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Impact of CFTR ΔF508 mutation on prostaglandin E_2 production and type-IIA phospholipase A_2 expression by pulmonary epithelial cells

Running title: CFTR inhibits PGE_2 release and $sPLA_2$-IIA expression

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Abstract

Cystic Fibrosis (CF) is characterized by an exacerbated inflammatory pulmonary response with excessive production of inflammatory mediators. We investigated here the impact of cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction on prostaglandin E_2 (PGE_2) production and type-IIA secreted phospholipase A_2 ($sPLA_2$-IIA) expression. We showed that both resting and LPS-stimulated human respiratory epithelial cell line bearing ΔF508 mutation on CFTR (CF cells), released more PGE_2 than control cell line. This was accompanied by an enhanced expression and activity of cyclooxygenase-2 (COX-2) in CF cells. PGE_2 release was attenuated after experimentally-induced re-trafficking of the ΔF508-CFTR at the plasma membrane. $sPLA_2$-IIA expression occurred at higher levels in CF cells than in control cells and was enhanced by LPS and PGE_2. Suppression of PGE_2 synthesis by aspirin led to an inhibition of LPS-induced $sPLA_2$-IIA expression. Higher activation of NF-κB was observed in CF cells compared to control cells and was enhanced by LPS. However, addition of PGE_2 or aspirin had no effect on NF-κB activation. LPS-induced $sPLA_2$-IIA expression was reduced by a NF-κB inhibitor. We suggest that the lack of the CFTR in the plasma membrane results in a PGE_2 overproduction and an enhanced $sPLA_2$-IIA expression. This expression is up-regulated by NF-κB and amplified by PGE_2 *via* a non identified signalling pathway.

Keywords: Cystic Fibrosis; Inflammation; Arachidonic Acid Metabolism; CFTR.

Introduction

Cystic Fibrosis (CF) is an autosomal recessive disorder caused by mutations of the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (25). CF patients express typical phenotype characterised by recurrent excessive inflammation and infection. Pulmonary infection is one of the most frequent processes that ultimately leads to pulmonary failure and death of the patients (5; 24). CFTR, a member of the ATP-binding cassette superfamily, contains two similar units, each including membrane-spanning domains and two nucleotide-binding domains linked by a single regulatory domain. It functions as a cyclic AMP (cAMP)-dependent chloride channel that regulates epithelial surface fluid secretion in the respiratory and gastrointestinal tracts (15). The deletion of the phenylalanine at position 508 (ΔF508) in the first nucleotide-binding domain of CFTR is the most common mutation (70% of the mutated alleles) in CF patients. The ΔF508-CFTR is unable to fold correctly and to assume its appropriate tertiary conformation, leading to its retention in the endoplasmic reticulum and to its degradation by the proteasome. However, if the CFTR is permitted to reach the cell membrane by artificial procedures, it becomes able to function as a cAMP-dependent chloride channel (6; 8).

The pulmonary disease in CF is characterized by an excessive inflammation in response to bacterial infection in particular by *Pseudomonas aeruginosa* (12). Bacterial lipopolysaccharides (LPS) are known to induce the expression of various genes

involved in inflammation, including those coding for phospholipase A_2 (PLA_2). The latter belongs to a family of enzymes that catalyse the hydrolysis of phospholipids at the sn-2 position, leading to the generation of lysophospholipids and free fatty acids, such as arachidonic acid (AA) (7). These products are the precursors of lipid mediators (platelet-activating factor and eicosanoids) endowed with various biological activities and involved in the pathophysiological changes observed in a number of inflammatory diseases (6; 3). PLA_2 are classified into two major classes: the low molecular weight secreted forms, termed $sPLA_2$, and the high molecular weight cytosolic forms, termed $cPLA_2$. In mammals, $sPLA_2$ are classified into several different groups including the type-IIA $sPLA_2$ ($sPLA_2$-IIA), which seems to play a role in the pathogenesis of various inflammatory and infectious diseases (29). $sPLA_2$-IIA has been also shown to exhibit anti-bacterial activity and to play a role in host defence immunity (29). Previous studies have shown the existence of a cross-talk between $cPLA_2$ and $sPLA_2$-IIA leading to the modulation of the expression of the latter *via* AA metabolite such as prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) (19). Clinical studies reported increased levels of eicosanoids in bronchoalveolar lavage fluid from CF patients (18) but whether this increase is a direct or a secondary consequence of CFTR dysfunction has not been investigated.

The present study was undertaken to investigate the impact of the ΔF508 mutation of CFTR on PGE_2 production and $sPLA_2$-IIA expression in human respiratory epithelial

cell lines. The rationale of this study is that the production of PGE_2 by CF pulmonary epithelial cells may up-regulate $sPLA_2$-IIA expression by these cells.

Materials and methods

Reagents

Dulbecco's modified Eagle's, minimum essential medium with Earle's salt and Ham F-12 cell culture media, Hank's balanced salt solution (HBSS), and trypsin-EDTA were from Invitrogen (Cergy-Pontoise, France). Foetal calf serum (FCS) was from Hyclone (Logan, UT). Phenylmethylsulfonyl fluoride (PMSF), benzamidin, dithiotreitol (DTT), ethylenediaminetetraacetic (EDTA), *Pseudomonas aeruginosa* LPS, Methyl arachidonyl flurophosphonate (MAFP), MG-132 and phorbol 12-myristate 13-acetate (PMA) were from Sigma (St. Louis, MO). LY311727 was a gift from Eli Lilly Co (Indianapolis, IN). The anti-p65 sub-unit and anti-COX-2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phosphoCREB antibody was purchased by Cell Signalling Technology (Beverly, MA). The anti-β-actin antibody was purchased from Sigma.

Cell culture

The human tracheal foetal epithelial cell lines CFT-2 and NT-1 were a kind gift of Dr Annick Paul (INSERM U402, Paris, France). CFT-2 cell line was derived from primary tracheal epithelia homozygous for the common cystic fibrosis mutation ΔF508, and NT-1 cell line was derived from normal primary tracheal epithelial cells (20). Both cell lines were grown in Dulbecco's modified Eagle's medium/Ham F-12 (v/v) supplemented with 10% FCS at 37°C in a 5% CO_2, 95% air. The human bronchial epithelial cell line IB3 and C38 cell lines, obtained from ATCC, were grown

in minimum essential medium with Earle's salt and L-glutamine supplemented with 10% FCS at 37°C in a 5% CO_2, 95% air. IB3 express mutant CFTR (ΔF508/W1282X) and C38 is derived from IB3 cells stably transfected with wild-type CFTR (33). The cells were pre-treated with various drugs (MAFP (21), LY311727 (27), aspirin, thapsigargin and MG-132 (9)) before incubation with LPS or PGE_2 as detailed in the figure legends.

RNA extraction and RT-PCR analysis

Cells were grown on a cell culture plate (Techno Plastic Products, Trasadingen, Switzerland), and total RNA was extracted using an RNeasy kit (Qiagen, Courtaboeuf, France). DNase treatment was performed using 3 μg of extracted RNA, 1 μl of DNase I (Amersham Biosciences, Orsay, France), and 0.5 μl of RNasin (Promega, Madison, WI) in a total volume of 20 μl in the manufacturer's buffer. cDNA were obtained by incubating RNA with 1 mM dNTP (Eurobio, Les Ulis, France), 1.5 μl of hexamers as primers, 20 units of RNasin (Promega), and 300 units of Moloney murine leukaemia virus reverse transcriptase RNase H minus (Promega) in a total volume of 40 μl in the manufacturer's buffer for 1 h at 42°C and 10 min at 70°C. PCR was performed using specific primers (Genset, Evry, France) for human $sPLA_2$-IIA (sense, 5′-AGG GAG GGA GGG TAT GAG AG-3′; antisense 5′-GAC AGG AAA GGA AGC CGC AC-3′), human COX-1 (sense, 5′-GCA TTG ACA CAA ACT CCC AGA AC-3′; antisense, 5′-TTC TTG CTG TTC CTG CTC CTG-3′) and human

COX-2 (sense, 5′-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3′; antisense, 5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′). As an internal control, we used primers for the detection of human β-actin (sense, 5′-AAG GAG AAG CTG TGC TAC GTC GC-3′; antisense, 5′-TCT AGA CTA ATT TGA ATT AGG TTG GTG TAG GAT GAC AAA C-3′). Amplifications were performed in a Peltier thermal cycler apparatus (MJ research, Watertown, MA) using Q-BioTaq™ polymerase (Qbiogene, Ilkirch, France). For the detection of $sPLA_2$-IIA, the thermocycling protocol was as follows: denaturation at 95°C for 45 s, annealing at 59°C, for 45 s for 40 cycles, and extension at 72°C, for human COX-1 the annealing temperature was 55°C for 36 cycles, for human COX-2 annealing temperature was 63°C for 38 cycles and for β-actin the annealing was 64°C for 28 cycles.

Nuclear extract and EMSA

Nuclear proteins were extracted from 2×10^6 cells. Briefly, cells were washed once and scraped in PBS containing 1 mM PMSF and 2 mM benzamidin before centrifugation for 5 min at 700 × g. The cells were suspended in 20 mM HEPES, pH 7, 10 mM KCl, 0.15 mM EDTA, 0.15 mM EGTA, 25% glycerol, 1% Nonidet P-40, and antiproteases; incubated for 5 min at 4°C; and then centrifuged for 5 min at 1250 × g at 4°C. The pellet (nuclear fraction) was resuspended in 10 mM HEPES, pH 8, 400 mM NaCl, 0.1 mM EDTA, 25% glycerol, and antiproteases; incubated for 30 min at 4°C under agitation; and centrifuged for 10 min 15000 × g at 4°C. Supernatant corresponding to the nuclear extract was quickly frozen at -80°C. The NF-κB double-

stranded oligonucleotides corresponded to an NF-κB binding site consensus sequence of 5′-GATCATGGGGAATCCCA-3′. The overhanging ends were γ-^{32}P-labeled with T4 polynucleotide kinase. Protein concentrations are determined by using Nanodrop spectrophotometer (Nyxor Biotech, Paris, France). Binding reactions were performed in a total volume of 20 μl for 20 min at room temperature by adding 5 μg of nuclear extract, 10 μl of 2X binding buffer [40 mM HEPES, pH 7, 140 mM KCl, 4 mM DTT, 0.02% Nonidet P-40, 8% Ficoll, 200 μg/ml BSA, 1 μg of poly(dI:dC)], and 1μl of labelled probe. For specificity control experiments, nuclear extracts were incubated 20 minutes with 50-fold excess of relevant unlabelled probe or irrelevant oligonucleotides sequence corresponding to 5′-CTAGATGCTGACACAGAACTCACTTTCCGCT-3′ before the addition of labelled probe. For supershift assay, 2 μg of a polyclonal anti-p65 antibody (Santa Cruz Biotechnology, Santa Cruz) were added, and the mixtures were incubated at 20 minutes room temperature. The specificity control of p65 antibody was performed by using an anti-phosphoCREB antibody (Cell Signalling, Beverly, MA). The loading protein control was performed by using KBF1 oligonucleotide which contains a half-κB site recognized by transcription factor CSL, a component of the Notch signalling pathway (13). The reaction mixtures were separated on a 5% polyacrylamide gel in 0.5% Tris / borate / EDTA buffer at 150 V for 2 h. Gels were dried and exposed for 2 to 12 h.

PGE_2 enzyme immunoassay

Epithelial cells were dispensed at 5 x 10^5 cells/well determined by trypan blue exclusion method. PGE_2 concentrations in culture medium or cell-free BALF were measured using a specific enzyme immunoassay kits purchased from Cayman Chemical Co (Ann Arbor, MI).

COX activity and Western blotting analysis

After treatment with LPS (1 μg/ml) for 6h, the total proteins were extracted and 30 μg of protein were fractionated on SDS-PAGE gel and transferred as described previously (14). The blot was then incubated with primary monoclonal anti-human COX-2 antibody (1:1000 in blocking reagent; Santa Cruz Biotechnologies, Santa Cruz, California) for 1 h at room temperature. The blot was subsequently washed with TBS-T and incubated with polyclonal anti-mouse immunoglobulin G coupled with horseradish peroxidase (HRP) (1:10000 in blocking buffer; Perbio) for 1 h at room temperature. After washings, HRP activity was developed using the ECL+ reagent kit (Amersham Biosciences, Little Chalfont, U.K.), and the corresponding luminescence was revealed by exposure of membranes to Kodak X-Omat AR films (Eastman Kodak, Rochester, NY). The position and molecular weight of COX-2 was validated by reference to Kaleidoscope Prestained Standards (Bio-Rad; *M*r range, 7600–216000). β-actin western blot analysis using specific antibody AC-74 (Sigma) was performed as internal control. For the measurement of COX activity the cells were incubated

with AA (10 μM) for 24 h, in the presence or in the absence of LPS, and then the levels of PGE_2 released were measured as indicated above.

CFTR cell ELISA

Cells were grown to confluent on 24-well plates. Following three washes with PBS, non-specific binding was blocked with 1 X TBS and 5% milk for 1 hour at 4°C. All incubations were followed by three washes with PBS. CFTR was detected by incubation with specific CFTR antibody CF-3 raised against first extra-cellular loops of human CFTR (1:500 in blocking buffer; Abcam Ltd, Cambridge, UK) at 4°C. Cells were then fixed with 3% paraformaldehyde for 15 minutes at 4°C. CFTR antibody was detected by incubation with an anti-mouse Ig-HRP (1:10000 in blocking buffer, Perbio, Rockford, IL) for 1 hour at 4°C. TMB substrate buffer was added at room temperature in the dark. The reaction was stopped with 2 N H_2SO_4 and color change of substrate was read at 450 nm (OD_{450}) in a plate reader. For the low-temperature CFTR re-trafficking studies, the cells were incubated at 28°C for 36 hours. For the thapsigargin studies, the cells were incubated with thapsigargin 1 μM for 90 min and the detection was performed 3 hours later. The results are expressed as subtraction OD_{450} values obtained with specific CFTR by OD_{450} values obtained with isotypic antibody (ΔOD_{450}).

$sPLA_2$ activity assay

The $sPLA_2$ activity was assayed using [^{3}H]-oleate-labelled membranes of *Escherichia coli*, following a modification of the method of Franson *et al.* (11). *E.coli* strain CECT 101 was seeded in medium containing 1% tryptone, 0.5% (w/v) NaCl, and 0.6% (w/v) sodium dihydrogen orthophosphate, pH 0.5, and grown for 6-8 h at 37°C in the presence of 5 μCi/ml [^{3}H]oleic acid (specific activity 10 Ci/mmol) until growth approached the end of the logarithmic phase. After centrifugation at 2500 × g for 10 min, the membranes were washed in buffer (0.7 M Tris-HCl, 10 mM $CaCl_2$, 0.1% BSA, pH 8.0), resuspended in saline and autoclaved for 30-45 min. After washing and centrifugation, the membranes were frozen at -80°C. The phospholipid fraction incorporated at least 95% of the radioactivity. Cell culture media (30 μl) were incubated with 20 μl of auto-claved oleate-labelled membranes in a final volume of 250 μl of 100 mM Tris-HCl, 1mM $CaCl_2$ buffer pH 7.5. Incubation proceeded for 30 min at 37°C with gently shaking to prevent sedimentation of membranes. The reaction was stopped by adding 100 μl of ice-cold solution of 0.25% BSA in saline to a final concentration of 0.07% (w/v). After centrifugation at 2500 × g for 10 min at 4°C, the radioactivity in the supernatants was determined by liquid scintillation counting. For the inhibition experiments, samples were pre-incubated with 20 μM LY311727 for 15 min at 37°C before addition of labelled membranes.

Calculation and statistical analyses

Data are expressed as mean ± sem of separate experiments, and statistical analyses were performed using unpaired Student's *t* test.

Results

Consequences of CFTR dysfunction on PGE_2 release and COX expression in epithelial cells

To determine the impact of CFTR on AA metabolism, we measured PGE_2 release by two pulmonary epithelial CF cell lines, CFT-2 and IB3, *versus* two control cell lines, NT-1 and C38, respectively. Basal PGE_2 release occurred at higher levels in both CF cell lines as compared to control cell lines (Fig 1A, 1B). Upon *P. aeruginosa* LPS stimulation, PGE_2 release was increased and remained higher in CF cells compared to control cells. This enhanced PGE_2 release was not due to a higher responsiveness of CF cells to LPS since similar results were observed when cells were stimulated with PMA instead of LPS. Indeed, PMA (50 ng/ml) induced the production of 519 ± 3.4 pg/ml and 622 ± 8.2 pg/ml ($n = 3$, $p < 0.05$) of PGE_2 from NT-1 and CFT-2 cells, respectively. The subsequent studies were performed with the NT-1 and CFT-2 cells because they produced much more PGE_2 than C38 and IB3 cells.

PCR and western blot analysis of cell extracts revealed a marked expression of COX-2, the inducible form of COX, whose intensity was more pronounced in CFT-2 as compared to NT-1 cells (Fig. 2). In contrast, the expression of the constitutive form COX-1 occurred at similar levels in the two cell lines (Fig. 2A). Similar results were observed in IB3 *versus* C38 cell lines (data not shown). COX activity was measured after the addition of AA (10 μM) for 24 h (Fig. 2D). This led to an increased release of

PGE_2 whose level was higher in CFT-2 as compared to NT-1 cells. Surprisingly, LPS had no significant effect either on COX-2 expression or on COX activity.

Consequences of ΔF508-CFTR membrane re-trafficking on PGE_2 production by epithelial cells

In the case of the ΔF508 mutation, the CFTR dysfunction is due to the retention of this molecule in the endoplasmic reticulum leading to its lack of expression at the plasma membrane. It is known that this mutated protein is functional and can be addressed experimentally at the cell membrane. Therefore, we examined whether experimental procedures (thapsigargin and low temperature) known to permit a normal intracellular traffic of ΔF508-CFTR (6; 8) are able to reduce the enhanced PGE_2 release observed in CFT-2 cells. Having established that CFTR was readdressed at the plasma membrane (Fig. 3), we looked at the synthesis of PGE_2 under those experimental conditions. Interestingly, we observed that both thapsigargin and low temperature reduced the levels of PGE_2 released by CFT-2 cells under LPS stimulation. However, there is no significant difference in PGE_2 release by unstimulated cells either after temperature and thapsigargin treatment (Fig. 4 and 5). These procedures also reduced although at a lesser extent the levels of PGE_2 release by NT-1 cells.

The consequences of PGE_2 release on $sPLA_2$-IIA expression in CF epithelial cells

As PGE_2 is known to modulate $sPLA_2$-IIA expression in different cell types (29), we next examined the consequences of the enhanced PGE_2 release on $sPLA_2$-IIA expression in CFT-2 and NT-1 cells. The results showed that CFT-2 cells secreted more $sPLA_2$ than NT-1 cells either at the basal level or upon stimulation with LPS or PGE_2 (Fig. 6A). This $sPLA_2$ activity was strongly inhibited after treating the incubation medium from LPS-stimulated CFT-2 cells with 20 μM of the $sPLA_2$-IIA inhibitor LY311727 (the levels of $sPLA_2$-IIA activity were 2610 ± 381 cpm/ml and 437 ± 246 cpm/ml of released oleic acid in untreated and LY311727-treated media, respectively ($n = 3$, $p < 0.05$)). Similarly, IB3 cells released more $sPLA_2$-IIA-like activity than C38 cells both in stimulated and non-stimulated conditions (Fig. 6B).

These findings were confirmed with the study of $sPLA_2$-IIA mRNA expression, which showed that, under basal conditions, CFT-2 cells expressed $sPLA_2$-IIA mRNA at a higher level than NT-1 cells (Fig. 7). This level was increased by the addition of LPS or PGE_2 to both cell types (Fig. 7). Pre-incubation of the cells for 24 h with 200 μM aspirin, known to inhibit PGE_2 synthesis, led to a marked inhibition of LPS-induced $sPLA_2$-IIA mRNA expression (Fig. 7) and reduced the levels of $sPLA_2$-IIA activity released in the medium (data not shown). We verified that aspirin was able to abolish PGE_2 release in both cell types under our experimental conditions (data not shown).

It was not possible to investigate the effect of thapsigargin on LPS-induced $sPLA_2$-IIA expression since the restoration of normal intra-cellular traffic of CFTR by thapsigargin is a transitory process which cannot be maintained for more than 8 h (8).

The role of NF-κB in ΔF508-CFTR-induced sPLA$_2$-IIA up-regulation in CFT-2 cells

In a next step, we investigated the possible implication of NF-κB in the enhanced expression of sPLA$_2$-IIA in CFT-2 cells as a consequence of CFTR dysfunction. EMSA analysis showed that, in the absence of any added stimuli, a higher nuclear translocation of NF-κB was observed in CFT-2 as compared to NT-1 cells (Fig. 8). The specificity of the complex was verified by inhibition with an excess of unlabelled relevant oligonucleotides and irrelevant unlabelled oligonucleotides containing a mutated NF-κB site. Moreover, supershift studies showed that an antibody directed against the NF-κB p65 subunit displaced this band, thus confirming that the complex belongs to the NF-κB family (Fig. 8A). This nuclear translocation was increased after the addition of LPS. However, PGE$_2$ had no effect on NF-κB activation (Fig. 8B). In addition, inhibition of PGE$_2$ synthesis by aspirin failed to interfere with LPS-induced NF-κB activation in these cells (Fig. 8C). Pre-incubation of the cells with MG-132, a NF-κB inhibitor, before addition of LPS, markedly reduced the sPLA$_2$-IIA mRNA levels in LPS-stimulated CFT2 and NT-1 cells (Fig. 7).

Discussion

In Cystic Fibrosis, the inflammatory component is responsible for the pulmonary failure and death of the patients (24). Lipid mediators including eicosanoids play a major role in the induction and/or progression of the inflammatory reaction in various models of inflammatory diseases (16). Previous studies reported an increase of eicosanoids concentrations in BALF, saliva and urine from CF patients (18; 17; 23), but the molecular mechanisms involved in this increase have not been investigated.

We showed here that CFT-2, a cell line bearing the ΔF508-CFTR mutation, produced more PGE_2 than the control cell line NT-1. This finding is not simply due to a genetic background difference between these cells since enhanced PGE_2 release was also observed in IB3 cells bearing the ΔF508-CFTR mutation, as compared to C38 cells, which are corrected IB3 cells complemented with wild-type CFTR. In all these cell lines, the production of PGE_2 was enhanced after LPS stimulation and remained higher in CF cells, as compared to corresponding cell controls. Taken together, these findings suggest that CFTR dysfunction leads to enhanced PGE_2 release and that the latter is amplified by LPS. These findings are in agreement with the report of Fink *et al*. (10) which showed that stimulation by isolates of *Burkholderia cepacia*, an important lung pathogen in CF patients, induces a release of PGE_2 at higher levels in IB3 cells than in C38 cells. However, the bacterial component(s) and the mechanisms involved in the induction of PGE_2 release by *B. cepacia* have not been analyzed in this study.

Therefore, it was of a great interest to investigate the mechanism responsible of the observed increase of PGE_2 release by CF cells. We examined the expression levels of COX-1 and COX-2, two enzyme isoforms involved in the conversion of AA to PGE_2. The results revealed that CFT-2 and IB3 cells constitutively expressed COX-2 at higher levels than NT-1 and C38 cells. In contrast, no significant difference was observed in the expression level of COX-1. However, although LPS clearly induced an increase in PGE_2 release it had no effect on the expression of COX-2 in all the cell lines studied. This is in agreement with studies of Rodgers *et al*. (26) which reported that bradykinin fails to induce COX-2 expression, although it was able to stimulate PGE_2 release by A549 cell line. Therefore, the observed increase in PGE_2 release by LPS-stimulated cells might probably due to the activation by LPS of the release of AA via a PLA_2-dependent process. Indeed, we observed that LPS induces a marked release of AA by NT-1 and CFT-2 cells. Using MAFP and LY311727, respectively $cPLA_2$ and $sPLA_2$-IIA inhibitors, we showed that $cPLA_2$ plays a major role in LPS-induced AA release both in NT-1 and CFT-2 cells. However, $sPLA_2$-IIA had no significant role in this release (Medjane *et al*. manuscript in preparation).

The previous study of Berguerand *et al* (2) showed that under stimulation with bradykinin, CFT-2 cells release more AA than NT-1 cells. This study suggested that CFTR inhibits $cPLA_2$ activity and that the retention of CFTR in the endoplasmic reticulum, which occurs in cells bearing ΔF508-CFTR mutation, would lead to the removal of this inhibition and consequently to enhanced AA release. Indeed, procedures that allow CFTR to reach the plasma membrane in CF cells reduced this

release (2). In agreement, the present study shows that re-trafficking of CFTR leads to a decrease of PGE_2 release in CFT-2 cells. On the same line, C38 cells, in which ΔF508-CFTR mutation is corrected by gene transfection, released less PGE_2 than IB3 cells. It is of note that PGE_2 release was also partially reduced after re-trafficking of CFTR in NT-1 cells. This might be due to the fact that even in normal cells, part of CFTR is retained in the endoplasmic reticulum. Indeed, only 30% of the total wild-type CFTR are detected in the plasma membrane of normal epithelial cells (31). In agreement, the present study shows that low-temperature or thapsigargin treatment also induced CFTR re-trafficking in NT-1 cells.

Taken together, these findings suggest that the enhanced PGE_2 release observed in CF cells is due both to increased expression of COX-2 and accumulation of free AA, as a consequence of CFTR dysfunction. However, as CF patients are known to overproduce other eicosanoids (e.g., thromboxanes, leukotrienes), which are not products of epithelial cells, it is likely that other mechanisms besides direct effect of CFTR are involved in the exacerbation of AA metabolism in CF.

We next examined the consequences of CFTR dysfunction on $sPLA_2$-IIA expression in epithelial cells and the possible implication of PGE_2 in this process. Indeed, previous studies have shown that PGE_2 regulates $sPLA_2$-IIA expression, either negatively or positively, depending on the cell type (29). Our study clearly shows that ΔF508-CFTR mutation leads to an increased synthesis and secretion of $sPLA_2$-IIA by CFT-2 cells. In agreement, IB3 cells released more $sPLA_2$-IIA than C38 corrected cells. Our study also clearly demonstrates that PGE_2 is involved, at least in part, in

the observed increase of sPLA$_2$-IIA expression in CFT-2 cells. Indeed, i) these cells release more PGE$_2$ than NT-1 cells, ii) added PGE$_2$ enhances sPLA$_2$-IIA expression in CFT-2 and NT-1 cells, and iii) aspirin, which inhibits PGE$_2$ synthesis, abrogates LPS-induced sPLA$_2$-IIA expression in both cell lines. Thus, based on our results and findings from other groups (2; 22), we suggest that the CFTR dysfunction in epithelial cells leads to an increase of free AA production and to a subsequent enhanced PGE$_2$ release. In turn, the latter induces an up-regulation of sPLA$_2$-IIA expression. This led us to investigate the signaling pathways that may link CFTR dysfunction to sPLA$_2$-IIA up-regulation.

Our findings revealed an enhanced nuclear translocation of NF-κB in CFT-2 as compared to NT-1 cells. This is in agreement with the previous studies of Venkatakrishnan *et al*. (30) reporting an exaggerated NF-κB activation in IB3 CF cells as compared to corresponding corrected C38 cells. We demonstrated that this abnormal activation is involved, at least in part, in the LPS-induced sPLA$_2$-IIA up-regulation in CFT-2 cells. Indeed, NF-κB translocation was increased by LPS, and LPS-induced sPLA$_2$-IIA expression was abolished by pre-treating the cells with MG-132, an inhibitor of NF-κB translocation. However, PGE$_2$-induced sPLA$_2$-IIA expression seems to occur via a NF-κB-independent process in both cell lines. Indeed, PGE$_2$ had no effect on NF-κB activation and inhibition of PGE$_2$ synthesis by aspirin failed to interfere with LPS-induced NF-κB activation in these cells. This led us to suggest that CFTR dysfunction results in an abnormal NF-κB activation leading to an up-regulation of sPLA$_2$-IIA expression. In parallel, CFTR dysfunction leads to an

increased PGE_2 release that induces $sPLA_2$-IIA expression via a NF-κB-independent pathway. Both NF-κB-dependent and -independent $sPLA_2$-IIA expressions are enhanced by LPS. The signaling pathway(s) and the transcription factors involved in PGE_2-induced $sPLA_2$-IIA expression in this cell system remains to be investigated. In particular, it would be of interest to examine the possible implication of cAMP/PKA and CEB/P or CREB in PGE_2-induced $sPLA_2$-IIA expression in CF cells. It is of interest to note that pulmonary tissue from CF mice have a different expression pattern of a number of genes involved in inflammation as compared to WT mice (32), suggesting that the genes other than that encoding for $sPLA_2$-IIA are also modulated by normal CFTR.

In conclusion, our study suggests that CFTR dysfunction in epithelial cells induces an enhanced AA metabolism leading to PGE_2 overproduction. This overproduction might have some pathophysiological consequences in CF lungs since PGE_2 has been shown to induce the expression of a number of cytokines including IL-6 and IL-8 by epithelial cells and T-lymphocytes (4; 26; 28), which may contribute to the exacerbation of inflammation. However, PGE_2 has also immunosuppressive functions since it has been shown to inhibit microbial phagocytosis and killing by alveolar macrophages (1). Thus, PGE_2 overproduction could contribute not only to enhance the inflammatory state of the lung but also to impair its defence against infection. Our studies clearly demonstrated that CF epithelial cells exhibit exaggerated synthesis and secretion of $sPLA_2$-IIA through an autocrine/paracrine process involving PGE_2. The fact that LPS enhances $sPLA_2$-IIA expression in CF cells

suggests that sPLA$_2$-IIA gene is probably up-regulated in CF patients during the episodes of infection by Gram negative bacteria, particularly by *P. aeruginosa*. The importance of this finding is linked to the fact that sPLA$_2$-IIA is known to exhibit pro-inflammatory and bactericidal properties and thus may play a role in lung host defence (29), The pathophysiological relevance of the induction of sPLA$_2$-IIA expression by bronchial epithelial cells in the context of CF remains to be investigated.

Acknowledgments

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Legends

Fig. 1: Production of PGE_2 by epithelial cell lines

(A) NT-1 and CFT-2 cells were stimulated for 24 h by *P. aeruginosa* LPS (1 μg/ml). Supernatants were collected and PGE_2 concentrations were determined by an enzyme immuno assay. The results are expressed as the mean ± sem of 3 distinct experiments performed in duplicate; *: CFT-2 *vs* NT-1 ($p < 0.05$); #: LPS *vs* control ($p < 0.05$). (B) C38 and IB3 cells were stimulated for 24 h by *P. aeruginosa* LPS (10 μg/ml). The results are expressed as the mean ± sem of three distinct experiments performed in duplicate; *: IB3 *vs* C38 ($p < 0.05$); #: LPS *vs* control ($p < 0.05$).

Fig. 2: Expression of COX-1 and COX-2 in NT-1 and CFT-2 cells

NT-1 and CFT-2 cells were stimulated or not by LPS (1μg/ml) for 6h. (A) Total RNA were analysed by RT-PCR with specific probes for COX-1, COX-2 and β-actin. (B) Quantitation of PCR signal intensity normalized against β-actin. (C) Total protein extracts were analysed by western blot with specific COX-2 and β-actin antibodies. (D) NT-1 and CFT-2 cells were incubated for 24h with arachidonic acid (10μM) and stimulated or not by LPS (1 μg/ml). PGE_2 concentrations were determined by enzyme immuno assay. The results are expressed as the means ± sem and are representative of three distinct experiments performed in duplicates. *: CFT-2 *vs* NT-1 ($p < 0.05$); #: treated *vs* control cells ($p < 0.05$).

Fig. 3: Effect of low-temperature and thapsigargin on ΔF508-CFTR and CFTR membrane re-trafficking

CFTR and ΔF508-CFTR were detected by specific CFTR antibody (CF3). NT-1 and CFT-2 cells were incubated at low-temperature (28°C) for 36 hrs (A) or pre-treated with thapsigargin (1 μM) for 90 min and incubated in fresh medium for 3 hrs (B) and cell ELISA was performed as detailed in the Methods. The results expressed were obtained after subtraction of OD_{450} values obtained with isotypic antibody (ΔDO_{450}) ±

sem. The figure is representative of three independent experiments performed in triplicate. *: NT-1 *vs* CFT-2 ($p < 0.05$), #: treated *vs* control cells ($p<0.05$).

Fig. 4: Effect of ΔF508-CFTR membrane re-trafficking induced by low-temperature culture on PGE_2 production by NT-1 and CFT-2 cells

NT-1 and CFT-2 cells were cultured at 28°C for 48 h and then incubated with LPS (1 μg/ml) for 24 h. Supernatants were collected and PGE_2 concentrations were determined by an enzyme immuno assay. The results are expressed as the mean ± sem of 3 distinct experiments performed in duplicate. *: NT-1 vs CFT-2 ($p < 0.05$); §: 28°C *vs* 37°C ($p < 0.05$).

Fig. 5: Effect of ΔF508-CFTR membrane re-trafficking induced by thapsigargin on PGE_2 production by NT-1 and CFT-2 cells

NT-1 and CFT-2 cells were pre-incubated with thapsigargin (1 μM) for 90 minutes and incubated with LPS (1 μg/ml) for 10 h. Supernatants were collected and PGE_2 concentrations were determined by an enzyme immuno assay. The results are the mean ± sem of 3 distinct experiments performed in duplicate. *: CFT-2 *vs* NT-1 ($p < 0.05$); #: LPS *vs* control ($p < 0.05$); §: LPS + thapsigargin *vs* LPS alone ($p < 0.05$).

Fig. 6: Measurement of $sPLA_2$-IIA secretion by NT-1 and CFT-2 cells

Supernatants were collected after 24 h incubation of the cells with LPS (1 μg/ml) or PGE_2 (10 μM). $sPLA_2$-IIA activity was measured as detailed in Methods. The results show the release of sPLA2-IIA activity by: A) CFT-2 *vs* NT-1 cells and B) IB3 *vs* C38 cells. They are expressed as percents of $sPLA_2$-IIA released by non stimulated NT-1 or C38 cells and are representative of three independent experiments performed in duplicate. *: CFT-2 *vs* NT-1 or IB3 *vs* C38 ($p < 0.05$); #: stimulated *vs* non stimulated corresponding cell line ($p < 0.05$).

Fig. 7: $sPLA_2$-IIA mRNA expression by NT-1 and CFT-2 cells

Total RNA were extracted 24 h after cell treatment with LPS (1 μg/ml) or PGE_2 (10 μM) and RT-PCR were performed with specific $sPLA_2$-IIA and β-actin primers. Cells were pre-incubated with aspirin (200 μM) for 24 h or with MG132 (1 μM) for 2 h before addition of LPS. (A) Electrophoretic migration of actin and sPLA2-IIA PCR products in ethydium bromide gel. (B) Densitometric analysis was carried out using the NIH Image version 1.62 software. The results are the means ± sem of three independent experiments. *: CFT-2 *vs* NT-1 ($p < 0.05$); #: LPS + inhibitor *vs* LPS alone ($p < 0.05$).

Fig. 8: NF-κB nuclear translocation in NT-1 and CFT-2 cells

Nuclear extracts were obtained and gel shift analysis was performed as described in Methods. (A) Shift analysis were performed by using (1) 50-fold excess of unlabelled oligonucleotide KBF1, (2) 50-fold excess of labelled irrelevant oligonucleotide, (3) supershift analyses were performed using a specific antibody direct against the p65 sub-unit, (4) relevant labelled oligonucleotide. (B) Control of anti-p65 antibody specificity was performed using an anti-phosphoCREB antibody with nuclear protein extract from CFT-2 cells. (C) NT-1 and CFT-2 cells were incubated with PGE_2 (10 μM) or LPS (1 μg/ml) for 1h and nuclear proteins were extracted. CSL was used as internal control. (D) NT-1 and CFT-2 cells were incubated with aspirin (200 μM) for 24 h or its vehicle then incubated with LPS (1 μg/ml) for 1h before extraction of nuclear proteins. The results are representative of 3 separate experiments.

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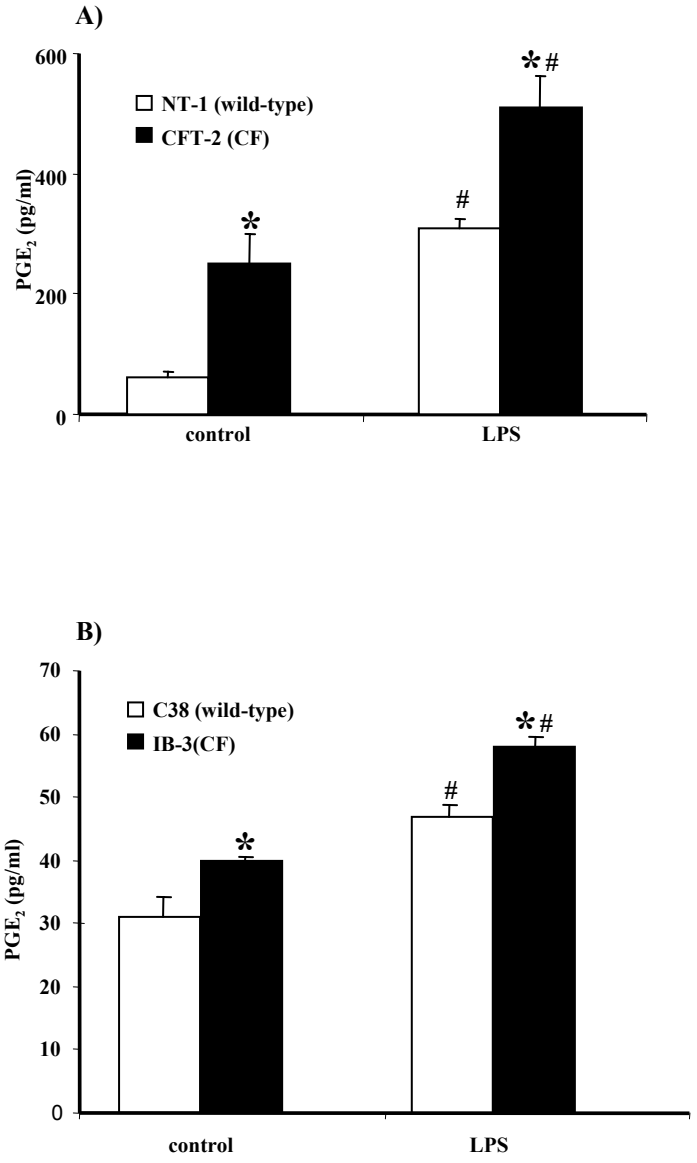
Fig. 1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400
200
0
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NT-1 (wild-type)
CFT-2 (CF)
*
*#
#
control
LPS
B)
70
60
50
40
30
20
10
0
PGE2 (pg/ml)
C38 (wild-type)
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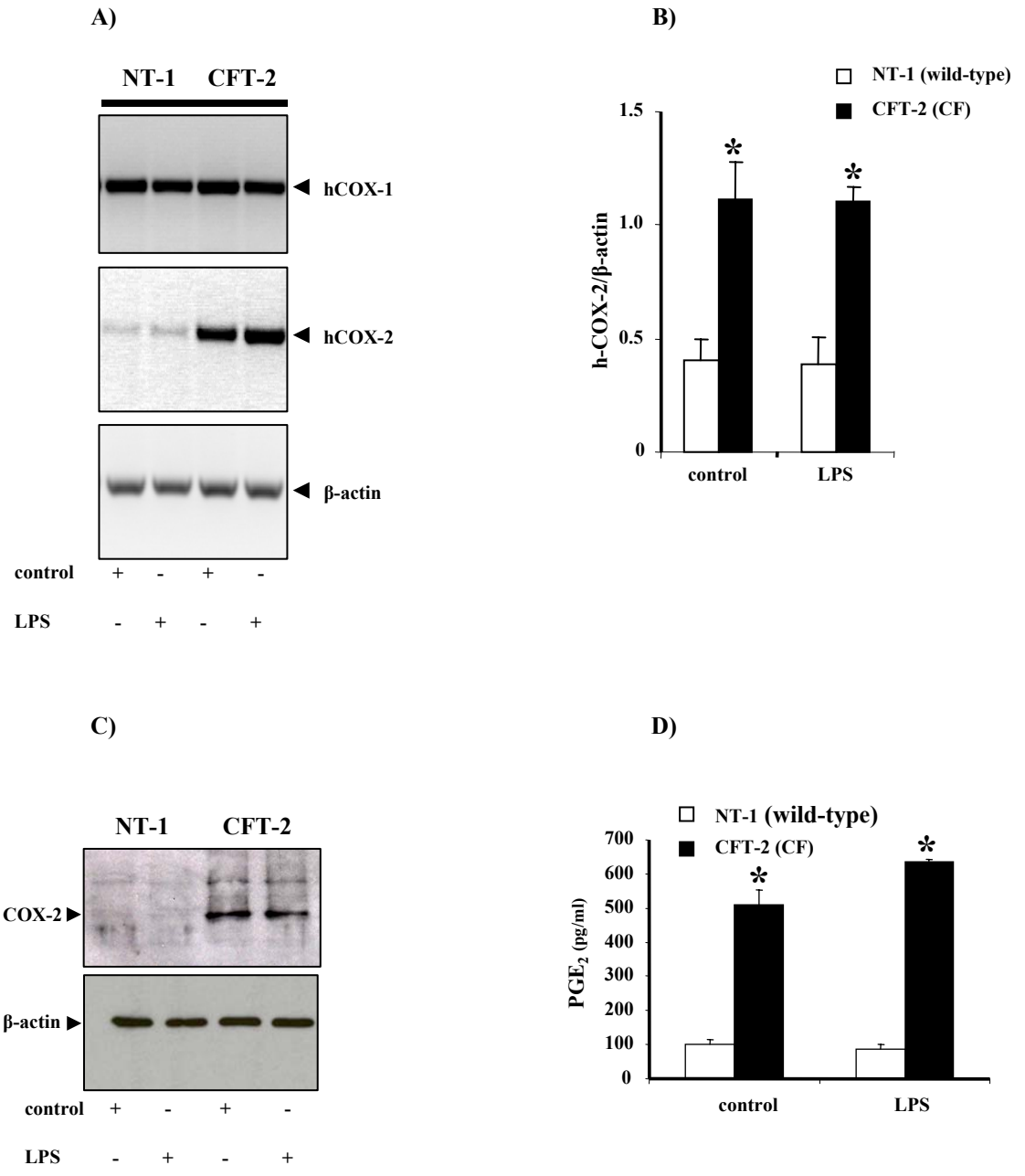
*
*#
#
control
LPS

Fig. 2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CFT-2
hCOX-1
hCOX-2
β-actin
control + - + -
LPS - + - +
B)
NT-1 (wild-type)
CFT-2 (CF)
h-COX-2/β-actin
1.5
1.0
0.5
0
*
*
control
LPS
C)
NT-1
CFT-2
COX-2
β-actin
control + - + -
LPS - + - +
D)
NT-1 (wild-type)
CFT-2 (CF)
PGE2 (pg/ml)
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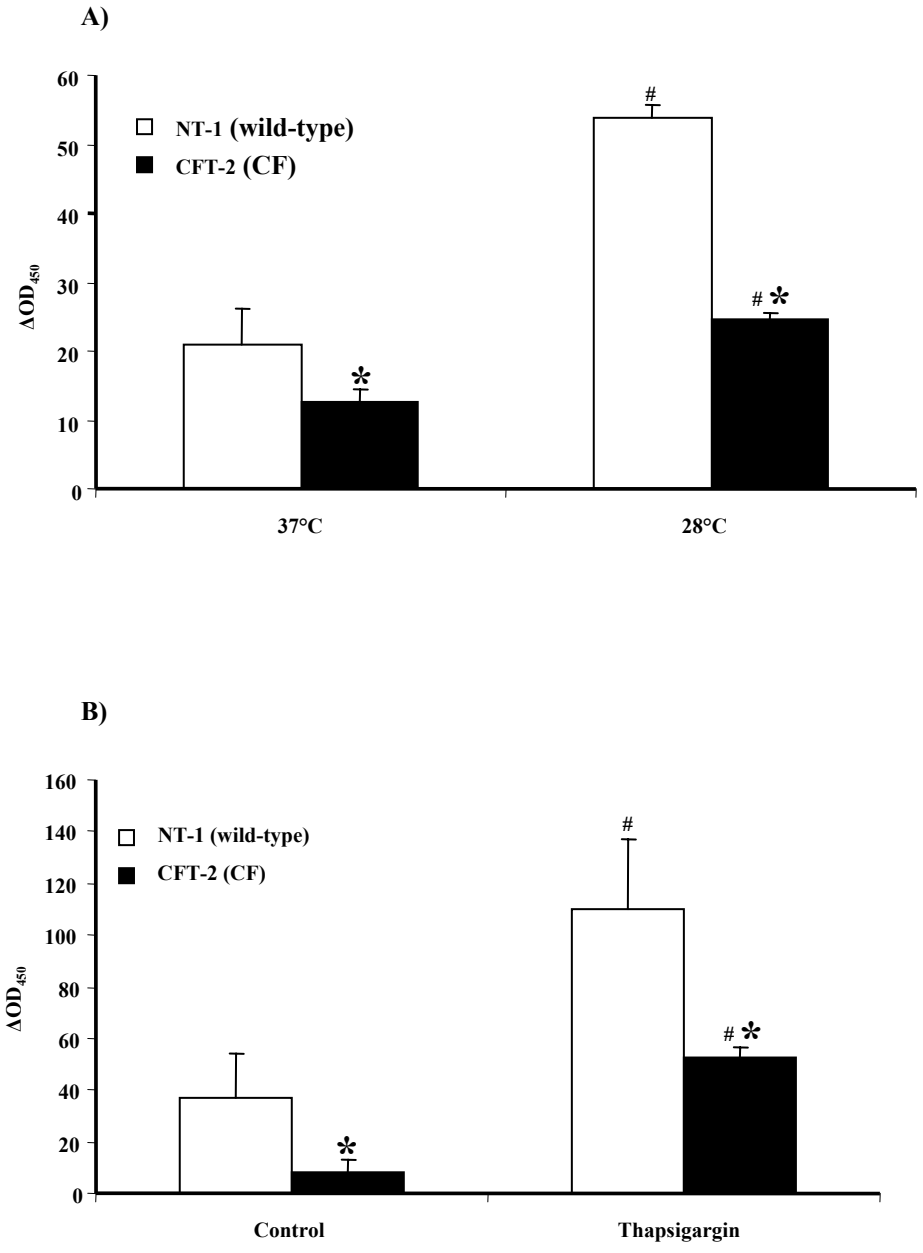
500
400
300
200
100
0
*
*
control
LPS

Fig. 3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60
50
40
30
20
10
0
ΔOD450
NT-1 (wild-type)
CFT-2 (CF)
#
#*
*
37°C
28°C
B)
160
140
120
100
80
60
40
20
0
ΔOD450
NT-1 (wild-type)
CFT-2 (CF)
#
#*
*
Control
Thapsigargin

Fig. 4

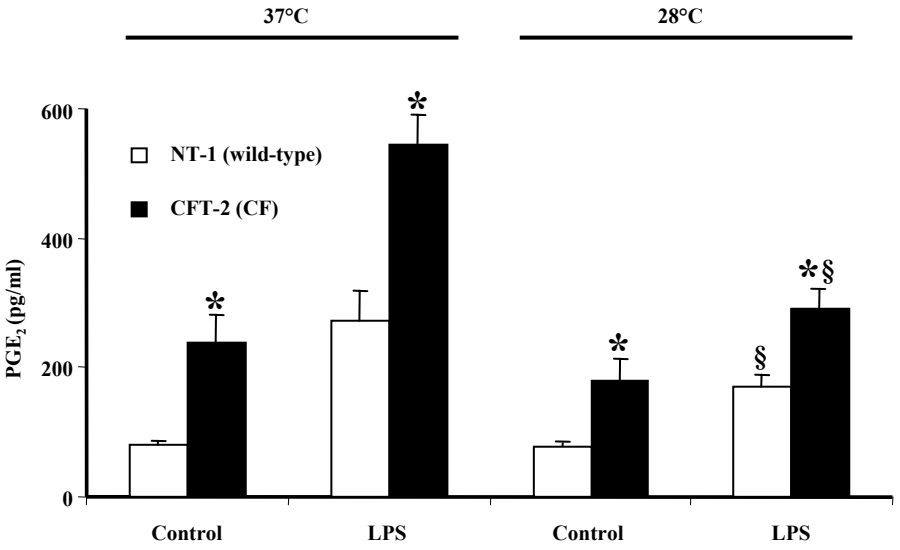
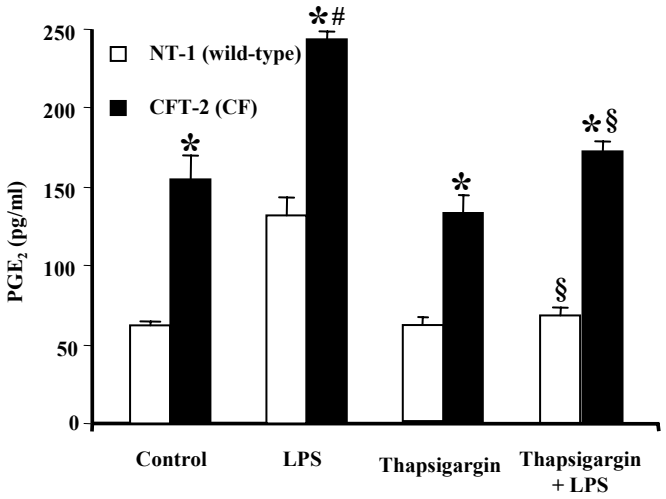

37°C
28°C
600
400
200
0
PGE2 (pg/ml)
NT-1 (wild-type)
CFT-2 (CF)
*
*
*
*§
§
Control
LPS
Control
LPS

Fig. 5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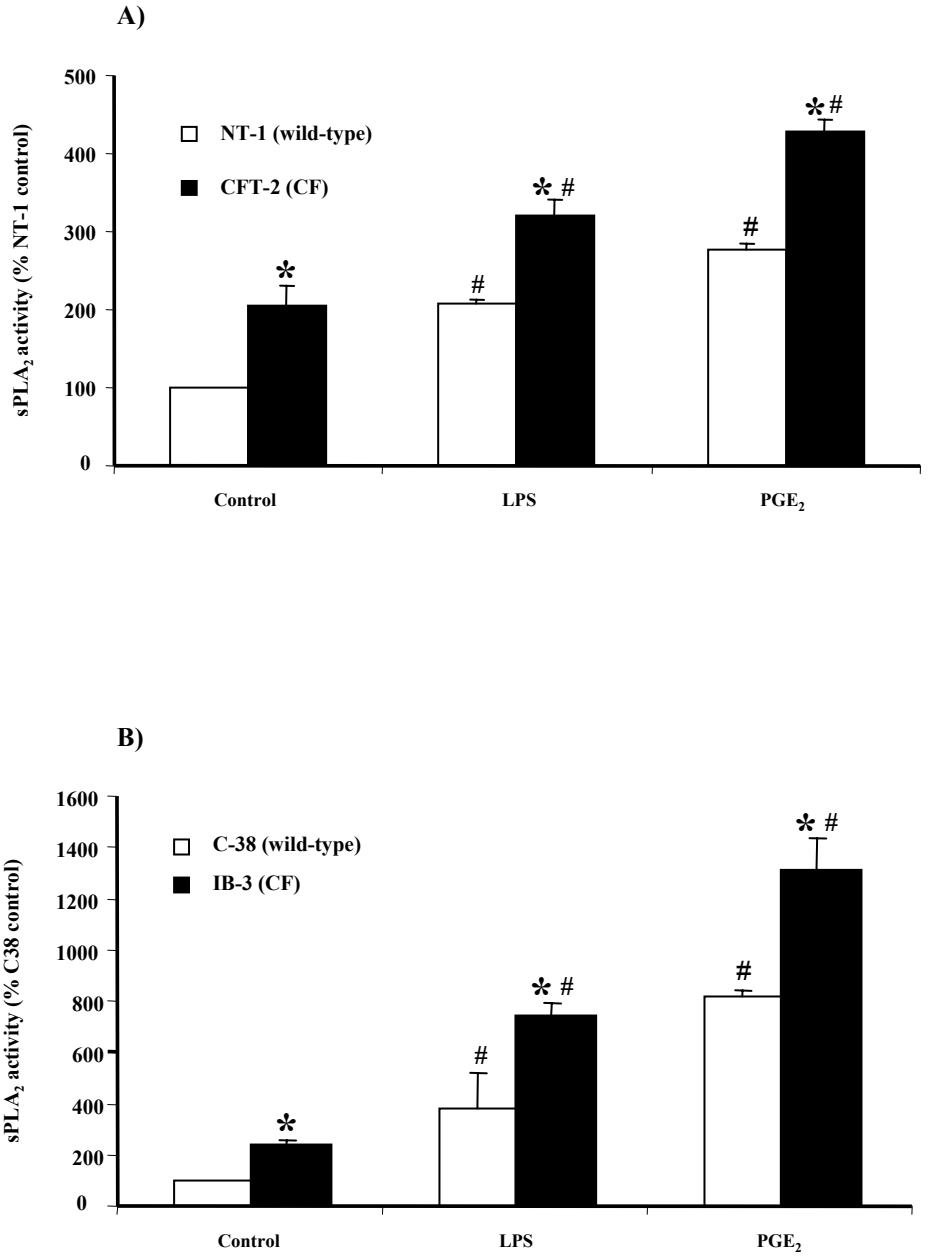
*#
*
*§
§
Control
LPS
Thapsigargin
Thapsigargin + LPS

Fig. 6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400
300
200
100
0
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NT-1 (wild-type)
CFT-2 (CF)
*
#
*#
#
*#
Control
LPS
PGE2
B)
1600
1400
1200
1000
800
600
400
200
0
sPLA2 activity (% C38 control)
C-38 (wild-type)
IB-3 (CF)
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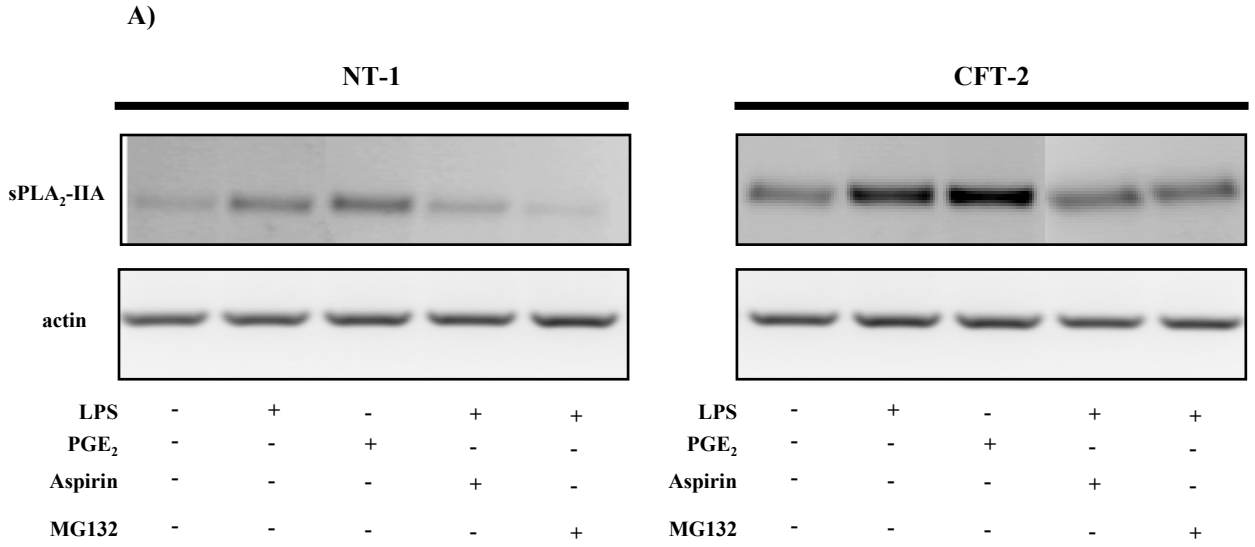
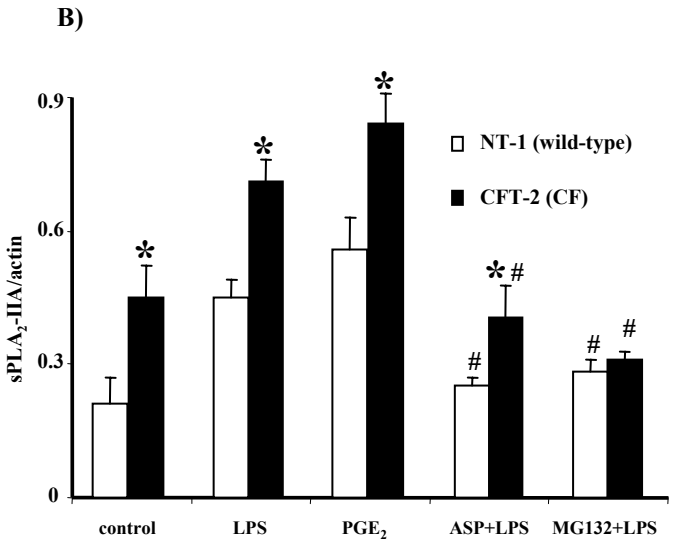
#
*#
#
*#
Control
LPS
PGE2

Fig. 7


A)
NT-1
CFT-2
sPLA2-IIA
actin
LPS - + - + + LPS - + - + +
PGE2 - - + - - PGE2 - - + - -
Aspirin - - - + - Aspirin - - - + -
MG132 - - - - + MG132 - - - - +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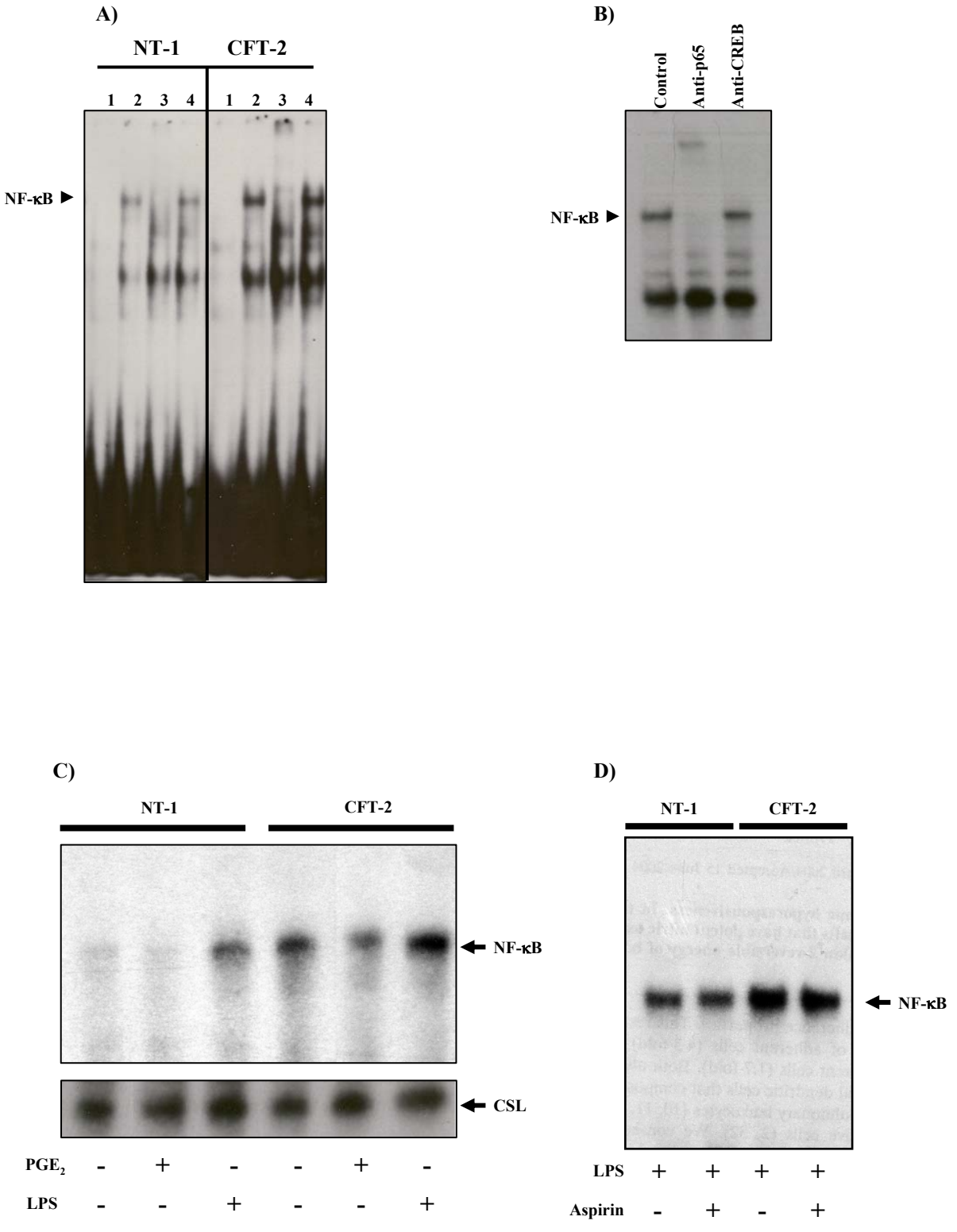
0.6
0.3
0
NT-1 (wild-type)
CFT-2 (CF)
control
LPS
PGE2
ASP+LPS
MG132+LPS

Fig. 8


A)
NT-1
CFT-2
1 2 3 4 1 2 3 4
NF-κB
B)
Control
Anti-p65
Anti-CREB
NF-κB
C)
NT-1
CFT-2
NF-κB
CSL
PGE2 - + - - + -
LPS - - + - - +
D)
NT-1
CFT-2
NF-κB
LPS + + + +
Aspirin - + - +