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Cigarette Smoke Inhibits Lipopolysaccharide-Induced Production of Inflammatory Cytokines by Suppressing the Activation of Activator Protein-1 in Bronchial Epithelial Cells[1]

Martti Laan,* Steven Bozinovski,* and Gary P. Anderson[2]*†

Chronic smoking is characterized by immunosuppressive changes in the airways, leading to chronic colonization with bacteria, which in turn may contribute to the chronic obstructive pulmonary disease. The mechanisms causing this immunosuppression, however, are poorly characterized. This study evaluated whether cigarette smoke can inhibit endotoxin (LPS)-induced inflammatory cytokine production in bronchial epithelial cells and, if so, what the mechanisms are behind this effect. Pretreatment with cigarette smoke extract (CSE) concentration dependently inhibited the LPS-induced GM-CSF and IL-8 protein release, which was accompanied by decreased expression of mRNA in human bronchial epithelial cells (Beas-2B). The increase of neutrophil chemotaxis induced by conditioned medium from LPS-treated Beas-2B cells was also suppressed by CSE. In addition, the activity of LPS-induced transcription factor AP-1, but not NF-κB, was down-regulated by CSE. Notably, at the concentrations used, CSE had no effect on number or viability of Beas-2B cells. These data indicate that cigarette smoke possesses immunosuppressive properties by down-regulating the bacterial pathogen-induced neutrophil-mobilizing cytokine production via suppression of AP-1 activation in the airways. Hence, this study suggests a novel mechanism by which cigarette smoke may contribute to chronic colonization and chronic obstructive pulmonary disease in smokers. ***The Journal of Immunology,*** **2004, 173: 4164–4170.**

Chronic obstructive pulmonary disease (COPD)[3] is one of the most common reasons of death worldwide, afflicting, as estimated by World Health Organization, over 600 million people. COPD encompasses emphysema, the destruction of lung parenchyma that causes impaired gas exchange and small airway collapse, and chronic obstructive bronchitis, a chronic inflammatory cellular process that leads to small airway obstruction (1, 2).

The single most important factor in the development of COPD is cigarette smoke (3). Although the precise mechanisms behind the pathogenesis of COPD are not known, the current hypothesis suggests that cigarette smoke causes airway inflammation by activating bronchial epithelium, macrophages, and neutrophils, which by releasing proteases and free oxygen radicals cause injury of parenchymal tissue and bronchioles and consequent impairment in gas exchange and airflow (reviewed in Ref. 4). In accordance with this hypothesis, an increased number of neutrophils and macrophages as well as inflammatory cytokines and proteases have been found in the airways of COPD patients (1, 5), and the inflammatory properties of cigarette smoke have been demonstrated in several in vitro as well as in vivo models (6–8). However, recently, it has been shown that chronic airway inflammation persists and does not resolve even in COPD patients who have quit smoking (9, 10). Thus, the development of persistent inflammation and subsequent COPD is likely to involve other factors and mechanisms than the inflammatory effect of cigarette smoke alone.

In addition to its direct, inflammation-inducing effect on airways, chronic smoking is also characterized by profound immunosuppressive changes, which impair host defense and increase susceptibility to infections. Hence, healthy smokers have a greater likelihood of having a lower respiratory tract illness as well as longer duration of symptoms than nonsmokers, a difference even more pronounced among HIV-1-positive subjects (11, 12). Also, more importantly, while the lower airways of the healthy patients are sterile, the airways of COPD patients are chronically colonized with potential respiratory pathogens, representing predominantly Gram-negative bacteria such as nontypeable *Haemophilus influenza*, *Moraxella catarrhalis*, and *Pseudomonas aeruginosa* (reviewed in Ref. 13). There is now increasing amount of evidence from in vitro and in vivo studies that the chronic bacterial colonization can stimulate host immune system and cause a low-grade infection that induces chronic airway inflammation (13). Also, clinical studies on the colonization in COPD patients have shown a strong correlation between bacterial load and inflammatory cytokine and protease levels in the airways (14) as well as a negative correlation between bacterial load and airflow (15). Thus, it is plausible that chronic colonization of the airways in smokers contributes to the progression of COPD and is also responsible for the persistent airway inflammation and airway damage in ex-smokers. The mechanisms behind the immunosuppressive effect of cigarette smoke and the consequent bacterial colonization, however, are presently poorly characterized.

LPS or endotoxin is a predominant, integral structural component of the outer membrane of Gram-negative bacteria and one of the most potent microbial initiators of inflammation (reviewed in Ref. 16). LPS is recognized by TLR4, a component of the innate immune response, evolved to recognize pathogen-associated molecular patterns. The subsequent inflammatory reaction is essential

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[3] Abbreviations used in this paper: COPD, chronic obstructive pulmonary disease; CSE, cigarette smoke extract.

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for host defense and involves production of inflammatory cytokines and chemokines, which in turn coordinate the recruitment of neutrophils and macrophages. Accordingly, it has been demonstrated that mice deficient in TLR4 are characterized by diminished inflammatory cytokine production as well as substantial delay in clearance of *H. influenza* (17). Thus, an inhibition of Gram-negative bacteria/LPS-induced and TLR4-mediated inflammatory response is likely to result in impaired clearance and colonization and/or generalization of bacterial infection (18).

In the airways, the first line of defense against bacteria is bronchial epithelium (19). Bronchial epithelial cells express TLR4 (20) and are also a major source of inflammatory cytokines such as GM-CSF and IL-8 (19). Production of these inflammatory cytokines is under the control of several transcription factors such as NF-κB and AP-1 (21) and is essential for neutrophil recruitment in several models of airway inflammation (22, 23). Because airway epithelium is also the primary site of action for cigarette smoke, the modulation of LPS-induced inflammatory cytokine production by cigarette smoke in epithelial cells is likely to reflect the initial changes of the innate immune response in the airways of smokers.

The aim of this study was to evaluate whether cigarette smoke inhibits the LPS-induced production of inflammatory cytokines GM-CSF and IL-8 in bronchial epithelial cells. The study also evaluated whether the diminished GM-CSF and IL-8 release by cigarette smoke is due to inhibited production of these cytokines and whether these changes are functionally significant. To establish the mechanism behind the inhibited cytokine production, the involvement of transcription factors NF-κB and AP-1 was also studied.

Materials and Methods

Materials

The BEAS-2B cell line was purchased from American Type Culture Collection (Manassas, VA). All cell culture medium and supplements were purchased from Invitrogen Life Technologies (Paisley, U.K.), unless otherwise indicated.

Culture of human bronchial epithelial cells

Beas-2B cells were cultured in 1:1 mixture of keratinocyte/serum-free medium supplemented with 25 mg of bovine pituitary extract and 2.5 μg of epidermal growth factor, and Eagle's MEM supplemented with 10% FCS, 2 mM L-glutamine, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 1.5 g/L sodium bicarbonate and antibiotics (5 μg/ml gentamicin, 100 μg/ml penicillin, 100 μg/ml streptomycin) at 37°C in a humidified atmosphere containing 5% CO_2 in air. Cells were cultured in uncoated Nunclon T75 flasks and, for experiments, transferred to 6- or 12-well Nunclon Multidishes (all from Nunc, Roskilde, Denmark). Immediately before the start of each experiment, the cell culture was washed with PBS and placed in fresh growth medium. In the experiments for real-time PCR, EMSA, or chemotaxis (see below), the cells were placed after washing in DMEM supplemented with 1% of FCS and 5 μg/ml gentamicin to reduce basal (inherent) cytokine release (24).

Preparation of cigarette smoke extract (CSE) and treatment of cells

CSE was prepared by a modification of a previously described method (25) using filtered Winfield (1.2 mg of nicotine, 16 mg of tar, 15 mg of CO; Phillip Morris, Moorabbin, Australia) cigarettes. The smoke was bubbled through 25 ml of cell culture medium by a syringe-driven apparatus. A 30-ml vol of smoke was drawn within 10 s, followed by a 20-s break. This process was repeated eight times per cigarette. We previously determined that this method of preparing CSE produces a consistent working solution by using OD and bioassays (activation of macrophage-like THP-1 cells).

Beas-2B cells were grown to confluence and washed with PBS before CSE or vehicle (the same medium without CSE) exposure. To mimic smoking of one cigarette, the cells were exposed to CSE for 15 min. The CSE was removed thereafter; the cells were washed with PBS, placed in fresh medium, and subsequently treated with LPS (1 μg/ml, *Escherichia coli* serotype 026:B6; Sigma-Aldrich, St. Louis, MO) or vehicle (PBS). Cell supernatants were collected at 2, 6, and 18 h for cytokine measurements.

In experiments with multiple CSE exposures, the cells were treated with CSE or vehicle for 15 min, followed by LPS treatment (see above), and then at every 2 h by CSE or vehicle for 15 min by removing the cell supernatant for the time of CSE exposure and replacing the same supernatant thereafter. A total of 20 μl of supernatant was collected before every exposure for cytokine measurements.

To study the effect of MAPKs on the inhibitory effect of CSE, the p38 inhibitor SB 203580 (10 μM; Sigma-Aldrich), the MEK1/2 inhibitor U0126 (10 μM; Cell Signaling Technology, Beverly, MA), and the PI3K inhibitor wortmannin (1 μM; Sigma-Aldrich) were used. The cells were pretreated with the inhibitors for 1 h, exposed to CSE for 15 min thereafter (see above), and subsequently treated with LPS (1 μg/ml) for 2 or 6 h.

ELISAs

Cell-free supernatants were collected and stored at −80°C. After thawing, the levels of IL-8 or GM-CSF were analyzed, according to the manufacturer's instructions, using commercial human IL-8 or human GM-CSF OptEIA ELISA sets (BD Pharmingen, San Diego, CA).

RNA extraction and real-time PCR

Beas-2B cells were grown to confluence in six-well plates and pretreated with CSE or vehicle, followed by treatment with LPS or vehicle for 20, 60, or 180 min. The cells were then harvested by scraping, centrifuged (1500 × *g* for 5 min), snap frozen in liquid nitrogen, and stored at −80°C. Total RNA was isolated from pooled samples of three separate experiments, according to the manufacturer's instructions, using the RNeasy kit (Qiagen, Helden, Germany). The purified total RNA prep was used as a template to generate first-strand cDNA synthesis using SuperScript II (Invitrogen Life Technologies). The reaction mix containing 1 μg of RNA, 250 ng of random hexamers (Promega, Madison, WI), and 10 mM dNTP mix was diluted to 12 μl in sterile water, heated to 65°C for 5 min, and chilled on ice for 1 min. First strand synthesis was then performed in 20 μl total reaction volume by adding 50 mM Tris.HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 40 U of RNaseout, and 200 U of Superscript II reverse-transcriptase enzyme at 42°C for 50 min. The reaction was inactivated by heating at 70°C for 15 min. cDNA was diluted 5-fold in sterile water and stored at −20°C before amplification.

The quantitative PCR was performed by ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA) using predeveloped Taqman probe/primer combinations for 18S rRNA, GM-CSF, and IL-8 optimized by the manufacturer. Taqman PCR was performed in 10-μl vol using AmpliTaq Gold polymerase and universal master mix (Applied Biosystems). Threshold cycle numbers were transformed using the ΔΔCt (threshold cycle) and relative value method, as described by the manufacturer, and were expressed relative to 18S rRNA, which was used as a housekeeping gene by multiplexing single reactions.

Neutrophil chemotaxis

Neutrophil chemotaxis assay was performed in Transwell 24-well plates (Corning Glass, Corning, NY) with 3-μm polycarbonate membrane filters, as previously described (26). Briefly, Beas-2B cells were grown to confluence in bottom wells of Transwell plates. The cells were pretreated with CSE (1.6 cigarettes/25 ml) or vehicle for 15 min, washed with PBS, and placed in fresh medium (DMEM with 1% FCS and 5 μg/ml gentamicin). Then the cells were treated with LPS (1 μg/ml) or vehicle and incubated for 3 h. Shortly before adding neutrophils to the upper compartment, the conditioned medium was transferred to wells without Beas-2B culture, to avoid adherence of migrated neutrophils to bronchial cells.

Neutrophils were isolated from peripheral blood of healthy volunteers. Whole heparinized blood was sedimented using Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden), to remove mononuclear cells and platelets. Erythrocytes were lysed in the remaining pellet using erythrocyte lysis buffer (Qiagen), according to the manufacturer's instructions. Then the cells were washed twice with DMEM and brought to the final concentration of 3×10^6/ml in DMEM (with 1% FCS and 5 μg/ml gentamicin). A total of 100 μl of this neutrophil suspension was added to the upper compartment of Transwells and incubated for 2 h in 5% CO_2 at 37°C. After incubation, 25 mM EDTA was added to the bottom wells for 5 min to release any cells that were adhered to the underside of the filter. Cell samples from the bottom wells were counted in duplicates by hemocytometer and expressed as total number of migrated cells/ml.

Activity of transcription factors AP-1 and NF-κB

Preparation of nuclear extracts. Beas-2B cells were grown to confluence in six-well plates and pretreated with CSE or vehicle, followed by treatment with LPS or vehicle for 20, 60, or 180 min. The cells were then harvested by scraping, centrifuged (1500 × *g* for 5 min), snap frozen in liquid nitrogen, and stored at −80°C. For preparation of nuclear extracts,

pooled samples from three separate experiments were resuspended in 200 μl of nuclear lysis buffer 1 (10 mM HEPES (pH 7.6), 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 5 mM 2-ME, 0.2% Nonidet P-40, and 0.2% protease inhibitor mixture set III (Calbiochem, La Jolla, CA)) for 10 min on ice. Nuclei were pelleted by centrifugation at 800 $\times$ *g* for 30 s and lysed in 100 μl of nuclear lysis buffer 2 (50 mM HEPES (pH 7.6), 400 mM KCl, 0.1 mM EDTA, 10% glycerol, 5 mM 2-ME, and 0.2% protease inhibitor mixture set III). Following a 30-min incubation on ice, the nuclei extract (supernatant) was retained following centrifugation for 10 min (400 $\times$ *g* at 4°C).

Electromobility shift assay. The following pairs of complementary oligonucleotides with 4 nt overhangs were used to generate dsDNA for NF-κB and AP-1 binding sites: AP-1 sense, 5′-CATGCGCTTGATGAGTCAGC CGGAA-3′, and AP-1 antisense, 5′-CTAGTTCCGGCTGACTCATCAA GCG-3′; NF-κB sense, 5′-CATGCAACAGAGGGGACTTTCCGAGAGG, and NF-κB antisense, 5′-CATGCCTCTCGGAAAGTCCCCTCTGTTG-3′. Complementary pairs of oligonucleotides (50 ng) were annealed and radiolabeled using 5 U of Klenow fragment (Promega) in the presence of 50 mM Tris-HCl (pH 7.2), 10 mM $MgSO_4$, 0.1 mM DTT, 0.2 mM dGTP, 0.2 mM dCTP, 0.2 mM dTTP, and 100 μCi of [α-^{32}P]dATP. Labeled probes were then separated from unincorporated isotope by size exclusion chromatography using Microspin G-25 columns (Amersham Biosciences). Nuclear extracts (5 μl) were used in binding assays in 20-μl reactions and incubated for 20 min at room temperature with labeled probe (2 μl, 50,000 cpm), distilled water (9 μl), and binding buffer (4 μl). For AP-1, the binding buffer contained 50 mM Tris.HCl (pH 7.5), 5 mM $MgCl_2$, 250 mM NaCl, 25 mM DTT, 2.5 mM EDTA, and 20% glucerol. For NF-κB, the binding buffer contained 50 mM HEPES (pH 7.9), 250 mM KCl, 12.5 mM DTT, 0.5 mM EDTA, 0.25% Nonidet P-40, and 50% glucerol. Fifteen microliters of binding reaction were loaded onto 7.5% polyacrylamide gel and run at 200 V for 40 min. Gels were then dried and exposed to autoradiography using an Intensifier screen at −80°C. Densitometry was performed using Kodak EDAS 1D image analysis software and expressed as net intensity of background-subtracted values.

Cell survival

Confluent Beas-2B cells were treated with increasing concentrations of CSE, 1% NaN_3 (positive control), or vehicle for 15 min; washed with PBS; and incubated for 18 h in complete cell culture medium. The cells were trypsinized thereafter, and cell number and viability were determined (in triplicates) by ethidium bromide/acridine orange fluorescent viability stains (Molecular Probes, Eugene, OR) using hemocytometer. Data are expressed as percentage of CSE-treated living cells to vehicle-treated living cells.

Results

The effect of CSE on LPS-induced inflammatory cytokine release

LPS induced a significant increase in GM-CSF and IL-8 proteins at 2, 6, and 18 h (Fig. 1). In the concentration range from 0.1 to 1.6 cigarettes/25 ml medium, pretreatment with CSE concentration dependently inhibited the LPS-induced release of GM-CSF at 2, 6, and 18 h (Fig. 1). In the same concentration range, CSE also inhibited the LPS-induced IL-8 release at 2 and 6 h, but not at 18 h (Fig. 1).

In the concentration range from 0.1 to 1.6 cigarettes/25 ml medium, the GM-CSF levels were undetectable in nearly all cell supernatants at 2, 6, or 18 h after treatment with CSE alone. The basal IL-8 levels were concentration dependently suppressed by CSE treatment alone at 2 h (Spearman correlation, $p < 0.001$, $r = -0.8$, $n = 24$; difference at 1.6 cigarettes/25 ml medium, vehicle 136.2 ± 5.8 vs CSE 27.0 ± 5.9), but had no significant effect at 6 ($p = 0.3$, $r = 0.2$, $n = 24$, difference at 1.6 cigarettes/25 ml medium: vehicle 534.5 ± 39.7 vs CSE 780.5 ± 129.8) or at 18 h ($p = 0.2$, $r = 0.3$, $n = 24$; difference at 1.6 cigarette/25 ml medium, vehicle 922.8 ± 112.4 vs CSE 1099.8 ± 112.8 pg/ml).

In separate experiments, responsiveness of primary human bronchial epithelial cells (Clonetics, Biowhittaker, Walkersville, MD) to LPS was also studied. In our hands, these cells were completely refractory to LPS in culture, presumably reflecting culture dedifferentiation, precluding replication of this finding in primary human cells (data not shown).

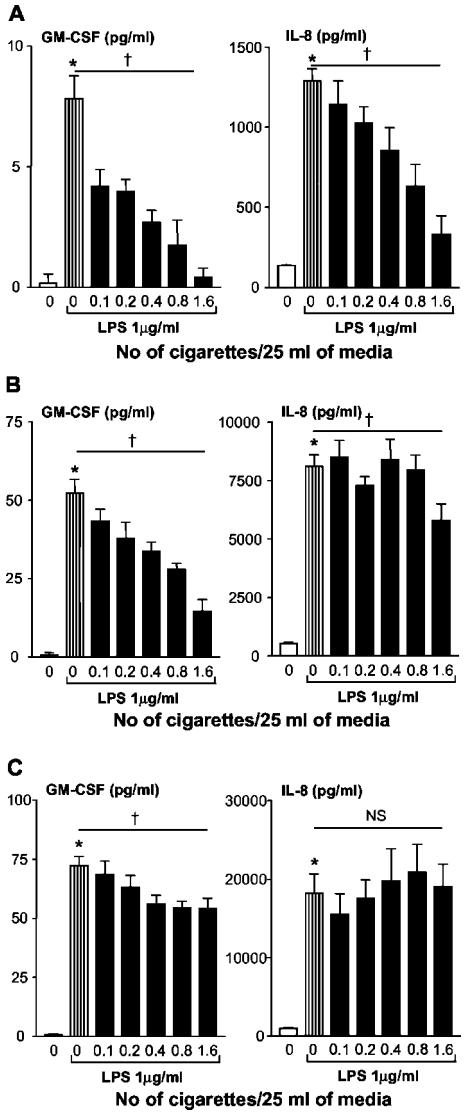

FIGURE 1. Effect of CSE on LPS-induced GM-CSF and IL-8 protein release in bronchial epithelial cells. Beas-2B cells were pretreated with CSE or vehicle for 15 min, followed by treatment with LPS (1 μg/ml) or vehicle, and incubated for 2 (*A*), 6 (*B*), or 18 h (*C*). The levels of GM-CSF and IL-8 were measured in cell supernatants by ELISA. Treatment with LPS significantly (Mann-Whitney, $p < 0.05$, $n = 4$) induced the release of GM-CSF and IL-8 at 2, 6, as well as 18 h as compared with vehicle. CSE concentration dependently inhibited the LPS-induced GM-CSF release at 2 (Spearman rank correlation $p < 0.001$, $r = -0.8$, $n = 20$), 6 ($p < 0.001$, $r = -0.9$), as well as 18 h ($p < 0.001$, $r = -0.8$). CSE concentration dependently inhibited the LPS-induced IL-8 release at 2 (Spearman rank correlation $p < 0.001$, $r = -0.8$, $n = 20$) and 6 h ($p < 0.05$, $r = -0.4$), but not at 18 h ($p = 0.3$, $r = 0.2$). Data are mean with SEM of separate experiments. *, $p < 0.05$ compared with vehicle; †, $p < 0.05$ for correlation between CSE concentration and GM-CSF or IL-8 concentration.

The effect of CSE on TNF-α-induced IL-8 release

In Beas-2B cells, treatment with 100 ng/ml TNF-α resulted in a significant (Mann-Whitney, $p < 0.05$, $n = 4$) increase in IL-8 release at 2 (vehicle 1.4 ± 0.3 vs TNF-α 844.9 ± 57.1 pg/ml), 6 (vehicle 3.2 ± 0.8 vs TNF-α 4567.6 ± 205.3 pg/ml), as well as 18 h (vehicle 8.7 ± 1.8 vs TNF-α 18 727.6 ± 270.4). Pretreatment with CSE (1.6 cigarettes/25 ml medium for 15 min) significantly (Mann-Whitney, $p < 0.05$, $n = 4$) inhibited the TNF-α-induced increase in IL-8 at 2 (CSE + TNF-α 100 ng/ml, 29.1 ± 3.7 pg/ml), but not at 6 (5096.8 ± 274.1 pg/ml) or 18 h (17 468.3 ± 2170.6 pg/ml).

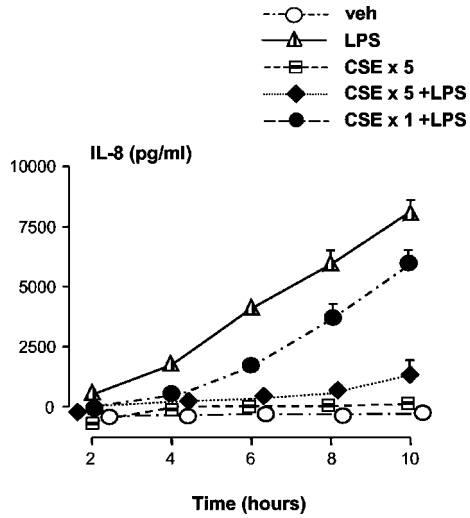

FIGURE 2. Effect of multiple CSE exposures on LPS-induced IL-8 protein release in bronchial epithelial cells. Beas-2B cells were exposured to CSE (1.6 cigarettes/25 ml medium) or vehicle for 15 min at every 2 h. After the first exposure, the cells were treated with LPS (1 μg/ml) or vehicle. The levels of IL-8 were measured in cell supernatants by ELISA. Multiple CSE exposure significantly inhibited the LPS-induced IL-8 release compared with LPS treatment at all time points measured (Mann-Whitney, $p < 0.05$, $n = 6$). At 6, 8, and 10 h, the IL-8 levels after multiple CSE plus LPS treatment were also significantly lower than the ones after single CSE plus LPS treatment (Mann-Whitney, $p < 0.05$, $n = 6$). Data are mean with SEM of separate experiments.

The effect of multiple CSE exposure on LPS-induced inflammatory cytokine release

Multiple 15-min CSE exposures at every 2 h resulted in a sustained inhibition of LPS-induced IL-8 release (Fig. 2).

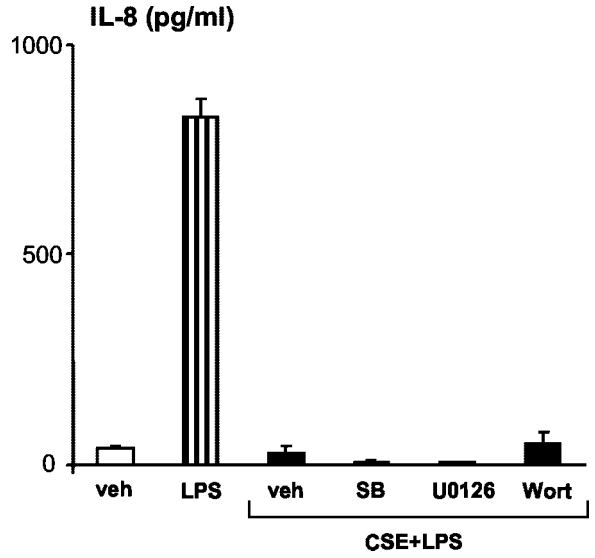

FIGURE 3. Effect of MAPK inhibitors on CSE-induced suppression of IL-8 release in bronchial epithelial cells. Beas-2B cells were pretreated with SB 203580 (SB, 10 μM), U0126 (10 μM), or wortmannin (Wort, 10 μM) for 1 h, and subsequently were exposed to CSE (1.6 cigarettes/25 ml medium) for 15 min, followed by LPS treatment for 2 h. The levels of IL-8 were measured in cell supernatants by ELISA. None of the MAPK inhibitors used had a significant effect on CSE-induced suppression of IL-8 release (Mann-Whitney, $p > 0.05$, $n = 4$). Data are mean with SEM of separate experiments.

The effect of MAPK inhibitors on CSE-induced inhibition of inflammatory cytokines

At 2 h, the p38 inhibitor SB 203580, the MEK1/2 inhibitor U0126, and the PI3K inhibitor wortmannin had no effect on CSE-induced inhibition of IL-8 release induced by LPS (Fig. 3). The same was true when other time points (6 h) or inflammatory cytokines (GM-CSF) were studied (data not shown).

The effect of CSE on LPS-induced inflammatory cytokine mRNA expression

LPS induced a >2500-fold increase in GM-CSF mRNA expression at 180 min in Beas-2B cells (Fig. 4*A*). This increase in GM-CSF expression was completely inhibited by pretreatment with CSE (Fig. 4*A*). LPS also induced a 2000-fold increase in IL-8 mRNA expression at 180 min in Beas-2B cells (Fig. 4*B*). This increase in IL-8 expression was also inhibited by CSE (Fig. 4*B*).

The effect of CSE on LPS-induced neutrophil chemotaxis

LPS treatment resulted in a significant release of neutrophil chemotactic factors in Beas-2B cells (Fig. 5.) This increase in neutrophil chemotaxis was inhibited by pretreatment with CSE (Fig. 5).

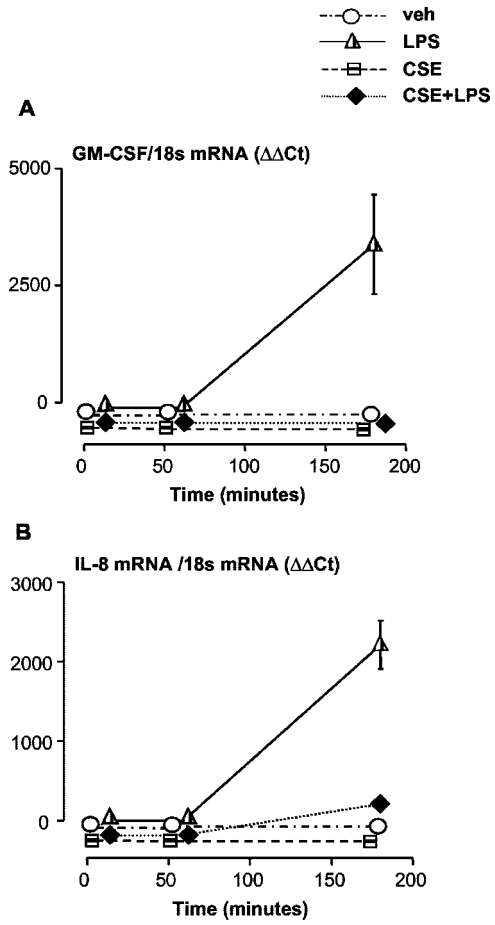

FIGURE 4. Effect of CSE on LPS-induced GM-CSF (*A*) and IL-8 (*B*) mRNA expression in bronchial epithelial cells. Beas-2B cells were pretreated with CSE (1.6 cigarettes/25 ml medium) or vehicle for 15 min, followed by treatment with LPS (1 μg/ml) or vehicle, and incubated for 20, 60, or 180 min. The expression of GM-CSF and IL-8 mRNA was detected by real-time PCR, as described in *Materials and Methods*. Treatment with LPS resulted in an increase in GM-CSF as well as IL-8 mRNA expression at 180 min. This increase was inhibited by pretreatment with CSE. Data are mean with SEM of triplicate measurements of pooled samples from three separate experiments.

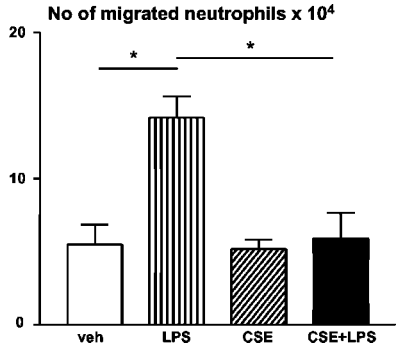

FIGURE 5. Effect of CSE on LPS-induced neutrophil chemotaxis in bronchial epithelial cells. Beas-2B cells were pretreated with CSE (1.6 cigarettes/25 ml medium) or vehicle for 15 min, followed by treatment with LPS (1 μg/ml) or vehicle, and incubated for 2 h. The conditioned medium from these cells was used as chemotactic stimuli for neutrophils using Transwell membranes. The conditioned medium from LPS-treated Beas-2B cells significantly (Mann-Whitney, $p < 0.05$, $n = 6$) increased neutrophil chemotaxis. This increase in neutrophil chemotaxis was inhibited by pretreatment with CSE (Mann-Whitney, $p < 0.05$, $n = 6$). Data are mean with SEM of six separate experiments; $*$, $p < 0.05$.

The effect of CSE on LPS-induced activation of transcription factors

LPS treatment resulted in a 3.5-fold increase in AP-1 activity in Beas-2B cells (Fig. 6). This increase in AP-1 activity was inhibited by pretreatment with CSE (Fig. 6).

In contrast, LPS treatment did not result in a remarkable increase in NF-κB activity in Beas-2B cells; neither was the basal activity of NF-κB remarkably altered by CSE (Fig. 7).

The effect of CSE on Beas-2B cell survival

In the concentration range used for inhibition studies, CSE had no effect on the number of living cells (1.6 cigarettes/25 ml medium, 18 h: 103.7 $\pm$ 5.9% compared with vehicle, $n = 4$). Neither was cell survival after cotreatment with LPS (1 μg/ml) and CSE (1.6 cigarettes/25 ml medium) different from the one after treatment with LPS alone (100.4 $\pm$ 6.6% compared with vehicle, $n = 4$). In contrast, treatment with 1% NaN_3 (positive control) for 15 min resulted in a significant reduction cell survival (21.0 $\pm$ 11.8% compared with vehicle, Mann-Whitney: $p < 0.05$, $n = 4$) (Fig. 5).

Discussion

It is known that chronic smoking impairs local immune defenses and causes bacterial colonization in the airways, which in turn may contribute to the development of COPD. It has been suggested that suppression of T cell as well as macrophage function or diminished mucociliary flow by cigarette smoke might be, at least partly, responsible for this weakened immune response (27–29). Whether cigarette smoke also alters the response to bacterial pathogens/LPS in bronchial epithelial cells and whether this involves the production of neutrophil-mobilizing cytokines have not been previously characterized.

It has been demonstrated that production of IL-8 as well as GM-CSF is critical for neutrophil recruitment, and inhibition of either of these cytokines results in reduced influx of neutrophils and also in increased susceptibility to respiratory infections (30, 31). Studies on the kinetics of the inflammatory responses in the airways in vivo have shown that LPS challenge causes an early increase in inflammatory cytokines, peaking at 2–3 h, followed by

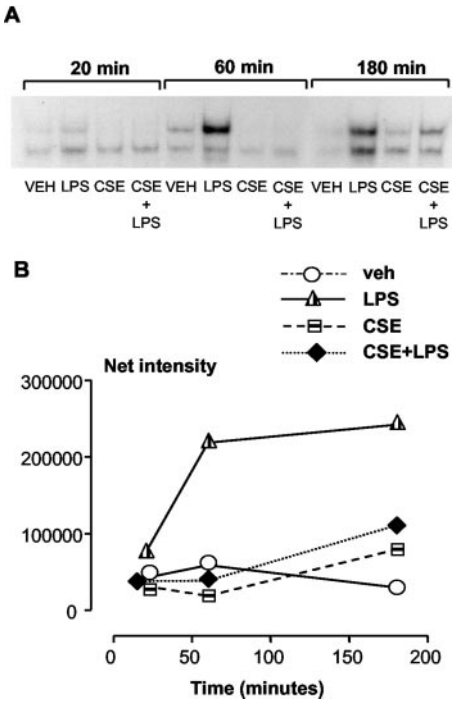

FIGURE 6. Effect of CSE on LPS-induced AP-1 activity in bronchial epithelial cells shown as autoradiograph (*A*) and net intersity of background subtracted values (*B*). Beas-2B cells were pretreated with CSE (1.6 cigarettes/25 ml medium) or vehicle for 15 min, followed by treatment with LPS (1 μg/ml) or vehicle, and incubated for 20, 60, or 180 min. The activity of AP-1 was detected by EMSA, as described in *Materials and Methods*. Treatment with LPS resulted in an increase in AP-1 activity at 60 as well as 180 min. This increase was inhibited by pretreatment with CSE. Data are mean values of pooled samples from three separate experiments.

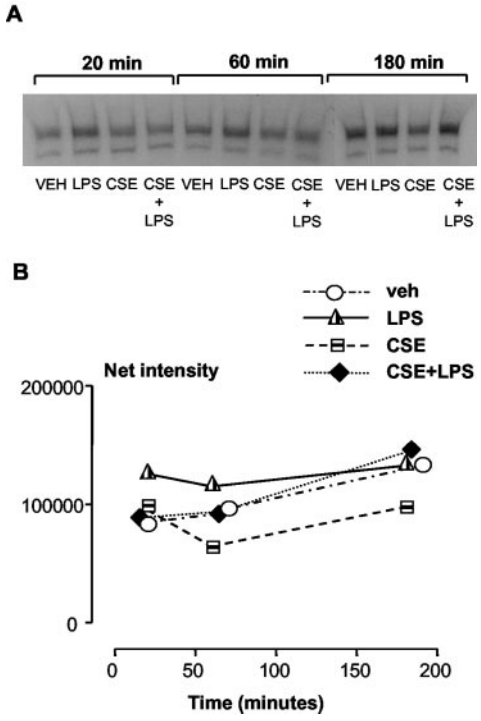

FIGURE 7. Effect of CSE on LPS-induced NF-κB activity in bronchial epithelial cells shown as autoradiograph (*A*) and net intensity of background subtracted values (*B*). Beas-2B cells were pretreated with CSE (1.6 cigarettes/25 ml medium) or vehicle for 15 min, followed by treatment with LPS (1 μg/ml) or vehicle, and incubated for 20, 60, or 180 min. The activity of NF-κB was detected by EMSA, as described in *Materials and Methods*. There was no major difference in NF-κB activity after LPS or CSE treatment at any of the time points studied. Data are mean values of pooled samples from three separate experiments.

a consequent neutrophil influx (32). This study shows that a single 15-min CSE exposure potently inhibits the LPS-induced early release of GM-CSF and IL-8, whereas the inhibitory effect weakens (GM-CSF) or disappears (IL-8) at later time points. As suggested by complete inhibition of LPS-induced GM-CSF and IL-8 mRNA by cigarette smoke, the decreased release of these neutrophil-mobilizing cytokines is due to diminished de novo production. This decrease in inflammatory cytokine production is also functionally significant, resulting in an inhibition of LPS-induced neutrophil chemotaxis.

This single CSE exposure used in this study aimed to mirror the effect of smoking one cigarette on innate immune response. An average smoker, however, smokes >13 cigarettes/day (33), and is thus likely to have the next cigarette before the recovery of the cytokine production. To address this issue, we used multiple 15-min CSE exposures with 2-h intervals, which resulted in a sustained inhibition of LPS-induced IL-8 release. Thus, as a consequence, smoking may result in a constant suppression of neutrophil mobilization in response to bacterial pathogens.

Clinical studies have shown that smoking-induced chronic bronchitis and COPD are characterized by increased levels of inflammatory cytokines and inflammatory cells in the airways. However, these changes reflect a disease state, which takes tens of years to develop, and therefore may not indicate the initiating mechanisms. It is therefore of considerable interest that a recent clinical study on acute effects of cigarette smoke in human healthy smokers, smoking two cigarettes, showed a decrease in airway neutrophil numbers at 3 h, consistent with our data (34). There is also increasing amount of evidence that chronic smoking causes colonization of the airways by bacteria and that this colonization contributes to the inflammatory changes in the airways (13–15, 35). It is thus plausible that the immunosuppressive effect of cigarette smoke, described in this study, may result in a delayed clearance and consequent colonization of bacteria, which, indirectly, results in chronic airway inflammation and contributes to the development of COPD.

Gram-negative bacteria such as *H. influenza*, *M. catarrhalis*, and *P. aeruginosa* comprise by far the most important group of colonizing bacteria seen in chronic smokers with COPD (13), and LPS is the most important component behind the innate response to these bacteria (16). Therefore, this study focuses on the effects of CSE on LPS-induced cytokine release in bronchial epithelial cells, which are the first line defense against bacteria in the lungs and also the primary site of cigarette smoke exposure. We also show that CSE inhibits the release of IL-8 induced by TNF-α, a common mediator of Gram-negative and Gram-positive (36) as well as viral (37) and fungal (38) infections. Thus, it is likely that CSE possesses broad immunosuppressive properties and may inhibit innate immune responses to a variety of different stimuli.

In bronchial epithelial cells, GM-CSF and IL-8 expression require activation of two distinct signaling pathways leading to activation of the transcription regulators AP-1 and NF-κB (21). In this study, we show that the AP-1 activation in Beas-2B cells is up-regulated by LPS and that pretreatment with CSE results in a transient inhibition of this LPS-induced increase in AP-1 activity. The activity of NF-κB, in contrast, although present even in unstimulated cells, is not altered either by LPS or CSE treatment. Thus, this study suggests a role for AP-1, but not NF-κB, in coordinating both the inflammatory effect of LPS and the immunosuppressive actions of cigarette smoke in bronchial epithelial cells. However, it cannot be ruled out that the basal activity of NF-κB is required for induction of inflammatory genes in this cell system.

Also, it has been shown that at certain concentrations, the treatment with CSE results in increased apoptosis and/or necrosis of bronchial epithelial cells and fibroblasts (39, 40). In this study, treatment with CSE, used in the concentration range and time frame sufficient for suppression of cytokine production, did not result in an increased cell death. Thus, the inhibitory effect by CSE seen in our study is unlikely to result from the toxic effects of cigarette smoke.

Previous studies on the effects of cigarette smoke have shown that CSE per se induces IL-8 release in bronchial epithelial cells (6, 41). In these studies, the irritant was left in contact with the cells for extended periods. However, during normal smoking the epithelium is exposed to an initially high concentration of smoke constituents, which immediately begins to decrease. Hydrophilic constituents, such as nicotine (42), clear very rapidly, whereas lipophilic molecules may be retained if they partition into the cell membrane lipid compartment, as has been demonstrated for the very well-established kinetic behavior of inhaled therapeutic molecules of differing lipophilicity (43). We therefore modeled our exposure on these known kinetic parameters. Accordingly, we have pulsed cells with boluses of CSE, mimicking single or multiple cigarette smoke exposures, and observed a slight decrease in basal IL-8 release at 2 h and no effect at later time points. Thus, although detailed analysis of this discrepancy requires further evaluation, it is likely that different exposure strategies alter the nature of this response.

In conclusion, this study shows that CSE inhibits the LPS-induced GM-CSF and IL-8 production in human bronchial epithelial cells, which is preceded and paralleled by inhibition of LPS-induced AP-1 activation. These data indicate that cigarette smoke might possess immunosuppressive properties in the airways by down-regulating the bacterial pathogen-induced production of neutrophil-mobilizing cytokines via inhibition of AP-1 activation. Thus, this study suggests a novel mechanism by which cigarette smoke may contribute to chronic colonization of the airways and development of COPD in smokers.

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