



# Cigarette smoke extract reduces VEGF in primary human airway epithelial cells

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**ABSTRACT:** Reduced vascular endothelial growth factor (VEGF) has been reported in bronchoalveolar lavage fluid and lungs of severe emphysema patients. Airway epithelial cells (AEC) are exposed to various environmental insults like cigarette smoke and bacterial infections, but their direct effect on VEGF production in well-differentiated primary human AEC remains unclear.

The current authors determined the effect of cigarette smoke extract (CSE) alone and in combination with *Mycoplasma pneumoniae* (*Mp*) on VEGF production in well-differentiated primary normal human bronchial epithelial (NHBE) and small airway epithelial cells (SAEC) in air-liquid interface cultures. Secretion and expression of VEGF were determined by ELISA and real-time RT-PCR, respectively. Cell growth, apoptosis, extracellular signal-regulated kinase (ERK)1/2 and protein kinase (PK)C signalling pathways were evaluated to further dissect VEGF regulation under CSE treatment.

CSE significantly reduced VEGF secretion in NHBE and SAEC. In SAEC, *Mp* alone significantly increased the VEGF, while the presence of CSE attenuated *Mp*-induced VEGF production. While ERK inhibitor reduced VEGF secretion only in NHBE, a PKC inhibitor significantly decreased VEGF secretion in both NHBE and SAEC.

In conclusion, direct cigarette smoke extract exposure significantly reduced vascular endothelial growth factor production in well-differentiated primary human airway epithelial cells, in part through modifying extracellular signal-regulated kinase 1/2 and protein kinase C signalling pathways.

**KEYWORDS:** Airway epithelial cells, cigarette smoke extract, extracellular signal-regulated kinase 1/2, protein kinase C, vascular endothelial growth factor

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death worldwide [1, 2]. Emphysema is one of the important pathological features in severe COPD, which is associated with irreversible loss of lung function largely due to destruction of alveolar structure including microvasculature [3]. Cigarette smoke (CS) is one of the major contributing factors in the development of emphysema.

Vascular endothelial growth factor (VEGF)-A (also known as VEGF) is a critical angiogenic factor in maintaining normal tissue vasculature and endothelial cell growth and survival [4]. CS exposure has been associated with reduced VEGF and VEGF receptor (VEGFR)-2 expression and VEGFR-2 activation in severe emphysema patient lungs and rodent lungs [5, 6]. Moreover, reduced VEGF has been clearly implicated in the destruction of alveolar wall components including microvasculature [6–9]. As the first line of lung host defence mechanism, airway epithelial cells

(AEC) are exposed to the highest level of CS and other environmental insults such as pathogens. However, the direct impact of CS on well-differentiated human primary AEC with relation to VEGF production has not been well studied.

Most previous studies aimed at determining the effects of CS on cell proliferation, apoptosis, inflammation and senescence have been performed using either immortal lung epithelial cell lines or primary large AEC cultured under submerged conditions [10–14]. AEC grown under submerged culture conditions are poorly differentiated and do not mimic major *in vivo* features such as mucociliary differentiation. BOUSSAT *et al.* [15] found that well-differentiated normal human bronchial epithelial (NHBE) cells produced significantly higher amounts of VEGF compared with transformed epithelial cell lines. However, it remains unclear whether CS exposure can directly decrease VEGF levels in well-differentiated primary human AEC. As small airways and lung parenchyma are

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## STATEMENT OF INTEREST

None declared.



the major affected sites in COPD, it is critical to evaluate the direct effect of CS on VEGF in well-differentiated small airway epithelial cells (SAEC). Findings from the SAEC will further reveal how CS affects distal lungs in COPD and illustrate whether SAEC and NHBE respond differently to CS exposure with regard to VEGF production.

Increased prevalence of bacterial colonisation has been reported in the airways of smokers with and without COPD [16, 17]. *Mycoplasma pneumoniae* (*Mp*), an atypical respiratory bacterium, is associated with upper as well as lower respiratory tract infections in smokers suffering from COPD [18–20]. The impact of bacterial infection on tissue remodelling (e.g. angiogenesis) in COPD patients is poorly understood. Therefore, the current authors also examined whether *Mp* can modify VEGF production in CS-exposed AEC.

Signalling pathways such as mitogen-activated protein kinase (MAPK) and protein kinase (PK)C have been shown to regulate VEGF expression in various cell types, including smooth muscle cells, human umbilical vein endothelial cells, retinal pigment epithelial cells, macrophages and renal glomerular epithelial cells [21–27]. CS and CS extract (CSE) have been shown to activate MAPK and PKC signalling pathways in lung epithelial cell lines (e.g. A549) and primary cells (e.g. NHBE) cultured under submerged conditions [28–32]. Although reduced VEGF expression is observed in severe emphysematous lungs, the molecular mechanisms involved in CS-mediated VEGF reduction remain unclear. In the present study, the role of MAPK and PKC signalling pathways in VEGF production was determined in well-differentiated human primary AEC that were treated with CSE.

The current results demonstrated that CSE significantly reduced VEGF expression in well-differentiated NHBE and SAEC. The inhibitory effect of CSE on VEGF was more pronounced in SAEC in the presence of *Mp* infection.

## METHODS

### Primary human AEC air-liquid interface culture

Primary human AEC (Clonetics NHBE and SAEC) were obtained from different healthy donors (Cambrex Bio Science, Walkersville, MD, USA). They were cultured in serum-free medium (Dulbecco's modified Eagle medium and bronchial epithelial cell basal medium at 1:1) supplemented with various growth factors, including insulin ( $0.4 \mu\text{g}\cdot\text{mL}^{-1}$ ), transferrin ( $5 \mu\text{g}\cdot\text{mL}^{-1}$ ), hydrocortisone ( $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ ), epinephrine ( $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ ), epidermal growth factor ( $10 \text{ ng}\cdot\text{mL}^{-1}$ ), bovine pituitary extract ( $52 \mu\text{g}\cdot\text{mL}^{-1}$ ), retinoic acid ( $30 \text{ ng}\cdot\text{mL}^{-1}$ ), bovine serum albumin ( $50 \mu\text{g}\cdot\text{mL}^{-1}$ ), gentamycin ( $50 \mu\text{g}\cdot\text{mL}^{-1}$ ) and amphotericin B ( $0.05 \mu\text{g}\cdot\text{mL}^{-1}$ ). Cells between passages 2 and 4 were plated onto 12-mm collagen-coated polyester transwell inserts (pore size  $0.4 \mu\text{m}$ ; Corning Inc., Corning, NY, USA) at  $4 \times 10^4$  cells $\cdot\text{cm}^{-2}$ . Within a week of submerged culture conditions these epithelial cells attained 100% confluence, and were then transferred into an air-liquid interface (ALI) condition by reducing apical surface medium volume to  $50 \mu\text{L}$ . Epithelial cells were maintained in an ALI culture condition for a week to induce mucociliary differentiation [33, 34]. On day 7 of ALI culture, CSE treatment was started and refreshed daily for up to 14 days. CSE was applied to the apical side of epithelial cells to mimic the *in vivo* pattern of CS exposure. At days 1, 7 and 14

after CSE treatments, basolateral supernatants were collected for VEGF ELISA, while cells were collected to evaluate VEGF mRNA expression, cell morphology, DNA content and activation of signalling pathways.

### CSE preparation

CSE was prepared fresh daily, as previously described but with slight modification [35–38]. Unfiltered research grade cigarettes (2R1) were purchased from Kentucky Tobacco Research and Development Center at the University of Kentucky (Louisville, KY, USA). Briefly, CSE was prepared by combusting one cigarette and bubbling mainstream cigarette smoke into 25 mL of sterile serum-free cell culture medium with the help of a peristaltic pump. This preparation was sterilised through a  $0.22\text{-}\mu\text{m}$  syringe filter and considered as 100% CSE. Complete cell culture medium was used to dilute 100% CSE to the required CSE concentrations, which were used within 30 min of CSE preparation.

### Mp infection in AEC

*Mp* strain 15531 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and was grown at  $37^\circ\text{C}$  in SP4 glucose broth. Adherent *Mp* was collected, then frozen at  $-80^\circ\text{C}$  for infection experiments [34]. Frozen *Mp* stock (passage 4) was resurrected in SP4 broth at  $37^\circ\text{C}$  for 2 h, and then used for infecting the epithelial cells at a concentration of 1 colony-forming unit (cfu) $\cdot\text{cell}^{-1}$  (a physiological dose) or  $4 \times 10^4$  cfu $\cdot\text{transwell}^{-1}$  at the apical side in the presence or absence of 20% CSE. Epithelial cells were infected with *Mp* once only at the beginning of CSE treatment (day 7 of ALI culture). On days 1, 7 and 14 post *Mp* infection, cells and supernatants were harvested for VEGF detection, and also cultured to verify *Mp* infection.

### ELISA for VEGF

VEGF secretion in basolateral supernatants from AEC was measured using a VEGF ELISA kit (ELISA Tech., Aurora, CO, USA) according to the manufacturer's instructions. A standard curve was generated using recombinant VEGF ( $0.05\text{--}2 \text{ ng}\cdot\text{mL}^{-1}$ ) to calculate VEGF concentrations in AEC supernatants.

### Quantitative real-time RT-PCR

Expression of human airway epithelial VEGF mRNA was determined by quantitative real-time RT-PCR. Total RNA was extracted using the Trizol reagent (Gibco BRL, Rockville, MD, USA). Reverse transcription was performed using  $1 \mu\text{g}$  of total RNA as described previously [34]. Human VEGF (Genbank accession number M27281.1) primers (forward 5'-CTTG-CCTTGCTGCTCTACC-3' and reverse 5'-CACACAGGATG-GCTTGAAG-3') and probe (5'-AGTTCATGGATGTCTATC-AGCGCAGCT-3') were designed using the Primer Express software (Applied Biosystems Inc., Foster City, CA, USA). Primers and probes for human glyceraldehyde-3-phosphate dehydrogenase (4326317E) and B-cell lymphoma (Bcl)-2 (assay ID HS00153350\_m1) were purchased as premixed solution from Applied Biosystems. Real-time PCR was performed using  $25 \mu\text{L}$  reaction mixture containing  $30 \text{ ng}$  cDNA, fluorogenic probe, primers and other components in the TaqMan RT-PCR kit. The comparative threshold cycle method was used to determine the relative gene expression levels in AEC.

### Total DNA quantification

To monitor cell growth of well-differentiated epithelial cells, total DNA content, which directly reflects the total number of cells, was determined using the Quant-iT dsDNA broad range (BR) kit from Molecular Probes (Eugene, OR, USA), according to the manufacturer's instructions. In brief, whole cell lysates were prepared from each transwell using 200  $\mu$ L of cell lysis solution (0.2% TritonX-100). Cell lysates (20  $\mu$ L) were added directly to 200  $\mu$ L of working Quant-iT dsDNA BR reagent. DNA content was determined by measuring fluorescence intensity using 510-nm/527-nm (excitation/emission) filters in a fluorescence plate reader. A standard curve was prepared using the  $\lambda$ DNA standards (50–1,000 ng·well<sup>-1</sup>) provided with the kit and used to determine the DNA content of cell lysates.

### AEC morphology

For cell morphology analysis, cells were fixed in ice-cold acetone and embedded in JB-4 polymer [33]. Cell sections of 2  $\mu$ m thickness were cut with a microtome and stained with

haematoxylin and eosin to examine the cell morphology, including cell integrity, nuclear shape and mucociliary differentiation, under a light microscope.

### Western blot analysis

To prepare whole cell lysates, NHBE or SAEC were lysed in Western blot lysis buffer containing 50 mM Tris-HCl (pH7.4), 0.15 M NaCl, 0.1% SDS, 1.0% Nonidet P-40 and 10 mM EDTA with 1% protease inhibitors and 1% phosphatase inhibitor cocktails. Equal amounts of protein samples were resolved on 10% or 12% SDS-PAGE gels, and transferred onto nitrocellulose membranes as described previously [33]. Antibodies against extracellular signal-regulated kinase (ERK)1/2, phospho-ERK1/2, c-Jun N-terminal kinase (JNK), phospho-JNK, p38MAPK and phospho-p38MAPK (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, and Cell Signaling Technology Inc., Danvers, MA, USA) were applied to the nitrocellulose membranes to detect phosphorylated and total ERK1/2, JNK and p38MAPK. Densitometry was performed using the NIH Image-J software, and the ratio of phospho-MAPK/total MAPK was used to determine MAPK activation levels.

### Statistical analysis

Statistical analyses were performed using Graphpad PRISM 4.0 software and data expressed as mean  $\pm$  SEM. Statistical significance between different groups was analysed using paired t-tests and ANOVA. A p-value of <0.05 was considered statistically significant.

## RESULTS

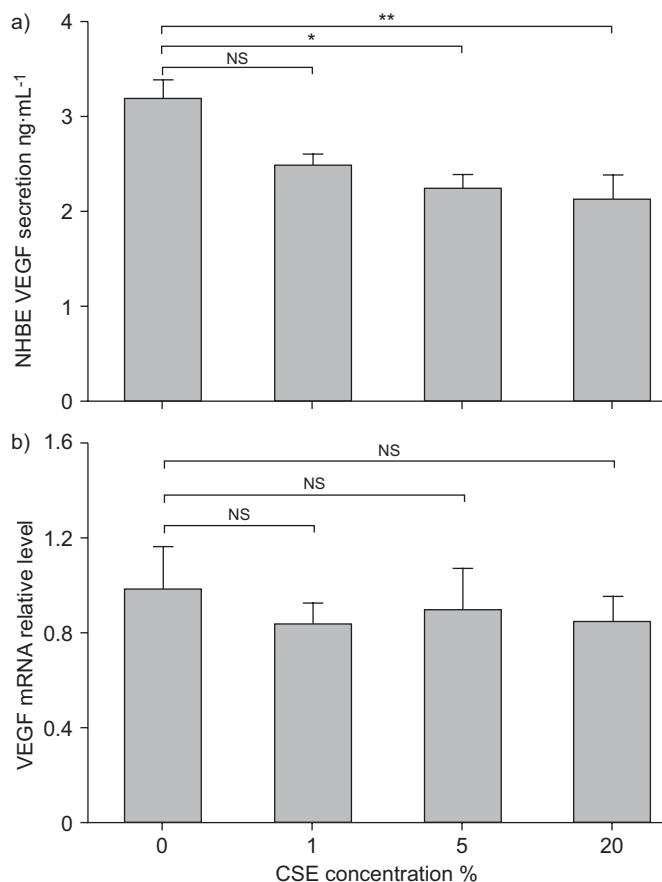
### Effect of CSE on VEGF production in human primary AEC

Initial experiments using different doses of CSE (1–20%) showed a significant decrease of VEGF protein secretion in NHBE after 7 days of CSE treatment compared with the untreated group (fig. 1a). CSE tended to decrease VEGF mRNA expression, but the differences between CSE-treated and -untreated cells were not statistically significant ( $p > 0.05$ ; fig. 1b). The current authors decided to use 20% CSE for subsequent experiments, as 20% CSE produced a maximal decrease in VEGF secretion without apparent cell damage (e.g. cell death or cell detachment from the transwell membrane).

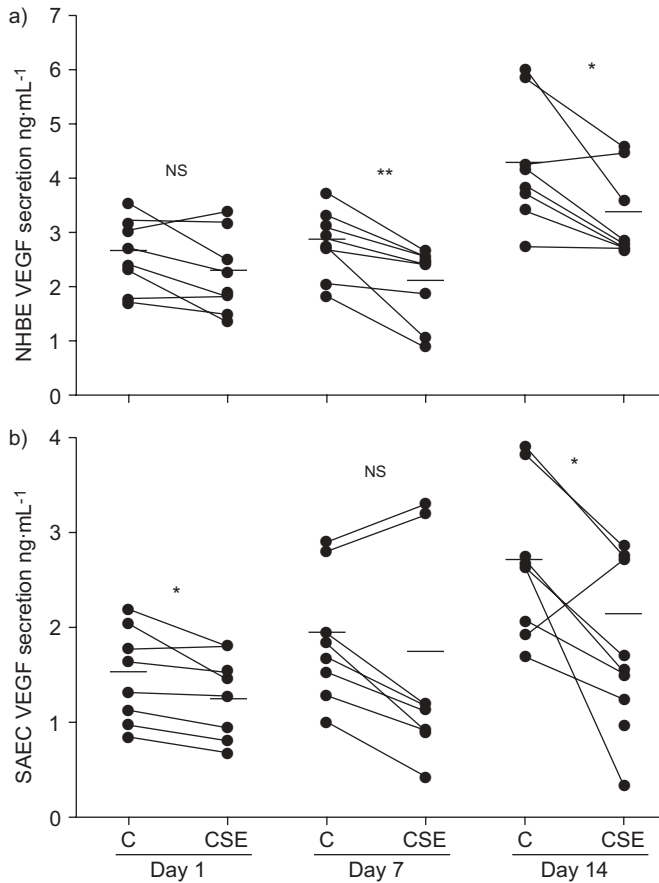
AEC were exposed to media alone or 20% CSE for 1, 7 and 14 days. Repeated (day 7 and 14) but not a single (day 1) CSE exposure significantly reduced VEGF in NHBE cells (fig. 2a). Unlike NHBE, both a single (day 1) and repeated (day 14) CSE exposure significantly reduced VEGF secretion in SAEC compared with untreated cells (fig. 2b).

### Effect of CSE on VEGF production in the presence of *Mp* infection in human primary AEC

*Mp* alone significantly increased VEGF secretion in SAEC but not in NHBE (table 1; fig. 3a and b). The presence of CSE significantly attenuated *Mp*-induced VEGF secretion in SAEC (fig. 3b). The inhibitory effect of CSE in combination with *Mp* on VEGF production occurred at earlier time-points (days 1 and 7) and was more pronounced in SAEC compared with NHBE (table 1). Furthermore, in response to simultaneous CSE and *Mp* exposure, the degree of VEGF reduction in SAEC was greater than that in NHBE (table 1).



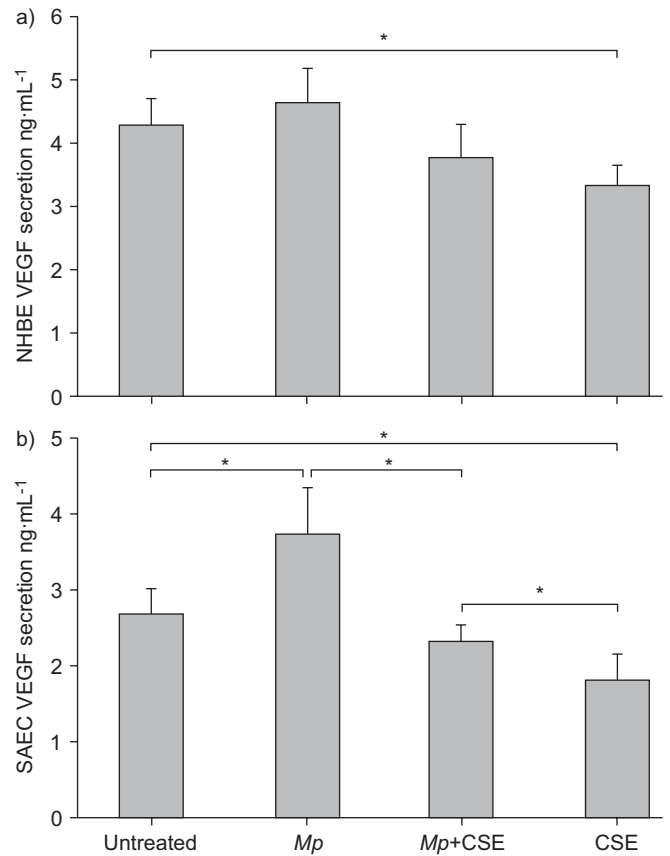
**FIGURE 1.** Dose response of cigarette smoke extract (CSE; 1–20%) on vascular endothelial growth factor (VEGF) a) secretion and b) mRNA expression in well-differentiated normal human bronchial epithelial (NHBE) cells. CSE was applied to the apical side of NHBE cells for 7 days. a) VEGF levels in basolateral supernatants were determined using an ELISA. CSE at 5 and 20% significantly reduced VEGF secretion compared with untreated cells. b) VEGF mRNA relative expression levels were determined using quantitative real-time RT-PCR. Data are expressed as mean  $\pm$  SEM (n=6). NS: nonsignificant (one-way ANOVA). \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ .



**FIGURE 2.** Cigarette smoke extract (CSE) exposure reduced vascular endothelial growth factor (VEGF) secretion in well-differentiated a) normal human bronchial epithelial (NHBE) cells and b) small airway epithelial cells (SAEC), compared with the respective untreated controls (C), after 1, 7 and 14 days of CSE treatments. Data are expressed as individual values (●) and means (horizontal bars); n=8. ns: nonsignificant (paired t-test). \*: p<0.05; \*\*: p<0.01.

**Effect of CSE on cell growth and apoptosis in human primary AEC**

To reveal the mechanisms underlying CSE-mediated VEGF reduction, the effect of 20% CSE on NHBE and SAEC growth or apoptosis was determined by examining cell morphology,



**FIGURE 3.** Effects of cigarette smoke extract (CSE) on vascular endothelial growth factor (VEGF) secretion in *Mycoplasma pneumoniae* (Mp)-infected well-differentiated a) normal human bronchial epithelial (NHBE) cells and b) small airway epithelial cells (SAEC). Epithelial cells were infected with Mp in the presence or absence of CSE for 14 days. Data are expressed as mean ± SEM (n=8). \*: p<0.05 (paired t-test).

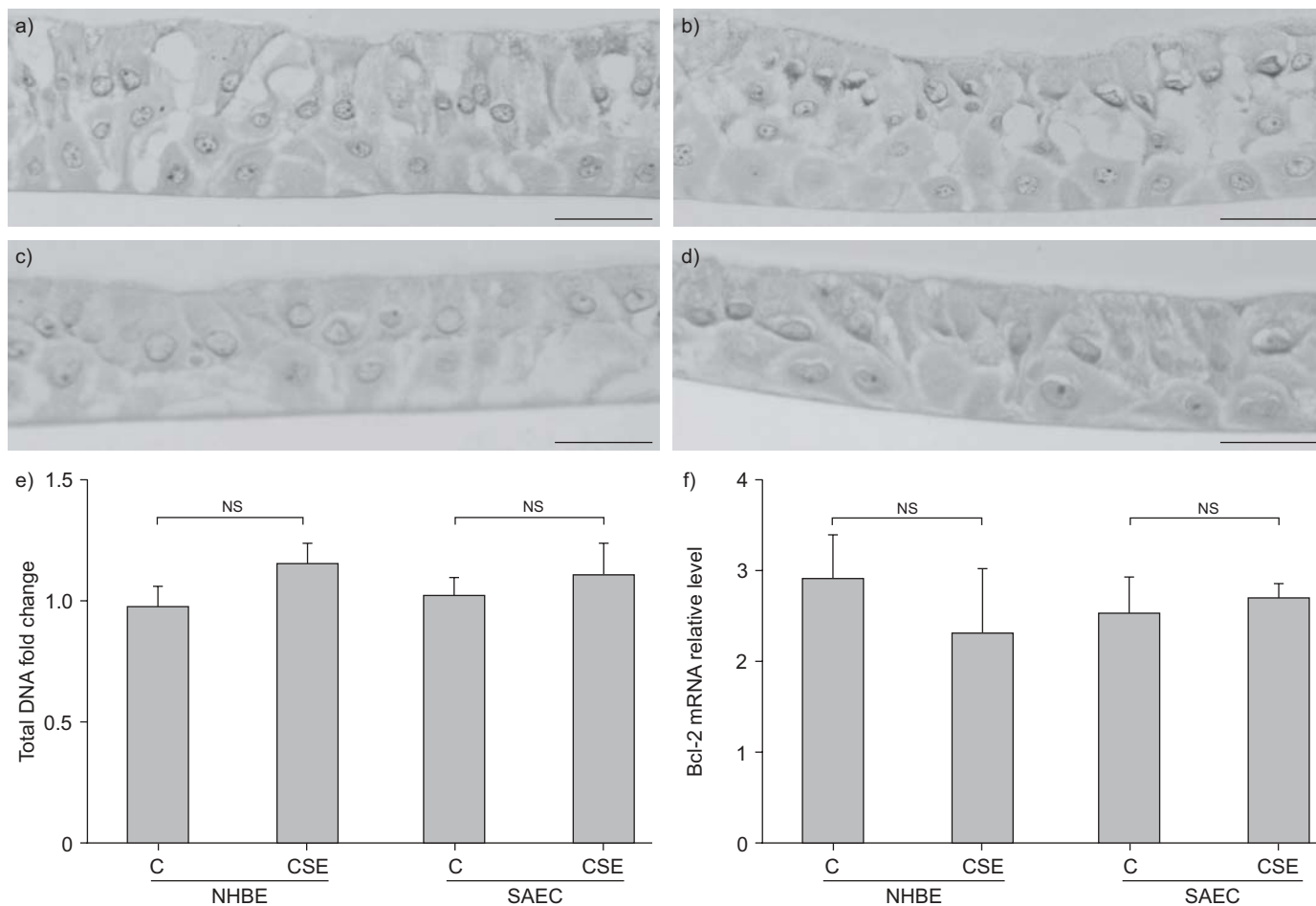
total DNA content and Bcl-2 mRNA expression, respectively (fig. 4). No apparent changes in the cell morphology were observed in NHBE or SAEC following CSE treatment, in comparison with their respective controls (fig. 4a–d). Similarly, no significant differences existed in the total DNA content of CSE-untreated and -treated cells, suggesting that 20% CSE

**TABLE 1** Change in vascular endothelial growth factor secretion following exposure to cigarette smoke extract (CSE), *Mycoplasma pneumoniae* (Mp) or both

	CSE				Mp				Mp+CSE			
	NHBE cells		SAEC		NHBE cells		SAEC		NHBE cells		SAEC	
	Change %	p-value	Change %	p-value	Change %	p-value	Change %	p-value	Change %	p-value	Change %	p-value
<b>Day 1</b>	86.9 ± 10	NS	81.6 ± 9.6	0.0023	94.9 ± 12.5	NS	109.5 ± 16.4	NS	90.4 ± 10	0.025	73.6 ± 7.2	0.022
<b>Day 7</b>	73.8 ± 9.1	0.001	80.7 ± 19.9	NS	109.5 ± 7.6	NS	129.0 ± 16.4	0.011	91.8 ± 8.1	NS	78.1 ± 13	0.048
<b>Day 14</b>	78.0 ± 6.8	0.019	66.6 ± 12.3	0.016	108.1 ± 12.4	NS	138.2 ± 22.2	0.028	88.5 ± 11.5	NS	85.8 ± 6.9	0.023

Data are presented as mean ± SEM, unless otherwise stated, and are comparisons with untreated cells. NHBE: normal human bronchial epithelial; SAEC: small airway epithelial cells; ns: nonsignificant.





**FIGURE 4.** Effects of cigarette smoke extract (CSE) on cell growth and apoptosis in normal human bronchial epithelial (NHBE) cells and small airway epithelial cells (SAEC). Cells were exposed to medium alone or 20% CSE for 14 days, and were harvested to examine cell morphology using haematoxylin and eosin staining. a) Untreated NHBE cells, b) CSE-treated NHBE cells, c) untreated SAEC and d) CSE-treated SAEC. e) Total DNA content was determined using whole cell lysates and expressed as fold change over untreated cells (C). f) B-cell lymphoma (Bcl)-2 mRNA relative expression levels in NHBE cells and SAEC were determined by quantitative real-time RT-PCR. Data are expressed as mean  $\pm$  SEM (n=4 to n=6). ns: nonsignificant (paired t-test). Scale bars=100  $\mu$ m.

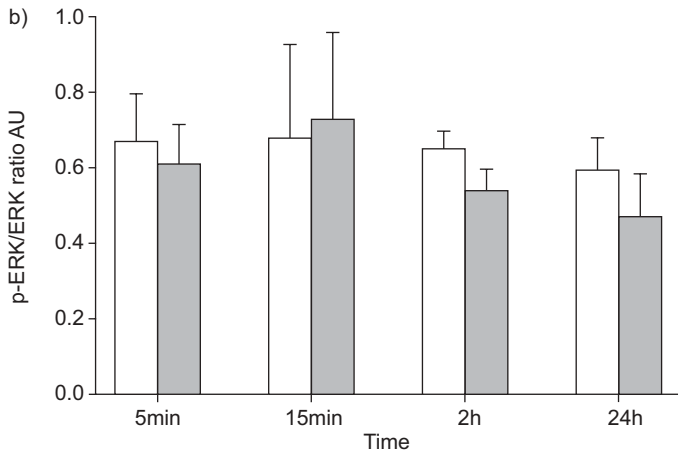
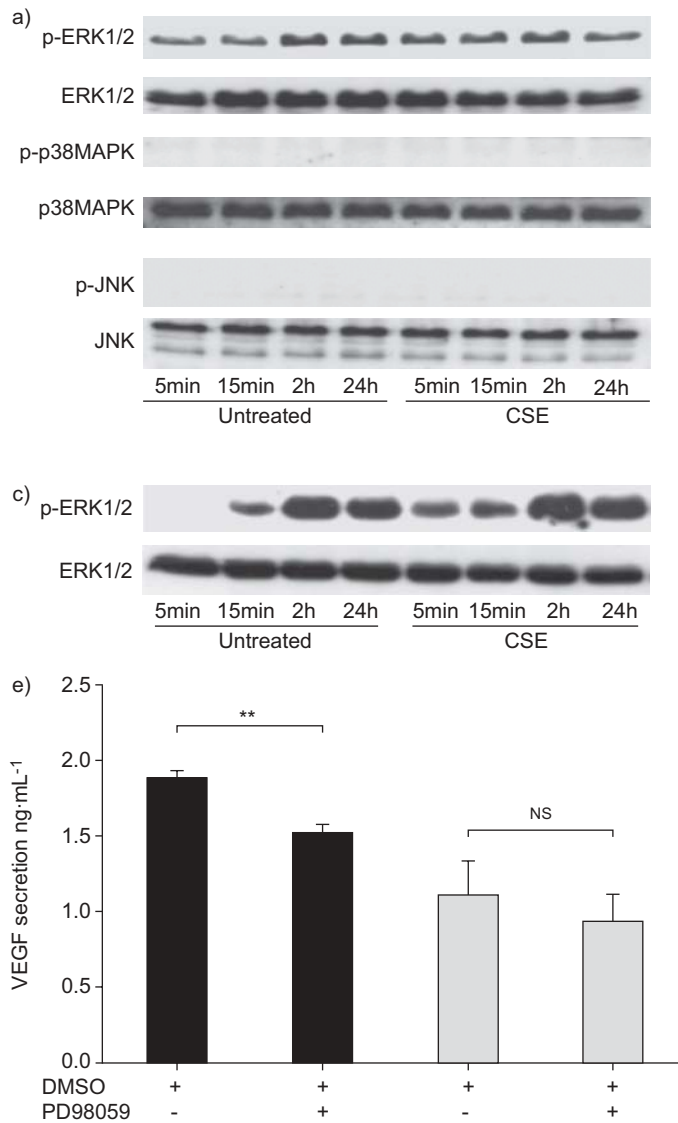
treatment did not increase cell growth or induce cell death in either NHBE or SAEC (fig. 4e). Bcl-2 is an anti-apoptotic protein, and overexpression of Bcl-2 has been shown to protect human airway epithelial cell line 1HAE0 and lung adenocarcinoma cell line A549 from corticosteroid- and hydrogen peroxide-induced apoptosis, respectively [39, 40]. In the present study, CSE did not significantly reduce or alter Bcl-2 mRNA expression compared with untreated cells (fig. 4f). Collectively, these data indicate that 20% CSE treatment did not have any noticeable effects on NHBE or SAEC growth and survival.

#### **Role of MAPK signalling in VEGF expression in human primary AEC**

To elucidate the role of individual MAPK signalling pathways in regulating VEGF expression in NHBE and SAEC, MAPK activation was examined at 5 min, 15 min, 2 h and 24 h following 20% CSE exposure. In NHBE cells, CSE exposure caused acute increase (after 15 min) but subsequent decrease (at 2 h and 24 h) in ERK1/2 phosphorylation, compared with untreated cells (at 24 h,  $p=0.066$ ). In the same time-frame, no

changes were observed in p38MAPK or JNK phosphorylation following CSE exposure (fig. 5a and b). Acute CSE exposure also increased ERK1/2 activation in SAEC but, unlike NHBE, ERK1/2 remained activated at 24 h compared with untreated cells (fig. 5c). Similar to the findings in NHBE, CSE did not alter p38MAPK and JNK activation in SAEC (data not shown).

To confirm a role of ERK1/2 signalling in the regulation of airway epithelial VEGF production, PD98059 (5  $\mu$ M; Calbiochem, San Diego, CA, USA), a specific inhibitor of MEK1/2 (the kinase upstream of ERK1/2), was applied to the cells 2 h prior to the CSE treatment. VEGF secretion and mRNA expression were measured after 24 h of CSE treatment. PD98059 pretreatment significantly reduced basal ERK1/2 phosphorylation in both NHBE and SAEC (fig. 5d). Treatment with PD98059 alone for 24 h significantly reduced VEGF secretion in NHBE cells, but not in SAEC (fig. 5e). PD98059 treatment together with 20% CSE further reduced VEGF in NHBE compared with either treatment alone, but the difference was not statistically significant compared with PD98059 alone (data not shown). PD98059 did not significantly



**FIGURE 5.** Role of mitogen-activated protein kinase (MAPK) in cigarette smoke extract (CSE)-mediated vascular endothelial growth factor (VEGF) reduction in normal human bronchial epithelial (NHBE) cells and small airway epithelial cells (SAEC). Cells were treated with either medium alone (□) or 20% CSE (■). Activation of three MAPK pathways (phosphorylated (p)-MAPK/total MAPK ratio) was determined at the indicated time-points by a) Western blot and b) densitometry in NHBE cells and c) in SAEC. CSE exposure acutely (at 15 min) increased extracellular signal-regulated kinase (ERK)1/2 phosphorylation in both NHBE cells and SAEC, but at 24 h it decreased ERK1/2 phosphorylation in NHBE cells. CSE did not alter p38MAPK or c-Jun N-terminal kinase (JNK) phosphorylation in NHBE cells or SAEC. d and e) Cells were treated with 5  $\mu$ M PD98059, a specific inhibitor of MEK1/2 (the kinase upstream of ERK1/2), for 2 and 24 h, or with 0.1% dimethyl sulphoxide (DMSO) as a vehicle control. d) Inhibition of ERK1/2 phosphorylation was verified by Western blot at 2 h. e) VEGF secretion was determined by ELISA at 24 h in NHBE cells (■) and SAEC (▒). Data are expressed as mean  $\pm$  SEM (n=6). AU: arbitrary units. \*\*: p < 0.01; NS: nonsignificant (paired t-test).

reduce VEGF mRNA expression in NHBE at 24 h post CSE treatment (data not shown).

### Role of PKC signalling in VEGF expression in human primary AEC

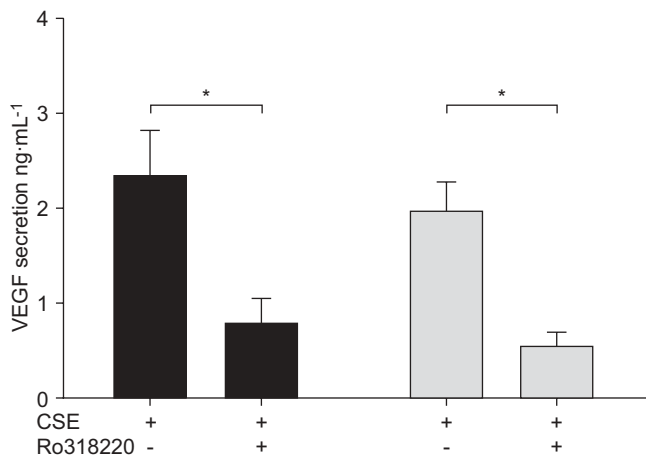
To understand the role of PKC signalling in regulating VEGF expression in NHBE and SAEC, cells were pretreated with the pan-PKC inhibitor Ro318220 (10  $\mu$ M; Calbiochem) for 2 h, followed by 20% CSE treatment for 24 h. VEGF secretion and relative mRNA expression were determined at 24 h post CSE treatment. Pharmacological inhibition of PKC significantly reduced VEGF secretion in NHBE and SAEC, suggesting that this pathway is required for VEGF production in both cell types (fig. 6). PKC inhibition also tended to reduce VEGF mRNA expression (up to 2.5-fold).

### DISCUSSION

The present study demonstrated that CSE exposure reduced VEGF expression in well-differentiated primary human large and small AEC. Inhibitory effects of CSE on VEGF secretion were more pronounced in SAEC in the presence of a bacterial

infection. Furthermore, the results revealed that ERK1/2 and PKC inhibition significantly reduced VEGF production, and might be in part responsible for CSE-mediated reduction of VEGF secretion in NHBE cells and/or SAEC.

CS or CS-related component exposure has been shown to induce a variety of biological responses, such as increased pro-inflammatory cytokine release, oxidative stress and apoptosis in lung epithelial cell lines or primary AEC under submerged culture conditions [10–14]. A comparative study among various transformed lung epithelial cell lines and primary epithelial cells cultured under submerged culture conditions suggested that primary AEC were more responsive to CSE than transformed cell lines with regard to the release of inflammatory cytokines such as interleukin (IL)-8 and IL-6 [11]. Most of the previous studies were performed under submerged culture conditions, which do not mimic many of the major *in vivo* features of AEC such as mucociliary differentiation. Unlike previous studies, the current study utilised well-differentiated AEC to unravel the impact of CSE on airway epithelial biology such as VEGF production. The present



**FIGURE 6.** Role of protein kinase (PK)C in cigarette smoke extract (CSE)-mediated vascular endothelial growth factor (VEGF) reduction in normal human bronchial epithelial cells (■) and small airway epithelial cells (■). Cells were pretreated with a PKC inhibitor, Ro318220 (10  $\mu$ M), for 2 h, followed by 20% CSE treatment for 24 h. VEGF secretion was determined by an ELISA at 24 h post CSE treatment. Data are expressed as mean  $\pm$  SEM ( $n=3$ ). \*:  $p<0.05$ .

results provide direct evidence that CSE exposure significantly reduced VEGF secretion in both large and small AEC, and thus have extended previous studies in whole lung tissues of severe emphysema patients as well as rodents [5, 6, 41]. The maximum inhibition of CSE on VEGF secretion in the current study was 27% for NHBE and 34% for SAEC. Since the absolute VEGF protein levels (up to 6 ng·mL<sup>-1</sup>) in cultured AEC were very high, a reduction of 27% or 34% in VEGF should be significant. In support of this notion, previous studies demonstrated 20–38% VEGF reduction in human emphysematous lungs compared with normal lungs [5, 6, 42]. Although the direct consequences of reduced VEGF from AEC with CS exposure were not explored in the current study, decreased VEGF has been clearly implicated in the destruction of lung parenchyma, including microvasculature, in severe emphysematous lungs [6, 8, 9].

Large AEC are exposed to the highest concentration of CS, but major pathological lesions in COPD patients exist in small airways and lung parenchyma. Hence, it is important to understand the effect of CS exposure on SAEC and to compare it with large AEC (e.g. NHBE cells). The current study is the first to determine the impact of CSE on VEGF production in both well-differentiated SAEC and NHBE cells. The results demonstrated that SAEC were more responsive to acute CSE exposure than NHBE cells, with regard to VEGF reduction. Although some reports have proposed certain differences of cellular population or gene expression, such as galectin-3 and Ki-67, in small and large AEC in COPD [43], no previous studies have addressed the molecular mechanisms responsible for the different responses to CS exposure between these two types of AEC. Further studies are needed to demonstrate the molecular basis upon which SAEC are more responsive to CSE exposure than NHBE cells with regard to VEGF reduction. Since both types of epithelial cells express different repertoires of nicotine receptors [44], it may be speculated that differing

levels of nicotine receptors on SAEC and NHBE cells may be involved, but this needs to be explored in future experiments.

Increased bacterial colonisation has been reported in the airways of asymptomatic smokers as well as smokers with COPD. Among various bacteria, *Mp*, an atypical respiratory pathogen, was detected in 6.7% of COPD patients [19]. The significance of *Mp* colonisation/infection in COPD is largely unknown. The current authors found that *Mp* alone significantly increased VEGF secretion in SAEC, but not in NHBE cells. Moreover, the presence of CSE abrogated *Mp*-induced VEGF secretion in SAEC, suggesting that persistent bacterial infection in a CS exposure milieu can significantly reduce VEGF secretion in lower airways, and may accelerate the loss of microvasculature, a pathological feature highly relevant to the development of emphysema in human lungs.

CS exposure has been shown to induce apoptosis, senescence, cell proliferation and inflammatory response in different cell types, including AEC cultured under submerged conditions [10–14]. In contrast to previous findings, the well-differentiated primary human AEC in the current experiments did not increase cell growth, death or apoptosis upon CSE exposure. Therefore, the current findings suggest that CSE-mediated reduction in VEGF secretion is an active process and might be occurring at the transcriptional and/or translational level. Although there was a significant decrease in the VEGF protein secretion in CSE-treated cells compared with untreated cells at different time-points, the decrease in VEGF mRNA levels did not attain a significant difference between CSE-treated and -untreated cells. The inconsistency of VEGF mRNA and protein data may reflect the timing effect after CSE treatment. In a study by KOYAMA *et al.* [45], VEGF protein, but not VEGF mRNA, was increased in lung epithelial A549 cells at 72 h following IL-1 $\beta$  stimulation. The VEGF mRNA level increased during the first 12 h of IL-1 $\beta$  stimulation when VEGF protein was not increased [45]. Therefore, it is not surprising that, after 24 h of CSE treatment, VEGF protein decreased in AEC but VEGF mRNA did not change significantly.

BOUSSAT *et al.* [15] showed that well-differentiated NHBE cells produced a noticeable amount of VEGF at the baseline, which increased over the culture time. However, the molecular mechanisms governing the regulation of VEGF expression in well-differentiated primary human AEC are still not clear. Signalling pathways, including MAPK and PKC, have been shown to regulate VEGF expression in different cell types [21–27]. In the present study, the role of MAPK pathways, including ERK1/2, JNK and p38MAPK, in regulating CSE-mediated decrease of VEGF secretion in well-differentiated AEC was examined. It was found that CSE exposure did not change JNK or p38MAPK phosphorylation/activation in either cell type, but an acute (15 min) increase followed by a decrease (by 24 h) in phospho-ERK/total ERK ratio was seen in CSE-treated NHBE cells compared with CSE-untreated cells. The current authors note that the Western blot data plus ERK inhibitor studies did not fully explain the significant difference in VEGF levels between CSE-treated and -untreated NHBE cells. Also, the ERK inhibition data did not explain the CSE-mediated decrease of VEGF in SAEC. Together, the data suggest that additional signalling pathways could also be involved in CSE-induced reduction of VEGF. Indeed, it was

confirmed that the PKC pathway may also contribute to regulation of VEGF production in NHBE and SAEC, by use of a pan-PKC inhibitor (fig. 6). Nonetheless, due to the complexity of VEGF regulation, further studies are warranted to investigate the role of additional signalling pathways, in order to better reveal the process of VEGF regulation following CS exposure.

The present study demonstrated that NHBE and SAEC differed in their responses to CSE with regard to VEGF production, and showed a role of the ERK1/2 pathway in the regulation of VEGF expression. Future experiments may need to focus on signalling molecules or pathways that are uniquely utilised by either type of epithelial cells to regulate VEGF synthesis. For example, the prostaglandin (PG)<sub>E2</sub> pathway, which is clearly involved in tumour angiogenesis [46, 47], may play an important role in airway epithelial VEGF production. The current authors observed an induction (1.9-fold increase) of mitochondrial PGE synthase-1 (mPGES-1; a key enzyme in PGE<sub>2</sub> synthesis) protein expression in SAEC, but not in NHBE, at day 7 post *Mp* infection (data not shown). This increased PGE<sub>2</sub> pathway activity could be responsible for the significant increase in VEGF production in SAEC following *Mp* infection. *Mp*-induced mPGES-1 expression in SAEC was reduced (2.7-fold) in the presence of CSE. This observation needs to be further explored.

The current study demonstrated that cigarette smoke extract exposure significantly reduced vascular endothelial growth factor secretion in well-differentiated primary human large and small airway epithelial cells. Inhibitory effects of cigarette smoke extract on vascular endothelial growth factor secretion were more pronounced in small airway epithelial cells in the presence of a bacterial infection. Greater reduction of vascular endothelial growth factor secretion from small airway epithelial cells following cigarette smoke extract exposure could contribute to the loss of microvasculature in lung parenchyma of chronic obstructive pulmonary disease patients. Persistent bacterial infections or colonisation in cigarette smoke-exposed lungs may further enhance the loss of microvasculature and subsequent development of emphysema.

## REFERENCES

- 1 State of Lung Disease in Diverse Communities: 2007. American Lung Association, August 2007. Available at [http://www.lungusa.org/site/c.dvLUK9O0E/b.308853/k.37D1/State\\_of\\_Lung\\_Disease\\_in\\_Diverse\\_Communities.htm](http://www.lungusa.org/site/c.dvLUK9O0E/b.308853/k.37D1/State_of_Lung_Disease_in_Diverse_Communities.htm).
- 2 Hartert TV, Gabb MG. The national and global impact of COPD. *Adv Stud Med* 2004; 4: S738–S743.
- 3 MacNee W. Pathogenesis of chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2005; 2: 258–266.
- 4 Gerber HP, McMurtrey A, Kowalski J, et al. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 1998; 273: 30336–30343.
- 5 Suzuki M, Betsuyaku T, Nagai K, et al. Decreased airway expression of vascular endothelial growth factor in cigarette smoke-induced emphysema in mice and COPD patients. *Inhal Toxicol* 2008; 20: 349–359.
- 6 Marwick JA, Stevenson CS, Giddings J, et al. Cigarette smoke disrupts VEGF165-VEGFR-2 receptor signaling complex in rat lungs and patients with COPD: morphological impact of VEGFR-2 inhibition. *Am J Physiol Lung Cell Mol Physiol* 2006; 290: L897–L908.
- 7 Spira A, Beane J, Pinto-Plata V, et al. Gene expression profiling of human lung tissue from smokers with severe emphysema. *Am J Respir Cell Mol Biol* 2004; 31: 601–610.
- 8 Kanazawa H, Yoshikawa J. Elevated oxidative stress and reciprocal reduction of vascular endothelial growth factor levels with severity of COPD. *Chest* 2005; 128: 3191–3197.
- 9 Kasahara Y, Tuder RM, Cool CD, Lynch DA, Flores SC, Voelkel NF. Endothelial cell death and decreased expression of vascular endothelial growth factor and vascular endothelial growth factor receptor 2 in emphysema. *Am J Respir Crit Care Med* 2001; 163: 737–744.
- 10 Ho YS, Chen CH, Wang YJ, et al. Tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induces cell proliferation in normal human bronchial epithelial cells through NF-κB activation and cyclin D1 up-regulation. *Toxicol Appl Pharmacol* 2005; 205: 133–148.
- 11 Kode A, Yang SR, Rahman I. Differential effects of cigarette smoke on oxidative stress and proinflammatory cytokine release in primary human airway epithelial cells and in a variety of transformed alveolar epithelial cells. *Respir Res* 2006; 7: 132.
- 12 Luppi F, Aarbiou J, van Wetering S, et al. Effects of cigarette smoke condensate on proliferation and wound closure of bronchial epithelial cells *in vitro*: role of glutathione. *Respir Res* 2005; 6: 140.
- 13 Moodie FM, Marwick JA, Anderson CS, et al. Oxidative stress and cigarette smoke alter chromatin remodeling but differentially regulate NF-κB activation and proinflammatory cytokine release in alveolar epithelial cells. *FASEB J* 2004; 18: 1897–1899.
- 14 Tsuji T, Aoshiba K, Nagai A. Cigarette smoke induces senescence in alveolar epithelial cells. *Am J Respir Cell Mol Biol* 2004; 31: 643–649.
- 15 Boussat S, Eddahibi S, Coste A, et al. Expression and regulation of vascular endothelial growth factor in human pulmonary epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2000; 279: L371–L378.
- 16 Qvarfordt I, Riise GC, Andersson BA, Larsson S. Lower airway bacterial colonization in asymptomatic smokers and smokers with chronic bronchitis and recurrent exacerbations. *Respir Med* 2000; 94: 881–887.
- 17 Sethi S, Maloney J, Grove L, Wrona C, Berenson CS. Airway inflammation and bronchial bacterial colonization in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2006; 173: 991–998.
- 18 Blasi F. Atypical pathogens and respiratory tract infections. *Eur Respir J* 2004; 24: 171–182.
- 19 Meloni F, Paschetto E, Mangiarotti P, et al. Acute *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* infections in community-acquired pneumonia and exacerbations of COPD or asthma: therapeutic considerations. *J Chemother* 2004; 16: 70–76.
- 20 Schito GC, Grazi G, Dainelli B, et al. Incidence of lower respiratory tract infections caused by *Mycoplasma*,



- Chlamydia and Legionella: an Italian Multicenter Survey. *J Chemother* 1994; 6: 319–321.
- 21 Chang HJ, Park JS, Kim MH, *et al.* Extracellular signal-regulated kinases and AP-1 mediate the up-regulation of vascular endothelial growth factor by PDGF in human vascular smooth muscle cells. *Int J Oncol* 2006; 28: 135–141.
  - 22 Xu H, Czerwinski P, Hortmann M, Sohn HY, Förstermann U, Li H. Protein kinase C $\alpha$  promotes angiogenic activity of human endothelial cells *via* induction of vascular endothelial growth factor. *Cardiovasc Res* 2008; 78: 349–355.
  - 23 Bian ZM, Elner SG, Elner VM. Thrombin-induced VEGF expression in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* 2007; 48: 2738–2746.
  - 24 Bian ZM, Elner SG, Elner VM. Regulation of VEGF mRNA expression and protein secretion by TGF- $\beta$ 2 in human retinal pigment epithelial cells. *Exp Eye Res* 2007; 84: 812–822.
  - 25 Riazzy M, Chen JH, Steinbrecher UP. VEGF secretion by macrophages is stimulated by lipid and protein components of OxLDL *via* PI3-kinase and PKC $\zeta$  activation and is independent of OxLDL uptake. *Atherosclerosis* 2008; [Epub ahead of print PMID: 18789802].
  - 26 Lee EY, Chung CH, Kim JH, Joung HJ, Hong SY. Antioxidants ameliorate the expression of vascular endothelial growth factor mediated by protein kinase C in diabetic podocytes. *Nephrol Dial Transplant* 2006; 21: 1496–1503.
  - 27 Jung YD, Liu W, Reinmuth N, *et al.* Vascular endothelial growth factor is upregulated by interleukin-1 $\beta$  in human vascular smooth muscle cells *via* the p38 mitogen-activated protein kinase pathway. *Angiogenesis* 2001; 4: 155–162.
  - 28 Hellermann GR, Nagy SB, Kong X, Lockey RF, Mohapatra SS. Mechanism of cigarette smoke condensate-induced acute inflammatory response in human bronchial epithelial cells. *Respir Res* 2002; 3: 22.
  - 29 Wyatt TA, Heires AJ, Sanderson SD, Floreani AA. Protein kinase C activation is required for cigarette smoke-enhanced C5a-mediated release of interleukin-8 in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 1999; 21: 283–288.
  - 30 Zhang Q, Adiseshaiah P, Reddy SP. Matrix metalloproteinase/epidermal growth factor receptor/mitogen-activated protein kinase signaling regulate fra-1 induction by cigarette smoke in lung epithelial cells. *Am J Respir Cell Mol Biol* 2005; 32: 72–81.
  - 31 Mercer BA, Kolesnikova N, Sonett J, D'Armiento J. Extracellular regulated kinase/mitogen activated protein kinase is up-regulated in pulmonary emphysema and mediates matrix metalloproteinase-1 induction by cigarette smoke. *J Biol Chem* 2004; 279: 17690–17696.
  - 32 Khan EM, Lanir R, Danielson AR, Goldkorn T. Epidermal growth factor receptor exposed to cigarette smoke is aberrantly activated and undergoes perinuclear trafficking. *FASEB J* 2008; 22: 910–917.
  - 33 Chu HW, Balzar S, Seedorf GJ, *et al.* Transforming growth factor- $\beta$ 2 induces bronchial epithelial mucin expression in asthma. *Am J Pathol* 2004; 165: 1097–1106.
  - 34 Chu HW, Thaikootathil J, Rino JG, *et al.* Function and regulation of SPLUNC1 protein in Mycoplasma infection and allergic inflammation. *J Immunol* 2007; 179: 3995–4002.
  - 35 Li CJ, Ning W, Matthey MA, Feghali-Bostwick CA, Choi AM. MAPK pathway mediates EGR-1-HSP70-dependent cigarette smoke-induced chemokine production. *Am J Physiol Lung Cell Mol Physiol* 2007; 292: L1297–L1303.
  - 36 Liu X, Conner H, Kobayashi T, *et al.* Cigarette smoke extract induces DNA damage but not apoptosis in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2005; 33: 121–129.
  - 37 Panayiotidis MI, Stabler SP, Allen RH, Ahmad A, White CW. Cigarette smoke extract increases S-adenosylmethionine and cystathionine in human lung epithelial-like (A549) cells. *Chem Biol Interact* 2004; 147: 87–97.
  - 38 Su Y, Han W, Giraldo C, De Li Y, Block ER. Effect of cigarette smoke extract on nitric oxide synthase in pulmonary artery endothelial cells. *Am J Respir Cell Mol Biol* 1998; 19: 819–825.
  - 39 Dorscheid DR, Wojcik KR, Sun S, Marroquin B, White SR. Apoptosis of airway epithelial cells induced by corticosteroids. *Am J Respir Crit Care Med* 2001; 164: 1939–1947.
  - 40 Ravid T, Tsaba A, Gee P, Rasooly R, Medina EA, Goldkorn T. Ceramide accumulation precedes caspase-3 activation during apoptosis of A549 human lung adenocarcinoma cells. *Am J Physiol Lung Cell Mol Physiol* 2003; 284: L1082–1092.
  - 41 Tang K, Rossiter HB, Wagner PD, Breen EC. Lung-targeted VEGF inactivation leads to an emphysema phenotype in mice. *J Appl Physiol* 2004; 97: 1559–1566.
  - 42 Wang YH, Bai CX, Mao L, Zhang M. [Expression of pulmonary vascular endothelial growth factor and vascular endothelial growth factor receptor in emphysema]. *Zhonghua Nei Ke Za Zhi* 2005; 44: 276–279.
  - 43 Pilette C, Colinet B, Kiss R, *et al.* Increased galectin-3 expression and intra-epithelial neutrophils in small airways in severe COPD. *Eur Respir J* 2007; 29: 914–922.
  - 44 West KA, Brognard J, Clark AS, *et al.* Rapid Akt activation by nicotine and a tobacco carcinogen modulates the phenotype of normal human airway epithelial cells. *J Clin Invest* 2003; 111: 81–90.
  - 45 Koyama S, Sato E, Tsukadaira A, *et al.* Vascular endothelial growth factor mRNA and protein expression in airway epithelial cell lines *in vitro*. *Eur Respir J* 2002; 20: 1449–1456.
  - 46 Liu M, Yang SC, Sharma S, *et al.* EGFR signaling is required for TGF- $\beta$ 1 mediated COX-2 induction in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2007; 37: 578–588.
  - 47 Shin VY, Wu WK, Chu KM, *et al.* Nicotine induces cyclooxygenase-2 and vascular endothelial growth factor receptor-2 in association with tumor-associated invasion and angiogenesis in gastric cancer. *Mol Cancer Res* 2005; 3: 607–615.