CIGARETTE SMOKE MODULATES RHINOVIRUS-INDUCED AIRWAY EPITHELIAL CELL CHEMOKINE PRODUCTION

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Running Head: Cigarette Smoke Alters Viral Inflammation

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ABSTRACT

Human rhinovirus (HRV) infections induce epithelial cell production of chemokines that may contribute to the pathogenesis of exacerbations of chronic obstructive pulmonary disease (COPD) and asthma. Cigarette smoking is the predominant risk factor for the development of COPD and also aggravates asthma symptoms. We examined if cigarette smoke extract (CSE) may modulate viral inflammation by altering the profile of HRV-induced epithelial chemokine production.

Purified HRV-16, and CSE were used to examine the effects on CXCL8 and CXCL10 production from both primary human bronchial epithelial cells and the BEAS-2B epithelial cell line.

Both CSE and HRV-16 induced CXCL8 production and, when used in combination, induced at least an additive production of CXCL8 compared to either stimulus alone. By contrast, CSE did not induce CXCL10 and markedly inhibited HRV-16-induced CXCL10 production. Inhibition of HRV-16-induced CXCL10 by CSE was mediated, at least in part, via transcriptional regulation. In contrast, the increased CXCL8 production seen with the combination of CSE and HRV-16 was not due to transcriptional regulation but was associated with CXCL8 mRNA stabilization.

Thus, CSE differentially modulates HRV-16-induced chemokine production from human airway epithelial cells in a manner that might be expected to alter inflammatory cell profiles.

INTRODUCTION

Human rhinovirus (HRV) infections are a major cause of exacerbations of both chronic obstructive pulmonary disease (COPD) and asthma [1]. Although the mechanisms by which HRV infections induce such exacerbations of lower airway diseases are not fully delineated, it is thought that infection of airway epithelial cells trigger epithelial responses that contribute to increased airway inflammation. In support of this, epithelial cells infected with HRV produce numerous chemokines, cytokines and host defense molecules [2]. Several of these chemokines, including CXCL8 and CXCL10, are also observed in increased amounts in airway secretions during *in vivo* HRV infections. Levels of both CXCL8 and CXCL10 correlate with symptom severity during HRV infections [3, 4]. CXCL8 is a potent chemoattractant for neutrophils, and neutrophil numbers in airway secretions have been shown to correlate with disease severity in COPD and during viral exacerbations of asthma [5, 6]. CXCL10 is a chemoattractant for activated lymphocytes and natural killer cells that have been linked to host antiviral defenses.

Cigarette smoke has profound effects on airway biology and is the major risk factor for the development of COPD [7]. In addition, some 25% of asthmatic subjects smoke [8], and these individuals have worse respiratory symptoms, more frequent hospitalizations and decreased quality of life, compared to asthmatic subjects who do not smoke [9]. Moreover, asthmatic subjects who smoke are also less responsive to corticosteroid treatment than non-smoking asthmatics [10].

Interestingly, it has been reported that both the frequency and severity of viral infections is greater in cigarette smokers compared to non-smokers [11, 12]. Although a few studies have investigated the combined effects of viruses and cigarette smoke, to date, there have been no studies looking at the effects of cigarette smoke on epithelial responses to HRV infection. The

current studies were performed to determine if, and how, cigarette smoke modulates HRV-induced chemokine production from human airway epithelial cells.

MATERIAL AND METHODS
Materials

The following reagents were purchased from the indicated suppliers: bronchial epithelial cell basal medium (BEBM) and additives to create serum-free bronchial epithelial cell growth medium (BEGM) (Lonza, Walkersville, MD); HBSS, TRIzol reagent, and FBS (Invitrogen, Burlington, ON, Canada); DNase I (Ambion, Austin, TX); TaqMan Master Mix, 20X GAPDH, RNase inhibitor and reverse transcriptase (Applied Biosystems, Foster City, CA); the firefly luciferase reporter plasmid pGL4 basic and passive lysis buffer (Promega, Madison, WI); Firefly luciferase assay kit (Biotium Inc., Hayward, CA); the selective p38 mitogen activated protein kinase, (p38 MAPK) inhibitor, SB203580 [4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H-imadazole] (Calbiochem, Gibbstown, NJ); TransIT-LT1 transfection reagent (Mirus, Madison, WI); antibodies against phospho-p38 and total-p38 (Cell Signalling Technology, Beverly, MA). All other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

Virus and cell lines

The BEAS-2B cell line was a gift from Dr. Curtis Harris (National Cancer Institute, Bethesda, MD). HRV type 16 (HRV-16), HRV type 1A (HRV-1A), H1-HeLa cells and WI-38 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). HRV-16 viral stocks were propagated in WI-38 cells, while HRV-1A stocks were propagated in H1-HeLa cells. Each viral stock was purified to remove soluble products of the propagating cells by sucrose density centrifugation as previously described [13]. Viral titers were also determined 5 days after inoculation of monolayers of WI-38 or H1-HeLa cells, as previously described [13].

Epithelial cell culture

Normal human lungs that were not used for transplant were obtained from a tissue retrieval service (International Institute for the Advancement of Medicine, Edison, NJ). Ethical approval to receive tissues was obtained from both the Conjoint Health Research Ethics Board of the University of Calgary and from the Internal Ethics Board of the International Institute for the Advancement of Medicine. All donors had negative serologies for HIV1/2, HTLV1/2, hepatitis A, B and C, and syphilis. In the current studies, lungs were used from 10 different donors (9 male, age range 18-56 years old). Of these, 6 subjects died of cerebral vascular events and 4 from head trauma. Lungs were received within 24-36 h after surgical removal. Primary human bronchial epithelial cells (HBE) were obtained by protease digestion of dissected airways as previously described [14]. HBE and BEAS-2B cells were grown in submersion culture in BEGM and incubated at 37ºC in 5% CO_2. Primary cell cultures achieved confluence in two weeks and the epithelial nature of cells was confirmed by cytokeratin staining [14]. Prior to stimulation, cells were cultured overnight in BEGM from which hydrocortisone was removed, and this medium was used for all experiments.

Preparation of cigarette smoke extract (CSE)

CSE was freshly prepared by a minor modification of previously published methods [15]. In brief, crude CSE was generated by bubbling one research grade cigarette (3R4F, College of Agriculture Reference Cigarette Program, University of Kentucky, USA) per 4ml of BEGM without hydrocortisone at a rate of 5 minutes per cigarette using a syringe apparatus. The crude CSE was filtered through a 0.22μm filter and subsequently adjusted with medium to an absorbance of 0.15 at 320nm. This solution was taken as 100% CSE. For experiments using aged CSE, the extract was prepared as described and left at 4°C for 24 h.

Viral infection and stimulation of epithelial cells

BEAS-2B cells were infected with HRV-16 at $10^{4.5}$ 50% tissue culture-infective dose ($TCID_{50}$) U/ml (multiplicity of infection (MOI) of ~0.1), while HBE were infected with $10^{5.5}$ $TCID_{50}$ U/ml (MOI ~1.0),. The higher dose used in HBE is needed to ensure robust responses, as it has been shown that, even at high doses of HRV, no more than 10% of HBE are infected [16]. Infections were performed in the presence or absence of CSE. All experiments were performed using 50% CSE unless stated otherwise. Cells were incubated at 34ºC in 5% CO_2. Viability of cells exposed to HRV-16, CSE or the combination was determined using both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays. BEAS-2B cells were pre-incubated with the selective p38 MAPK inhibitor, SB203580 (3μM), or vehicle control (DMSO) for 1h at 37ºC before stimulation with HRV-16 and/or CSE.

Transfection of epithelial cells with poly[I:C]

Polyionosinic-polycytodylic acid (poly[I:C]) (0.1μg) was transfected into cells in BEBM with no additives using TransIT. After 1 hour, cells were washed with HBSS and cultured in medium with or without CSE for 24 h.

Real Time RT-PCR

CXCL8 and CXCL10 mRNA expression analysis was performed by real time RT-PCR. For each sample, 400ng of input RNA was reverse transcribed into cDNA, followed by PCR amplification in the presence of specific primers and probes for the gene of interest or the housekeeping gene GAPDH. Primer and probe sequences for CXCL8 and CXCL10 have been described previously

[4, 17]. In each case, a synthetic first-strand cDNA amplicon was used to generate standard curves to permit absolute quantification of mRNA. Data were expressed as femtograms or attograms calculated from the standard curve after correction for minor variations in GAPDH levels.

CXCL8 mRNA stability studies

BEAS-2B cells were exposed to HRV-16, CSE or the combination for 2 h and actinomycin D was added at a final concentration of 10µg/ml. Cellular RNA was then isolated at various times for analysis of CXCL8 mRNA levels.

ELISAs

CXCL8 and CXCL10 were assayed by ELISA using matched antibody pairs according to the manufacturer's protocol (R&D Systems, Minneapolis, MN).

CXCL8 and CXCL10 promoter constructs

The 972-bp CXCL10 promoter construct has been previously described [4]. A 720-bp CXCL8 promoter construct, corresponding to the sequence from -712 to +8 (relative to the transcriptional start site) of the 5'-flanking region of the human CXCL8 gene, was generated from genomic DNA using forward 5'-CGG<u>GGTACC</u>TATAGTCAGTCCTTACATTGC-3" and reverse 5'-CCC<u>AAGCTT</u>CTTATGGAGTGCTCCGGTGGC-3' primers incorporating the *Kpn*I and *Hind*III restriction sites (underlined) used for insertion into the reporter plasmid. Both promoters were cloned upstream of the inducible firefly luciferase gene in pGL4 basic.

Transient transfection and luciferase assay

The CXCL8 (0.2μg) or the CXCL10 promoter construct (1μg) were transfected into sub confluent (40-50%) monolayers of BEAS-2B cells as previously described [17]. Cells were washed stimulated with HRV-16, CSE or the combination for either 5h (CXCL8) or 24h (CXCL10) and luciferase activity was measured as described [17].

Western blotting for p38 MAPK activation

Cell extraction, SDS-PAGE and probing with the phospho-p38 MAPK and total-p38 MAPK Ab were performed as previously described [17].

Statistical analysis

Normally distributed data were analyzed using either paired t tests or one-way ANOVA with student Newman-Keuls post hoc analysis. Data that were not normally distributed were analyzed using Kruskal-Wallis ANOVA followed by Wilcoxon matched-pairs signed-rank test. To determine whether there was synergy between HRV-16 and CSE, the sum of HRV-16 alone and CSE alone was compared with HRV-16 + CSE. Paired t tests or Wilcoxon matched-pairs signed-rank tests were used to determine differences. Data that had two independent variables were analyzed using two-way repeated measures ANOVA with Bonferroni's multiple comparison post hoc analysis. Values of $p < 0.05$ were considered significant.

RESULTS
CSE does not affect cell viability or viral titre.

Concentrations of 50% CSE or lower alone or in combination with HRV-16 did not affect viability of HBE or BEAS-2B cells as assessed using both MTT (see Figure A in the Supplementary data) and LDH assays (data not shown). To determine effects of CSE on viral replication, cells were exposed for 1 h to HRV alone or HRV +CSE for 1 h. Cells were washed and fresh medium, with or without CSE, added. Viral titres in supernatants obtained after 24h, expressed as Log $TCID_{50}$, were not different between cells exposed to HRV-16 alone (6.0 ± 0.04) and HRV-16 plus CSE (5.9 ± 0.35). Values are mean $\pm$ SEM of three separate experiments (see Figure B in the Supplementary data).

CSE and HRV-16 each induce CXCL8 production and induce at least additive production when used in combination.

Based on preliminary time course studies (see Figure C in the Supplementary data) CXCL8 mRNA levels were measured at 5 h post stimulation, while protein levels were assessed at 24 h after stimulation. HRV-16 alone and CSE each induced CXCL8 mRNA and protein from both HBE (Figure 1A and B) and BEAS-2B cells (Figure 1C and D). The combination of HRV-16 and CSE induced levels of CXCL8 mRNA and protein that were significantly greater than those induced by either stimulus alone. CXCL8 mRNA and protein induced by the combination of CSE and HRV-16 in BEAS-2B cells was synergistic compared to the two stimuli individually (Figure 1C and D). Synergistic induction of CXCL8 mRNA by HRV-16 + CSE was also observed in HBE (Figure 1A). By contrast, CXCL8 protein production from HBE by CSE + HRV-16 was not significantly greater than the sum of responses to each stimulus individually (Figure 1B). The effects of CSE alone or in combination with HRV-16 on CXCL8 protein

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production was concentration dependent (see Figure D in the Supplementary data) but synergistic induction was only observed at 50% CSE.

CSE inhibits HRV-16-induced CXCL10 expression.

HRV-16 induced CXCL10 mRNA and protein expression in HBE and BEAS-2B cells (Figure 2A-D). By contrast, CSE alone did not induce production of CXCL10 from either cell type, and CSE markedly inhibited HRV-16-induced CXCL10 mRNA and protein expression. In HBE, both HRV-16-induced CXCL10 mRNA and protein were significantly inhibited (Figure 2A and B).   In BEAS-2B cells, HRV-16 induced CXCL10 protein production also was significantly inhibited (Figure 2D). The CSE-mediated inhibition of HRV-16 induced CXCL10 mRNA expression in BEAS-2B cells (Figure 2C) just failed to achieve statistical significance (p = 0.054). The inhibition of HRV-16-induced CXCL10 by CSE was concentration dependent, with concentrations of CSE as low as 0.005 % still significantly inhibiting HRV-16-induced CXCL10 production (see Figure E in the Supplementary data).

CSE also modulates chemokine production induced by HRV-1A.

To determine if the effects of CSE on viral production of epithelial chemokines was unique for HRV-16, we also used the minor group rhinovirus serotype, HRV-1A as a stimulus in BEAS-2B cells. CSE also induced additive induction of CXCL8 when added with HRV-1A. As was the case with HRV-16, CSE inhibited HRV-1A-induced production of CXCL10 (see Figure F in the Supplementary data).

Induction of CXCL8 and inhibition of CXCL10 is unaffected by aging CSE.

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Both freshly prepared CSE and aged CSE induced comparable levels of CXCL8 protein, either alone or in the combination with HRV-16 (Figure 3A). Both fresh CSE or aged CSE when used in combination with HRV-16 synergistically induced CXCL8. In addition, both freshly prepared CSE and aged CSE significantly inhibited HRV-16-induced CXCL10 to a comparable extent (Figure 3B).

CSE inhibits HRV-16-induced and poly[I:C]-induced CXCL10 promoter activation.

To determine if CSE modulated HRV-16-induced CXCL10 transcription, promoter-luciferase construct studies were performed. As expected, HRV-16 induced CXCL10 promoter activity (Figure 4A). CSE alone did not induce promoter activation and, indeed, significantly suppressed basal promoter drive. In addition, CSE significantly inhibited HRV-16 induced CXCL10 promoter activation (Figure 4A).

We have previously shown that HRV-induced epithelial production of CXCL10 is dependent upon viral replication, and that synthetic dsRNA (poly[I:C]), a mimic for viral dsRNA generated during the replication cycle also induces CXCL10 production [4, 18]. Poly [I:C] caused robust production of CXCL10 protein in both HBE and BEAS-2B cells and, in both cell types, CSE significantly inhibited these responses (see Figure G in the Supplementary data). CSE also significantly inhibited CXCL10 promoter activation induced by poly[I:C] (Figure 4B).

CSE alone does not induce CXCL8 promoter activation and does not enhance HRV-16-induced CXCL8 promoter activation.

As expected, HRV-16 alone activated the CXCL8 promoter. Surprisingly, not only did CSE alone not induce CXCL8 promoter activation, but it significantly inhibited HRV-16-induced promoter drive (Figure 5).

The combination of CSE and HRV-16 increases stability of CXCL8 mRNA.

Preliminary data demonstrated that synergistic induction of CXCL8 steady state mRNA was first observed at 2 h after stimulation (data not shown). Thus, we used a 2 h exposure before addition of actinomycin D. In cells treated with either CSE alone or HRV-16 alone, CXCL8 mRNA decayed, with 50% loss occurring between 2 and 3 h in each case (Figure 6). In cells exposed to the combination of CSE and HRV-16, however, a significant stabilization of CXCL8 mRNA was observed, such that there was no significant degradation of CXCL8 mRNA over the 3 h time period studied (Figure 6).

The effects of CSE alone or in combination with HRV-16 on the p38 MAPK pathway.

Inhibition of the p38 MAPK pathway reduces HRV-16 induced CXCL8 production from epithelial cells [17], and the p38 MAPK pathway has been linked to stabilization of CXCL8 mRNA [19]. In agreement with our earlier studies, HRV-16 induced rapid phosphorylation of p38 MAPK. Consistent with an earlier report [20], CSE also modestly induced phosphorylation of p38 MAPK in BEAS-2B cells. The combination of HRV-16 and CSE induced additive phosphorylation of p38 MAPK at 30 minutes (as assessed by densitometry), but the response to the combination was less than additive at later time points (Figure 7A). The selective p38 MAPK inhibitor, SB203580, significantly inhibited CSE-induced CXCL8 protein production as well as CXCL8 production in response to the combination of HRV-16 and CSE (Figure 7B).

DISCUSSION

We provide clear evidence that CSE modulates epithelial responses to HRV-16 infection. Although it has been previously reported that CSE itself induces CXCL8 production from epithelial cells [21], our data are the first to show more than additive induction by the combination of CSE and HRV infection. Indeed, synergistic induction was seen for mRNA and protein production from BEAS-2B cells and mRNA from HBE. Protein release from HBE failed to achieve significance for synergy, probably due to the wide range of CXCL8 protein production seen between individual primary HBE donors. By contrast, CSE did not induce CXCL10 production from epithelial cells and strikingly inhibited HRV-induced CXCL10 production. These interactions were not unique for HRV-16, as CSE caused similar modulation of chemokine responses induced by the minor group serotype, HRV-1A. If similar responses occur *in vivo*, the increases in CXCL8 would be expected to favour a neutrophilic response in the airway and might lead to worse clinical outcomes, given that airway neutrophil numbers correlate with disease severity both in COPD and during viral exacerbations of asthma [5, 6]. Similarly, if reduced production of CXCL10 occurs in response to cigarette smoke *in vivo*, this could also worsen clinical outcomes. CXCL10 is a selective chemoattractant for type 1 lymphocytes and NK cells that play a role in antiviral defenses, and CXCL10 levels are induced during viral exacerbations of asthma [22], possibly as part of the host antiviral defense. Although antinflammatory drugs, such as corticosteroids [23], and host defense molecules such as nitric oxide [18], suppress HRV-induced epithelial production of CXCL10, these treatments also inhibit a range of other cytokines, including CXCL8. Thus, the differential effects of CSE is somewhat unusual. It has been shown that CXCL10 deficient mice show increased viral replication and mortality in response to some viruses [24]. Thus cigarette smoke induced reductions in CXCL10 could impair host defenses against HRV. Interestingly mice exposed to

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cigarette smoke and infected with influenza show increased viral titers in their lungs compared to animals infected without smoke exposure [25]. The recent demonstration that CSE also reduces lipopolysaccaride induced epithelial production of CXCL10 [26], raises the possibility that host defenses against bacterial infections could also be impaired by cigarette smoke.

The ability of CSE to modulate HRV-16-induced epithelial chemokine production does not appear to be mediated by unstable, volatile components of CSE, as very comparable results were observed using CSE prepared 24 h prior to use. Interestingly, CSE was markedly more potent in suppressing viral induction of CXCL10 than in inducing or enhancing CXCL8 production, suggesting that differing mechanisms may be involved. It is known that CXCL8 can be induced in epithelial cells by HRV-16 in a manner that does not require viral replication but involves direct signalling via ICAM-1 [27]. By contrast, HRV-induced CXCL10 production is absolutely dependent upon viral replication [4]. Nonetheless, suppression of epithelial CXCL10 production by CSE is not mediated by effects on viral replication. Not only does CSE not alter viral titres from infected cells, but induction of CXCL10 by synthetic dsRNA is also inhibited by CSE. The effects of CSE are mediated, at least in part, at the level of transcription since CXCL10 promoter activation by both HRV-16 infection and dsRNA exposure is inhibited by CSE. Interestingly, basal activation of the CXCL10 promoter also was inhibited in the presence of CSE. This suggests that CSE either induces repressive factors that suppress both basal and HRV-induced transcription, inhibits responses triggered by constitutively active transcription factors, or that CSE directly impacts the basal transcriptional complex. Further studies are needed to delineate the mechanisms of transcriptional repression of CXCL10 by CSE.

Although CSE enhanced epithelial expression of both CXCL8 mRNA and protein, it did not induce CXCL8 promoter activation and, indeed, inhibited HRV-16-induced CXCL8

promoter drive, despite the more than additive induction of steady state CXCL8 mRNA. This indicated that transcriptional regulation did not underlie the more than synergistic induction of CXCL8 by HRV-16 and CSE. It is well established, however, that expression of CXCL8 is also regulated at the level of mRNA stability, and it has been shown that activation of the p38 MAPK pathway leads to stabilization of CXCL8 mRNA [19]. We demonstrated that treatment with the combination of HRV-16 and CSE led to a marked stabilization in CXCL8 mRNA compared to either stimulus alone. Both stimuli induced phosphorylation of p38 and, at the earliest time point this induction by the combination of stimuli was additive. The fact that induction by the combination of HRV-16 and CSE became less than additive at later time points when mRNA was still stable, raises questions about whether p38 is the sole mechanism regulating mRNA stability. Nonetheless, selective inhibition of the p38 pathway reduced CXCL8 production by each stimulus alone, with significant effects on CSE and particularly on CXCL8 production by the combination of HRV and CSE, clearly indicating that this pathway plays a role in the synergistic induction of CXCL8 induction by these stimuli.

In summary, we demonstrate for the first time that CSE can modulate HRV-induced chemokine production from airway epithelial cells. CSE in combination with HRV-16 leads to an at least additive induction of CXCL8 and this synergy is mediated, at least in part, at the level of mRNA stabilization, with some role for the p38 MAPK pathway. CSE is more potent in suppressing HRV-16-induced production of CXCL10 and this effect is mediated, at least in part, at the level of transcriptional control. Additional studies are needed to further delineate the mechanisms by which CSE modulates viral induction of these chemokines. If cigarette smoke modulates viral responses in the same way in vivo, this would suggest that patients who smoke would have increased neutrophilia and decreased recruitment of lymphocytes populations

compared to nonsmokers. This change in inflammatory pattern could lead to worse exacerbations of lower airway diseases, such as COPD and asthma.

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FIGURE LEGENDS

FIGURE 1. CSE and HRV-16 induce CXCL8 alone and at least additively increase CXCL8 in combination in airway epithelial cells. **Panel A**: CXCL8 mRNA levels were determined at 5h in HBE (n=10). **Panel B**: CXCL8 protein levels were determined at 24h in HBE (n=9). **Panel C**: CXCL8 mRNA levels were determined at 5h in BEAS-2B cells (n=6). **Panel D**: CXCL8 protein levels were determined at 24h in BEAS-2B cells (n=10). Asterisks indicate a significant difference between HRV-16 + CSE and the sum of values from each treatment alone. Values are means ±SEM.
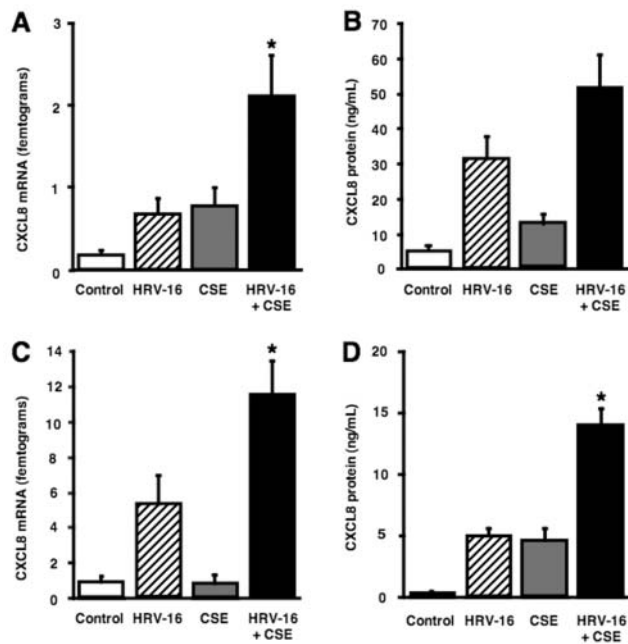
FIGURE 2. CSE inhibits HRV-16-induced CXCL10 in airway epithelial cells. Panel A: CXCL10 mRNA (n=10) and **Panel B**: CXCL10 protein (n=10) levels were determined at 24h in HBE. **Panel C**: CXCL10 mRNA (n=9) and **Panel D**: CXCL10 protein (n=10) levels were determined at 24h in BEAS-2B cells. Asterisks indicate a significant inhibition with HRV-16 + CSE compared to HRV-16 alone. Values are means ±SEM.
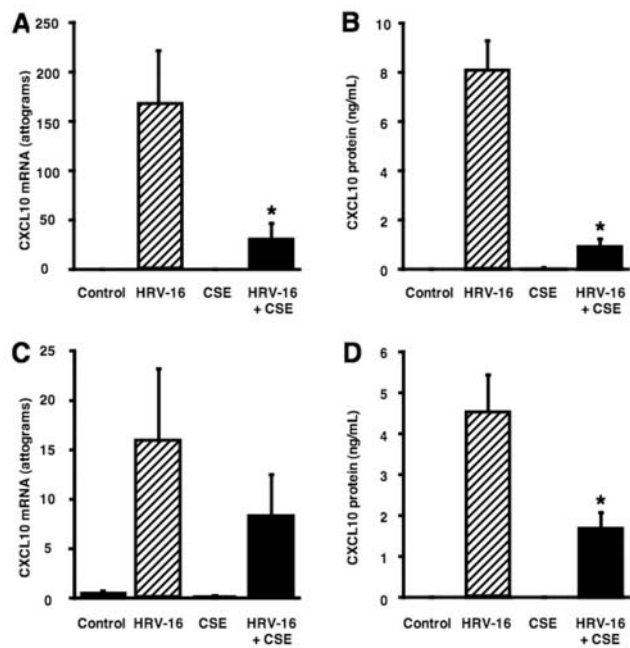
FIGURE 3. The effects of freshly prepared versus aged CSE alone or in combination with HRV-16 on CXCL8 and CXCL10 production in BEAS-2B cells. **Panel A**: CXCL8 (n=8) and **Panel B**: CXCL10 (n=8) protein levels were determined at 24h. Asterisks indicate a significant difference between HRV-16 + CSE or HRV-16 + aged CSE and HRV-16 alone. Values are means ±SEM.
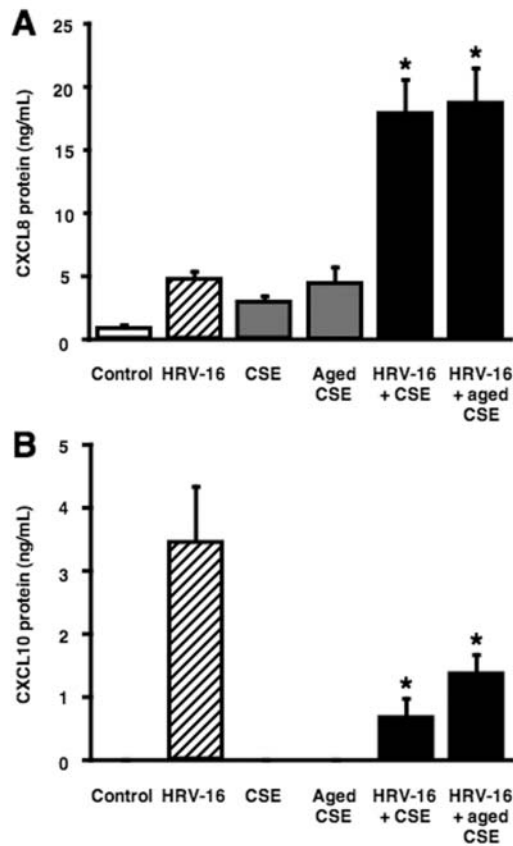
FIGURE 4. CSE inhibits both HRV-16-induced and poly[I:C]-induced CXCL10 promoter activation in BEAS-2B cells. Panel A: Cells stimulated with HRV-16, CSE or the combination (n=14). **Panel B**: Cells were transfected with poly[I:C] in the presence or absence of CSE (n=6). Asterisks indicate a significant inhibition with HRV-16 + CSE compared to HRV-16 alone or with poly[I:C] + CSE compared to poly[I:C] alone. Values are means ± SEM.
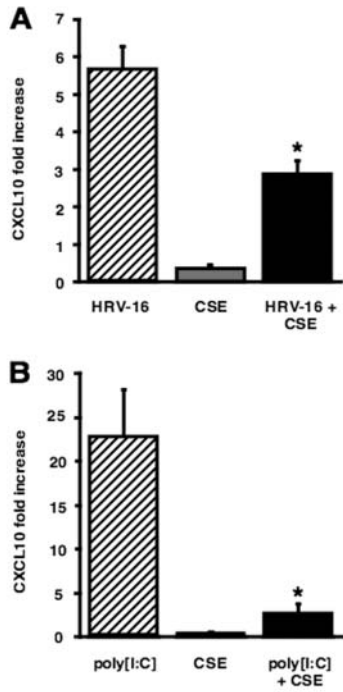
FIGURE 5. CSE in the combination with HRV-16 does not enhance CXCL8 promoter activation in BEAS-2B cells. Cells were stimulated with HRV-16, CSE or the combination. Asterisk indicates a significant inhibition with HRV-16 + CSE compared to HRV-16 alone. Values are means ± SEM (n=6).
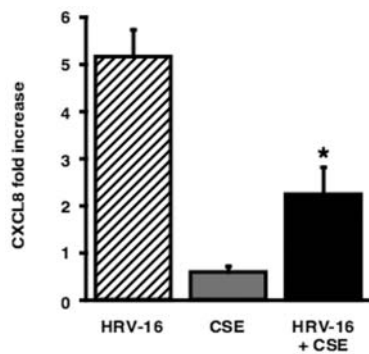
FIGURE 6. The combination of HRV-16 and CSE leads to stabilization of CXCL8 mRNA in BEAS-2B cells. Cells were stimulated for 2h with HRV-16, CSE or the combination, and then actinomycin D (10µg/mL) was added. Total cellular RNA was extracted at the times shown and CXCL8 mRNA levels assayed. Data are expressed as a percentage of CXCL8 mRNA at time 0 for each stimulus. Asterisks indicate a significant difference between HRV-16 + CSE and HRV alone. Values are means ± SEM (n=4).
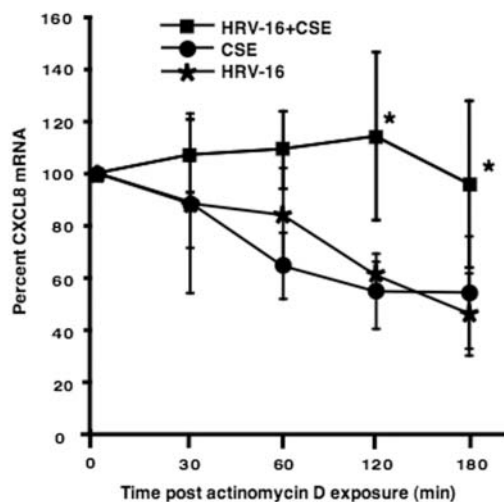
FIGURE 7. Activation of the p38 MAPK pathway and the effects of pathway inhibition on CXCL8 production in BEAS-2B cells. Panel A: Cells were exposed to medium, CSE, HRV-16 or the combination for the times indicated and probed using a specific antibody to phospho-p38 MAPK (representative of 3 separate experiments). Total p38 MAPK was assayed to determine equal loading. **Panel B**: Cells were incubated in the presence of SB203580, or vehicle control

(DMSO), for 1 h before stimulation with CSE, HRV-16 or the combination. Asterisks indicate a significant inhibition with stimulus + SB203580 compared with stimulus alone. Values are means ± SEM (n=3)