Cigarette Smoke Decreases Innate Responses of Epithelial Cells to Rhinovirus Infection

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Exposure to cigarette smoke is associated with a significant increase in the risk for respiratory viral infections. The airway epithelium is the primary target for both cigarette smoke and respiratory viral infection. We investigated the effects of cigarette smoke on the response of airway epithelial cells to rhinovirus infection. We found that pre-exposure of BEAS-2B cells or primary normal human bronchial epithelial cells (NHBEs) to cigarette smoke extract (CSE) reduced the induction of mRNA of the chemokines CXCL10 and CCL5 by either the viral mimic polyinosine-polycytidylic acid (Poly I:C) or human rhinovirus 16 (HRV-16) infection. The HRV-16-induced release of CXCL10 and CCL5 was also significantly suppressed by CSE. Activation of the IFN mediator STAT-1 and the activation of JNK by poly I:C and HRV-16 were partially suppressed by pre-exposure to CSE. In contrast, the poly I:C-induced and HRV-16-induced phosphorylation of ERK1/2 was unaffected by CSE. HRV-16-stimulated IFN- β mRNA was also significantly reduced by CSE. Because suppression of the IFN response to viral infection was associated with increased viral production, we assessed HRV-16 RNA concentrations. Exposure to CSE resulted in an increase in HRV-16 RNA at 48 hours after the infection of BEAS-2B cells. These data demonstrate that exposure to CSE alters the response of airway epithelial cells to HRV infection, leading to decreased activation of the IFN-STAT-1 and SAP-JNK pathways, the suppression of CXCL10 and CCL5 production, and increased viral RNA. A diminished, early epithelial-initiated antiviral response to rhinovirus infection could contribute to the increased susceptibility of subjects to prolonged respiratory viral infections after exposure to cigarette smoke.

Keywords: cigarette smoke; respiratory infections; rhinovirus; epithelial cells

Cigarette smoke is a significant public health problem, and one of the main preventable causes of morbidity and mortality (1). Adverse effects of cigarette smoke include increased risk of heart disease, cancer, and chronic obstructive lung disease. Overwhelming evidence exists that cigarette smoke is also associated with a significant increase in the risk of respiratory infections, and smoking is associated with both increased prevalence and severity of upper respiratory infections (URIs) (2–5). Exposure to cigarette smoke is a major risk factor for the development of URIs in children (6), and was identified as a risk factor in lower respiratory tract complications of rhinovirus infection in older adults (7). Furthermore, a recent study found that patients hospitalized because of rhinovirus-induced exacerbations of asthma were significantly more likely

CLINICAL RELEVANCE

Cigarette smoking is associated with a substantial increase in the risk for clinically relevant respiratory infections. Rhinovirus infections are the most common cause of acute upper respiratory illnesses, and are linked to exacerbations of asthma and chronic obstructive pulmonary disorder. Our study suggests that cigarette smoke blunts the response of airway epithelial cells to rhinovirus infection, which may explain the increased susceptibility of smokers to respiratory viral infections.

to be current smokers than nonsmokers (odds ratio, 11.2) (8). However, the mechanisms by which exposure to cigarette smoke increases the risk of respiratory infections are not entirely clear.

Cigarette smoke affects the first lines of defense within the airway, i.e., enhancing airway epithelial permeability, increasing tissue disruption, and impairing mucociliary clearance (9). Increased numbers of neutrophils and macrophages as well as proinflammatory cytokines are found within the airway after either acute or chronic cigarette smoke exposure (10–12). Several groups showed that exposure to cigarette smoke increases airway inflammation and worsens outcomes in influenza-infected mice (13, 14). Furthermore, exposure to cigarette smoke suppressed virally induced Th1 cytokine production and increased viral expression in a mouse model of respiratory syncytial viral infection (15). Cigarette smoke was also shown to exert detrimental effects on the immunologic host defenses that may interfere with effective and efficient antimicrobial responses (16). Several groups reported that cigarette smoke can inhibit basal and stimulated cytokine production from cultured cell lines and primary cells (17–20). In addition, alveolar macrophages isolated from smokers exhibited a reduced secretion of cytokines (TNF- α , IL- β , and IL-6) and chemokines (CCL5 and CXCL8) after stimulation with LPS (21).

These results suggest that cigarette smoke may disrupt the initial innate immune response required to resolve a respiratory viral infection in an efficient and timely manner. Given that respiratory viral infections are important causes of in both chronic obstructive lung disease and asthma (22), and that the airway epithelium is the primary site of rhinovirus infection as well as the site of the initial inflammatory response (23), we investigated whether cigarette smoke altered the response of human airway epithelial cells to infection with rhinovirus. First, we assessed the response of airway epithelial cells to polyinosine-polycytidylic acid (poly I:C, a double stranded (ds) RNA analogue used as a mimic of viral infection) after pre-exposure to cigarette smoke. Next, we assessed the effects of cigarette smoke on the ability of human airway epithelial cells to respond to infection with rhinovirus (RV), measuring the expression of RV-induced cytokines, the activation of RV-induced signaling pathways, and the RV RNA load.

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MATERIALS AND METHODS

Cell Cultures and Treatments

BEAS-2B cells (American Type Culture Collection, Manassas, VA) and normal human bronchial epithelial cells (NHBEs; Lonza, Gaithersburg, MD) were grown as previously described (24). Subconfluent cells were treated with cigarette smoke extract (CSE) or control media for 24 hours and then treated with poly I:C (Invivogen, San Diego, CA), as indicated. Subconfluent cells were infected with human rhinovirus (HRV)–16 at 1.5×10^4 of the 50% tissue culture infective dose (TCID₅₀) after 24 hours in hydrocortisone-free BEGM.

Preparation of CSE

Two research-grade cigarettes (IR3F; Tobacco and Health Research Institute, University of Kentucky, Lexington, KY) were “smoked” using a modified 60-ml syringe apparatus, bubbling smoke through 2.5 ml of DMSO (Sigma-Aldrich, St. Louis, MO). The smoke/DMSO solution was diluted in media, filtered through a 0.22- μ m filter, and termed “CSE.” Control media were generated by bubbling air through 2.5 ml of DMSO, and were then diluted and filtered.

Preparation of HRV-16

HRV-16 (American Type Culture Collection) was propagated in WI-38 cells (American Type Culture Collection), and purified as previously described (25).

Isolation of RNA and Quantitative Real-Time PCR

Total RNA was isolated and assessed by quantitative real-time PCR, as previously described (24). The primer and probes for β -actin were previously described (24). Primers for CCL5 included: forward, 5'-TGACCAGGAAGGAAGTCAGC-3'; and reverse, 5'-AGCCGATTTTTCATGTTTGC-3'. Primers for CXCL10 included: forward, 5'-GAGCCTACAGCAGAGGAACC-3'; and reverse, 5'-AAGGCAGCAAATCAGAATCG-3'. Probes (BioSearch Technologies, Novato, CA) for CCL5 included 5' FAM-CCGCCGTCTCAACCCCTCAC-3' BHQ1; and for CXCL10, 5' FAM-tccagtctcagcaccatgaatcaaa-3' BHQ1. IFN- β , IFN- λ , IFN- ϵ , and IFN- α 1 mRNA were assessed using gene expression kits (Applied Biosystems, Foster City, CA). IFN values were assessed by relative quantification, using the 2(-Delta Delta C(T)) method (26).

ELISA

Protein levels of CXCL10, CCL5, and IFN- β in cell supernatants were determined by ELISA (R&D Systems, Minneapolis, MN). The minimal detectable dose for CXCL10 was 0.41 pg/mL, for CCL5 the minimal detectable dose was 1.7 pg/ml, and for IFN- β the minimal detectable dose was 25 pg/mL.

Cell Lysate, Nuclear Extract, and Immunoblotting

Cell lysates were isolated and immunoblotting was performed as described elsewhere (24). Nuclear protein was extracted using a nuclear extraction kit (Panomics, Fremont, CA).

For immunoblotting phospho-interferon regulator factor 3 (IRF3), phospho-signal transducer and activator of transcription 1 (STAT-1), phospho-jun c-terminal kinase (JNK), or phospho-extracellular signal-regulated kinase (ERK1/2) (Cell Signaling Technology, Danvers, MA), specific antibodies were used, followed by goat anti-rabbit or goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (Pierce, Rockford, IL). Blots were reprobed with antibodies specific for β -actin, TATA binding protein (Abcam, Cambridge, MA), total STAT-1, Stress activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK), or ERK-1/2 (Cell Signaling Technology).

Cell Cytotoxicity Assay

The CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega Madison, WI) was used as directed to measure lactate dehydrogenase (LDH) quantitatively in cell culture supernatants. Total cellular LDH was assessed from lysed cells.

Data Analyses

Data are expressed as mean $\pm$ standard error of the mean. Groups were compared using an unpaired Wilcoxon signed-rank test, with

$P \leq 0.05$ considered significant. For assessing gene expression, the mean of the poly I:C-induced or HRV-16-induced gene expression in control cells was calculated as 100% for that experiment. The mRNA of each sample was calculated as a percentage of the 100% level. Percent change was used to normalize the maximum induction of gene expression by poly I:C and HRV between separate experiments, because this maximum induction varied between passages of BEAS-2B cells. Data from three independent experiments, each performed in triplicate, were used for analyses.

RESULTS

CSE Decreases Poly I:C Induction of CXCL10 and CCL5 mRNA

The chemokines CXCL10 and CCL5 are among the most highly induced cytokines after infection of airway epithelial cells with RV (27). To begin to assess whether cigarette smoke interferes with antiviral responses, the human airway epithelial cell line BEAS-2B was pretreated with CSE or control media for 20 hours, and then stimulated with the viral surrogate poly I:C or control media for 6 hours. Concentrations of CXCL10 and CCL5 mRNA were measured by quantitative real-time PCR.

Poly I:C stimulated a significant increase in both CXCL10 and CCL5 mRNA levels, but pretreatment with CSE alone did not increase either CXCL10 or CCL5 mRNA levels (data not shown). CSE pretreatment did, however, decrease the response to poly I:C stimulation (Figure 1). As a percentage of the mRNA level in control poly I:C-treated cells, CSE pretreatment lowered the levels of both CCL5 ($44.7\% \pm 9.3\%$, $P = 0.057$) and CXCL10 ($62.7\% \pm 9.8\%$, $P = 0.019$) (Figure 1) induced by poly I:C.

Figure 1. Effects of cigarette smoke extract (CSE) on polyinosine-polycytidylic acid (poly I:C)-induced CCL5 and CXCL10 mRNA. BEAS-2B cells were treated for 20 hours with CSE or control media, followed by stimulation with poly I:C (2.5 μ g/ml) for 6 hours. Total RNA was isolated, cDNA was generated, and concentrations of CCL5, CXCL10, and β -actin mRNA were assessed using quantitative real-time PCR. CCL5 and CXCL10 mRNA concentrations were normalized to β -actin mRNA concentrations. The concentration of poly I:C CCL5 and CXCL10 mRNA in CSE-pretreated cells was calculated as the percentage of poly I:C-induced CCL5 and CXCL10 detected in control cells. Data are from three experiments, each performed in triplicate. Asterisk indicates statistical significance.

CSE Decreases HRV-16 Induction of CXCL10 and CCL5

Because CSE affected the induction of CCL5 and CXCL10 mRNA by poly I:C, we assessed whether CSE would also alter the response of BEAS-2B cells to HRV-16 infection with respect to CCL5 and CXCL10 induction. BEAS-2B cells were pretreated with either CSE media or control media for 20 hours, and then infected with HRV-16 (1.5×10^4 TCID₅₀) or mock-infected. Total mRNA was collected 48 hours after infection, and concentrations of CXCL10 and CCL5 mRNA were assessed using quantitative real-time PCR (Figure 2A).

Infection with HRV-16 induced a large increase in CCL5 mRNA (11.0 ± 1.6 fold, $P < 0.0001$) and CXCL10 mRNA (18.5 ± 4.4 fold, $P < 0.0001$) in cells pretreated with control media. CSE itself did not stimulate either CCL5 or CXCL10. CSE pretreatment significantly reduced the induction of CCL5 and CXCL10 mRNA by HRV-16 (Figure 2A). The HRV-16-induced CXCL10 mRNA concentrations in CSE-pretreated cells were $48.2\% \pm 5.6\%$ of the HRV-16-induced CXCL10 mRNA in control media-pretreated cells ($P < 0.0005$). The HRV-16-induced CCL5 mRNA concentrations in CSE-pretreated cells were $38.6\% \pm 1.2\%$ of the CCL5 mRNA concentrations induced by HRV-16 in control media-pretreated cells ($P < 0.0001$; Figure 2).

To explore this observation further, we assessed the effects of CSE on the HRV-16-induced release of CXCL10 and CCL5 protein from BEAS-2B cells (Figure 2B). In supernatants from cells treated with control media, low but detectable concentrations of CXCL10 (8.5 ± 1.4 pg/ml) were measured. However, CCL5 concentrations were below the limit of detection. The treatment of cells with CSE media alone did not increase CXCL10 or CCL5 protein (data not shown). In control cells, infection with HRV-16 induced an increase in the mean concentration of CXCL10 to 55.4 ± 8.6 pg/ml, whereas CCL5 concentrations were increased to 16.2 ± 2.1 pg/ml. Consistent with observations at the mRNA level, CSE significantly decreased the concentrations of HRV-16-induced CXCL10 and CCL5 released from cells. The HRV-16-induced release of CXCL10 in CSE-pretreated cells was $68.6\% \pm 5.9\%$ of the release in HRV-16-infected cells pretreated with control media ($P < 0.005$), whereas the HRV-16-induced release of CCL5 in CSE-pretreated cells was $21.6\% \pm 5.5\%$ of the release measured in HRV-16 infected in control cells ($P < 0.005$).

CSE Suppresses Poly I:C-Mediated Activation of IRF-3 in BEAS-2B cells

The previous data demonstrated that CSE is able to reduce the response of epithelial cells to poly I:C stimulation or HRV-16 infection, decreasing the release of the cytokines CCL5 and CXCL10. The activation of IRF-3 was associated with the response of many cells to viral infection (28). As such, we assessed the effects of CSE on the activation of IRF-3 by poly I:C stimulation and HRV-16 infection.

BEAS-2B cells were treated with poly I:C for 0.5, 2, and 4 hours. Nuclear extracts were isolated and assessed by immunoblotting for phospho-IRF-3. TATA binding protein was measured as a nuclear protein loading control. Poly I:C induced a robust phosphorylation and nuclear translocation of IRF-3 within 4 hours of stimulation. BEAS-2B cells were then exposed to CSE or control media for 20 hours and treated with poly I:C for 4 hours, and nuclear extracts were assessed for phospho-IRF-3 (Figure 3). Pretreatment with CSE appeared to decrease poly I:C-induced nuclear phospho-IRF-3, compared with poly I:C control-media non-pretreated cells. Thus, CSE appears to disrupt the activation of IRF-3 by dsRNA.

Next we assessed phospho-IRF-3 concentrations in nuclear extracts isolated from BEAS-2B cells infected with HRV-16 (1.5×10^4 TCID₅₀) for 4, 24, and 48 hours. We were unable to detect either phospho-IRF-3 or total IRF-3 at any time within the nuclear extracts of BEAS-2B cells after infection with HRV (data not shown).

CSE Suppresses Poly I:C-Induced and HRV-16-Induced Phosphorylation of STAT-1

Activation of the Type I IFN pathway is believed to be a critical event in the initial innate immune response of a cell to viral infection (28). Therefore, we assessed the effects of CSE on the activation of the IFN-dependent pathway JAK/STAT by assessing the phosphorylation of STAT-1 after stimulation with poly I:C or infection with HRV-16.

Initially, BEAS-2B cells were treated with poly I:C (2.5 μ g/ml) for 0.5, 1, 2, and 4 hours or infected with HRV-16 (1.5×10^4 TCID₅₀) for 24 and 48 hours, and phosphorylated STAT-1 in total cell lysates was assessed by immunoblotting. The phosphorylation of STAT-1 was evident only at 2 hours after poly I:C stimulation and at 48 hours after HRV-16 infection. After

Figure 2. Effects of CSE on human rhinovirus (HRV)-16-induced increase in CCL5 and CXCL10. BEAS-2B cells were grown to 40% confluence, treated for 20 hours with CSE or control media, and infected with HRV-16 for 48 hours. Cell supernatants were collected, and total RNA was isolated from cells. (A) CCL5, CXCL10, and β -actin mRNA concentrations were assessed by quantitative real-time PCR, with CCL5 and CXCL10 concentrations normalized to β -actin for each sample. The concentration of CCL5 and CXCL10 mRNA in HRV-16-infected samples treated with CSE was calculated as a percentage of the concentration of CCL5 or CXCL10 mRNA detected in HRV-16-infected samples treated with control media (percentage of HRV-16 stim. mRNA). (B) CCL5 and CXCL10 protein concentrations in culture supernatants were assessed by ELISA. The concentration of CCL5 and CXCL10 protein in HRV-16-infected samples treated with CSE was calculated as a percentage of the concentration of CCL5 or CXCL10 protein detected in HRV-16-infected samples treated with control media (percentage of HRV-16 stim. protein). These data are from three experiments, each performed in triplicate. Asterisk indicates statistical significance.

concentrations in culture supernatants were assessed by ELISA. The concentration of CCL5 and CXCL10 protein in HRV-16-infected samples treated with CSE was calculated as a percentage of the concentration of CCL5 or CXCL10 protein detected in HRV-16-infected samples treated with control media (percentage of HRV-16 stim. protein). These data are from three experiments, each performed in triplicate. Asterisk indicates statistical significance.

Figure 3. Effects of CSE on poly I:C induction (pIC) of phospho-IRF-3 (pIRF-3) nuclear translocation. BEAS-2B cells were grown to confluence, treated for 20 hours with control or CSE media, and then stimulated with poly I:C (2.5 μ g/ml) for 4 hours. Nuclear extracts were isolated and assessed for phospho-IRF-3 and TATA binding protein (TBP), as described in MATERIALS AND METHODS. NS, nonstimulated. This is representative of three separate experiments, each performed in duplicate.

establishing the kinetics of the activation of STAT-1 in our system, we assessed the effects of pretreating BEAS-2B cells for 20 hours with CSE on the ability of poly I:C and HRV-16 to induce phospho-STAT-1. As shown in Figures 4 and 5, CSE pretreatment appears to decrease both the poly I:C-induced and HRV-16-induced phosphorylation of STAT-1.

Effects of CSE on Poly I:C-Induced and HRV-16-Induced Activation of the MAP Kinases, SAPK/JNK and ERK1/2

Activation of the mitogen-activated protein (MAP) kinase kinases SAPK/JNK and ERK was shown to be involved in the regulation of virally induced gene expression (29–31). SAPK/JNK is involved in the regulation of β -defensin and activates activating transcription factor 2 (ATF2), a component

of the Type I IFN response, whereas ERK is involved in the regulation of RV-induced CXCL10 and respiratory syncytial virus (RSV)-induced CCL5. Therefore, we assessed the effects of CSE on the poly I:C-induced and HRV-16-induced activation of SAPK/JNK and ERK1/2.

Initially we determined the optimum time point to assess poly I:C-induced and HRV-16-induced phospho-JNK and phospho-ERK1/2. Total cell lysates were collected from BEAS-2B cells treated for 0.5, 1, 2, and 4 hours with poly I:C (2.5 μ g/ml) or infected with HRV-16 for 24 and 48 hours, and assessed by immunoblotting for phospho-JNK and phospho-ERK1/2. Poly I:C-induced phospho-SAPK/JNK and phospho-ERK1/2 were detected at 0.5, 1, and 2 hours after treatment, whereas HRV-16-induced phospho-SAPK/JNK and phospho-ERK1/2 were detected 48 hours after infection.

Phospho-SAPK/JNK and phospho-ERK1/2 were then assessed in poly I:C-stimulated BEAS-2B cells pretreated with either CSE or control media (Figure 4). Pretreatment with CSE appears to reduce the concentration of poly I:C-induced phospho-SAPK/JNK at all time points (0.5, 1, or 2 hours). In contrast, the concentration of poly I:C-induced phospho-ERK1/2 was unaffected by CSE pretreatment.

We then assessed the impact of CSE on HRV-16-induced SAPK/JNK and ERK activation (Figure 5). As in the pattern seen after poly I:C stimulation, the HRV-16-induced phosphorylation of SAPK/JNK appeared to be reduced by pretreatment with CSE. However, CSE had no effect on HRV-16-induced phospho-ERK1/2.

CSE Modulates the Response of Primary Human Airway Epithelial Cells to Poly I:C and RV Infection

To assess whether the effects of CSE observed in the BEAS-2B cell line with respect to HRV infection may be relevant to primary human airway epithelial cells, we extended our studies to NHBEs. First we assessed the impact of CSE on poly I:C-induced CXCL10 and CCL5 mRNA. Because we previously observed that NHBEs are more sensitive to poly I:C stimulation

Figure 4. Effects of CSE on poly I:C-induced phospho-STAT-1 (pSTAT1), phospho-SAPK/JNK (pJNK), and phospho-ERK1/2 (pERK). BEAS-2B cells were treated (Pre-Rx) for 20 hours with either CSE or control media (CM), followed by stimulation with poly I:C (2.5 μ g/ml) for 0, 0.5, 1, and 2 hours. Total cell lysates were assessed by immunoblotting for phospho-STAT-1 (isoform A at 91 kD, and isoform B at 84 kD), phospho-SAPK/JNK (JNK1 at 46 kD, and JNK2/3 at 54 kD), and phospho-ERK1/2 (ERK1 at 44 kD, and ERK2 at 42 kD), stripped, and assessed for total STAT-1, total JNK, or total ERK1/2 as appropriate, and then stripped and assessed for control protein β -actin. In this example, the same blot was used to assess ERK1/2 and SAPK/JNK. This is representative of three separate experiments, each performed in duplicate.

Figure 5. Effects of CSE on HRV-16-induced phospho-STAT-1, phospho-SAPK/JNK1/2, and phospho-ERK1/2. BEAS-2B cells were grown to 40% confluence, treated with CSE or control media for 20 hours, and infected with HRV-16 for 48 hours. Total cell lysates were assessed by immunoblotting for phospho-STAT-1 (isoform A at 91 kD, and isoform B at 84 kD), phospho-SAPK/JNK1/2 (JNK1 at 46 kD, and JNK2/3 at 54 kD), and phospho-ERK1/2 (ERK1 at 44 kD, and ERK2 at 42 kD), stripped, and assessed for total STAT-1, ERK1/2, or SAPK/JNK1/2 and then the control protein β -actin. In this example, the same blot was used to assess SAPK/JNK1/2 and ERK1/2. This is representative of three separate experiments, each performed in duplicate.

than are BEAS-2B cells, we analyzed the expression of CXCL10 and CCL5 mRNA by quantitative real-time PCR after the stimulation of NHBEs with three doses of poly I:C (which did not cause visibly cytotoxic effects in cells). As expected, CXCL10 mRNA was not evident in unstimulated cells, but was induced in a dose-dependent manner by poly I:C (Figure 6A). Low concentrations of CCL5 mRNA were detected in untreated NHBE samples, and poly I:C induced a dose-dependent increase in CCL5 mRNA levels. Based on these data, we used a poly I:C dose of 0.5 μ g/ml in subsequent experiments.

Next we assessed the effects of CSE on the ability of poly I:C to induce both CXCL10 and CCL5 in NHBE cells (Figure 6B). We assessed three different doses of CSE: 1:1,000, 1:2,000, and 1:4,000. Similar to what we observed in the BEAS-2B cell line (data not shown), exposure of NHBE cells to CSE diluted 1:1,000 reduced the amount of CXCL10 and CCL5 mRNA induced by poly I:C. At this dose of CSE, poly I:C-induced CXCL10 mRNA concentrations were reduced by 37%, whereas poly I:C-induced CCL5 mRNA concentrations were reduced by 22%. CSE at a dilution of 1:2,000 was less effective at suppressing the induction of CXCL10 and CCL5 by poly I:C, causing a decrease in poly I:C-induced CXCL10 and CCL5 of 10% or less. The suppressive effect of CSE on poly I:C-induced CXCL10 or CCL5 was no longer evident at a dilution of 1:4,000.

Using NHBEs from two separate donors, we assessed the effects of CSE at a dose of 1:1,000 on the ability of HRV-16 to induce CXCL10 and CCL5 mRNA (Figure 6C). In control media-treated NHBE cells from both donors, HRV-16 infection induced a large increase in CXCL10 and CCL5 mRNA

expression at 48 hours. Treatment with CSE (1:1,000) significantly reduced the HRV-16-induced increase in CXCL10 and CCL5 mRNA levels in NHBEs from both donors ($P < 0.01$). The experiment was performed four times, twice with cells from each donor.

CSE Alters the Induction of IFN- β by HRV-16 in Airway Epithelial Cells

Because the pathways involved in the IFN response appeared to be suppressed by the exposure of BEAS-2B cells to CSE, we assessed whether CSE affected the induction of IFN- β by HRV-16 (Figure 7A). Using quantitative real-time PCR, we observed a consistent induction of IFN- β mRNA at 48 hours after infection with HRV-16 in three separate experiments (each in triplicate). After the exposure of BEAS-2B cells to CSE (1:1,000), the induction of IFN- β mRNA by HRV-16 was significantly attenuated ($P < 0.0001$). We also assessed the effects of CSE on the induction of IFN- β mRNA in NHBE cells. In NHBEs from two different donors, HRV-16 induced an increase in IFN- β mRNA. This induction was significantly suppressed by exposure of the cells to CSE (1:1,000) ($P < 0.005$). CSE had no effect, however, on basal IFN- β mRNA levels in uninfected BEAS-2B or NHBE cells (data not shown).

We also assessed whether the HRV-16-induced expression of other IFN genes, IFN- λ , IFN- α 1 and IFN- ϵ . IFN- λ mRNA was not detected in either control or HRV-16-infected cells, whereas no change in either IFN- α 1 or IFN- ϵ mRNA levels was evident after infection with HRV-16 (data not shown). Although we detected an increase in IFN- β mRNA after the HRV-16 infection of BEAS-2B cells, we could not detect any IFN- β protein in cell supernatants before or after infection with HRV-16.

Exposure to CSE Increased HRV-16 RNA Levels in HRV-16-Infected BEAS-2B Cells

The production of CXCL10 and CCL5 was linked to effective viral clearance, and blocking the IFN JAK-STAT pathway was shown to result in increased RV replication within the cell. Therefore, we asked whether the exposure of cells to CSE affects the concentration of HRV-16 within the cell. To assess this, we analyzed HRV-16 RNA expression by real-time RT-PCR in BEAS-2B cells pretreated for 20 hours with CSE or control media and then infected with HRV-16 (Figure 7C). At 24 hours after infection, similar amounts of HRV-16 RNA were detected in CSE and control media-treated cells. However, 48 hours after infection, we observed significantly higher concentrations of HRV-16 RNA ($P < 0.01$) in cells pretreated with CSE compared with cells pretreated with control media.

Impact of Poly I:C, HRV-16, and CSE on Cell Viability

Cell viability was assessed using the LDH cytotoxicity assay. In all instances, cell viability was $> 90\%$. No differences were evident in the level of cell death between control, poly I:C, or HRV-16-infected BEAS-2B, or NHBE cells, in either the absence or presence of CSE.

DISCUSSION

Cigarette smoke exposure is associated with an increased prevalence of respiratory viral infections as well as a prolonged duration of viral infection and worse outcomes for the host (2–5). Epithelial cells are the primary target of HRV infection, and as such, their response is critical to the launch of an effective early innate immune response. We hypothesized that cigarette

Figure 6. Effects of CSE on poly I:C and HRV-16 induction of CXCL10 and CCL5 in primary human airway epithelial cells. (A) Confluent monolayers of normal human bronchial epithelial cells (NHBEs) were stimulated for 6 hours with poly I:C at 0.05, 0.1, and 0.5 $\mu\text{g/ml}$. Total RNA was isolated, and CXCL10, CCL5, and β -actin mRNA concentrations were assessed by quantitative real-time PCR. CCL5 and CXCL10 mRNA concentrations were normalized to β -actin. (B) In separate experiments, confluent monolayers of NHBEs were treated for 20 hours with CSE diluted 1:1,000, 1:2,000, and 1:4,000 in basal media, followed by stimulation with 0.5 $\mu\text{g/ml}$ of poly I:C for 6 hours. Total RNA was isolated, and CCL5, CXCL10, and β -actin mRNA concentrations were assessed by quantitative real-time PCR. (C) Subconfluent monolayers of NHBEs were treated for 20 hours with CSE diluted 1:1,000 in hydrocortisone-free media and then infected with HRV-16 for 48 hours. Isolated RNA was assessed for the expression of CXCL10, CCL5, and β -actin mRNA by quantitative real-time PCR. CXCL10 and CCL5 mRNA concentrations were normalized to β -actin. The experiment was performed in triplicate, and repeated four times with NHBEs from two donors (i.e., twice with NHBEs from each donor). Concentrations of normalized CXCL10 or CCL5 were calculated as described in MATERIALS AND METHODS. Asterisk indicates statistical significance.

smoke exposure alters the response of epithelial cells to RV infection.

The composition of cigarettes and cigarette smoke was extensively assessed by analytical chemistry and shown to be a complex mixture of components including hydrocarbons, oxygen-containing components, and nitrogen-containing components (32). The *in vivo* human airway epithelial transcriptome in bronchial cells from smokers was found to contain altered expressions of numerous genes (33). Several studies indicated that *in vitro* exposure to CSE reflects *in vivo* changes in the airways of humans who smoke. For example, Winkler and colleagues reported a significant overlap in changes in gene expression observed in alveolar macrophages isolated from the airways of smokers and those genes regulated after *in vitro* exposure of monocyte-derived macrophages to cigarette smoke extract (34).

The RV infection of epithelial cells was shown to induce a wide range of cytokines and chemokines, including CCL5 and CXCL10 (27). CCL5 and CXCL10 participate in the induction and propagation of inflammation. However, they are also important mediators of the early innate antiviral response (35). CXCL10 and CCL5 recruit CD8^+ T cells and natural killer cells to the site of infection. These cells release IFN- γ and TNF- α , cytokines known to be important for the clearance of respiratory viral infections (36).

A link between adequate viral induction of CXCL10 and effective clearance of respiratory viral infections was demonstrated in an animal model of virally induced airway inflammation (37). In a rat model of parainfluenza-induced chronic airway inflammation, high expression of CXCL10 was associated with protection from virus-induced airway inflammation and fibrosis. This was attributed to the ability of CXCL10 to recruit activated T cells to the site of infection, and to induce

the production of IFN- γ . A decreased IFN response in patients with asthma was associated with an increased susceptibility to exacerbations of the airway (38, 39). A role for CCL5 in effective host clearance of respiratory viral infection was also reported. CCL5-deficient mice were immune-compromised, and were unable to clear parainfluenza infections (40). In that study, CCL5 was necessary for the survival of infected macrophages, and was required for host survival after infection with influenza. Furthermore, CCL5 and CXCL10 were each shown to inhibit viral replication directly (41, 42).

Because the evidence indicates that the viral induction of CCL5 and CXCL10 is linked to an effective early innate immune response, we assessed the effects of cigarette smoke exposure on poly I:C-induced (a dsRNA viral mimic) and HRV-16-induced CCL5 and CXCL10 production in airway epithelial cells. The BEAS-2B cell line, a transformed human airway epithelial cell line, has become a model *in vitro* system for investigating the early effects of HRV infection in airway epithelial cells (43). Observations of many of the effects of HRV on the BEAS-2B cell line were shown to reflect the effects of natural and experimental HRV infection *in vivo* in human subjects (43, 44). As such, we initially assessed the effects of CSE on HRV infection using the BEAS-2B cell line.

Pre-exposure to CSE significantly attenuated both the CCL5 and CXCL10 responses after stimulation with poly I:C or infection with HRV-16. Reduced chemokine responses were evident at the mRNA and protein levels. In addition, we showed that CSE reduced the induction by poly I:C and HRV-16 of CXCL10 and CCL5 in primary human bronchial epithelial cells.

Well-differentiated airway epithelial cells were shown to be more resistant to HRV infection than the same cells in undifferentiated monolayers (45). However, a recent study

Figure 7. Effects of CSE on HRV-16-induced IFN- β and HRV-16 RNA in human airway epithelial cells. (A) Subconfluent monolayers of BEAS-2B and NHBEs were treated with CSE or control media for 20 hours, and then infected with HRV-16 for 48 hours. Total RNA was isolated, and IFN- β mRNA concentrations were assessed by real-time quantitative PCR, normalized to β -actin mRNA concentrations. Concentrations of normalized CXCL10 or CCL5 were calculated

as described in MATERIALS AND METHODS. (B) BEAS-2B cells were grown to 40% confluence, treated with CSE or control media for 20 hours, and infected with HRV-16 for 24 or 48 hours. Total RNA was isolated, cDNA was generated, and the concentration of HRV-16 RNA was assessed by quantitative real-time PCR and normalized to the concentration of β -actin mRNA detected in each sample. HRV-16 RNA concentrations determined after 24 hours in control media-treated cells were taken as 100%. Data are from three experiments, each performed in triplicate. Asterisk indicates statistical significance.

reported that various types of differentiated bronchial epithelial cells differ in their susceptibility to RV infection (46). Whereas cells at the superficial layer were relatively resistant to RV infection, basal cells were more sensitive. Furthermore, in injured epithelial cells, HRV replication was increased. Thus, differentiated epithelial cells with intact tight junctions may not relate to the *in vivo* situation of the asthmatic airway in which the epithelial layer is damaged. Based on this finding, it is difficult to determine how undifferentiated epithelial cells grown in a submerged monolayer compare with a damaged epithelium in the human airway with respect to RV infection.

The HRV infection of epithelial cells is reported to result in the rapid generation of Type I IFN, a subsequent activation of the JAK2-STAT1 pathway, and the induction of a wide range of IFN-responsive genes (47). Interfering with the Type I IFN pathway during the HRV infection of epithelial cells was associated with an impaired activation of antiviral genes and the delayed clearance of viral infection. The treatment of airway epithelial cells during HRV infection with a neutralizing antibody against IFN- β significantly impaired the induction of IFN-responsive genes, including CXCL10 (47). In addition, disruption of the JAK-STAT pathway was shown to block the antiviral response of epithelial cells after infection with HRV. Although Chen and colleagues found that IFN- β was necessary, as discussed previously, another group reported that HRV-induced CXCL10 is independent of IFN production (48). Here, we show that CSE impaired HRV-16-induced STAT-1 phosphorylation, and that it also significantly reduced the HRV-16-induced expression of IFN- β mRNA in BEAS-2B and NHBE cells. Decreased HRV-induced IFN- β was associated with increased HRV replication in epithelial cells isolated from patients with asthma (39). Based on this finding, we assessed whether the reduced IFN response to HRV in CSE-exposed cells affected the replication of HRV-16. In our system, we observed increased HRV-16 mRNA concentrations in epithelial cells exposed to CSE. In preliminary work, we observed that CSE also increased HRV-16 mRNA concentrations in NHBEs from a single donor. We are continuing these experiments in NHBE cells from multiple donors to confirm this observation.

We failed, however, to detect the induction of IFN- β protein release from HRV-16-infected BEAS-2B cells. This is consistent with HRV inducing a very low level of IFN- β mRNA in A549 cells (49), and with the HRV induction of IFN- β mRNA, but not detectable IFN- β protein, in cell supernatants (48). Nevertheless, we observed the phosphorylation of STAT-1 as well as increased IFN- β mRNA after infection with HRV-16, suggesting either that (1) HRV infection did induce the production of IFN- β , but at concentrations too low for detection by our assays; or (2) STAT-1 is activated by a non-Type I IFN mechanism. As such, we postulate that disruption by CSE of the JAK-STAT pathway may account for the decreased response of epithelial cells to RV infection.

The transcription factor IRF-3 is significantly involved in the activation of the innate immune response to many viral infections, and is required for the viral induction of IFN- β (50). As expected, we found that pre-exposure to CSE inhibited the poly I:C-mediated activation of IRF-3. This is consistent with a recent study showing that cigarette smoke inhibited the poly I:C-induced activation of IRF-3, impaired the ability of poly I:C to inhibit viral replication, and diminished IFN-induced STAT-1 phosphorylation (51). However, we failed to find evidence of IRF-3 activation after infection with HRV-16. In agreement with our observations, a recent report showed that the HRV infection of A549 cells did not result in an activation of IRF-3 (49). Although many effects of poly I:C on airway epithelial cells are consistent with the effects of HRV infection, such as the induction of CXCL10 and CCL5, these data remind us that results obtained using a dsRNA surrogate may not always reflect the effects of infection with HRV. This is not surprising, because HRV engages the receptor intercellular adhesion molecule 1 (ICAM-1) to enter the cell, and infection with HRV exposes the cell not only to dsRNA but also single stranded (ss) RNA, which may play a role in regulating the cellular response to infection through the activation of ssRNA-dependent signaling pathways.

The HRV infection of airway epithelial cells also induces the activation of two MAP kinases, JNK and ERK1/2. The activation of JNK is critical for the activation of ATF2, a transcription

factor involved in the induction of the IFN response. Therefore, the disruption of this pathway could have important consequences for the clearance of a viral infection. As with the activation of phospho-STAT1, we observed decreased HRV-16-induced phosphorylation of JNK after exposure of cells to CSE. Disruption of the JNK pathway was shown to block bacterial induction of the antimicrobial peptide β -defensin (31), whereas CSE inhibits the bacterial induction of defensin in epithelial cells (52). Furthermore, lower concentrations of human β -defensin-2 were detected within the airways of current or former smokers with acute pneumonia, compared with patients with acute pneumonia and who had never smoked (52). Together with our data, these studies suggest that disruption of the JNK pathway is the mechanism through which CSE blocks the microbial induction of β -defensins.

In contrast to the observations regarding STAT-1 and JNK, we observed that the poly I:C-induced and HRV-16-induced phosphorylation of ERK1/2 was unaffected by the CSE treatment of BEAS-2B cells. This is particularly interesting in view of a recent publication showing that activation of the MAP kinase kinase (MEK)–ERK pathway by HRV-16 actually inhibits CXCL10 production (30). Thus, after exposure to cigarette smoke, HRV-16 can still effectively induce the MEK–ERK pathway, exerting an inhibitory effect on the expression of CXCL10.

Evidence is accumulating that cigarette smoke alters the responses of airways to microbial infections. Diminished LPS or the IL-1-induced secretion of cytokines from alveolar macrophages isolated from smokers was reported (53–55), and the exposure of alveolar macrophages isolated from nonsmokers to cigarette smoke rendered them hypo-responsive to endotoxin challenge (14, 20). In contrast, the treatment of a monocytic cell line with LPS in the presence of cigarette smoke enhanced the LPS-induced activation of p38, ERK, and JNK. This discrepancy may be attributable to the different cell types used or the length of time that cells were exposed to cigarette smoke. Here, we observed decreased virally induced cytokines as well as the decreased activation of the signaling intermediates STAT-1 and JNK in cells pre-exposed to CSE. This is comparable to reports showing that cigarette smoke suppresses LPS-induced cytokine production from macrophages and bronchial epithelial cells as well as TNF- α -induced cytokines from airway smooth muscle cells.

Our results with CSE complement previous reports stating that asthmatic epithelial cells are dysfunctional in their response to HRV infection (38, 39, 56). The HRV infection of airway epithelial cells isolated from patients with asthma was more productive, with increased HRV replication observed, along with a decrease in the IFN response. In our study, the IFN response to HRV in epithelial cells was evidently disrupted by cigarette smoke, with a decrease in activation of the JAK2–STAT1 pathway, as well as a significant suppression of the HRV-16-induced increase of IFN- β mRNA concentrations.

In conclusion, our study suggests that exposure to cigarette smoke blunts the response of airway epithelial cells to RV infection, leading to increased viral replication and production, potentially by interfering with the IFN pathway and the transcriptional activation of antiviral genes. The blunted response of epithelial cells during the early phase of respiratory viral infection could, in part, explain the increased susceptibility of smokers and children exposed to cigarette smoke to URIs, as well as the decreased ability of these subjects to clear URIs efficiently.

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