Cigarette Smoke Extract Reduces VEGF in Primary Human Airway Epithelial Cells

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Abstract

Reduced VEGF has been reported in bronchoalveolar lavage fluid (BALF) 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.

We 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) under the 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 1/2 (ERK1/2) and protein kinase C (PKC) signaling 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.

We conclude that direct CSE exposure significantly reduced VEGF production in well-differentiated primary human AEC, which is in part through modifying ERK1/2 and PKC signaling pathways.

Abbreviations used

ALI   Air-liquid interface

BAL   Bronchoalveolar lavage

CS   Cigarette smoke

CSE   Cigarette smoke extract

ERK1/2   Extracellular signal-regulated kinase 1 and 2

JNK   c-Jun NH2-terminal kinase

MAPK   Mitogen activated protein kinase

Mp   *Mycoplasma pneumoniae*

NHBE   Normal human bronchial epithelial cells

p38MAPK   p38 Mitogen activated protein kinase

PKC   Protein kinase C

SAEC   Small airway epithelial cells

VEGF   Vascular endothelial growth factor

INTRODUCTION

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-A (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 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 defense mechanism, airway epithelial cells 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 airway epithelial cells (AEC) with relation to VEGF production has not been well studied.

Most of the previous studies aimed at determining the effects of CS on cell proliferation, apoptosis, inflammation and senescence have been performed by using either immortal lung epithelial cell lines or primary large AEC cultured under the submerged conditions [10-14]. AEC grown under the submerged culture conditions are poorly differentiated, and do not mimic major *in vivo* features such as mucociliary differentiation. Boussat and colleagues found that well-differentiated NHBE produced significantly higher amount of VEGF compared to transformed epithelial cell lines [15]. However, it remains unclear whether CS exposure can directly decrease VEGF levels in well-differentiated primary human bronchial epithelial cells. As small airways and lung parenchyma are 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 colonization 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 remodeling (e.g., angiogenesis) in COPD patients is poorly understood. Therefore, we also examined if Mp can modify VEGF production in CS-exposed AEC.

Signaling pathways such as mitogen activated protein kinase (MAPK) and protein kinase C (PKC) 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 signaling pathways in lung epithelial cell lines (e.g., A549) and primary cells (e.g., NHBE) cultured under the 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 this study, we determined the role of MAPK and PKC signaling pathways in VEGF production in well-differentiated human primary airway epithelial cells that are treated with CSE.

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

METHODS

Primary human airway epithelial cell air-liquid interface (ALI) culture

Primary human airway epithelial cells (Clonetics NHBE and SAEC) were obtained from different healthy donors (Cambrex Bio Science, Walkersville, MD). They were cultured in serum free medium (DMEM:BEBM, 1:1) supplemented with various growth factors including insulin (0.4 µg/ml), transferin (5 µg/ml), hydrocortisone (0.5 µg/ml), epinephrine (0.5µg/ml), epidermal growth factor (10 ng/ml), bovine pituitary extract (52 µg/ml), retinoic acid (30 ng/ml), BSA (50 µg/ml), gentamycin (50 µg/ml) and amphoterisin B (0.05 µg/ml). Cells between passages 2 to 4 were plated onto 12 mm collagen-coated polyester transwell inserts (pore size, 0.4 µm, Corning Inc., Corning, NY) at 4×10^4 cells/cm^2. Within a week of submerged culture condition these epithelial cells attained 100% confluence, and were then transferred into an ALI condition by reducing apical surface medium volume to 50 µl. 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 post-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 signaling pathways.

Cigarette smoke extract (CSE) preparation

CSE was prepared fresh daily as previously described with slight modification [35-38]. Unfiltered research grade cigarettes (2R1) were purchased from Kentucky Tobacco Research and Development Center at University of Kentucky, Louisville, KY. 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 sterilized through a 0.22 μm 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 minutes of CSE preparation.

Mycoplasma pneumoniae (Mp) infection in airway epithelial cells

Mp strain 15531 was obtained from American Type Culture Collection (ATCC, Manassas, VA), and was grown at 37°C in SP4 glucose broth. Adherent Mp was collected, and frozen at -80 °C for infection experiments [34]. Frozen Mp stock (passage 4) was resurrected in SP4 broth at 37°C for two hours, and then used for infecting the epithelial cells at a concentration of 1 colony forming unit [cfu]/cell (a physiologic dose) or $4x10^4$ cfu/transwell 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.

Enzyme linked immunosorbant assay (ELISA) for VEGF

VEGF secretion in basolateral supernatants from airway epithelial cells was measured using a VEGF ELISA kit (ELISA Tech., Aurora, CO) as per manufacturer's instructions. Standard curve was generated using recombinant VEGF (0.05 – 2 ng/ml) 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). Reverse transcription was performed using 1 μg of total RNA as described

previously [34]. Human VEGF (Genbank accession number: M27281.1) primers (Forward 5'-CTTGCCTTGCTGCTCTACC-3' and Reverse 5'-CACACAGGATGGCTTGAAG-3') and probe (5'-AGTTCATGGATGTCTATCAGCGCAGCT-3') were designed using the Primer Express software (Applied Biosystems). Primers and probes for human GAPDH (4326317E) and B-cell lymphoma-2 (Bcl-2) (assay ID HS00153350_m1) were purchased as premixed solution from the Applied Biosystems. Real-time PCR was performed using 25 µl reaction mixture containing 30 ng cDNA, fluorogenic probe, primers and other components in the TaqMan RT-PCR kit. The comparative threshold cycle (C_T) method was used to determine the relative gene expression levels in airway epithelial cells.

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 kit from Molecular Probes (Eugene, Oregon) per manufacturer's instruction. In brief, whole cell lysates were prepared from each transwell using 200 µl of cell lysis solution (0.2% TritonX-100). Cell lysates (20 µl) were added directly to 200 µl of working Quant-iT dsDNA BR reagent. DNA content was determined by measuring fluorescence intensity using ex510nm/em527nm filters in a fluorescence plate reader. Standard curve was prepared using λDNA standards (50 – 1000 ng/well) provided with the kit and used to determine DNA content of cell lysates.

Airway epithelial cell morphology

For cell morphology analysis, cells were fixed in ice-cold acetone and embedded in JB-4 polymer [33]. Cell sections of 2 µm 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, 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 phospho-ERK1/2, ERK1/2, phospho-JNK, JNK, phospho-p38MAPK and p38MAPK (Santa Cruz Biotechnology Inc., Santa Cruz, CA, and Cell Signaling Technology Inc., Danvers, MA) 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 ratio of phosphorylated-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 means ± SEM. Statistical significance between different groups was analyzed using the 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 airway epithelial cells

Our 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 to 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). We decided to use 20% CSE for our 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).

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

Effect of CSE on VEGF production in the presence of Mp infection in human primary airway epithelial cells

Mp alone significantly increased VEGF secretion in SAEC, but not in NHBE (Table 1, fig. 3a and 3b). Presence of CSE significantly attenuated Mp-induced VEGF secretion in SAEC (fig. 3b). Inhibitory effect of CSE in combination of Mp on VEGF production occurred at earlier time points (days 1 and 7) and was more pronounced in SAEC as compared to NHBE (Table 1). Further, in response to simultaneous CSE and Mp exposure, the degree of VEGF reduction in SAEC was greater than that in NHBE (Table 1).

Effect of CSE on cell growth and apoptosis in human primary airway epithelial cells

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, total DNA content and B-cell lymphoma 2 (Bcl-2) mRNA expression, respectively (fig. 4). No apparent changes in the cell morphology were observed in NHBE as well as SAEC following CSE treatment in comparison with their respective controls (fig. 4a-4d). Similarly, no significant differences existed in total DNA content of CSE-untreated and -treated cells, suggesting that 20% CSE treatment did not increase cell growth or induce cell death in both NHBE and 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 our study, CSE did not significantly reduce or alter Bcl-2 mRNA expression as compared to 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 signaling in VEGF expression in human primary airway epithelial cells

To elucidate the role of individual MAPK signaling pathway in regulating VEGF expression in NHBE and SAEC, we examined MAPK activation at 5 min, 15 min, 2 h and 24 h following 20% CSE exposure. In NHBE, CSE exposure acutely increased, but subsequently tended to decrease ERK1/2 phosphorylation at 2 h and 24 h compared with untreated cells (at 24 h, $p = 0.066$). In the same time frame, we did not observe any changes in p38MAPK or JNK phosphorylation following CSE exposure (fig. 5a and fig. 5b). Acute CSE exposure also increased ERK1/2 activation in SAEC, but unlike NHBE, ERK1/2

remained activated at 24 h compared to 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 signaling in the regulation of airway epithelial VEGF production, PD98059 (5 μM), a specific MEK1/2 (upstream of ERK1/2) inhibitor, 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 along with 20% CSE further reduced VEGF in NHBE compared with either treatment alone, but was not statistically significant from PD98059 alone (data not shown). PD98059 did not significantly reduce VEGF mRNA expression in NHBE at 24 h post-CSE treatment (data not shown).

Role of PKC signaling in VEGF expression in human primary airway epithelial cells

To understand the role PKC signaling in regulating VEGF expression in NHBE and SAEC, cells were pretreated with the pan PKC inhibitor Ro318220 (10 μM) 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 cells (fig. 6). PKC inhibition also trended to reduce (up to 2.5-fold) VEGF mRNA expression.

DISCUSSION

This study demonstrated that CSE exposure reduced VEGF expression in well-differentiated primary human large and small airway epithelial cells. Inhibitory effects of CSE on VEGF secretion were more pronounced in SAEC in the presence of a bacterial infection. Further, our 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 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 airway epithelial cells 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 IL-8 and IL-6 [11]. Most of those previous studies were performed under the submerged culture conditions, which do not mimic many of the major *in vivo* features of AEC such as mucociliary differentiation. Unlike previous studies, our current study utilized well-differentiated AEC to unravel the impact of CSE on airway epithelial biology such as VEGF production. Our 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 inhibitions of CSE on VEGF secretion in the current study are 27% for NHBE and 34% for SAEC. Since the absolute VEGF protein levels (up to 6 ng/ml) in cultured airway epithelial cells are very high, a reduction of 27% or 34% in VEGF should be significant. In support of this notion, previous studies demonstrated 20–38% of VEGF reduction in human emphysematous lungs as compared to normal lungs [42-44].

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Although the direct consequences of reduced VEGF from airway epithelial cells with cigarette smoke 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 airway epithelial cells 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 to large airway epithelial cells (e.g., NHBE). This is the first study to determine the impact of CSE on VEGF production in both well-differentiated SAEC and NHBE. Our results demonstrated that SAEC were more responsive to acute CSE exposure than NHBE 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 airway epithelial cells in COPD [45], none of the studies has addressed the molecular mechanisms responsible for the different responses to CS exposure between these two types of airway epithelial cells. We realize that future studies are needed to demonstrate the molecular basis upon which SAEC are more responsive to CSE exposure than NHBE with regard to VEGF reduction. Since both types of epithelial cells express different repertoire of nicotine receptors [46], we may speculate that differing levels of nicotine receptors on SAEC and NHBE may be in part involved, but this needs to be explored in future experiments.

Increased bacterial colonization 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 colonization/infection in COPD is largely unknown. We found that Mp alone significantly increased VEGF secretion in SAEC, but not in NHBE. 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 pathologic 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 airway epithelial cells cultured under the submerged condition [10-14]. Unlike previous findings, well-differentiated primary human AEC in our experiments did not increase cell growth, death or apoptosis upon CSE exposure. Therefore, our 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 is a significant decrease in the VEGF protein secretion in CSE-treated cells compared to CSE-untreated cells at different time points, decrease in VEGF mRNA levels could 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 S and colleagues, VEGF protein, but not VEGF mRNA, was increased in lung epithelial A549 cells at 72 hr following IL-1ß stimulation. Instead, VEGF mRNA level increased during the first 12 hr of IL-1ß stimulation when VEGF protein was not increased (47). Therefore, it is not surprising that after 24 h of CSE treatment, VEGF protein decreased in airway epithelial cells, but VEGF mRNA did not change significantly.

Boussat and colleagues had shown that well-differentiated NHBE produced noticeable amount of VEGF at the baseline that increases over the culture time. However, the molecular mechanisms governing the regulation of VEGF expression in well-differentiated primary human AEC are still not clear [15]. Signaling pathways including mitogen activated protein kinase (MAPK) and PKC have been shown to regulate VEGF expression in different cell types [21-27]. In the present study, we examined the role of MAPK pathways including ERK1/2, JNK and p38MAPK in regulating CSE-mediated decrease of VEGF secretion in

well-differentiated airway epithelial cells. We found that CSE exposure did not change JNK or p38MAPK phosphorylation/activation in both cell types, but we did see an acute (15 min) increase, followed by a decrease (24 h) in p-ERK/total ERK ratio in CSE-treated NHBE cells compared to CSE-untreated cells. We realize that our Western blot data as well as ERK inhibitor studies did not fully explain the significant difference of 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, our data suggest that additional signaling pathways could also be involved in CSE-induced reduction of VEGF. Indeed, we confirmed that PKC pathway may also contribute to VEGF production in NHBE and SAEC using a pan-PKC inhibitor (fig. 6). Nonetheless, due to the complexity of VEGF regulation, further studies are warranted to investigate the role of additional signaling pathways to better reveal the process of VEGF regulation following cigarette smoke exposure.

We demonstrated that NHBE and SAEC differed in their responses to CSE with regard to VEGF production, and the role of ERK1/2 pathway in the regulation of VEGF expression. Future experiments may need to focus on signaling molecules or pathways that are uniquely utilized by either type of epithelial cells to regulate VEGF synthesis. For example, prostaglandin E_2 (PGE$_2$) pathway that is clearly involved in tumor angiogenesis [48-49] may play an important role in airway epithelial VEGF production. We observed an induction (1.9-fold increase) of mitochondrial prostaglandin E synthase-1 (mPGES-1, a key enzyme in PGE$_2$ synthesis) protein expression in SAEC, but not in NHBE, at day 7 post-Mp infection (data not shown). This increased PGE$_2$ pathway 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.

Our current study demonstrated that CSE exposure significantly reduced VEGF secretion in well-differentiated primary human large and small airway epithelial cells. Inhibitory effects of CSE on VEGF secretion were more pronounced in SAEC in the presence of a bacterial infection. Greater reduction of VEGF secretion from SAEC following CSE exposure could contribute to the loss of microvasculature in lung parenchyma of COPD patients. Persistent bacterial infections or colonization in CS-exposed lungs may further enhance the loss of microvasculature and subsequent development of emphysema.

Acknowledgements

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Table 1. Percent change in VEGF secretion in NHBE and SAEC following CSE, Mp or Mp + CSE exposure compared to untreated cells

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

NHBE = normal human bronchial epithelial cells; SAEC = small airway epithelial cells; CSE = cigarette smoke extract; Mp = *Mycoplasma pneumoniae*; NS = Not significant.

Figure Legends

Fig. 1 Dose response of cigarette smoke extract (CSE, 1 – 20%) on VEGF secretion and mRNA expression in well-differentiated NHBE cells. CSE was applied to the apical side of NHBE cells for 7 days. (a) – VEGF levels in basolateral supernatants were determined by using an ELISA. CSE at 5% and 20% significantly reduced VEGF secretion compared to untreated cells. (b) – VEGF mRNA relative expression levels were determined by using quantitative real-time RT-PCR. Data are expressed as Means ± SEM (n = 6). NS = Not significant (One-way ANOVA).

Fig. 2 CSE exposure reduced VEGF secretion in well-differentiated NHBE cells (a) and SAEC (b) as compared to the respective untreated controls (CT) after 1, 7 and 14 days of CSE treatments. Data are expressed as individual values along with means (horizontal bars) in the graphs (n = 8). *, $p < 0.05$; **, $p < 0.01$; and NS = Not significant (paired t test).

Fig. 3 Effects of CSE on VEGF secretion in Mp-infected well-differentiated NHBE cells (a) and SAEC (b). Epithelial cells were infected with Mp in the presence or absence of CSE for 14 days. Data are expressed as Means ± SEM (n = 8). *, $p < 0.05$ (paired t test).

Fig. 4 Effects of CSE on cell growth and apoptosis in NHBE cells and SAEC. Cells were exposed to medium alone or 20% CSE for 14 days, and were harvested to examine cell morphology using hematoxylin and eosin (H&E) staining (original magnification, 200x) in untreated NHBE cells (a), CSE-treated NHBE cells (b), untreated SAEC (c), and CSE-treated SAEC (d). (e) – Total DNA content was determined using whole cell lysates and expressed as fold change over untreated cells. (f) – B-cell lymphoma-2 (Bcl-2) mRNA expression relative

levels in NHBE cells and SAEC were determined by quantitative real-time RT-PCR with data expressed as Mean ± SEM (n = 4-6). NS = not significant (paired *t* test).

Fig. 5 Role of MAPK in CSE-mediated VEGF reduction in NHBE cells and SAEC. Cells were treated with either medium alone or 20% CSE. Activation of all three MAPK pathways (p-MAPK/total MAPK ratio) was determined at indicated time points by Western blot and densitometry in NHBE cells (a - b) and SAEC (c). CSE exposure acutely (15 min) increased ERK1/2 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 JNK phosphorylation in NHBE cells as well as SAEC. For inhibitor studies, cells were treated with PD98059 (5 μM), a specific inhibitor of MEK1/2 (upstream of ERK1/2) for 2 and 24 h. (d) – Inhibition of ERK1/2 phosphorylation was verified by Western blot at 2 h. (e) – VEGF secretion was determined by an ELISA at 24 h. Data are expressed as Means ± SEM (n = 6). **, p <0.01; NS = Not significant (paired *t* test).

Fig. 6 Role of PKC in CSE-mediated VEGF reduction in NHBE cells and SAEC. Cells were pretreated a PKC inhibitor Ro318220 (10 μM) for 2 h, followed by 20% CSE treatment for 24 h. VEGF secretion was determined by an ELISA at 24 h post-CSE treatment in NHBE cells and SAEC. Data are expressed as Means ± SEM (n = 3).
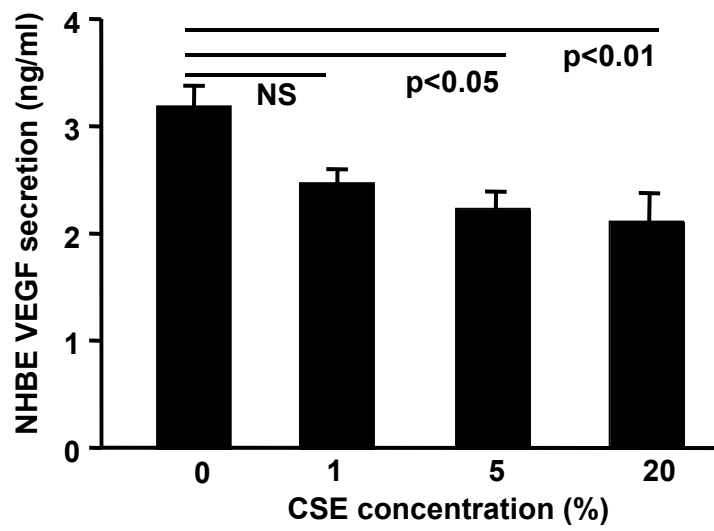
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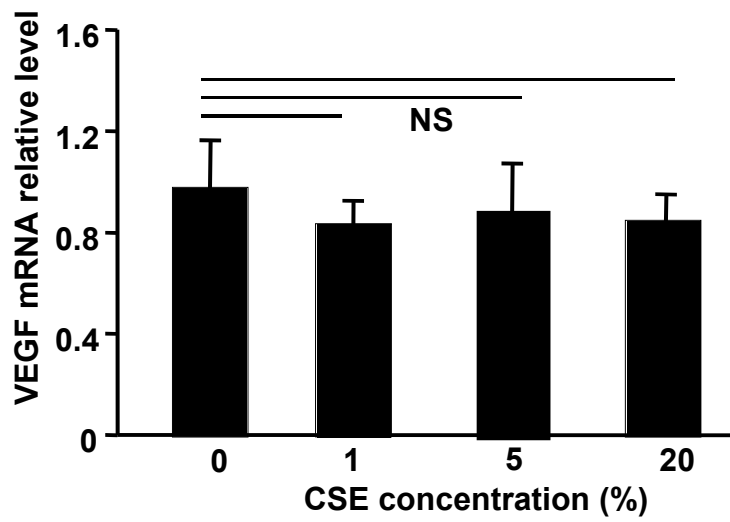
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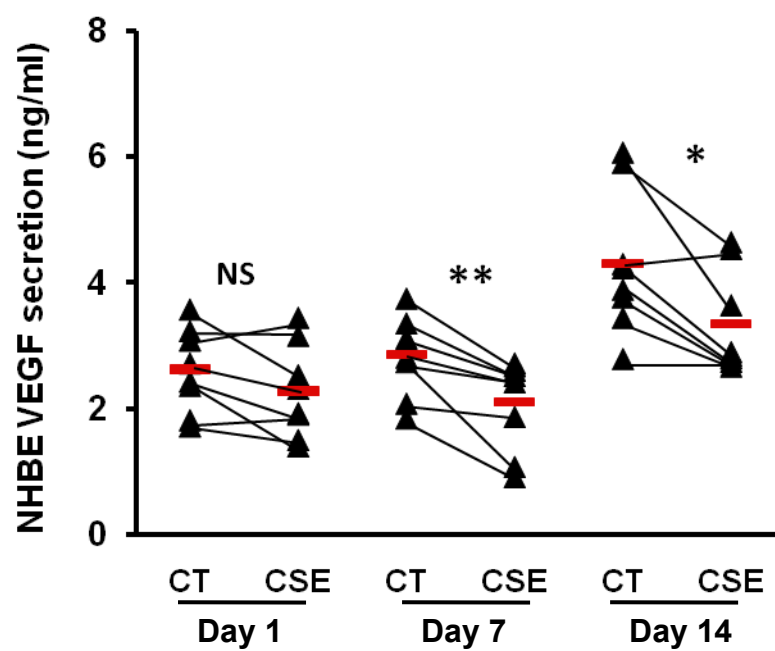
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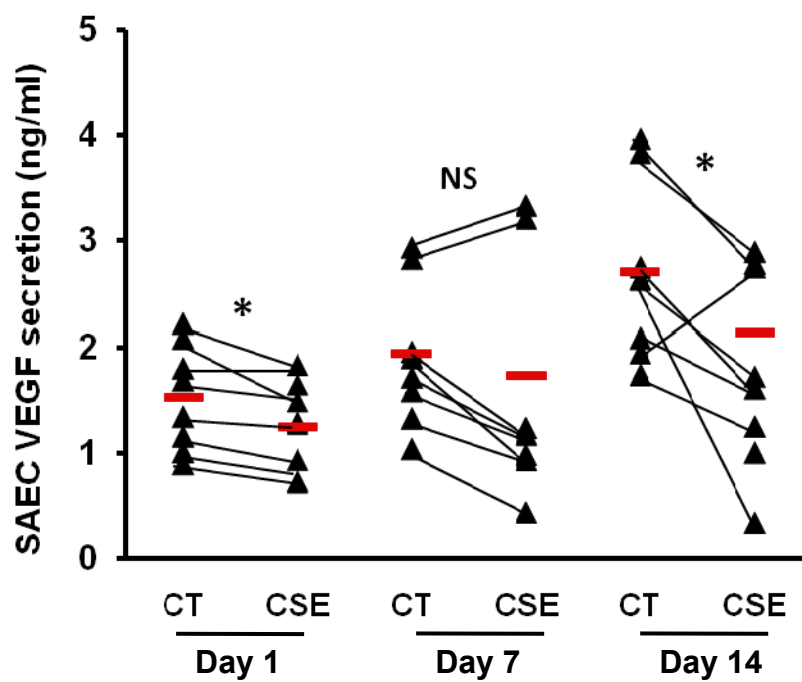
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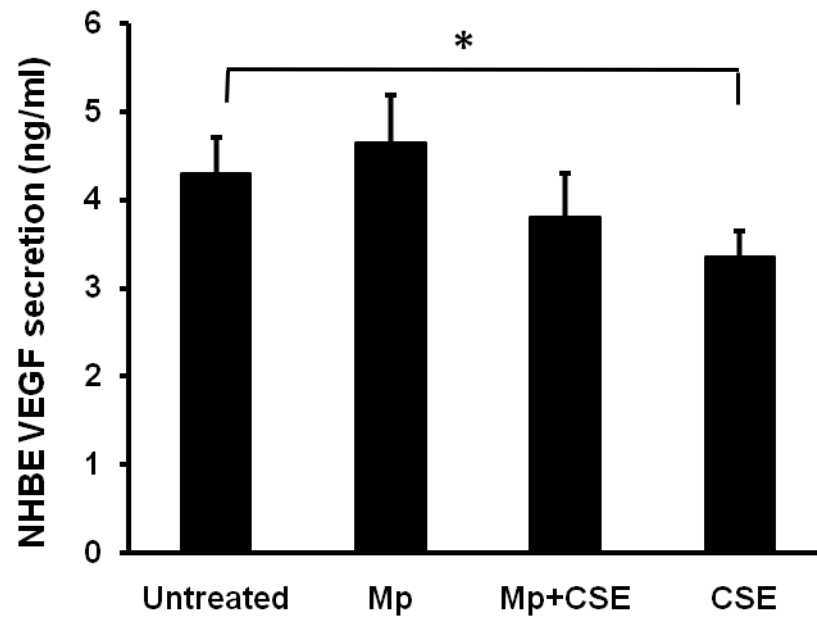
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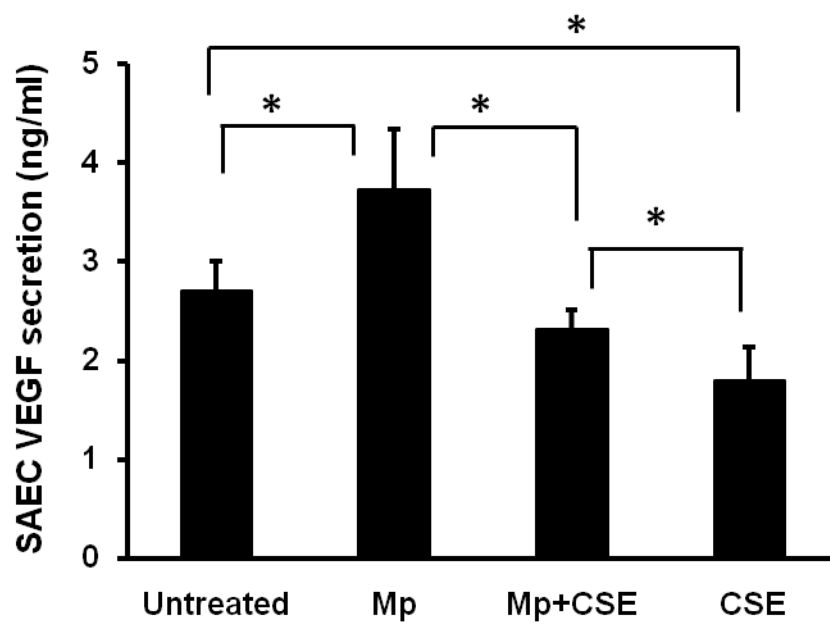
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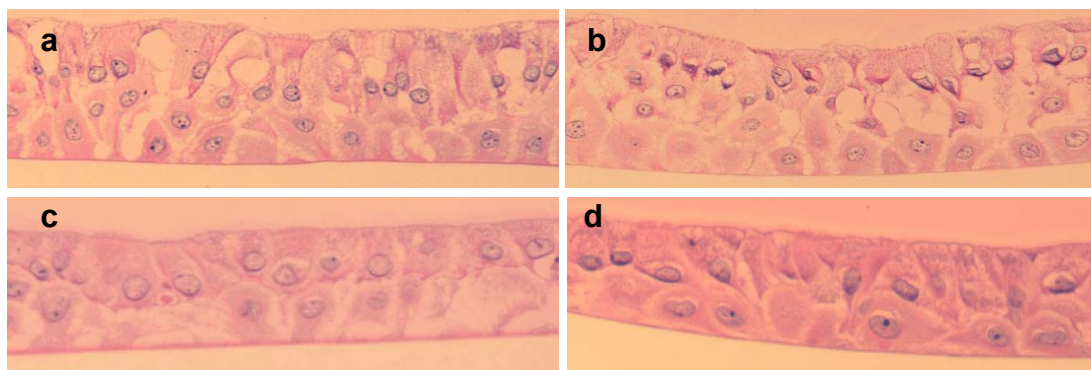
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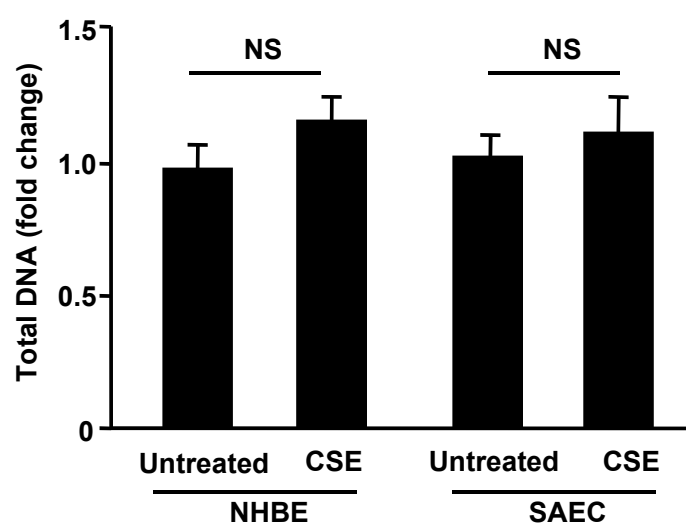
Fig. 4e

Fig. 4f

Fig. 5a

Fig. 5b

Fig. 5c

NHBE　　　**SAEC**

DMSO (0.1%)	+	+	+	+
PD98059 (5 μM)	−	+	−	+

Fig. 5d

DMSO (0.1%)	+	+	+	+
PD98059 (5 μM)	−	+	−	+

Fig. 5e

| CSE | + | + | | + | + |
| Ro318220 (10 µM) | − | + | | − | + |

Fig 6