Ibuprofen Modulates NF- κ B Activity but Not IL-8 Production in Cystic Fibrosis Respiratory Epithelial Cells

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For editorial comment see p. 189

Key Words

Corticosteroids • Cystic fibrosis • Dexamethasone • Epithelial cells • Ibuprofen • IL-1 β • IL-8 • NF- κ B • NSAIDS • TNF- α • Transcription factors

Abstract

Background: High-dose ibuprofen is clinically effective in cystic fibrosis (CF); however, its molecular mechanisms are poorly understood. **Objective:** To test the hypothesis that clinically relevant concentrations of ibuprofen suppress activation of nuclear factor (NF)- κ B and thus down-regulate stimulated interleukin (IL)-8 production in CF respiratory epithelial cells. **Methods:** The majority of experiments were conducted in CFTE29o– cells (F508del-mutated CF transmembrane regulator, CFTR). Key experiments were confirmed in CFBE41o– cells (F508del-mutated CFTR) and 1HAEo– cells (wild-type CFTR). NF- κ B and IL-8 were stimulated with tumour necrosis factor (TNF)- α or IL-1 β . NF- κ B and IL-8 suppression by ibuprofen (480 μ M) was compared to dexamethasone (5 nM). **Results:** Both TNF- α and IL-1 β activated NF- κ B and stimulated IL-8 production. Both ibuprofen and dexamethasone demonstrated comparably modest suppression of NF- κ B transcriptional activity. However, ibuprofen had no effect on stimulated IL-8 mRNA and protein. By contrast, dexamethasone significantly down-regulated stimulated IL-8 mRNA and protein. **Conclusions:** The pres-

ent data do not support the hypothesis that ibuprofen down-regulates IL-8 production in response to TNF- α and IL-1 β in CF respiratory epithelium. Suppression of NF- κ B transcriptional activity does not discriminate between anti-inflammatory drugs with or without effects on IL-8 production. We speculate that NF- κ B-independent mechanisms may be responsible for anti-IL-8 effects of dexamethasone.

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Introduction

Cystic fibrosis (CF) is a lethal genetic disease characterised by progressive lung damage due to chronic infection and exaggerated airway inflammation. Current anti-inflammatory therapies for CF include corticosteroids [1–3] and ibuprofen [4, 5], a non-steroidal anti-inflammatory drug (NSAID). These drugs slow decline in pulmonary function and exert other beneficial clinical effects. Unfortunately, serious adverse effects (corticosteroids [6]), or concerns thereof (ibuprofen [7]) have limited their clinical use. Therefore, there is a need to develop novel and safer anti-inflammatory drugs for CF. A better un-

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derstanding of molecular mechanisms of existing anti-inflammatory therapies could help to create future drugs.

The inflammation in CF airways is neutrophil dominated. The factors released by activated neutrophils are believed to cause lung damage. Neutrophils are recruited to CF airways by overabundant interleukin (IL)-8, which is the principal neutrophil-chemo-attracting agent [8]. For instance, in CF nasal epithelium, the presence of neutrophils correlated well with the expression of IL-8 mRNA [9].

CF respiratory epithelium secretes exaggerated amounts of IL-8, either spontaneously [10, 11] or in response to pro-inflammatory cytokines [12, 13]. Down-regulation of IL-8 production in CF respiratory epithelium could attenuate neutrophil-dominated inflammation and its negative consequences in the CF lung.

In CF, ibuprofen is administered at high doses to achieve a specific range of concentrations in serum (50–100 mg/l [4, 5]). These concentrations diminish neutrophil migration [14]. At these concentrations, ibuprofen is believed to target intracellular pathways beyond suppression of prostaglandin synthesis [7, 15]. In particular, ibuprofen has been shown to down-regulate production of leukotriene 4 via yet unknown mechanisms [16]. Further, several CF-unrelated studies indicate that high-dose ibuprofen suppresses activity of nuclear factor (NF)- κ B [17–19]. NF- κ B is the major transcription factor activating IL-8 production in response to many CF-relevant inflammatory factors, such as tumour necrosis factor (TNF)- α and IL-1 β [20]. The suppression of NF- κ B activation and subsequent down-regulation of IL-8 could explain the decrease of neutrophil migration in CF patients treated with ibuprofen [14].

We hypothesised that CF-relevant concentrations of ibuprofen suppress NF- κ B activation in response to TNF- α and IL-1 β and thus down-regulate stimulated IL-8 production in CF respiratory epithelium. We tested this hypothesis in a respiratory epithelial cell line (CFTE29o–) [21] which expresses the most common CF mutation (F508del) [22]. Key experiments were confirmed in another CF respiratory epithelial cell line (CFBE41o–; F508del) [23], as well as in a 1HAEo– respiratory epithelial cell line expressing wild-type CF transmembrane regulator (CFTR) [24]. Anti-IL-8 activity of high-dose ibuprofen was compared to that of dexamethasone, a corticosteroid, which has a well-established record of IL-8 down-regulation in respiratory epithelium [25]. Both drugs were used at clinically relevant concentrations: ibuprofen at 100 mg/l (480 μ M) [4, 5] and dexamethasone at 1.7 μ g/l (5 nM) [26, 27].

Materials and Methods

Reagents

Ibuprofen was from Cayman Chemicals. Similar to the clinical drug, it is a racemic (i.e. 1:1) mixture of S(+) and R(–) enantiomers of ibuprofen. Dexamethasone was from Sigma. Both drugs were prepared as stocks in absolute ethanol (200 and 1 mM, respectively) and stored at –20°C. Ethanol was used as a diluent control in these experiments. S(+)-ibuprofen, the more active enantiomer of ibuprofen, was from Sigma. S(+)-ibuprofen was solubilised freshly in PBS (4.85 mM), and PBS was used as a diluent control. Human recombinant TNF- α and IL-1 β were from BD Biosciences. Unless otherwise specified, reagents were obtained from Sigma.

Cell Lines

For the majority of experiments, we used the CFTE29o– cell line expressing F508del-mutated CFTR [21]. Key experiments were confirmed in CFBE41o– cell line (F508del) [23] and 1HAEo– cell line, a non-CF cell line [24]. All cell lines were a kind gift from Dr. Dieter Gruenert (University of California at San Francisco).

Basal cell culture was maintained in coated [21, 28] flasks in minimal essential medium supplemented with 10% fetal bovine serum, antibiotics and 2 mM L-glutamine. Subconfluent cells were trypsinised [21], collected and counted. Unless otherwise specified, cell concentration was adjusted to 0.25×10^6 viable (trypan blue exclusion) cells/ml. Cells were plated, cultured overnight to 95–100% confluency, and used in the experiments in antibiotics-free minimal essential medium supplemented with 0.5% bovine serum albumin and 2 mM L-glutamine.

IL-8 ELISA

Cells grown on 24-well plates were pre-incubated (30 min to 16 h) with culture medium with or without test drugs. Then, TNF- α or IL-1 β were added (10 ng/ml final solution), and cells were stimulated for 1 h. After removal of inflammatory stimuli, cells were cultured in the presence or absence of test drugs for another 4 h. Cell culture supernatants were collected and analysed for IL-8 by ELISA (BD Biosciences).

IL-8 mRNA Expression

Cells grown on 6-well plates were pre-incubated for 30 min with culture medium with or without test drugs and stimulated with TNF- α or IL-1 β (both at 10 ng/ml) for 1 h. At the end of stimulation, cells were lysed with TRIzol (Invitrogen). Total RNA was extracted, and 1 μ g of total RNA was reverse transcribed with QuantiTect reverse transcription kit (Qiagen). IL-8 mRNA expression was quantified by real-time quantitative PCR (qPCR). Primer sets (IL-8 and β -actin) and QuantiFast Sybr Green PCR kit were from Qiagen. PCR reactions (25 μ l) were conducted using 1.5 μ l of cDNA on Mx3000 qPCR system (Stratagene). The comparative threshold cycle (C_T) method was used for relative quantification of PCR products [29] after IL-8 was normalised to β -actin ($\Delta C_t = C_{t_{IL-8}} - C_{t_{\beta-actin}}$). IL-8 mRNA data in treated samples were expressed in fold changes over controls: fold change_{treated sample} = $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_{t_{treated sample}} - \Delta C_{t_{control}}$.

NF- κ B p65 Nuclear Translocation and Binding Activity

Nuclear translocation of NF- κ B p65 in response to TNF- α or IL-1 β was confirmed by immunostaining experiments.

Fig. 1. TNF- α and IL-1 β stimulate IL-8 in CFTE29o- (a) and CFBE41o- (b) cells. CF respiratory epithelial cells were stimulated for 1 h with culture medium (basal), or 10 ng/ml TNF- α (TNF) or IL-1 β (IL-1). After removal of stimuli, cells were cultured for 4 h, and IL-8 in supernatants was measured by ELISA. n = 3–4, * p < 0.05 vs. basal. Data are means + SEM.

CFTE29o- cells were plated onto 12-mm glass coverslips and grown as above. Cells were stimulated with TNF- α or IL-1 β (both at 10 ng/ml) for 1 h and used in immunofluorescent staining. Cells were washed with PBS and fixed for 30 min in methanol-free 4% formaldehyde, freshly prepared from 16% stock (Polysciences). Then, cells were permeabilised for 20 min with pre-chilled (-20°C) absolute methanol and washed with PBS. Unspecific binding was blocked by 1% BSA in PBS (30 min). NF- κB p65 was immunodetected using rabbit anti-p65 antibody (Santa Cruz, 1:200 in 1% BSA, overnight staining), biotinylated goat anti-rabbit antibody (Santa Cruz, 1:200, 45 min) and streptavidin-Alexa Fluor 488 conjugate (Invitrogen, 1:100, 45 min). Immunofluorescence was visualised and documented using Axio Imager fluorescence microscope from Zeiss.

For NF- κB p65 binding activity, CFTE29o- cells were grown on 60-mm culture dishes, pre-incubated for 30 min with culture medium with or without test drugs, and stimulated with TNF- α or IL-1 β (both at 10 ng/ml) for 1 h. Cell nuclei were obtained immediately after 1 h stimulation using Nuclear Extraction kit (Active Motif). Protein concentrations were quantified using BCA protein assay (Pierce Endogen). Five-microgram nuclear extracts were used in the NF- κB p65 Trans AM assay (Active Motif).

NF- κB Transcriptional Activity: Transient Transfection and Reporter Gene Assay

For a high-efficiency transient transfection, we used a Nucleofector II device (Amaxa). CFTE29o- cells were collected by trypsinisation, pelleted by centrifugation (90 g, 10 min) and counted. Two million viable cells were re-pelleted by centrifugation (90 g, 10 min) and reconstituted in 100 μl of Nucleofector Solution V (Nucleofector Kit V, Amaxa). Cells were electroporated in the presence of 1.0 μg of NF- κB firefly luciferase Rapid Response Reporter (Promega) and 0.1 μg of internal control (*Renilla* luciferase reporter, HSV-TK constitutive promoter; Promega) using program T-020. The NF- κB firefly luciferase Rapid Response Reporter contains five NF- κB -binding elements. This reporter also contains protein degradation sequences for a quicker reporter turnover and a more accurate assessment of NF- κB transcriptional activity.

Transfected cells were plated onto a 96-well plate (quadruplicate wells/outcome) at a concentration of 1.0×10^6 viable cells/

ml. Cells were cultured for 24 h, pre-incubated for 30 min with culture medium with or without test drugs, and stimulated with TNF- α or IL-1 β (both at 10 ng/ml) for 1 h. After removal of inflammatory stimuli, cells were cultured with or without test drugs for another 4 h and lysed with Passive Lysis Buffer (Promega). Luminescence was quantified using Dual-Luciferase Reporter Assay System (Promega) on Glomax microplate luminometer (Promega). Relative luciferase activity was calculated by normalising firefly luciferase activity against *Renilla* luciferase activity.

This transfection protocol provided high ($\sim 80\%$) transfection efficiency as judged by transfection experiments with plasmids encoding for green fluorescent protein (data not shown).

Statistical Analysis

Statistical analysis was conducted with Statistica 6.0 software (StatSoft). The results are presented as means + SEM. Differences were assessed by the Mann-Whitney U non-parametric test. In some graphs, data are presented as percent or fold change over respective controls. For these graphs, statistical significance was calculated prior to presentation as percent or fold change. The level of statistical confidence was set at $p < 0.05$.

Results

TNF- α and IL-1 β Stimulate IL-8 and NF- κB

In CFTE29o- cells, stimulation for 1 h with TNF- α or IL-1 β significantly up-regulated IL-8 production, as detected 4 h later ($p < 0.05$ vs. basal, both comparisons; fig. 1a). The magnitude of IL-8 up-regulation was comparable between both inflammatory stimuli (fig. 1a).

CFBE41o- cells slightly up-regulated IL-8 production in response to stimulation with 10 ng/ml TNF- α . However, this up-regulation did not reach statistical significance ($p > 0.05$ vs. basal; fig. 1b). In contrast, CFBE41o- significantly increased IL-8 production after stimulation with IL-1 β ($p < 0.05$ vs. basal; fig. 1b). The magnitude of

Fig. 2. TNF- α and IL-1 β stimulate NF- κ B activation and IL-8 mRNA transcription in CFTE290- cells. CFTE290- cells were stimulated for 1 h with culture medium (basal), or 10 ng/ml TNF- α (TNF) or IL-1 β (IL-1). Immediately after stimulation, NF- κ B p65 nuclear translocation was documented by immunofluorescence (**a**) and nuclear extracts were analyzed for NF- κ B p65 binding activity by TransAM assay (**b**). $n = 3-4$, * $p < 0.05$ vs. basal.

c Immediately after stimulation, cells were analysed for IL-8 mRNA expression by qPCR. The IL-8 mRNA expression of cells stimulated with TNF- α or IL-1 β was expressed in fold change over basal. $n = 3-5$, * $p < 0.05$ vs. basal, ** $p < 0.01$ vs. basal. **d** Cells expressing NF- κ B luciferase reporter were stimulated for 1 h, cultured for 4 h without stimuli and analysed for luciferase activity. $n = 7-8$, ** $p < 0.01$ vs. basal. Data are means + SEM.

IL-8 up-regulation that we observed 4 h after stimulation with 10 ng/ml IL-1 β (12-fold increase) was comparable to the one reported by John et al. [30] in response to 100 ng/ml TNF- α (estimated 12- to 15-fold increase, 24 h after stimulation). These findings suggest that CFBE410- cells are weak-to-moderate responders to TNF- α and vigorous responders to IL-1 β .

The activation of NF- κ B by two inflammatory stimuli was followed in CFTE290-cells because this cell line was a vigorous responder to both TNF- α and IL-1 β . First, we documented nuclear translocation of NF- κ B p65 after 1 h of stimulation with either inflammatory stimulus (fig. 2a). This observation was further confirmed by increased p65-binding activity in nuclei of stimulated CFTE290- cells ($p < 0.05$, both comparisons; fig. 2b).

As expected, the NF- κ B p65 nuclear translocation and increase in binding activity was associated with increased IL-8 mRNA transcription. This was true for both TNF- α

and IL-1 β ($p < 0.01$ or < 0.05 vs. basal, respectively; fig. 2c). Further, both TNF- α and IL-1 β increased NF- κ B transcriptional activity in CFTE290- cells (luciferase reporter studies; $p < 0.01$ vs. basal, both comparisons; fig. 1d).

To summarise, CFTE290- cells responded with activation of NF- κ B and up-regulation of IL-8 production after stimulation with both TNF- α and IL-1 β . Therefore, this cell line was used for the majority of subsequent studies.

Ibuprofen Suppresses NF- κ B Transcriptional Activity, but Not Stimulated IL-8 Production

Respiratory epithelial cell lines were pre-incubated with clinically relevant concentrations of ibuprofen or dexamethasone, and stimulated with TNF- α or IL-1 β . Ibuprofen was tested as a racemic mixture [i.e. a 1:1 mixture of S(+):R(-) enantiomers], or as S(+)-ibuprofen (the more active enantiomer).

In the first experiment, CFTE29o- and CFBE41o- cells were pre-incubated for 30 min with diluent or test drugs and stimulated for 1 h with TNF- α or IL-1 β . Then, inflammatory stimuli were removed, and cells were incubated with diluent or test drugs for 4 h. After this incubation, IL-8 production was quantified by ELISA.

Ibuprofen had no effect on stimulated IL-8 production in CFTE29o- or CFBE41o- cells ($p > 0.05$ vs. diluent + TNF or diluent + IL-1; fig. 3a–c). In marked contrast, dexamethasone significantly decreased stimulated IL-8 production in both CFTE29o- ($p < 0.01$ vs. diluent + TNF or diluent + IL-1; fig. 3a, b) and CFBE41o- cells ($p < 0.05$ vs. diluent + IL-1; fig. 3c). In CFTE29o- cells, the anti-IL-8 potency of ibuprofen was not increased by a longer (90 min to 16 h) pre-incubation with this drug ($p > 0.05$ vs. diluent + TNF, data not shown).

We next wanted to rule out that insensitivity to high-dose ibuprofen in CFTE29o- and CFBE41o- cells was due to CF gene defect. Therefore, we tested this drug in 1HAEo- cell line which expresses wild-type CFTR. Similar to CF cell lines, ibuprofen failed to decrease stimulated IL-8 production in 1HAEo- cells ($p > 0.05$ vs. diluent + TNF; fig. 3d).

We also tested whether S(+)-ibuprofen, the more active enantiomer of ibuprofen, would be capable of suppressing IL-8 production. S(+)-ibuprofen makes up two thirds of total ibuprofen in serum [31]; therefore, 100 mg/l of total ibuprofen contain 66 mg/l (320 μ M) of S(+)-ibuprofen. At this concentration, S(+)-ibuprofen did not suppress stimulated IL-8 production in CFTE29o- cells ($p > 0.05$ vs. diluent + TNF; fig. 3e).

The lack of IL-8 suppression by ibuprofen was corroborated by studies on IL-8 mRNA expression in CFTE29o- cells. Specifically, ibuprofen did not suppress IL-8 mRNA expression after 1-hour stimulation with TNF- α ($p > 0.05$ vs. diluent + TNF; fig. 4a).

By contrast, dexamethasone significantly decreased TNF- α -stimulated IL-8 mRNA expression ($p < 0.05$ vs. diluent + TNF; fig. 4a).

In subsequent experiments, we compared the effects of both ibuprofen and dexamethasone on NF- κ B activation. In the CFTE29o- cell line, neither ibuprofen nor dexamethasone suppressed NF- κ B p65 binding activity after 1 h of stimulation with TNF- α ($p > 0.05$ vs. diluent + TNF, both comparisons; fig. 4b). Interestingly, both ibuprofen and dexamethasone moderately decreased NF- κ B transcriptional activity in luciferase reporter studies ($p < 0.05$ ibuprofen + TNF vs. diluent + TNF, $p < 0.01$, dexamethasone + TNF vs. diluent + TNF; fig. 4c). The magnitude of these decreases did not differ significantly

between both drugs ($p > 0.05$; fig. 4c) and was within the range observed in previous reports [32–34].

To summarise, both high-dose ibuprofen and dexamethasone moderately decreased NF- κ B transcriptional activity, yet only dexamethasone significantly decreased stimulated IL-8 production in CF respiratory epithelial cells.

Discussion

High-dose ibuprofen is effective clinically in CF; however, its molecular mechanisms are poorly understood. In the present study, we tested the hypothesis that high-dose ibuprofen suppresses NF- κ B activity and thus decreases stimulated IL-8 production in CF respiratory epithelium. Anti-IL-8 activity of ibuprofen was compared to that of dexamethasone, a well-established anti-IL-8 drug.

Both high-dose ibuprofen and dexamethasone modestly suppressed NF- κ B transcriptional activity in CF respiratory epithelial cells, but only dexamethasone significantly down-regulated stimulated IL-8 at both mRNA and protein levels. The lack of IL-8 down-regulation by high-dose ibuprofen was documented in two CF respiratory epithelial cells, CFTE29o- and CFBE41o- cells, and in one respiratory epithelial cell line expressing wild-type CFTR, 1HAEo-.

Interestingly, we observed that CF respiratory epithelial cell lines demonstrate differential response to inflammatory stimuli. Specifically, CFTE29o- cells vigorously up-regulated IL-8 in response to both TNF- α and IL-1 β , whereas CFBE41o- cells responded only to IL-1 β in the present study. John et al. [30] have observed that CFBE41o- respond to 100 ng/ml TNF- α . The ten times higher concentration of TNF- α and a longer incubation time after the stimulation (24 vs. 4 h in our study) are the likely reasons behind this discrepancy between our and their data. The heterogeneity of inflammatory responses among CF respiratory epithelial cell lines underscores the necessity of testing several cell lines and different inflammatory stimuli, such as done in the present study.

Since high-dose ibuprofen is incapable of down-regulating stimulated IL-8 in CF respiratory epithelium, there must be alternative molecular mechanisms for ibuprofen in CF. These molecular mechanisms may involve inactivation of the transcription factor C/EPB homologous protein (CHOP). In CF, this transcription factor appears to be involved in at least two pathways.

The first pathway is directly related to the F508del mutation. In this mutation, accumulation of misfolded CFTR

Fig. 3. Dexamethasone (Dex; 5 nM) but not 480 μ M ibuprofen or 320 μ M S(+)-ibuprofen (Ibu) down-regulate IL-8 production in respiratory epithelial cells stimulated with TNF- α or IL-1 β . **a** CFTE290- cells were pre-treated for 30 min with diluent (Dil) or test drugs, and stimulated for 1 h with 10 ng/ml TNF- α (TNF). After removal of TNF, CFTE290- cells were incubated with Dil or test drugs for 4 h, and IL-8 in supernatants was measured by ELISA. The IL-8 data of cells treated with test drugs + TNF were expressed as percent of Dil + TNF. n = 6, ** p < 0.01 (Dex + TNF vs. Dil + TNF), *** p < 0.01 (Dex + TNF vs. Ibu + TNF). **b** CFTE290- were pre-treated for 30 min with Dil or test drugs, and stimulated for 1 h with 10 ng/ml IL-1 β (IL-1). After removal of IL-1, cells were processed as described in **a**. The IL-8 data of cells treated with test drugs + IL-1 were expressed as percent of Dil + IL-1. n = 6, ** p < 0.01 (Dex + IL-1 vs. Dil + IL-1), *** p <

0.01 (Dex + IL-1 vs. Ibu + IL-1). **c** CFBE410- cells were pre-treated for 30 min with Dil or test drugs, and stimulated for 1 h with 10 ng/ml IL-1. After removal of IL-1, cells were processed as described in **a**. The IL-8 data of cells treated with test drugs + IL-1 were expressed as percent of Dil + IL-1. n = 4, * p < 0.05 (Dex + IL-1 vs. Dil + IL-1), *** p < 0.05 (Dex + IL-1 vs. Ibu + IL-1). **d** 1HAEo- cells were pre-treated for 30 min with Dil or test drugs, and stimulated for 1 h with 10 ng/ml TNF. After removal of TNF, cells were processed as described in **a**. The IL-8 data of cells treated with Ibu + TNF were expressed as percent of Dil + TNF. n = 5. **e** CFTE290- cells were pre-treated for 30 min with Dil or S(+)-ibuprofen [S(+)-Ibu] and stimulated for 1 h with 10 ng/ml TNF. After removal of TNF, cells were processed as described in **a**. The IL-8 data of cells treated with test drugs + TNF were expressed as percent of Dil + TNF. n = 4. Data are means + SEM.

causes endoplasmic reticulum (ER) stress [35]. This ER stress leads to chronic IL-8 overproduction by CF respiratory epithelium [36] via activation of the transcription factor CHOP [37] in the absence of inflammatory stimuli. One could speculate that ibuprofen may clinically sup-

press this inflammatory pathway by blocking CHOP activity, as it was demonstrated with regard to NS398 [38].

Next, ibuprofen, as a COX2 inhibitor, could work by suppressing prostaglandin (PG) E₂ synthesis in CF respiratory epithelium. PGE₂ stimulates IL-8 production in

Fig. 4. Dexamethasone (Dex; 5 nM) suppresses NF- κ B transcriptional activation and IL-8 mRNA transcription, whereas 480 μ M ibuprofen (Ibu) only suppresses NF- κ B transcriptional activity in CFTE290- cells stimulated with TNF- α (TNF). **a** Cells were pre-treated for 30 min with diluent (Dil) or test drugs, and stimulated for 1 h with 10 ng/ml TNF. Immediately after stimulation with TNF, cells were analyzed for IL-8 mRNA expression by qPCR. The IL-8 mRNA expression in cells treated with test drugs + TNF was expressed in fold change over Dil + TNF. $n = 4$, * $p < 0.05$ (Dex + TNF vs. Dil + TNF). **b** Immediately after stimulation with TNF,

nuclear extracts were analyzed for NF- κ B p65 binding activity by TransAM assay. $n = 5$. **c** Cells expressing NF- κ B firefly luciferase reporter were pre-treated with Dil or test drugs for 30 min and stimulated for 1 h with 10 ng/ml TNF. After removal of TNF, cells were incubated with Dil or test drugs for 4 h, and analyzed for luciferase activity. The NF- κ B transcriptional activity in cells treated with test drugs + TNF was expressed in percent of Dil + TNF. $n = 7$, * $p < 0.05$ (Ibu + TNF vs. Dil + TNF), ** $p < 0.01$ (Dex + TNF vs. Dil + TNF). Data are means + SEM.

CF respiratory epithelial cells by activating CHOP [38]. In contrast to TNF- α and IL-1 β , PGE₂ up-regulates IL-8 only after prolonged (36-hour) stimulation [38]. Further, the magnitude of IL-8 up-regulation in response to PGE₂ (~1.5- to 2-fold increase) [38] is significantly less prominent compared to the IL-8 up-regulation by TNF- α or IL-1 β (in the present study: at least 10-fold increase for both stimuli after 1 h of stimulation). Therefore, it is possible that ibuprofen is ineffective against acute and strong stimuli (e.g. TNF- α or IL-1 β), but capable of down-regulating IL-8 production in CF respiratory epithelium in response to chronic stimuli, such as ER stress or exposure to PGE₂. This suppression of PGE₂-related pathway was demonstrated with another NSAID, NS-398 [38]. Decrease in IL-8 production due to diminished ER stress or attenuated response to PGE₂ could explain the in vivo suppression of neutrophil migration [14]. Alternatively, high-dose ibuprofen may directly target neutrophils and affect their functional properties, such as migration potential [39].

Next, clinical effects of ibuprofen may not be related to NF- κ B or CHOP. There is a recent report demonstrating that ibuprofen at clinically relevant concentrations (300 μ M) enhances CFTR function [40].

CFTR is the ion channel whose malfunction is believed to trigger or aggravate the clinical disease because of the hindered transport of ions, electrolytes and antioxidants [41, 42]. Augmentation of CFTR function is expected to attenuate CF lung disease.

In addition, high-dose ibuprofen was reported to down-regulate cAMP activation in CF respiratory epithelium [38]. Suppression of cAMP-dependent pathway could be another anti-inflammatory mechanism of ibuprofen.

Both CF respiratory epithelial cell lines (CFTE290- and CFBE41o-) responded well to treatment with dexamethasone by decreasing stimulated IL-8 production. Our data are consistent with previous reports in the literature that CF cell lines and primary cultures are responsive to corticosteroids [43–45]. There are also publications reporting insensitivity of CF tracheobronchial gland serous cells to corticosteroids [46, 47]. Since tracheobronchial gland serous cells are abundant in CFTR [48], these cells can innately exist at a higher pro-inflammatory state than mucosal cells, and hence be less sensitive to corticosteroids.

The present findings show that modulation of NF- κ B transcriptional activity in CF respiratory epithelial cells

not always results in a down-regulation of IL-8 production in response to TNF- α and IL-1 β . Therefore, our data suggest that anti-inflammatory drugs require NF- κ B-independent mechanisms to suppress stimulated IL-8 production in CF respiratory epithelium.

These NF- κ B-independent mechanisms can encompass other transcription factors or post-transcriptional mechanisms.

In respiratory epithelium, stimulation with TNF- α activates both NF- κ B and activator protein (AP)-1 [49]. Dexamethasone has been reported to suppress AP-1 binding activity [50]. The IL-8 promoter contains an AP-1 binding site [51], and IL-8 production can be up-regulated by activation of AP-1 [20]. Therefore, dexamethasone can down-regulate IL-8 production in CF respiratory epithelium by suppressing AP-1 activity. By contrast, we are not aware of any reports on such effects of ibuprofen on AP-1.

Alternatively, IL-8 production can be regulated by dexamethasone post-transcriptionally, e.g. by modulation of mRNA stability [52]. Modulation of IL-8 mRNA stability by dexamethasone could explain that only this drug affected the IL-8 mRNA expression while both ibuprofen and dexamethasone equally suppressed the NF- κ B transcriptional activity. We are not aware of any reports demonstrating the mRNA-destabilizing effects of ibuprofen.

There are some limitations to our study. First, the present in vitro experiments were conducted in cell lines, and not in primary cell culture. Second, we used inflammatory cytokines to elicit IL-8 response. It would be of interest to confirm the lack of anti-IL-8 activity of high-dose ibuprofen in CF respiratory epithelium exposed to bacterial pathogens, such as *Pseudomonas aeruginosa*.

In conclusion, suppression of NF- κ B transcriptional activity by high-dose ibuprofen does not result in a decreased IL-8 production in response to TNF- α or IL-1 β in CF respiratory epithelial cells. Other biochemical mechanisms are likely to be responsible for the beneficial clinical effects of this drug in CF. The anti-IL-8 activity of dexamethasone appears to be dissociated from its effects on NF- κ B and may involve NF- κ B-independent mechanisms. More studies are needed to specify these mechanisms in CF respiratory epithelium.

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