Airway Epithelial Cell Apoptosis and Inflammation in COPD, Smokers and Non-Smokers

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Hypothesis: We hypothesised that primary bronchial epithelial cells (PBECs) from subjects with COPD respond differently to *Pseudomonas aeruginosa* lipopolysaccharide (PA LPS) after cigarette smoke extract (CSE) exposure than PBECs obtained from smokers without airflow obstruction (SWAO) and non-smokers (NS).

Methods: PBECs from 16 COPD subjects, 10 SWAOand 9 NS were cultured at air-liquid interface. Cultures were incubated with CSE prior to stimulation with PA LPS. IL-6 and IL-8 were measured by ELISA and Toll-like receptor 4 expression by FACS. Activation of NF-κB was determined by western blotting and ELISA, and MAPK and caspase-3 activity by western blotting. Apoptosis was evaluated using Annexin-V staining and the terminal transferase-mediated dUTP nick end-labeling (TUNEL) methods.

Results: Constitutive release of IL-8 and IL-6 was greatest from the COPD cultures. However, CSE pre-treatment followed by PA LPS stimulation reduced IL-8 release from COPD PBECs, but increased it from cells of SWAOand NS. TLR-4 expression, MAPK and NF-κB activation in COPD cultures were reduced after CSE treatment, but not in the SWAOor NS groups, which was associated with increased apoptosis.

Conclusions: CSE attenuates inflammatory responses to LPS in cells from people with COPD but not those from non-smoking individuals and those who smoke without airflow obstruction.
INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a global health problem and a major cause of death.[1] Exacerbations of COPD secondary to infection are a significant cause of morbidity, mortality and burden on health care costs. The relationship between smoking, infection, inflammation and COPD is not well understood.

IL-6 and IL-8 responses of primary bronchial epithelial cells (PBECs) to stimulation with cigarette smoke extract (CSE) have been of particular interest.[2] IL-8 is an important neutrophil chemoattractant and has been implicated in inflammatory lung disease,[3] and is often used as a measure of inflammation in cell culture research.[4] Cigarette smoke induces IL-6 in lung tissue [5] and levels relate to COPD disease severity.[6] Systemically, IL-6 induces skeletal muscle wasting,[7] and probably heightens exacerbation risk,[8] both of which are major co-morbidities in COPD.

NF-κB is an important transcriptional factor in the regulation of inflammatory genes in the airways of people with COPD.[9] Previous investigators have reported a reduced activation of NF-κB in PBECs after exposure to CSE,[10] while others have reported an increased expression in bronchial biopsies obtained from smokers and COPD subjects.[9] Activated NF-κB can have pro-apoptotic effects in certain cells, yet be protective of apoptosis in others.[11] Furthermore, CSE has been reported to induce apoptosis in PBECs [12] and primary nasal epithelial cells by some,[13] but not by other investigators.[14] Furthermore, recent research using PBECs indicated that CSE induces necrosis rather than apoptosis.[15]

CSE provides a tool to explore the impact cigarette smoke has on PBEC cultures and facilitates our understanding of crucial intracellular signalling pathways. There is no consensus for the capacity for CSE to induce a pro-inflammatory response in epithelial cells. There is considerable diversity in the methods adopted to prepare CSE and no agreed gold standard. However, the weight of evidence would support that CSE has predominantly pro-inflammatory [2, 16-17] as opposed to immunosuppressive effects[18-19] on bronchial epithelial cells. Although there is evidence that nasal epithelial cells can act as a satisfactory
substitute for bronchial epithelial cells for certain end-points, we have recently shown that bronchial epithelial cells respond differently to nasal epithelial cells to CSE treatment.[20]

In this study we investigated the responses of PBECs obtained from subjects with COPD, smokers with no airflow obstruction (SWAO) and non-smoking control subjects to CSE treatment. To address this, we cultured cells at air-liquid interface (ALI), which most closely resembles the \textit{in vivo} environment, and stimulated cells with \textit{Pseudomonas aeruginosa} lipopolysaccharide (PA LPS) as a surrogate for infection, with or without pre-treatment with CSE. We aimed to determine the effects of CSE on inflammatory responses to PA LPS, and levels of apoptosis in COPD epithelial cells compared to SWAO and NS.
METHODS

Study Subjects

16 subjects with a diagnosis of COPD according to the British Thoracic Society guidelines (13 of whom were current smokers, 3 ex-smokers), 10 SWAO and 9 NS were included. Subject demographics are detailed in table 1. All study subjects provided written informed consent to have a fibre-optic bronchoscopy during which between 4 and 6 bronchial brushings obtained under direct visual guidance by means of a protected brush were obtained from the third generation bronchus. Brushings were placed in bronchial epithelium growth medium and transported to the laboratory for culture. Exclusion criteria included other significant pulmonary pathology including lung carcinoma. None of the patients had taken oral corticosteroids for at least 8 weeks prior to the study, and no individual had bronchodilator reversibility of greater than 10%. Patients with COPD had no recent exacerbations and current treatments are shown in figure 1. The 10 SWAO required a bronchoscopy for clinical requirements such as persisting haemoptysis, but no abnormalities were evident during the procedure. The 9 NS took part on a voluntary basis. This study was approved by the Office for Research Ethics Committees Northern Ireland (REC: 09/NIR03/42).

Cell Culture

PBECs were initially expanded in bronchial epithelial growth medium (BEGM, Promocell, Germany) in purified bovine collagen coated collagen coated (PureCol; Advanced Biomatrix) T10 flasks and then T75 flasks for expansion with Penicillin Streptomycin antibiotics (Invitrogen, USA) and Primocin (Invivogen, USA). Cells were confirmed to be epithelial in origin by randomly staining cultures by immunocytochemical staining for cytokeratin expression (data not shown). All experiments were performed in ALI cultures by seeding cells onto collagen coated Transwells (Corning Inc, USA) at a seeding density of 1.5 x 10^5 cells per well and grown until cultures became confluent and developed tight junctions. At this stage the apical media was removed and the cell cultures fed basolaterally only on alternate days for 28 days.
CSE

CSE was prepared by a modification of the method of Richter et al.[16] One commercial Marlboro Red cigarette (0.8 mg nicotine; 10 mg Tar; 10 mg carbon monoxide) was combusted with a modified syringe-driven apparatus. The smoke was bubbled through 25 ml of media over 5 minutes by drawing 35-ml volume of smoke every 15 s. The resulting suspension was filtered through a 0.2 µm pore-size filter to remove large particles and bacteria. This solution was regarded as “100% CSE” and was freshly generated for each experiment, and subsequently serially diluted with culture medium to obtain a final 5% working concentration.

Immunofluorescence

Selected cultures were stained with a rabbit anti-E-Cadherin primary antibody to demonstrate the presence of tight junctions. Separate cultures were exposed to both a 1:200 dilution of rabbit anti-MUC5AC primary antibody and a mouse anti-acetylated alpha tubulin antibody to demonstrate the presence of goblet cells and cilia respectively. Images were captured and visualized using LAS AF (Leica) acquisition software. Full details are provided in the supplementary file.

FACS

TLR-4 was determined using FACS by staining permeabilised cells with phycoerthrin (PE)-conjugated anti-TLR-4 monoclonal antibodies (eBioscience, USA). The results obtained with specific antibodies were compared with those using isotype-matched control antibodies. Analysis of 10,000 events was performed using an Epics XL flow cytometer (Beckman Coulter, UK Ltd).

Apoptosis was analysed using Annexin V (Av) and Propidium Iodide (PI) staining (eBioscience, UK). For experiments using CSE, cells were treated with 5% CSE for 24 h and the amount of apoptosis and/or necrosis was measured. Events which were positive for Av, but negative for PI were considered to be early apoptotic, events positive for both Av and PI
late apoptotic, and events positive for PI alone necrotic. Full details are provided in the supplementary file.

**TUNEL Assay**

After treating PBECs with CSE for 24 h, cultures were analysed for apoptosis using the Click-It TUNEL assay (Invitrogen, UK). Cells, after being fixed and permeabilised, were exposed to a reaction cocktail overnight at room temperature. Cells were then treated with a reaction buffer additive mixture for 30 minutes, and cells mounted onto glass coverslips with mounting media and DAPI. Ten randomly selected high power fields at a final magnification of x 100 were counted and the results expressed as the total number of apoptotic cells was divided by the total number of cells per field. Full details are provided in the supplementary file.

**Western blots**

Western blotting was used to determine phosphorylation of p38, ERK and JNK1 MAPK, cleaved caspase 3, IκB-α and phospho-NF-κB after 4 h stimulation with PA LPS, with or without 5% CSE pre-treatment for 24 h. Full details are provided in the supplementary file.

**ELISA**

Cytokine concentrations were measured from aliquots of basolateral and apical culture medium after appropriate stimulation as outlined in the results section using commercial IL-6 and IL-8 ELISA kits from R&D Systems Europe according to manufacturer’s instructions (Abingdon, UK). A chromogenic endotoxin quantification kit was used for the detection of bacterial endotoxins according to manufacturer’s instructions (Thermoscientific, UK).

**TransAM NF-κB Assay**

Nuclear extracts were prepared using a nuclear extraction kit from Active Motif (Belgium, UK) according to manufacturer’s instructions. Details are provided in the supplementary file.
The Active Motif Trans-AM NF-κB ELISA kit (Belgium, UK) was used to determine the levels of p65 in nuclear extracts. In brief, 2 µg of nuclear extract, diluted to 20 µL, was added to the wells coated with oligonucleotides containing the NF-κB consensus binding site. The primary antibodies used to detect NF-κB recognise an epitope on p65 that is accessible only when NF-κB is activated and bound to its target DNA. After the addition of secondary antibodies conjugated with HRP and substrate, absorbance was read at 450 nm (with a reference wavelength at 650 nm). In order to monitor for specificity, competitive binding assays were performed. Wild-type or mutated consensus oligonucleotides were added to the wells containing immobilised oligonucleotides before the addition of nuclear extracts.

**Statistics**

Statistical analysis was performed using SPSS version 17.0 (SPSS inc., Chicago, IL, USA). Data are presented as median values ± interquartile range. Comparisons between groups were performed using the nonparametric Kruskal-Wallis test for multiple comparisons and the Mann-Whitney test for two groups. A p value of less than 0.05 was considered significant.
RESULTS

Cell culture

Cells were successfully cultured in Transwells, developed tight junctions and had a cobblestone appearance when examined using a phase contrast light microscope. Mucus secretion was apparent after approximately 7 days in culture. After 14 days, there was an increase in the number of ciliated cells, and after 28 days in culture a marked increase in their number was apparent (Figure S1).

CSE

The optical density of a 5% CSE concentration at 450 nM did not vary significantly when comparing a series of 5% CSE preparations (OD approx. 0.25). Preparing CSE using a single cigarette in 25 ml of media produced a sufficiently consistent preparation. Previous investigators have reported similar findings.[18] Endotoxin was not detected in our 5% CSE preparation.

Soluble mediator release

Constitutive and stimulated release of IL-8 and IL-6 was used to determine epithelial cell activation. Protein concentration was not significantly different from Transwells selected at random determined using a BCA assay (data not shown), and so soluble mediator release was expressed in pg/ml rather than normalised to protein concentration. Concentrations of PA LPS (Sigma-Aldrich, UK) higher than 50µg/ml were cytotoxic and lower concentrations released less IL-8. The release of both IL-8 and IL-6 after stimulation with 50 µg/ml PA LPS from cultures obtained from control subjects and SWAO was heightened after pre-treatment with CSE for 24 h. In the COPD cultures, 5% CSE pre-treatment mitigated the stimulatory effects of PA LPS (Figure 1).

In separate experiments, cells obtained from a non-smoking subjects were incubated with various concentrations of CSE (1% - 50%) for 24 h. CSE stimulated the cultures to release
IL-8 up to a 5% concentration, subsequently falling at higher concentrations due to cytotoxicity (Figure 2).

**CSE induces apoptosis in Primary Bronchial Epithelial Cell Cultures**

Apoptosis was confirmed using the TUNEL assay. For the representative image (Figure 3), the number of apoptotic cells determined by this technique was 4% for the healthy subject and 19% for the corresponding patient with COPD after cells were exposed to CSE for 24 h.

Using the FACS technique, also after treatment with 5% CSE for 24 h in the COPD cells, 51% of cells remained viable (Av and PI negative), 19% were undergoing early apoptosis, 26% undergoing late apoptosis, and 4% necrotic (Figure 4). In separate experiments, cells from each of the three study groups (again after treatment with CSE for 24 h) were stained with Annexin V to determine any differences in the levels of apoptosis between the groups. CSE induced the highest percentage of apoptosis from the COPD cultures, with the least amount from the NS. Representative dot plots are shown in Figure 5.

**CSE cleaves full length Caspase-3 in Primary Bronchial Epithelial Cell Cultures**

Using an antibody against the cleaved fragment of caspase 3 (17 kDa), we demonstrated by Western blotting that treatment with PA LPS and CSE increased the amount of active caspase-3, whereas PA LPS alone did not. Furthermore, the COPD cultures had the greatest amount of cleaved caspase-3 after PA LPS and CSE stimulation. Cleaved caspase:actin ratio as determined by densitometry and a representative blot are shown in Figure 5.

In separate experiments, COPD cultures were treated with increasing concentrations of PA LPS (0-50 µg/ml) and CSE (5-50%) and caspase-3 was determined using an antibody against full length caspase (35 kDa) and its cleaved fragment (17 kDa). PA LPS alone did not cleave full length caspase, but there was definite cleavage using a 5% CSE, and increasingly so with higher CSE concentrations (Figure S2).

**CSE Reduces TLR-4 Expression in COPD cell cultures**

To examine whether CSE regulates the expression of TLR-4, PBEC cultures were treated with PA LPS [50 µg/ml], with or without pre-treatment with 5% CSE for 24 h. Although
there was no change in the absolute values for mean fluorescence intensity (MFI) for TLR-4
in the cultures from control cultures after CSE treatment, CSE treatment reduced the MFI for
TLR-4 in the COPD cultures. Representative histograms are shown in Figure 7.

CSE Reduces MAPK Activation and NF-κB in COPD cell cultures

Western blotting showed a reduced phosphorylation of p38, JNK and ERK MAPK in the
COPD cultures after treatment with 5% CSE for 24 h. All MAPK were activated, even
without stimulation, with no further significant increase after PA LPS stimulation (Lanes 1-4
in all blots; Figure S3-5). However, levels of phosphorylated p38, ERK and JNK were
reduced with 24 h 5% CSE pre-treatment and PA LPS stimulation in the COPD cultures
(Lanes 5-8 in all blots; Figure S3-5). The reduced activation of MAPK was not evident in the
control cultures. The reduced activation was not as pronounced for p38 compared with JNK
or ERK, and so this was also measured using FACS which confirmed a reduced MFI for
phospho-p38 after 5% CSE treatment (Figure S3).

There was a significant increase phosho-NF-κB protein level from whole cell lysates after
stimulation with 50 µg/ml PA LPS for all 3 groups. This was further heightened in the NS
and SWAO groups after pre-treatment with 5% CSE. In contrast, in the COPD cultures, pre-
treatment with 5% CSE reduced PA LPS induced NF-κB activation. phosho-NF-κB:actin
ratio as determined by densitometry and a representative blot are shown in Figure 8. Levels
of IκB-α decreased as phosho-NF-κB increased. Repeating experiments and determining
levels of p65 NF-κB from nuclear extracts using an ELISA based method demonstrated
similar results (Figure 9).
DISCUSSION

In this series of experiments we have demonstrated that the stimulated release of IL-6 and IL-8 from cultures obtained from controls and SWAO is amplified by 5% CSE pre-treatment, but this was not apparent in the COPD group. Our results also show cellular immunosuppressive effects of 5% CSE in the COPD cultures. These findings suggest that COPD PBECs are more susceptible to the immunosuppressive effects of CSE which may explain, in part, the increased susceptibility to this particular group to respiratory infections.

In our experiments, 5% CSE reduced the expression of TLR-4. Similar findings were recently reported using a human bronchial epithelial cell line, where CSE treatment lead to a down-regulation of TLR-4 expression, which was associated with a corresponding increase in the release of IL-8. Internalization of the receptor was proposed as the mechanism, as there was a parallel increase in the expression of TLR-4 in premeabilised cells.[4] However, the reported effects of CSE on TLR-4 expression are not entirely consistent. Although there was a dose dependent down-regulation of TLR-4 mRNA and protein expression in A549 cells, after stimulation with CSE[21], a publication the following year suggested that CSE increases TLR-4 expression in BEAS-2B cells.[22] CSE can also affect other important receptors, such as the RIG-1 receptors, by down-regulating their surface expression.[23] Therefore, although our data indicate that CSE downregulates TLR-4 expression in COPD PBECs, we cannot be absolutely confident that this is the sole mechanism of its immunosuppressive effects. It is conceivable that CSE also affects other receptors, which may effect cell stimulation independent of TLR-4.

Activation of NF-κB regulates gene expression, promotes cell survival, and serves to protect cells from apoptosis in murine B cells and in a number of cell lines.[24-25] Furthermore, activation of NF-κB through a TLR-dependent activation by hyaluronan in airway epithelial cells has also been shown to be protective of apoptosis.[26] Interestingly, as the absolute amount of TLR-4 was reduced in the COPD epithelial cell cultures after treatment with CSE, which was associated with a reduction in the activation of all MAPKs, is a potential mechanism of apoptosis in these particular cultures to CSE treatment. However, other data
does not support this concept, as treating PBECs obtained from healthy subjects with CSE for 48 h lead to inhibition of NF-κB activation without any evidence of apoptosis, necrosis, or caspase-3 activation.[14]

As well as differences in soluble mediator release, we have demonstrated differences in phosphorylated NF-κB in our study groups. Although treating PBECs obtained from healthy control subjects, and SWAO with PA LPS increased the amount of phosphorylated NF-κB, this was not the case for the COPD ALI cultures. Furthermore, the COPD ALI cultures had a reduced activation of all MAPK after treatment with 5% CSE. The latter observation was demonstrated in parallel with a reduced expression of TLR-4, and a diminished expression of the transcription factor, phosphorylated NF-κB.

The use of CSE for *in vitro* studies has been criticized as an unsatisfactory model for the long term, low concentration smoke which smokers are exposed to.[27] Concentrations of CSE used can range from 100% CSE for 15 minutes[18] to 1% CSE for 24 hours (in those studies which use a single cigarette to prepare the initial “100%” stock CSE)[28]. Other investigators have prepared CSE using multiple cigarettes ranging from 2 cigarettes up to as many as 5.[13, 29] Many of these studies assessed soluble mediator release and a single time-point, but two studies highlight that caution is required as CSE can delay LPS-induced release of IL-8 and GM-CSF in primary epithelial cells, but yet it was not abolished completely at later time points.[18-19] Therefore, if earlier time points were used in isolation, the findings would misleadingly suggest that CSE was solely immunosuppressive. There was unacceptable cytotoxicity at concentrations of CSE greater than 5% (Figure S2), so we opted to use this preparation of CSE to study soluble mediator release from the ALI cultures.

The majority of the subjects with COPD used in this study were current smokers. Our data suggest that the increase in apoptosis, and the increase in caspase-3 activation relative to SWAO and control subjects in response to CSE exposure was not related to cigarette smoking *per se*. It appears that the development of COPD itself further contributes to the increased susceptibility of the bronchial epithelium to the cytotoxic effects of CSE.
Alternatively the epithelial cells from these individuals may be intrinsically more vulnerable to apoptosis.

Caspase activation has not consistently been shown to be important in CSE induced apoptosis,[30] and in fact cell death can occur by mechanisms which are independent of caspase activity.[31] It is feasible that CSE alters the pro- and anti-apoptotic factors which are important players in this increasingly complicated cell death pathway. CSE alters levels of phosphorylated NF-κB in PBEC ALI cultures, and our data suggest that a heightened NF-κB activation may be protective of apoptosis in this context.

Although it could be argued that the process of cell culturing may alter the epithelial phenotype through the multiple cycles of proliferation involved in achieving ALI cultures, recent research suggests this is unlikely to be the case, at least for primary nasal epithelial cells.[32] In addition, there are varied methods used to obtain and establish airway epithelial cell cultures, each with their own merits and limitations. Furthermore, the airways of individuals with COPD may not be sterile. We have discussed these important aspects in the supplementary file.

To conclude, although airway epithelial cells from patients with COPD exhibit a constitutional pro-inflammatory phenotype, these cells have a diminished inflammatory response to CSE compared to control subjects. Furthermore, these cells have an increased susceptibility to undergo apoptosis. It is not clear if this abnormality results from a change to the cells rendered by the disease process, or whether these particular cells are fundamentally more vulnerable to cell death. Regardless of which explanation holds true, this particular abnormality at least suggests that therapies aimed at reducing the amount of apoptosis may be beneficial. The development inhibitors of apoptosis are realistic ambitions. There are already broad spectrum inhibitors of apoptosis in clinical trials for the treatment of liver disease and represent the first in their class of agents.[33] Furthermore, this data emphasises the importance of smoking cessation in COPD subjects in order to minimise loss of epithelial cells and to maintain integrity of the bronchial epithelium. Furthermore, our findings support
the notion that cigarette smoke reduces innate lung defence, and has the capacity to increase susceptibility to respiratory infections.
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Values are means ± SEM.

Abbreviations used: FEV₁: forced expiratory volume in one second; FVC: forced vital capacity, ICS: Inhaled corticosteroid, LABA: Long acting beta agonist

Table 1. Characteristics of the subject population.
Figure 1. (a) IL-8 and (b) IL-6 release from apical supernatants in PBEC ALI cultures from COPD subjects, smoking subjects and healthy control subjects after prolonged after treatment with PA LPS [50 µg/ml], with or without pre-treatment with 5% CSE for 24 h. Well differentiated PBEC ALI cultures were treated with PA LPS [50 µg/ml] for 24 h, with or without pretreatment with 5% CSE (or vehicle) for 24 h (n=7 for each group). Apical supernatants were collected and assessed for IL-8/IL-6 by ELISA. Data are displayed as median ± IQR. *p<0.05.

Figure 2. IL-8 dose response from control PBEC ALI cultures after prolonged treatment with CSE. Well differentiated PBEC ALI cultures from (a) NS and (b) COPD subjects were treated 1%, 2%, 5%, 10%, 20% or 50% CSE (or
vehicle) for 24 h. Supernatants from apical segment were collected and assessed for IL-8 by ELISA (n=5 for each group). Data are displayed as median ± IQR. *p<0.05.

**Figure 3.** Effect of 5% CSE on apoptosis in PBEC cultures from healthy subjects and smokers from ALI Cultures determined using the TUNEL assay. Representative images of bronchial epithelial cells from NS subjects or SWAO were grown on coverslips and treated with (a) PBS or (b) DNase I solution for negative and positive control respectively. Cultures obtained from (c) a NS subject and from (d) a COPD subject were treated with 5% CSE for 24 hrs and the number of apoptotic cells counted using the Click-iT reaction according to manufacturer’s instructions.
Figure 4. Annexin-V/Propidium Iodide analysis in of 5% CSE treatment in PBEC ALI COPD Cultures. In each plot, horizontal axis represents intensity of staining for Annexin V and vertical axis intensity of staining for PI (determined in the FL1 and FL3 plot respectively, both logarithmic scale). (a) Untreated cells stained with isotope control, (b) cells treated with staurosporin 2 \( \mu \)M for 8 h (c) cells treated with 0.1% Triton for 8 h, and (d) cells treated with 5% CSE for 24 h. For cells treated with 5% CSE, 51% remained viable, 19% were undergoing early apoptosis, 26% undergoing late apoptosis, and 4% were necrotic.

<table>
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Figure 5. Annexin-V analysis in PBEC ALI COPD Cultures demonstrating the effects of 24 h incubation with 5% CSE on cell viability. Representative dot-plots demonstrating the percentage of cells staining positive for Annexin V. Dot plots correspond to (a) NS, (b) SWAO and (c) a COPD subject. The percentage of cells staining positive in each quadrant are indicated in the table.
Figure 6. Effect of PA LPS and 5% CSE on caspase-3 activation in PBEC ALI cultures determined by western blotting. (a) Lanes 1-3 represent treatment of ALI cultures from a NS subject with media, 50 µg/ml PA LPS (4 h), and 5% CSE (24 h) and subsequently 50 µg/ml PA LPS (4 h) respectively. Lanes 4-6 represent equivalent stimulation in ALI cultures obtained from a SWAO subject, and lanes 7-9 ALI cultures from a subject with COPD. The upper blot represents the large fragment of caspase-3 resulting from cleavage (17 kDa) and the lower beta-actin. (b) Cleaved caspase:actin ratio (n=5 for each group). Data are displayed as median ± IQR. *p<0.05.
Figure 7. **Representative histogram of amount of intracellular TLR-4 in PBEC ALI cultures after stimulation with PA LPS with or without 5% CSE pre-treatment.** Well differentiated PBEC ALI cultures from (a, b) a subject with COPD and cultures from a (c, d) a SWAO were incubated with 5% CSE for 24 h and then stimulated with PA LPS [50 µg/ml] for 4 h. Separate PBEC ALI cultures from a COPD subject were treated either with or without a 5% CSE alone for 24 h (e, f). Cells were then fixed, permeabilised and stained with a PE conjugated antibody against TLR-4 or equivalent isotype-matched control. Mean fluorescence Intensity is shown (n=4 for each group). Data are displayed as median ± IQR. *p<0.05.
Figure 8. **Effect of LPS ± 5% CSE on NF-κB activation in PBEC ALI cultures determined by western blotting.** Western blot of p65 NF-κB and IκB-α protein expression (with beta-actin loading control) in PBEC ALI cultures of control NS, SWAO and COPD patients after treatment with 50 µg/ml PA LPS for 4 h or 5% CSE treatment for 24 h followed by treatment with 50 µg/ml PA LPS for 4 h. Lanes 1-3 represent treatment of NS PBEC’s with media alone, 50 µg/ml PA LPS (4 h) and 50 µg/ml PA LPS (4 h) with 5% CSE pre-treatment (24 h). Lanes 4-6 represent equivalent treatments in an ALI culture from a SWAO and 7-9 in those from a COPD subject. (b) phosho-NF-κB:actin ratio (n=5 for each group). Data are displayed as median ± IQR. * p<0.05.
Figure 9. Effect of LPS ± 5% CSE on NF-κB activation in PBEC ALI cultures determined using the TransAM NF-κB kit. Nuclear extracts from PBEC ALI cultures of control NS, SWAO and COPD patients after treatment with 50 μg/ml PA LPS for 4 h or 5% CSE treatment for 24 h followed by treatment with 50 μg/ml PA LPS for 4 h were assayed for NF-κB p65 activation using the TransAM NF-κB p65 Kit. Results shown are from wells assayed in duplicate.

REFERENCES


