $\text{Cl}^-$  concentration indicated for each data set), pH 7.4. IL-8 levels were assayed in 6-h supernatants (A) and cell lysates (B) collected from cell cultures exposed to either hypotonic (85 mM), isotonic (115 mM), or hypertonic (170 mM)  $\text{Cl}^-$  concentrations, respectively, as indicated. Values in ELISA assays (pg IL-8/ $10^6$  cells/h) represent means  $\pm$  SD of eight  $\Delta$ F508 homozygous CF HBG vs four non-CF HBG cell cultures, respectively, with each assayed in triplicate. \*\*,  $p < 0.05$  compared with hypotonic (85 mM) solution, \*\*\*,  $p < 0.001$ .

The intensities of bands were compared on the basis of adjusted volume (mean optical density  $\times$  area in square millimetres).

#### Nuclear protein extraction and EMSAs

Nuclear extracts were prepared and analyzed after the non-CF and CF HBG cells were incubated in different saline conditions, as described previously (9). Briefly,  $5\text{--}6 \times 10^6$  cells were suspended in 1.5 ml of lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM,  $\text{MgCl}_2$ , 10 mM KCl, 0.5 mM DTT (Sigma) and 0.1% Nonidet P-40 (Boehringer Mannheim)). The homogenate was centrifuged at 10,000 rpm, and the resulting pellet was resuspended in 30  $\mu\text{l}$  of lysis buffer (20 mM HEPES, 420 mM NaCl, 1.5 mM  $\text{MgCl}_2$ , 0.2 mM EDTA, 25% [v/v] glycerol, and 0.5 mM DTT). This suspension was incubated for 20 min at  $4^\circ\text{C}$  followed by centrifugation at 14,000 rpm for 10 min. To minimize proteolysis, all buffers contained 0.5 mM PMSF, 5  $\mu\text{g}/\text{ml}$  aprotinin, 1  $\mu\text{g}/\text{ml}$  chymostatin, 4  $\mu\text{g}/\text{ml}$  pepstatin, 5  $\mu\text{g}/\text{ml}$  leupeptin, and 0.1 mg/ml  $\alpha$ -1 antitrypsin (Boehringer Mannheim). The consensus  $\kappa\text{B}$  DNA sequence was used for the EMSA (5'-AGT TGA GGG GAC TTT CCC AGG C-3'; Promega, Madison, WI). The oligonucleotide was radiolabeled by the T4 polynucleotide kinase (Pharmacia Biotech, Paris, France) enzyme with [ $\alpha$ - $^{32}\text{P}$ ]ATP. Nuclear extracts (4  $\mu\text{g}$ ) were incubated with 50 kcpm of  $^{32}\text{P}$ -labeled NF- $\kappa\text{B}$  oligonucleotide in binding reaction mixture (20% Ficoll, 175 mM NaCl, 300 mM KCl, 0.05% Nonidet P-40, pH 7.0) in a final volume of 15  $\mu\text{l}$ . After 30 min on ice, the protein-DNA complexes were electrophoresed on a nondenaturing 5% polyacrylamide gel in a  $1 \times$  TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA). Gels were then dried under vacuum and exposed at  $-80^\circ\text{C}$  with autoradiographic film. In competition studies and supershift assays, a 100-fold molar excess of unlabeled oligonucleotide or 1  $\mu\text{g}$  of Abs was added to the binding reaction mixture as indicated, before the addition of the labeled  $\kappa\text{B}$  probe. Identification of the different NF- $\kappa\text{B}$  heterodimeric proteins was conducted by incubating the nuclear extracts with polyclonal Abs against the NF- $\kappa\text{B}$  proteins NF- $\kappa\text{B}$ 1 (p50) and the Rel (p65) RelA (Santa Cruz Biotechnology) before the addition of the labeled  $\kappa\text{B}$  probe. These Abs were added to the above reaction mixture at a concentration of 10  $\mu\text{g}/100 \mu\text{l}$ . All samples were then incubated at room temperature for 1 h before gel loading.

#### Statistical analysis

Results were expressed as means  $\pm$  SD. Each data point was confirmed in triplicate at least, and each cell culture experiment was performed at least three times. Differences in IL-8 levels were analyzed by the Student's  $t$  test for paired and unpaired samples.

## Results

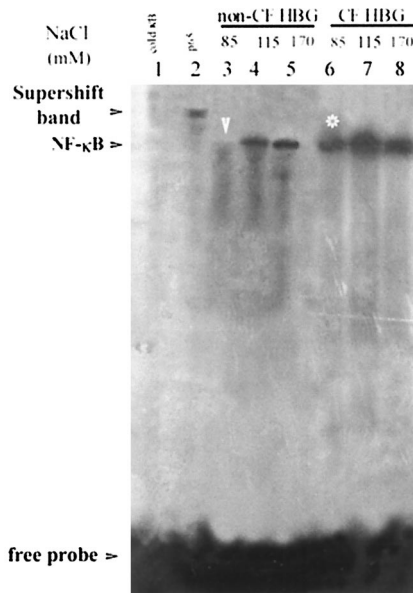
### IL-8 release by human non-CF and CF HBG cells is increased with increasing extracellular NaCl content

Previous studies have shown that established CF respiratory epithelial cell lines (20) as well as human CF bronchial submucosal

tissues in situ and primary cultures of CF bronchial gland cells (9, 21) constitutively expressed significantly high levels of proinflammatory cytokines, particularly the chemokine IL-8. IL-8 is one of the most potent neutrophilic chemoattractants in human CF airways (22, 23). To further mimic the in vivo situation, in which a change in NaCl concentration occurs in CF bronchial liquids (13–15) as a result of CFTR deficiency (i.e., 85 mM in non-CF vs 120–170 mM in CF), we examined whether the primary  $\Delta$ F508 homozygous CF and non-CF HBG cells displayed any differential expression in the IL-8 production and release in response to the relevant electrolyte concentration in the extracellular milieu. As shown in Fig. 1A, when the extracellular NaCl content increased (from 85 mM to 170 mM), we observed a significant 2-fold increase ( $p < 0.01$ ) in IL-8 release from non-CF HBG cells. Interestingly, exposure of CF HBG cells to isotonic (115 mM) and hypertonic (170 mM) NaCl resulted in a statistically ( $p < 0.001$ ) significant 3.0- and 5.0-fold increase in IL-8 release compared with similarly treated non-CF HBG cells. We also observed that the increased IL-8 release in CF HBG cells was paralleled by an increased intracellular IL-8 levels according to the extracellular NaCl content (Fig. 1B). Taken together, these findings clearly show that CF HBG cells are characterized by a higher susceptibility to induce IL-8 production and secretion in response to extracellular iso- and hypertonic NaCl changes when compared with that obtained from similarly treated non-CF HBG cells.

### Extracellular NaCl content is a potent activator of NF- $\kappa\text{B}$ expression

Therefore, it was of interest to determine whether or not variations in extracellular NaCl concentration affected NF- $\kappa\text{B}$  activation in the primary non-CF and CF HBG cell cultures. Nuclear extracts of non-CF and CF HBG cells previously incubated either in isotonic (85 mM) or hypotonic (115 mM) or hypertonic (170 mM) NaCl solutions were prepared and incubated with an end  $^{32}\text{P}$ -labeled DNA oligonucleotide containing the recognition site of NF- $\kappa\text{B}$ . As demonstrated by EMSA (Fig. 2), no evidence of activated NF- $\kappa\text{B}$  was found in primary non-CF HBG cells treated with the hypotonic (85 mM) NaCl solution (Fig. 2, lane 3). In contrast, when increasing the extracellular NaCl concentration from hypotonic (85 mM) to isotonic (115 mM) and hypertonic (170 mM), we noted a marked NF- $\kappa\text{B}$ -DNA binding activity in a gradual manner



**FIGURE 2.** NF- $\kappa$ B binding activity in human non-CF vs  $\Delta$ F508 homozygous CF bronchial gland epithelial cells after their exposure to increasing extracellular NaCl content. EMSA binding activity in nuclear protein extracts from non-CF HBG (lane 3–5) and CF HBG cells (lane 6–8) after their exposure to either hypotonic (85 mM), isotonic (115 mM), or hypertonic (170 mM) Cl<sup>-</sup> concentration, respectively, is as indicated. Note that the exposure of the non-CF HBG cells to hypotonic (85 mM) NaCl solution does not induce NF- $\kappa$ B activation (as shown by arrowheads), compared with CF HBG cells in which evidence of high endogenous NF- $\kappa$ B activity is present (as indicated by the asterisk). To demonstrate the specificity of binding of the NF- $\kappa$ B oligonucleotide, a 100-fold molar excess of unlabeled NF- $\kappa$ B (lane 1, cold  $\kappa$ B) was used to compete with the labeled NF- $\kappa$ B probe. The addition of Ab to Rel A (p65 subunit) component (lane 2, p65) caused a supershift, as indicated. The results are representative of three different experiments.

in non-CF HBG cells (Fig. 2, lanes 4 and 5). Surprisingly, high amounts of activated NF- $\kappa$ B were always detected in CF HBG cells for each of the hypo-, iso-, and hypertonic salt solutions (Fig. 2, lanes 6–8). Compared with non-CF HBG cells maintained in either isotonic (115 mM) or hypertonic (170 mM) NaCl solutions (Fig. 2, lanes 4 and 5), a higher NF- $\kappa$ B-DNA binding activity was demonstrated in the nuclear protein extracts from similarly treated CF HBG cells (Fig. 2, lanes 7 and 8), with a mean increase of 2.5- and 3.0-fold, respectively, as evaluated by densitometric analyses (data not shown). The specificity of NF- $\kappa$ B-DNA binding was confirmed in competition experiments with a 100-fold excess of (cold  $\kappa$ B) NF- $\kappa$ B oligonucleotide, which led to a complete inhibition of binding activity (Fig. 2, lane 1). Moreover, supershift assays confirmed the presence of p65 subunits of NF- $\kappa$ B (Fig. 2, lane 2). A similar result was observed with the addition of Abs to the p50 subunit of human NF- $\kappa$ B (data not shown).

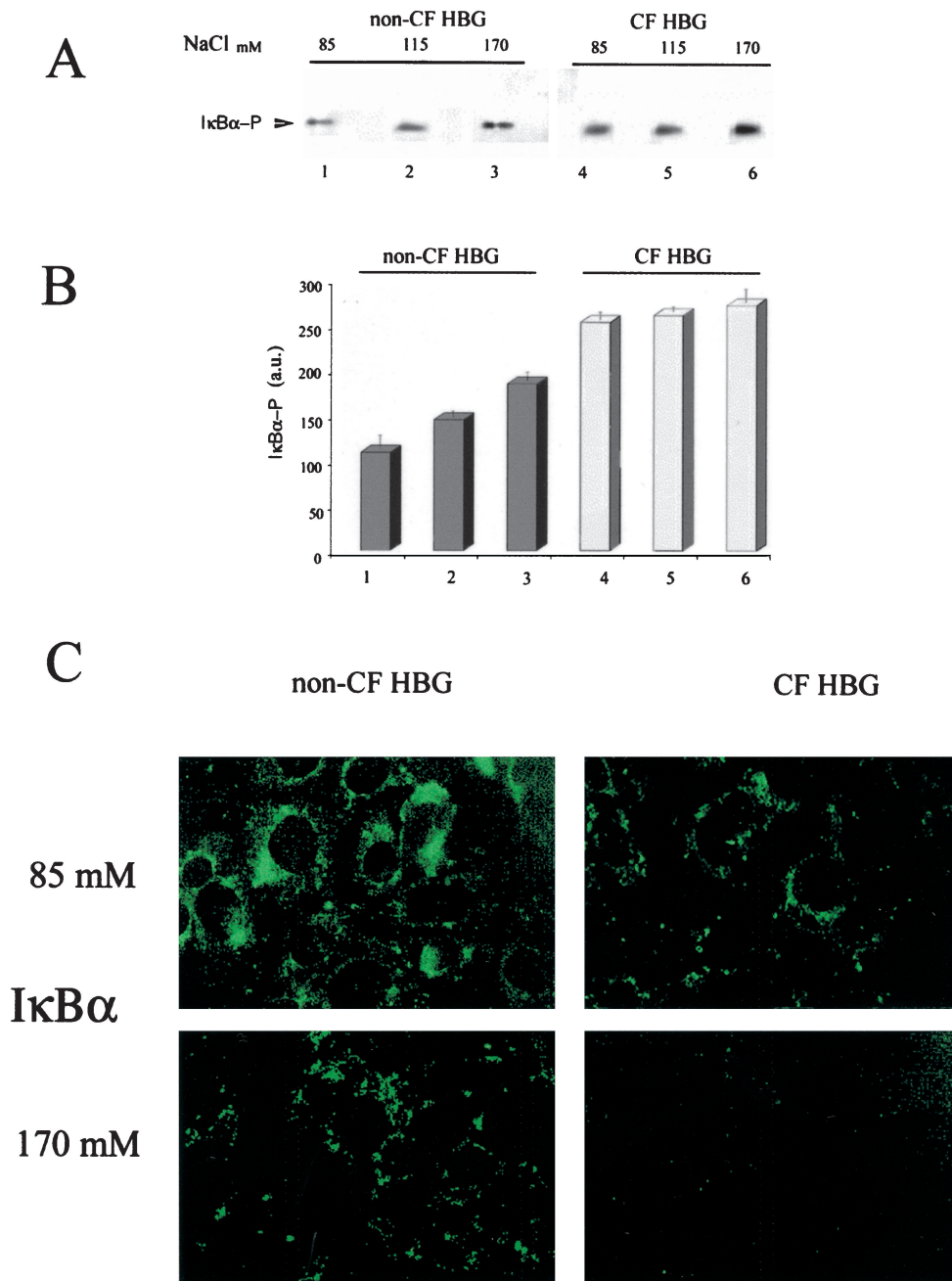
#### Effects of extracellular NaCl content on I $\kappa$ B $\alpha$ and I $\kappa$ B $\beta$ protein in non-CF and CF HBG cells

Activation of NF- $\kappa$ B occurs via phosphorylation of two serine residues of I $\kappa$ B $\alpha$  at positions 32 and 36 (24). This has been shown to enable conjugation with ubiquitin followed by proteasome-mediated degranulation of I $\kappa$ B, resulting in the release of active NF- $\kappa$ B (25). To measure I $\kappa$ B $\alpha$  phosphorylation, we used a phospho-specific anti-I $\kappa$ B $\alpha$ -Ab that detects I $\kappa$ B $\alpha$  only when activated by phosphorylation at Ser<sup>32</sup>. In a set of experiments, we examined the phosphorylation status of I $\kappa$ B $\alpha$  over the hypo-, iso-, and hyper-

tonic salt conditions in similarly treated non-CF and CF HBG cells (Fig. 3). In hypotonic (85 mM) NaCl solution, little or no phosphorylated I $\kappa$ B $\alpha$  was detected in non-CF HBG cells (Fig. 3A, lane 1). In contrast, when we increased the extracellular NaCl content from 85 mM to 115 mM and 170 mM NaCl, we noted a marked increase of phosphorylated I $\kappa$ B $\alpha$  in non-CF HBG cells (Fig. 3A, lanes 2 and 3). Image analysis of digitized Western blots demonstrated that 115 mM and 170 mM NaCl solutions increased the phosphorylated I $\kappa$ B $\alpha$  level by 25 and 75%, respectively ( $p < 0.05$ ), compared with that observed with the 85 mM NaCl solution (Fig. 3B, lanes 2 and 3, compared with lane 1). In striking contrast to non-CF HBG cells in which levels of phosphorylated I $\kappa$ B $\alpha$  were salt dependent, high levels of phosphorylated I $\kappa$ B $\alpha$  were found in CF HBG cells even in hypotonic (85 mM) NaCl solution and did not change in accordance to the hypo-, iso-, and hypertonic salt conditions (Fig. 3A, lanes 4–6). Image analysis of digitized Western blots of phosphorylated I $\kappa$ B $\alpha$  showed at least a 220% increase of phosphorylated I $\kappa$ B $\alpha$  in CF HBG cells regardless of the hypo-, iso-, and hypertonic salt conditions, compared with the value obtained with the hypotonic (85 mM) NaCl solution in non-CF HBG cells (Fig. 3B, lanes 4–6, compared with lane 1). Consistent with the results of the phosphorylation state of I $\kappa$ B $\alpha$ , the increase of NaCl content from 85 mM to 170 mM resulted in a marked decrease of I $\kappa$ B $\alpha$  protein in the cytoplasm of non-CF HBG cells, as determined by immunofluorescence analysis (Fig. 3C). In similarly treated CF HBG cells, little or no cytosolic I $\kappa$ B $\alpha$  protein was found in either the hypotonic (85 mM) or hypertonic (170 mM) NaCl treatment groups (Fig. 3C), confirming our previous study in which we reported a constitutive lack of cytosolic I $\kappa$ B $\alpha$  protein expression in CF HBG epithelial cells in vivo and in vitro (9). To assess whether extracellular NaCl content might directly modulate the expression and/or degradation of the cytoplasmic NF- $\kappa$ B regulatory protein, I $\kappa$ B $\beta$ , in non-CF and CF HBG cells, Western blots of I $\kappa$ B $\beta$  protein and image analysis of digitized Western blots were conducted (Fig. 4). Surprisingly, in contrast to the lack of I $\kappa$ B $\alpha$  protein expression in CF HBG cells (Fig. 3C and Ref. 9), I $\kappa$ B $\beta$  protein was detected in CF HBG cells. We showed that the I $\kappa$ B $\beta$  protein levels in CF HBG cells in the hypotonic (85 mM) NaCl solution were >250% higher than in similarly treated non-CF HBG cells and decreased (by a 30% reduction) in the hypertonic (170 mM) NaCl solution (Fig. 4, lane 6 compared with lane 4).

#### Effects of extracellular NaCl content on I $\kappa$ B kinases $\alpha$ and $\beta$ expression in non-CF and CF HBG cells

To determine whether NaCl-induced increases in phosphorylated I $\kappa$ B $\alpha$  and I $\kappa$ B $\alpha$  degradation were related to enhanced IKK- $\alpha$  and IKK- $\beta$ , Western blot analyses of both IKK- $\alpha$  and IKK- $\beta$  were performed from cytoplasmic extracts of CF and non-CF HBG cells similarly treated with the hypo-, iso-, and hypertonic NaCl solutions. In good agreement with the results of phosphorylated I $\kappa$ B $\alpha$  levels described in Fig. 3, A and B, the NaCl-induced IKK- $\alpha$  protein level increased in a gradual manner with increasing extracellular NaCl content in non-CF HBG cells (Fig. 5, A and C, lanes 1–3). Image analysis of digitized Western blots of the IKK- $\alpha$  protein demonstrated that both isotonic (115 mM) and hypertonic (170 mM) NaCl solutions increased the IKK- $\alpha$  levels by 25 and 75%, respectively, in non-CF HBG cells ( $p < 0.05$ ), respectively, compared with the IKK- $\alpha$  level observed in hypotonic (85 mM) NaCl solution, (Fig. 5C, lanes 2 and 3 compared with lane 1). In agreement with the results reported in Fig. 3, in which we showed a high level of phosphorylated I $\kappa$ B $\alpha$  in CF HBG cells, we concomitantly observed high levels of IKK- $\alpha$  kinase in CF HBG cells,



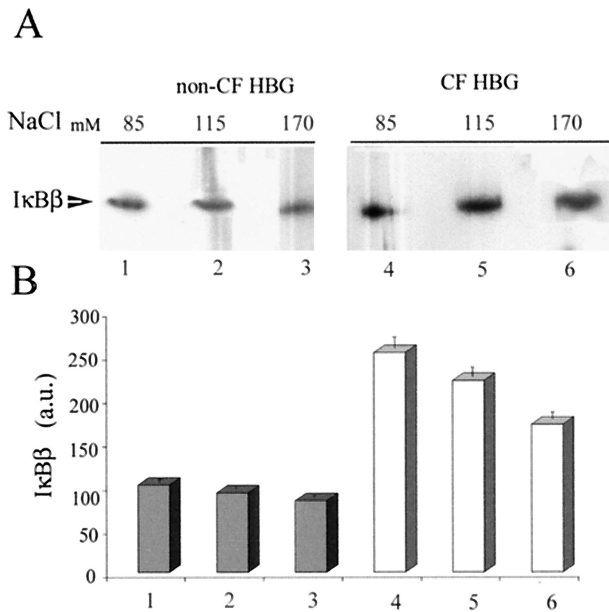
**FIGURE 3.** Expression of phosphorylated IκBα (IκBα-P) protein levels in human non-CF and CF bronchial gland epithelial cells after their exposure to increasing extracellular NaCl content. *A*, Equal amounts of cytoplasmic protein from non-CF and CF HBG cells were analyzed for levels of IκBα-P by Western blotting with polyclonal phospho-specific anti-IκBα Abs. *B*, Densitometric analyses of the data represented in *A*, combined with three similar studies, expressed in arbitrary units (a.u.). Densitometric results are reported as percentage of IκBα-P levels compared with those obtained from non-CF HBG cells placed in hypotonic (85 mM) NaCl solution (*lane 1*). *C*, The expression of cytosolic IκBα protein in non-CF and CF HBG cells exposed to either hypotonic (85 mM) or hypertonic (170 mM) Cl<sup>-</sup> concentration was determined by immunofluorescence using specific Abs to IκBα. Images were taken with a Carl Zeiss Axiophot microscope, and representative fields of three independent experiments are shown. Original magnification is ×400.

regardless of the salt conditions (Fig. 5A, lanes 4–6). Image analysis of digitized Western blots of IKK-α demonstrated that the IKK-α levels in CF HBG cells were increased by >300% in all salt conditions compared with the IKK-α level obtained in non-CF HBG cells treated with hypotonic (85 mM) NaCl solution (Fig. 5C, lanes 4–6 compared with lane 1). We further investigated whether variations in extracellular NaCl content directly affected the IKK-β levels in non-CF and CF HBG cells. In striking contrast to the high IKK-α levels that were found to be salt sensitive in non-CF and insensitive in CF HBG cells, we detected low IKK-β levels in both

non-CF and CF HBG cells and found no significant alteration in IKK-β levels, regardless of the saline conditions (Fig. 5, B and D, lanes 7–12).

## Discussion

Given that the chemokine IL-8 has been reported to play a major role in the early inflammatory pathogenesis in the airways of CF patients before the manifestation of bacterial infection (4, 9, 26, 27), it is important to elucidate the mechanisms by which IL-8

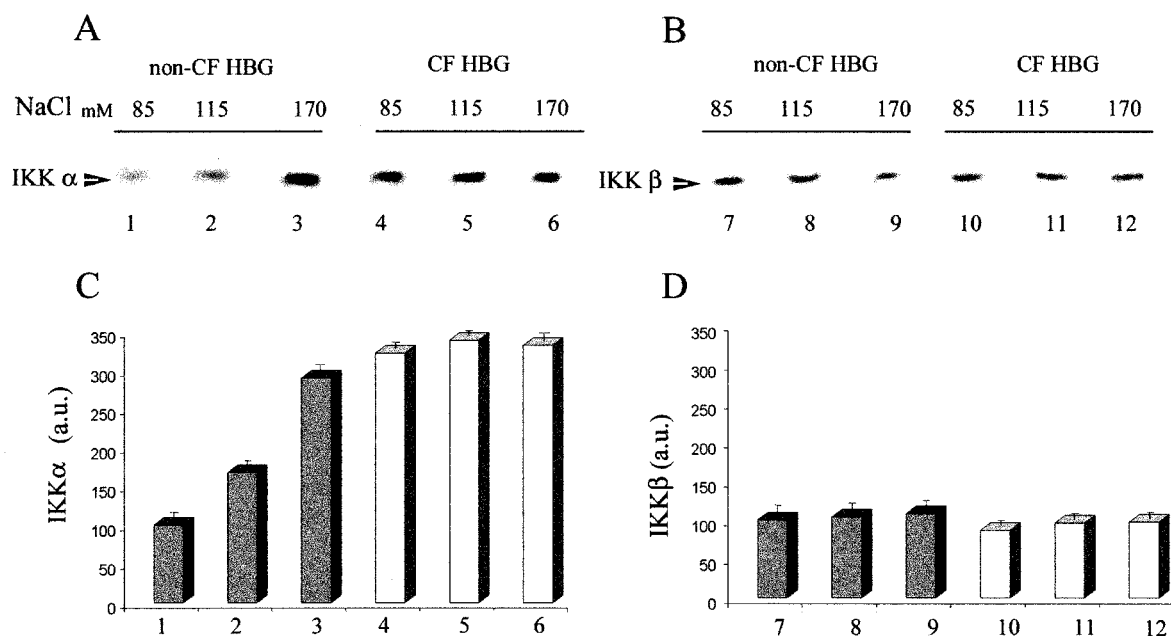


**FIGURE 4.** Expression of IκBβ protein levels in human non-CF and CF bronchial gland epithelial cells after their exposure to increasing extracellular NaCl content. *A*, Equal amounts of cytoplasmic protein from non-CF and CF HBG cells were analyzed for IκBβ protein by Western blotting. *B*, Densitometric analyses of the data represented in *A*, combined with three similar studies, expressed in arbitrary units (a.u.). Densitometric results are reported as percentage of the IκBβ level compared with those obtained from non-CF HBG cells placed in hypotonic (85 mM) NaCl solution (*lane 1*). One representative experiment of three independent experiments is shown.

inducers act upon human airway epithelial cells to modulate IL-8 production. In CF airway epithelial cells, loss of CFTR function results in abnormal transepithelial electrolyte and fluid transport

(1–3, 5). It has also been suggested that the CFTR malfunction may change the airway surface fluid composition from a normally hypotonic (85 mM) to a hypertonic (>115 mM) NaCl solution so that salt-sensitive antibacterial peptides are ineffective in the CF airway tract (5, 14, 15).

In the present study, we have now found evidence that increased NaCl concentrations induce an increase of IL-8 release (up to 5-fold) by CF bronchial gland cells compared with similarly treated non-CF bronchial gland cells. This is the first study to report the ability of extracellular dose-dependent NaCl to regulate IL-8 production by human non-CF and CF airway epithelial cells. In the presence of hypotonic (85 mM) NaCl concentration, the IL-8 production by CF bronchial gland cells was already higher than that by non-CF bronchial gland cells. More importantly, our data show that, compared with non-CF bronchial gland cells, CF bronchial gland cells were highly susceptible to induce increased IL-8 production with both isotonic (115 mM) and hypertonic (170 mM) NaCl solutions. Therefore, in *in vivo* situations, isotonic and hypertonic airway fluids, when present in CF patients, may represent a stimulus for bronchial gland IL-8 production, in addition to other stimuli provided by the presence of bacteria and/or bacterial exoproducts (20, 21). Consequently, isotonic and hypertonic airway fluids could increase the IL-8 amounts produced by CF bronchial gland cells and at least partially account for the recent findings that IL-8 levels and neutrophils in bronchoalveolar lavage fluids from infected children with CF were significantly increased compared with those from infected children with other chronic respiratory diseases, even when patients were matched for the pathogen present and for bacterial colony counts (27). In CF airway fluid, the effects of abnormal NaCl concentration on bronchial gland functions could provide another causal link between loss of CFTR function and CF airway disease. If abnormal ionic composition is present in CF airway fluid, as a primary abnormality occurring because of loss of CFTR function, then the effects on bronchial gland functions



**FIGURE 5.** Expression of IKK-α and IKK-β protein levels in human non-CF and CF bronchial gland epithelial cells after their exposure to increasing extracellular NaCl content. *A* and *B*, Equal amounts of cytoplasmic protein from non-CF and CF HBG cells were analyzed for IKK-α and IKK-β protein by Western blotting using specific anti-IKK-α and IKK-β mAbs, respectively. *C* and *D*, Densitometric analyses of the data represented in *A*, combined with three separate studies, expressed in arbitrary units (a.u.). Densitometric results are reported as percentage of IKK-α and IKK-β levels compared with those obtained from non-CF HBG cells placed in hypotonic (85 mM) NaCl solution (*lane 1* and *7*, respectively). One representative experiment of three independent experiments is shown.



that we have observed could contribute to the early and sustained inflammatory pathogenesis of CF disease, even before development of bacterial infections.

The correlation between the levels of IL-8 production, the inhibitor factor  $\text{I}\kappa\text{B}\alpha$  degradation, and nuclear translocation of NF- $\kappa\text{B}$  suggests a causal relationship in normal but not in CF bronchial gland epithelial cells. Several molecules are involved in NF- $\kappa\text{B}$  activation such as a network of kinases (IKK- $\alpha$ , IKK- $\beta$ , NIK, additional proteins) constituting several hierarchical modules that ultimately lead to the phosphorylation of  $\text{I}\kappa\text{B}$  (24, 25). Stimulus-induced degradation of  $\text{I}\kappa\text{B}$ s by the proteasome requires the phosphorylation of these inhibitor proteins at specific residues (25). For example,  $\text{I}\kappa\text{B}\alpha$  is phosphorylated following activation at the amino acids Ser<sup>32</sup> and Ser<sup>36</sup> (24). This was the reasoning behind our examination of whether elevated NaCl concentrations interfered with the phosphorylation of  $\text{I}\kappa\text{B}\alpha$ . Indeed, using a phospho-specific Ab, we were able to clearly demonstrate that isotonic (115 mM) and hypertonic (175 mM) NaCl solutions induced the phosphorylation of  $\text{I}\kappa\text{B}\alpha$  in a dose-dependent manner in normal but not CF bronchial epithelial cells. Surprisingly, we observed that the hypotonic (85 mM) NaCl solution significantly decreased the IL-8 production by CF bronchial gland cells with no significant inhibition of activated NF- $\kappa\text{B}$ . At this point, it should be mentioned that our studies showed that even at the lowest (85 mM) NaCl concentration  $\text{I}\kappa\text{B}\beta$  protein levels in CF bronchial gland cells were higher than those observed in non-CF HBG cells, suggesting that the decrease of IL-8 production by CF HBG cells in the hypotonic (85 mM) NaCl solution may occur by preventing the degradation of the  $\text{I}\kappa\text{B}\beta$  protein. One of the critical steps in the activation of the NF- $\kappa\text{B}$  pathway is the phosphorylation of the  $\text{I}\kappa\text{B}$  protein by two recently discovered  $\text{I}\kappa\text{B}$  kinases, IKK- $\alpha$  and IKK- $\beta$ , which leads to its degradation (10, 11, 24). Numerous studies on the role of the IKK complex in the NF- $\kappa\text{B}$  regulation have been limited to immortalized cell lines, and there is little information on their function in normal cells or cells derived from the site of human inflammatory diseases. The IKK complex represents a potential convergence point for multiple signaling stimuli that activate NF- $\kappa\text{B}$ . We hypothesized that IKK is a key signal transduction kinase that coordinates this process in human airway epithelial cells. Our results support the conclusion that extracellular milieu NaCl content selectively increases the IKK- $\alpha$  level in a concentration-dependent manner (but not IKK- $\beta$ ) in non-CF bronchial gland cells. Surprisingly, higher levels of IKK- $\alpha$  were found in CF bronchial gland cells at any extracellular NaCl concentration, suggesting the constitutive lack of  $\text{I}\kappa\text{B}\alpha$  protein that we had previously reported in CF bronchial gland epithelial cells in vivo and in vitro (9) may be mediated in part by the high constitutive IKK- $\alpha$  protein expression (not for IKK- $\beta$ ) that we demonstrate in the present study. Therefore, the IKK complex, especially IKK- $\alpha$  seems to be responsible, in part, for the constitutive IL-8-mediated NF- $\kappa\text{B}$  activation in CF bronchial gland cells. The constitutive activation of NF- $\kappa\text{B}$  associated with the lack of  $\text{I}\kappa\text{B}\alpha$  protein in CF HBG cells contrasts with the inducible feature of this pathway reported in many epithelial cell types (25). Therefore, it is reasonable to speculate that inhibition of IL-8 secretion through the overexpression of a nondegradable form of  $\text{I}\kappa\text{B}\alpha$  in airway epithelial cells could prevent neutrophil recruitment and transepithelial inflammatory cell migration in CF airways, as recently suggested for human intestinal epithelial cells (28). Jobin et al. (28) have recently reported that viral-delivered mutant  $\text{I}\kappa\text{B}\alpha$ , transferred into both transformed HT-29 cells as well as primary human colonic epithelial cells, blocked the production of multiple proinflammatory molecules, including IL-8, by inhibiting the NF- $\kappa\text{B}$  activation pathway.

These apparently contradictory results in the regulation of NIK signaling pathway between the non-CF and CF bronchial gland cells suggests that, in CF gland cells, the regulation of IL-8 production may induce other mechanisms involving distinct triggering molecular entities. For example, p38 mitogen-activated protein kinase (MAPK) pathways have been demonstrated to play an important role in the control of IL-8 production mediated by high salt concentrations in human PBMC and THP-1 monocyte-like cells (29, 30). High salt concentrations induced IL-8 at the transcriptional level, and this induction of IL-8 was inhibited by the p38 MAPK inhibitor SB 20508 (30), indicating the involvement of the MAPK transduction pathway. These investigators also found that IL-8 was not increased by the addition of glycerol, which achieved extracellular osmolarity equivalent to those of the media containing Na<sup>+</sup>, Cl<sup>-</sup> dissociable ions. The authors further concluded that hyperosmolarity itself is not a sufficient signal to activate IL-8. With respect to the mechanism by which extracellular NaCl content affects CF bronchial gland cells, further investigations are required to determine whether variations in tonicity and/or ionic imbalance differently affect the IL-8 production in CF and non-CF bronchial gland cells through differential activation of NIK and/or p38 MAPK pathways. It is also possible that a loss of CFTR function in CF bronchial gland cells may modify the NF- $\kappa\text{B}$  binding activity. Recently, Schwiebert et al. (31) suggested that complementation of CF respiratory surface epithelial cells with wild-type CFTR to correct the CFTR defect restored cytokine induction of RANTES but not IL-8 expression via a NF- $\kappa\text{B}$ -mediated pathway. In CF bronchial gland epithelial cells, investigation of the mutant CFTR-dependent mechanisms related to the signal transduction pathways for IL-8 expression will provide a better understanding of the inflammatory responses to the changes of the NaCl content in CF human airways.

In summary, our data show that isotonic and hypertonic airway fluids are capable of stimulating and/or maintaining high levels of NF- $\kappa\text{B}$  activation through the IKK- $\alpha$  pathway and subsequent high IL-8 production in HBG epithelial cells. We also demonstrate for the first time that primary CF HBG epithelial cells exhibit constitutive NF- $\kappa\text{B}$  binding activity and high levels of IKK- $\alpha$  at any extracellular hypo-, iso-, and hypertonic NaCl content. Given the proinflammatory functions of NF- $\kappa\text{B}$ , the IKK complex could represent a potential therapeutic target for CF human airway disease. Although our findings remain to be investigated in in vivo models, they may be informative with respect to inflammatory processes in which excessive NaCl-induced IL-8 secretion by airway epithelial cells plays a determinant role in the onset of early and chronic airway mucosal inflammation in CF patients.

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