# Cigarette Smoke Alters Respiratory Syncytial Virus–Induced Apoptosis and Replication

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**Individuals exposed to cigarette smoke have a greater number and severity of viral infections, including respiratory syncytial virus (RSV) infections, than do nonsmokers, but the cellular mechanism is unknown. Our objective was to determine the mechanism by which cigarette smoke augments viral infection. We hypothesize that cigarette smoke causes necrosis and prevents virus-induced cellular apoptosis, and that this is associated with increased inflammation and viral replication. Primary airway epithelial cells were exposed to cigarette smoke extract for 2 days, followed by 1 day of RSV exposure. Western blot detection of cleaved caspases 3 and 7 showed less apoptosis when cells were treated with cigarette smoke before viral infection. This finding was confirmed with ELISA and TUNEL detection of apoptosis. Measures of cell viability, including propidium iodide staining, ATP assay, and cell counts, indicated that cigarette smoke causes necrosis rather than virus-induced apoptosis. Using plaque assay and fluorescently-labeled RSV, we showed that although there were less live cells in the cigarette smoke–pretreated group, viral load was increased. The effect was inhibited by pretreatment of cells with N-acetylcysteine and aldehyde dehydrogenase, suggesting that the effect was primarily mediated by reactive aldehydes. Cigarette smoke causes necrosis rather than apoptosis in viral infection, resulting in increased inflammation and enhanced viral replication.**

**Keywords:** respiratory syncytial virus; apoptosis; necrosis; cigarette smoke

Apoptosis, or programmed cell death, is a host cell mechanism that limits viral replication and inflammation (1–4). Thus, the type of cell death that occurs in response to viral infection (apoptosis versus necrosis) is an important determinant of viral survival, replication, and inflammation. Cigarette smokers experience an increased number and severity of viral infections, including respiratory syncytial virus (RSV) infections (5). In U.S. Army recruits, smokers had more upper respiratory infections than nonsmokers (6). Female smokers in the Israeli army had a 60% risk of influenza, compared with a 41.6% risk in nonsmokers (7). In addition, neonates and children exposed to cigarette smoke have a higher incidence and severity of viral infection (8–11). These epidemiologic studies suggest that smoking is a risk factor for severe viral infection; however, the mechanism(s) for this are not known.

The studies performed to date in animals have shown variable effects of cigarette smoke on viral infections. In one study, mice were exposed to cigarette smoke and then infected with influenza A. Exposure to smoke did not increase the viral load (12). In another study, exposure to carbon black and acrolein impaired elimination of influenza A virus from the lungs (13). Finally, neonatal mice were exposed to smoke, followed by subsequent RSV infection. This exposure led to increased viral gene expression (14).

Cigarette smoke contains thousands of compounds, and many effects of cigarette smoke have been attributed to electrophilic α,β-unsaturated aldehydes like acrolein and reactive oxygen species (ROS). Both acrolein and ROS can deplete cellular glutathione. This is the mechanism by which acrolein inhibits neutrophil caspase activation and apoptosis (15, 16). Cigarette smoke depletes glutathione by irreversibly reacting with glutathione derivatives and binding them to acrolein (17). Acrolein also converts lymphocyte apoptosis to necrosis (18). In B lymphoblastoid and Jurkat T cells, acrolein directly alkylates caspase-8 and inhibits its activity (19). Finally, in airway epithelial cells, cigarette smoke extract, which contains both acrolein and ROS, disturbs mitochondrial function, decreasing the capacity of mitochondria for ATP synthesis (20), and cigarette smoke extract reduces ATP levels in both subconfluent and confluent bronchial epithelial cell cultures (21). Since apoptosis is an energy-requiring process, this depletion of ATP should convert apoptosis to necrosis. Cigarette smoke components can alter apoptosis in disparate ways, depending on the specific component and cell type.

Apoptosis, compared with necrosis, is an efficient way to rid the airway of virally infected cells, and it is associated with a less significant inflammatory response. In addition, when necrotic cells burst, virus is released and not contained as in apoptosis. In this study, we show that cigarette smoke causes necrosis rather than apoptosis and that this is associated with increased viral replication. Treatment with N-acetylcysteine (NAC) and aldehyde dehydrogenase partially reverses this alteration of cell death from apoptosis to necrosis, suggesting a role for reactive aldehydes.

### CLINICAL RELEVANCE

Individuals exposed to cigarette smoke have a greater number and severity of viral infections, but the cellular mechanism is unknown. Our study shows that cigarette smoke causes necrosis rather than apoptosis in viral infection, resulting in increased inflammation and enhanced viral replication.

## MATERIALS AND METHODS

N-Acetyl-L-cysteine, PEG-superoxide dismutase, PEG-catalase, superoxide dismutase, catalase, Nω-nitro-L-arginine methyl ester (L-NAME), aldehyde dehydrogenase, 0.4% trypan blue solution, and mouse monoclonal antibody to β-actin were obtained from Sigma (St. Louis, MO). Spermine NONOate was obtained from Caymen Chemical (Ann Arbor, MI). Protease inhibitors were obtained from Roche Applied Science (Mannheim, Germany). Rabbit polyclonal antibody to cleaved caspase 3 (Asp 175) #9664 and cleaved caspase 7 (Asp 198) #9491 were obtained from Cell Signaling Biotechnology (Beverly, MA). Mouse monoclonal

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$IgG_{2a}$ (DO-1) antibody to p53 was obtained from Santa Cruz Biotechnology (sc-126). Rabbit polyclonal antibody to XIAP (21278) was obtained from Abcam (Cambridge, MA).

### Human Tracheobronchial Epithelial Cells

Human tracheobronchial epithelial (hTBE) cells were obtained from Dr. Joseph Zabner and the Cell Culture Core under a protocol approved by the University of Iowa Institutional Review Board. Epithelial cells were isolated from tracheal and bronchial mucosa by enzymatic dissociation and cultured in Laboratory of Human Carcinogenesis (LHC)-8e medium on plates coated with collagen/albumin for study up to passage 10 as described previously (22). For infection, cells at 50 to 80% confluence were treated with human RSV strain A2 (MOI 2). Viral stocks were obtained from Advanced Biotechnologies, Inc. (Columbia, MD). The initial stock ($1.1 \times 10^9$ TCID50) was aliquoted and kept frozen at −135°F, and a fresh aliquot was thawed for each experiment. Fluorescent viral stocks (rrRSVBN1 $1.7 \times 10^6$ plaque-forming units [pfu]/ml and rgRSV224 is $2.5 \times 10^6$ pfu/ml) were obtained from Mark Peeples (Ohio State University, Columbus, OH) and Peter Collins (NIH), and their synthesis and use has been previously described (23, 24).

### Vero Cells

Vero cells were cultured in minimum essential medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), penicillin-streptomycin (Gibco/Invitrogen), L-glutamine (Gibco/Invitrogen), non-essential amino acids (Gibco/Invitrogen), and sodium pyruvate (Gibco/Invitrogen).

### Preparation of Cigarette Smoke Extract

Cigarette smoke extract solution was prepared using a modification of the method of Blue and Janoff (25). Ten ml of (LHC)-8e media were drawn into a 60-ml plastic syringe. Through one opening of a stopcock, 40 ml of cigarette smoke were drawn into the syringe and mixed with the medium by vigorous shaking for 1 minute. The process was repeated with one cigarette used for each 10 ml of medium. The generated cigarette smoke extract solution was filtered (0.22 μm) to remove large particles (26–29). The resulting solution was designated a 100% cigarette smoke extract solution. A 2% solution refers to 20 microliters of cigarette smoke extract per milliliter of media.

### Cell Protein Isolation and Western Blot Analysis

Twenty micrograms of protein were mixed 1:1 with 2× sample buffer, and Western blot analysis was performed as previously described (30).

### Cytokine Measurements

Primary human tracheobronchial epithelial cells were plated at approximately 80% confluence and exposed to various concentrations of cigarette smoke extract. Supernatants were collected and frozen at −70°C. Human IL-6 and IL-8 concentrations were determined using DuoSet enzyme-linked immunosorbent assay (ELISA) kits from R&D systems (Minneapolis, MN).

### ELISA

Cell Death Detection ELISA Plus (Roche, Mannheim, Germany) was used to measure the nucleosomal fragmentation, a characteristic of apoptosis, according to the manufacturer's instructions.

### TUNEL Assay

hTBE cells were plated in collagen-coated LabTek Chamber Slide (2-well Permanox) System from Electron Microscopy Services (Hatfield, PA). TUNEL staining for apoptotic nuclei was performed using the DeadEnd Fluorometric TUNEL system (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, cells were fixed in 4% paraformaldehyde for 25 minutes and then permeabilized with 0.2% Triton X-100 solution for 5 minutes. Labeling reactions were performed with 350 μl of rTdT incubation buffer for 60 minutes at 37°C in a humidified chamber. Color development was done with diaminobenzidine for 8 minutes. Vectashield Mounting Medium with Fluorescence with DAPI (H-1200) was applied (Burlingame, CA). Positively stained apoptotic nuclei were observed using the Leica DMI6000B light and fluorescent microscope (Bannockburn, IL).

### Cell Survival Assays

Cell death was determined using Guava ViaCount reagent (Guava Technologies, Hayward, CA), which contains propidium iodide and a cell-permeable dye for nucleated cells LDS-751, following the manufacturer's protocol and as described previously (30). Cells were mobilized using 0.05% trypsin and not scraped, and trypsin was inhibited immediately after cell mobilization was visually confirmed. Guava PCA flow cytometer (Guava Technologies, Hayward, CA) was used to identify nucleated cells (total cells) and propidium iodide–stained nucleated cells (dead cells) to determine the percent of cell death. Cellular ATP was measured using the CellTiter-Glo Luminescent Viability Assay (Promega) and was performed according to the manufacturer's instructions. Trypan blue staining was performed by making a 10% trypan blue solution with cell media and viewing live/dead cells under a light microscope.

### Fluorescent RSV/DAPI Staining

hTBE cells were plated at 80% confluence in a Lab-Tek Chamber Slide (2-well Permanox) System (Electron Microscopy Services) and treated with cigarette smoke extract or control media for 48 hours followed by green fluorescent RSV for 48 hours. Cells were fixed with zinc formalin for 10 minutes, and Vectashield Mounting Medium with Flurescence with DAPI (H-1200) was applied (Burlingame, CA). Cells were viewed with Leica DMI6000B light and fluorescent microscope (Bannockburn, IL).

### Plaque Assay

Viral titers of hTBE RSV-infected cells were measured by standard plaque assay as previously described (30).

### Statistical Analysis

Statistical analysis was performed on the cell death assay, the ELISA, the ATP assay, and the plaque assay data. Significant differences between two groups were determined by Student's *t* test, and significant differences between more than two groups were confirmed by one-way ANOVA with a Bonferroni's test for multiple comparisons using Graphpad statistical analysis software (La Jolla, CA). Variability is expressed by standard error of the mean.

## RESULTS

### Effect of Cigarette Smoke Extract on Airway Epithelial Cells

We determined the toxicity and inflammatory effects of cigarette smoke extract on airway epithelial cells independent of RSV infection. We exposed cells to various concentrations of cigarette smoke extract for 48 hours to evaluate the effect of cigarette smoke extract up until the point of RSV addition. We performed an ATP assay in which the amount of ATP reflects cell viability (more ATP, more viability). As Figure 1A shows, the ATP is depleted slightly at a 4% cigarette smoke extract concentration and markedly at 5% and above. We also performed trypan blue staining to determine cell viability, and as shown in Figure 1B, the percent cell death rises markedly at a 3% cigarette smoke extract concentration and higher. Microscope photographs in Figure 1C show increased cell death and decreased cell density at 3% cigarette smoke extract and above. No induction of cytokines/chemokines like IL-6 (Figure 1D) and IL-8 (data not shown) was seen with cigarette smoke extract alone. Therefore, we selected a 2% cigarette smoke extract concentration for the subsequent experiments because this concentration demonstrates little toxicity or inflammation secondary to the cigarette smoke extract exposure alone at the time RSV was added. Similar toxicity curves have been published in the literature (31).

### Cigarette Smoke Prevents RSV- and Staurosporine-Induced Caspase 3 and 7 Activation

hTBE cells were plated in an adherent monolayer and exposed to a sequence of conditions for a total of 72 hours. The groups were as follows: control media (72 h), 2% cigarette smoke extract for

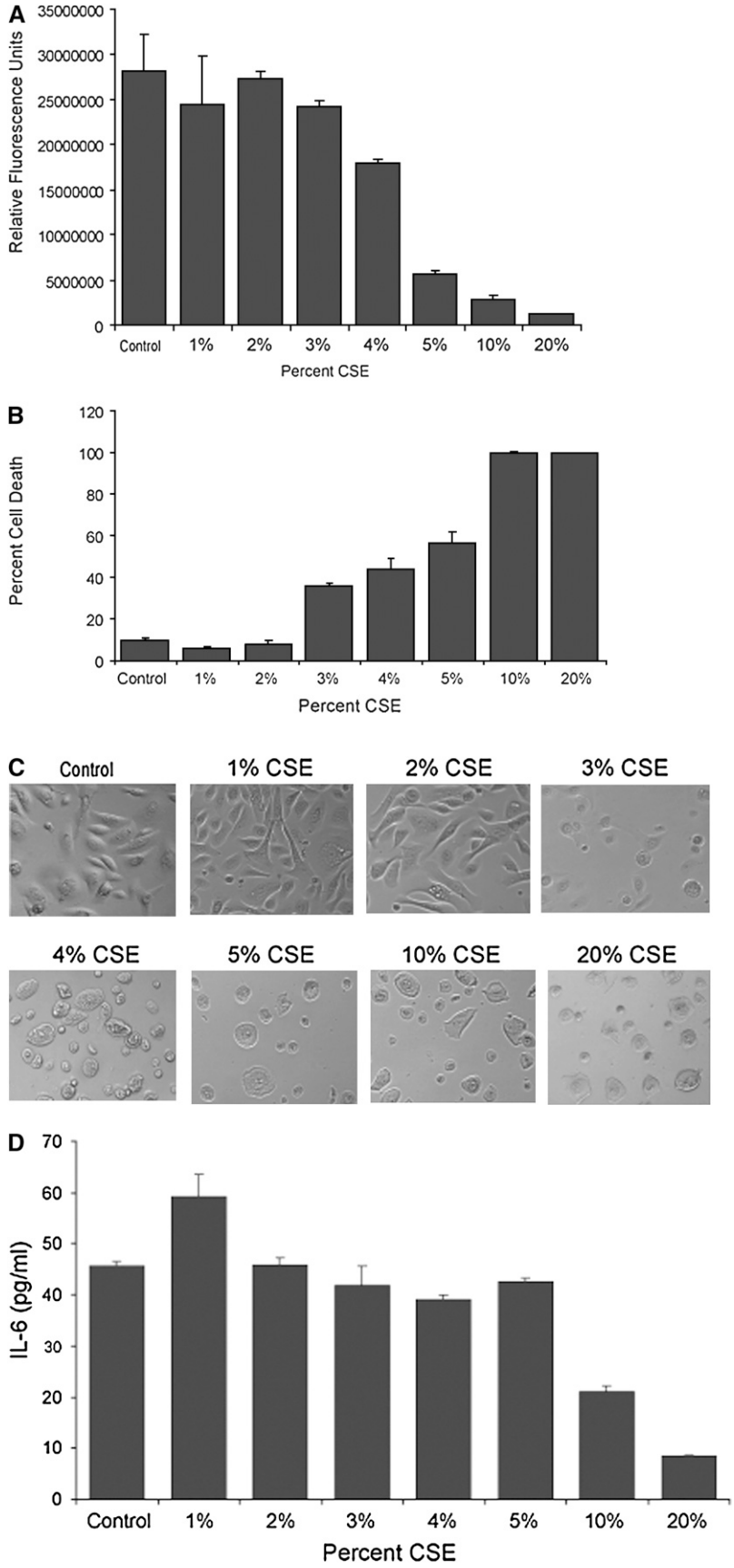


***Figure 1.*** Dose-toxicity and cytokine data for cigarette smoke extract (CSE). Human tracheobronchial epithelial (hTBE) cells were plated in an adherent monolayer and exposed to various concentrations of cigarette smoke extract as indicated for 48 hours. (*A*) ATP reagent was added, and relative intensity was quantified using a luminometer. (*B*) Trypan blue solution (0.4%) was filtered and added to the cell media in a 10% solution. Alive and dead cells were counted, and percent cell death was calculated. (*C*) Light micrographs were obtained. (*D*) Human IL-6 was determined by ELISA.

72 hours, RSV (MOI 2) for 24 hours (48 h of control media followed by 24 h of RSV), or 2% cigarette smoke extract for 48 hours followed by 24 more hours of both cigarette smoke extract (CSE) and RSV (MOI 2) (Figure 2A). The cells were then harvested for whole cell protein, and Western blots for cleaved caspase 3 and cleaved caspase 7 were performed. As Figure 2B demonstrates, RSV exposure alone for 24 hours leads to increased cleaved caspase 3 and 7 proteins. Pretreatment with cigarette smoke extract for 48 hours prevents the appearance of cleaved caspase 3 and 7 products after 24 hours of RSV infection. Similar results were seen with Western blots performed for cleaved caspase 8 and cleaved caspase 9 (data not shown). These results indicate that cigarette smoke prevents RSV-induced activation of caspases.

We next wanted to see whether cigarette smoke extract would prevent caspase activation by a nonviral stimulus. We exposed hTBEs to staurosporine, a known activator of caspases and inducer of apoptosis. hTBE cells were plated in an adherent monolayer and exposed to control media (72 h), 2% cigarette smoke extract for 72 hours, 48 hours of control media followed by 20 μM staurosporine for 24 hours, or 2% cigarette smoke extract for 48 hours followed by 24 hours of both cigarette smoke extract and 20 μM staurosporine. The cells were then harvested for whole cell protein, and Western blots for cleaved caspase 3 and 7 were performed. As shown in Figure 2C, cigarette smoke extract attenuates cleaved caspase 3 and 7 production, even with the known apoptosis inducer, staurosporine. These data demonstrate that cigarette smoke exposure limits caspase activation after both viral (RSV) and nonviral stimuli (staurosporine). Figure 2D demonstrates that some reduction is evident after 24 hours of pretreatment with cigarette smoke extract before viral infection, but

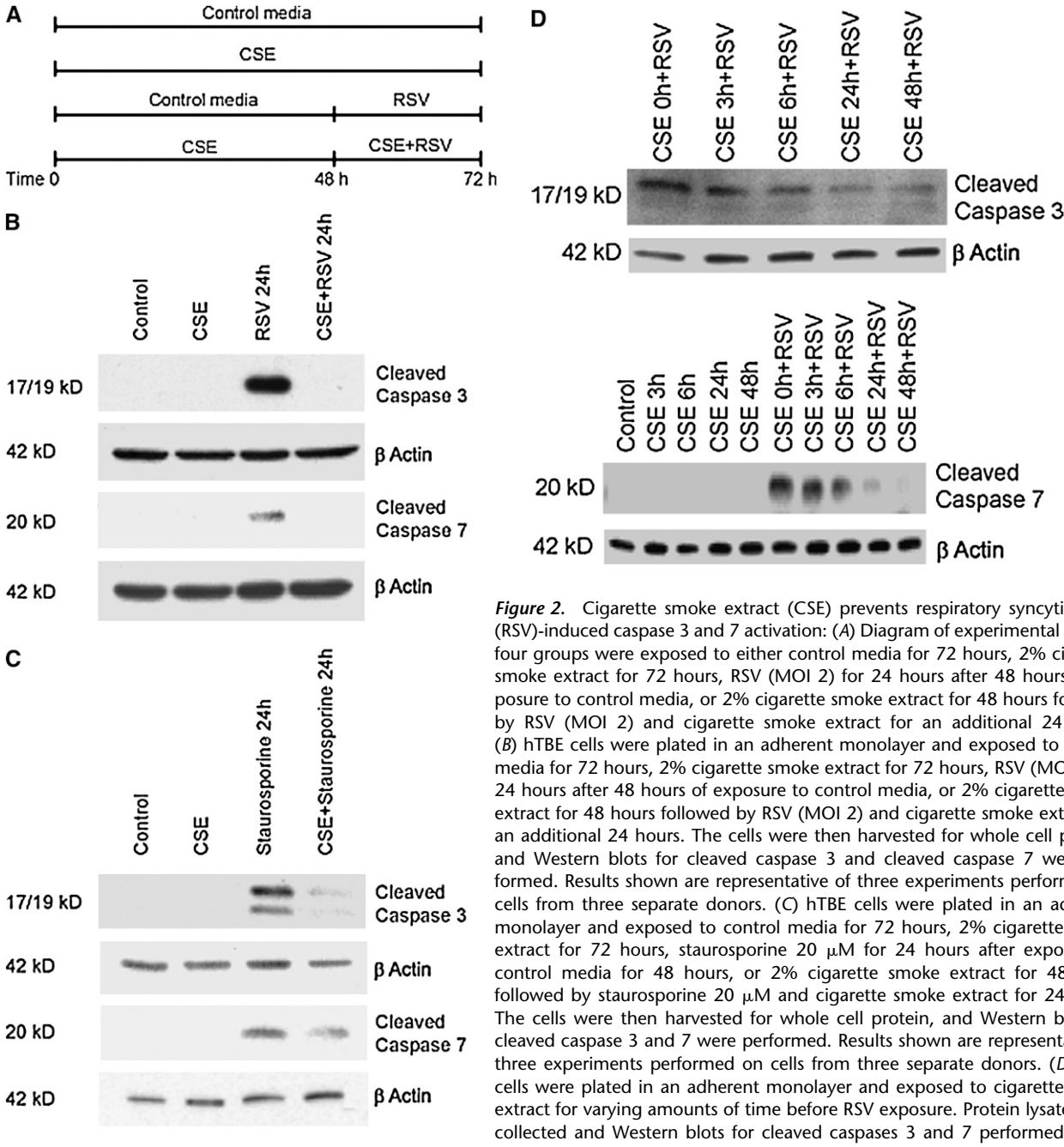


***Figure 2.*** Cigarette smoke extract (CSE) prevents respiratory syncytial virus (RSV)-induced caspase 3 and 7 activation: (*A*) Diagram of experimental design: four groups were exposed to either control media for 72 hours, 2% cigarette smoke extract for 72 hours, RSV (MOI 2) for 24 hours after 48 hours of exposure to control media, or 2% cigarette smoke extract for 48 hours followed by RSV (MOI 2) and cigarette smoke extract for an additional 24 hours. (*B*) hTBE cells were plated in an adherent monolayer and exposed to control media for 72 hours, 2% cigarette smoke extract for 72 hours, RSV (MOI 2) for 24 hours after 48 hours of exposure to control media, or 2% cigarette smoke extract for 48 hours followed by RSV (MOI 2) and cigarette smoke extract for an additional 24 hours. The cells were then harvested for whole cell protein, and Western blots for cleaved caspase 3 and cleaved caspase 7 were performed. Results shown are representative of three experiments performed on cells from three separate donors. (*C*) hTBE cells were plated in an adherent monolayer and exposed to control media for 72 hours, 2% cigarette smoke extract for 72 hours, staurosporine 20 μM for 24 hours after exposure to control media for 48 hours, or 2% cigarette smoke extract for 48 hours followed by staurosporine 20 μM and cigarette smoke extract for 24 hours. The cells were then harvested for whole cell protein, and Western blots for cleaved caspase 3 and 7 were performed. Results shown are representative of three experiments performed on cells from three separate donors. (*D*) hTBE cells were plated in an adherent monolayer and exposed to cigarette smoke extract for varying amounts of time before RSV exposure. Protein lysates were collected and Western blots for cleaved caspases 3 and 7 performed. Some reduction in caspase cleavage is evident after 24 hours of pretreatment with cigarette smoke extract, but 48 hours of cigarette smoke extract exposure before RSV infection is needed to see a marked and reliable reduction in both cleaved caspases 3 and 7.

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### Cigarette Smoke Extract Does Not Alter p53 or XIAP

Our previous work has shown that RSV decreases p53 protein and delays apoptosis in airway epithelial cells, so we next looked at whether cigarette smoke extract is altering apoptosis via decreased p53 protein. Figure 3 demonstrates that RSV does decrease p53 as we previously showed, but cigarette smoke extract does not alter the p53 protein independently of this virally mediated reduction in p53 protein. We then evaluated whether cigarette smoke extract is altering caspase activity by increasing the expression of inhibitors of apoptosis (IAPs). One IAP protein, X chromosome linked IAP (XIAP), inhibits the activity of several caspases, including caspase-3 and caspase-7 (32, 33). Again, Figure 3 demonstrates that cigarette smoke extract does not increase XIAP to decrease caspase-3 and caspase-7 activation. The mechanism for cigarette smoke–induced reduction in apoptosis is not related to alterations in p53 or XIAP protein.

### Cigarette Smoke Extract Prevents RSV-Induced Apoptosis

Having shown that cigarette smoke prevents RSV-induced caspase activation, we asked if it blocks apoptosis. We performed an ELISA, which detects release of histones and DNA to the cytosol (a marker of apoptosis). We found that there was significant apoptosis in cells exposed to RSV for 24 hours. In the cells pretreated with cigarette smoke extract and then exposed to RSV, there was a significant decrease in apoptosis (Figure 4A). Next we performed a TUNEL assay with DAPI staining of nuclei. This assay detects DNA strand breakage, a feature of apoptosis. DNase was used as a positive control. As shown in Figure 4B, significant TUNEL staining was observed in the RSV-treated group, but minimal TUNEL staining was observed in the cigarette smoke extract or cigarette smoke extract plus RSV groups. These experiments further support the idea that apoptosis is occurring in the RSV-infected cells but not in the RSV-infected cells pretreated with cigarette smoke extract.

### Cigarette Smoke Extract Pretreatment of RSV-Infected Cells Leads to Cell Death

If the cigarette smoke extract–pretreated, RSV-infected cells are not undergoing apoptosis, they are either living longer or are dying by a different mechanism. We next determined the overall cell viability of cigarette smoke-exposed and RSV-infected cells. First, we performed cell counts using a Coulter counter, both on total cells (cells in the supernatant plus adherent cells) and adherent cells alone. We found that the total number of cells did not vary among all groups, indicating that cigarette smoke extract does not alter cell proliferation (Figure 5A). There is a slightly greater proportion of cells in the supernatant in the cigarette smoke–exposed, RSV-infected cells, indicating some loss of attachment after the cigarette smoke exposure (Figure 5B). Next we performed a cell viability assay, using flow-cytometric analysis of propidium iodide–stained cells (Figure 5C). We found that although there is less apoptosis in the cells exposed to cigarette smoke extract and then exposed to RSV, there is approximately three times more cell death as measured by propidium iodide staining. These data can be reconciled with the data in Figure 4A by recognizing that when cells undergo early apoptosis, plasma membrane integrity is maintained. Cells can already demonstrate pyknosis and nuclear fragmentation (as detected by ELISA and TUNEL assay) while the plasma membrane is still intact, and the cell is not permeable to propidium iodide (34). It is also important to note that while cigarette smoke extract alone and cigarette smoke extract followed by RSV infection do cause some cell toxicity (15–20% cell death), sufficient cells (80–85%) are still alive to produce cleaved caspases if induced. Figure 5C indicates the decrease in cleaved caspase 3 in the cigarette smoke–pretreated, RSV-infected group in Figure 2B is due to less caspase activation and not less caspase production due to lack of viable cells. The viability of the cigarette smoke extract–exposed cells is also evident in Figure 7B. In further evaluation of overall cell death, we performed an ATP assay. As shown in Figure 5D, there is significantly less ATP in the cells pretreated with cigarette smoke extract and then exposed to RSV when compared with the RSV-infected cells alone, again indicating that there is ATP depletion and more overall cell death in the cigarette smoke–pretreated cells. In fact, there is less ATP in the cigarette smoke–only treated cells, suggesting that the cigarette smoke extract may set up a low ATP environment where apoptosis cannot occur. We show by multiple methods that while cigarette smoke prevents RSV-induced caspase activation and apoptosis in airway epithelial cells, some of the cells (15–20%) are still dying by another mechanism, most likely necrosis. The remaining cells have less ATP but are clearly viable at this point. Although we did not directly show that the inhibition of apoptosis is related to levels of ATP, it is known that loss of cellular ATP prevents apoptosis because this mode of cell death requires a large amount of cellular energy (34–37). We are unaware of any studies showing less ATP levels in airway epithelial cells of cigarette smokers *in vivo*, and such a translational study is the subject of future work.

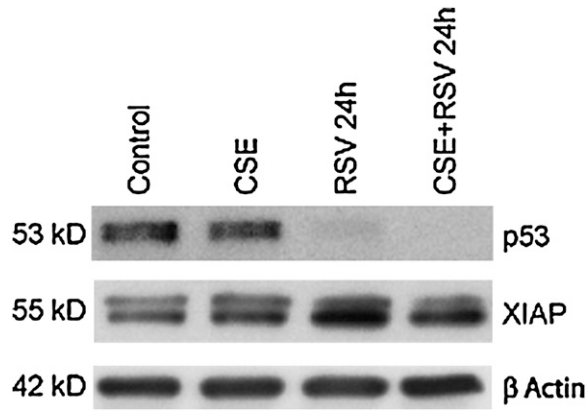


***Figure 3.*** Cigarette smoke extract (CSE) does not alter p53 or XIAP. hTBE cells were plated in an adherent monolayer and exposed to control media for 72 hours, 2% cigarette smoke extract for 72 hours, RSV (MOI 2) for 24 hours after exposure to control media for 48 hours, or 2% cigarette smoke extract for 48 hours followed by RSV (MOI 2) and cigarette smoke extract for an additional 24 hours. The cells were then harvested for whole cell protein, and Western blots for p53 and XIAP were performed. Results shown are representative of three experiments performed on cells from three separate donors.

### N-Acetylcysteine Limits the Effect of Cigarette Smoke Extract on RSV-Infected Cells

Acrolein and reactive oxygen species (ROS) are significant components of cigarette smoke. Acrolein has been shown to convert apoptosis to necrosis in other cells types (15, 16, 18, 19), so we investigated whether an agent known to inhibit the activity of reactive aldehydes, like acrolein, and ROS alter the effect of cigarette smoke extract on the cells. hTBE cells were plated in an adherent monolayer and exposed to control media (72 h), 2% cigarette smoke extract (CSE) for 72 hours, RSV (MOI 2) for 24 hours (48 h of control media followed by 24 h of RSV), or 2%

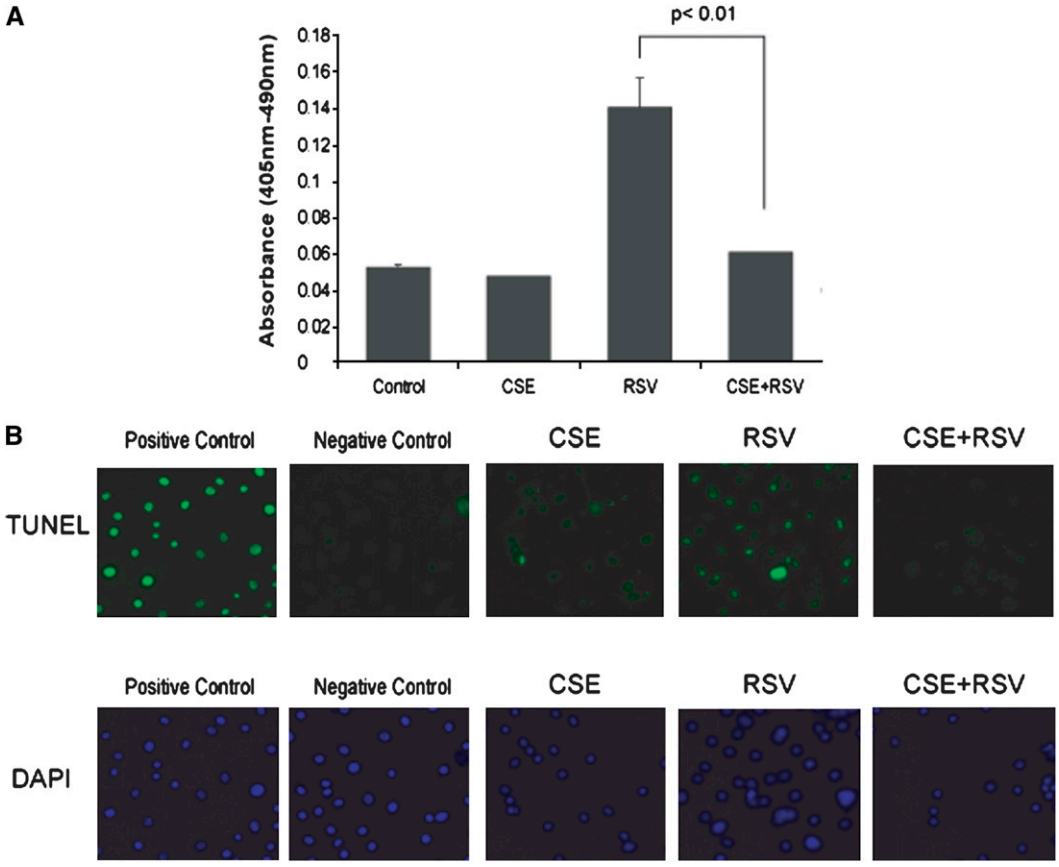


***Figure 4.*** Cigarette smoke extract (CSE) prevents RSV-induced apoptosis. (*A*) hTBE cells were plated in an adherent monolayer and exposed to control media for 72 hours, 2% cigarette smoke extract for 72 hours, control media for 48 hours followed by RSV (MOI 2) for 24 hours, or 2% cigarette smoke extract for 48 hours followed by RSV (MOI 2) and 2% cigarette smoke extract for 24 hours. Cells were then lysed and ELISA performed to detect nucleosomal fragments, indicative of apoptosis. One-way ANOVA with a Bonferroni's test for multiple comparisons shows a statistically significant difference between cells exposed to cigarette smoke extract and RSV as compared cells only exposed to RSV ($P < 0.01$). Results shown are representative of three experiments performed in triplicate on cells from three separate donors. (*B*) Cells exposed to the same conditions were then evaluated using a TUNEL assay. TUNEL staining of DNA strand breakage (indicative of apoptosis) shows there is significant TUNEL staining in cells exposed to RSV but not those exposed to cigarette smoke extract alone or cells treated with cigarette smoke extract before RSV infection.

cigarette smoke extract for 48 hours followed by 24 more hours of both cigarette smoke extract and RSV (MOI 2), with and without 2 mM N-acetylcysteine. The cells were then harvested for whole cell protein, and Western blot for cleaved caspase 3 was performed. This decrease in apoptosis seen in cells pretreated with cigarette smoke extract is reduced by pretreatment with N-acetylcysteine in cigarette smoke–exposed cells (Figure 6A). Western blots were also performed for cleaved caspase 7, but a reliable change such as that seen with cleaved caspase 3 was not observed; therefore, we conclude that cleaved caspase 3 is the major effector caspase involved in our system.

Since N-acetylcysteine is a nonspecific inhibitor of many components of cigarette smoke, including reactive aldehydes, reactive oxygen species, and nitric oxide, we next determined which components of cigarette smoke extract are depleting ATP and leading to necrosis in our system. We again exposed cells to control media (72 h), 2% cigarette smoke extract (CSE) for 72 hours, RSV (MOI 2) for 24 hours (48 h of control media followed by 24 h of RSV), or 2% cigarette smoke extract for 48 hours followed by 24 more hours of both cigarette smoke extract and RSV (MOI 2). We pretreated these four groups with inhibitors of various cigarette smoke components: aldehyde dehydrogenase for reactive aldehydes, N-acetylcysteine, LNAME for nitric oxide, PEG-superoxide dismutase and PEG-catalase for intracellular reactive oxygen species, and superoxide dismutase and catalase for extracellular reactive oxygen species. As shown in Figure 6B, no changes in ATP are seen when pretreated with these inhibitors in the control or RSV groups. However, when these inhibitors are used to pretreat the cigarette smoke extract–exposed group, only the aldehyde dehydrogenase and the N-acetylcysteine show a statistically significant prevention of ATP depletion. When these inhibitors are used to pretreat the cigarette smoke extract– and RSV-exposed group, the aldehyde dehydrogenase, N-acetylcysteine, PEG-superoxide dismutase/PEG-catalase, and superoxide dismutase/catalase show a statistically significant prevention of ATP depletion. These data suggest that reactive aldehydes cause depletion of ATP and subsequent necrosis under cigarette smoke extract conditions. Clearly, the system is complex, and other components of cigarette smoke, such as extracellular and intracellular reactive oxygen species, may also be depleting ATP under conditions of viral infection and cigarette smoke exposure.

### Cigarette Smoke Increases Viral Load

Since necrosis facilitates viral replication while apoptosis is known to inhibit viral replication (1–4), we evaluated whether cigarette smoke increases viral load. First, hTBE cells were plated in an adherent monolayer and exposed to control media for 48 hours, followed by fluorescent RSV (MOI 2) for 48 hours or 2% cigarette smoke extract for 48 hours, followed by both 2% cigarette smoke extract and fluorescent RSV (MOI 2) for 48 hours. At least 48 hours of fluorescent virus infection is needed for the viral genes to be expressed and the fluorescent proteins to be visible. As shown in Figure 7A, pretreatment with cigarette smoke extract demonstrated more red-fluorescent RSV after 48 hours of infection. Figure 7B shows that pretreatment with cigarette smoke extract also allows for more green fluorescent viral load, and DAPI staining confirms that this increase in viral load and percent infection is greater even despite a 15 to 20% reduction in total cell number. To confirm that the viral load was

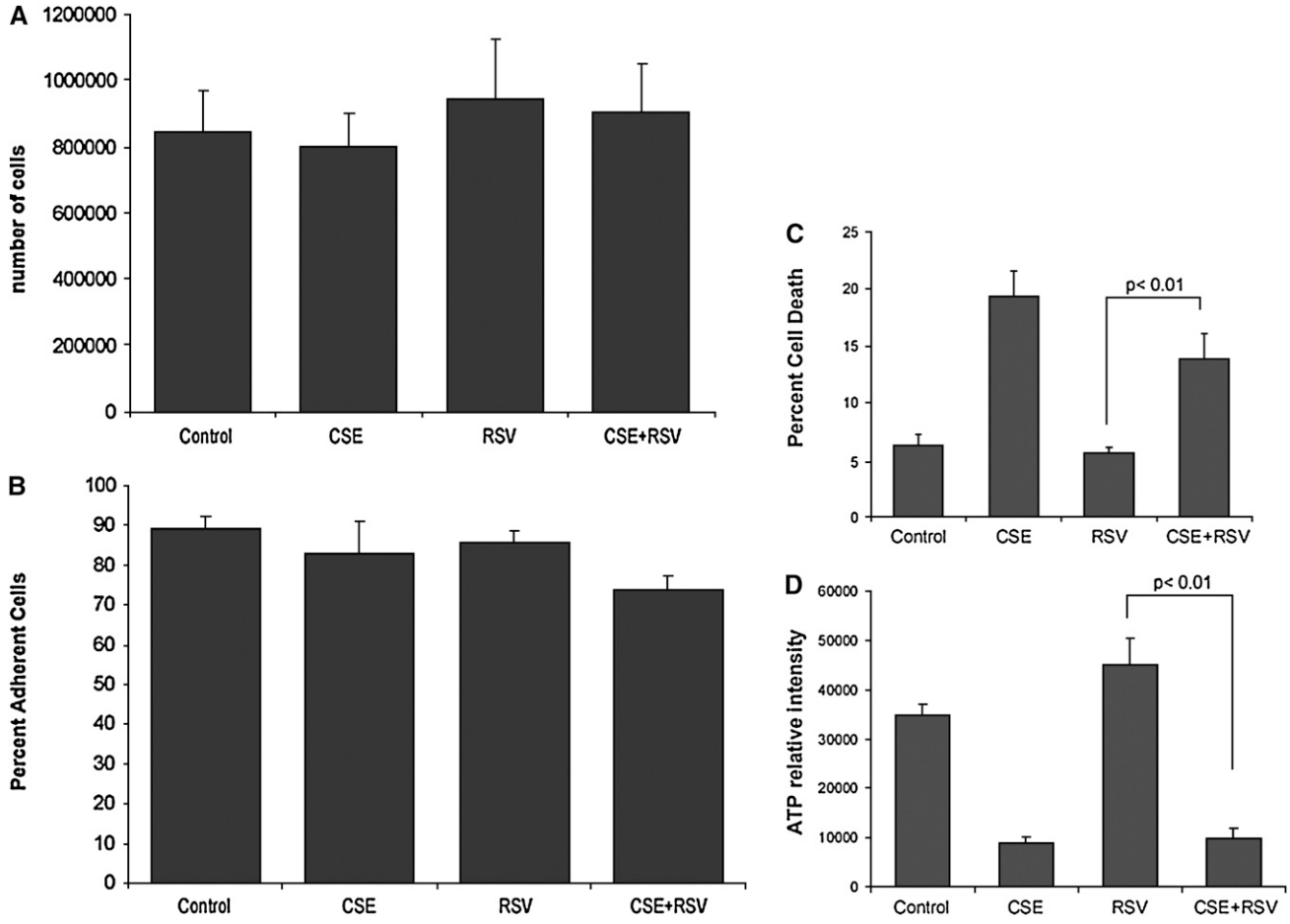


***Figure 5.*** Cigarette smoke extract (CSE) pretreatment of RSV-infected cells leads to cell death. hTBE cells were plated in an adherent monolayer and exposed to control media for 72 hours, 2% cigarette smoke extract for 72 hours, RSV (MOI 2) for 24 hours after exposure to control media for 48 hours, or 2% cigarette smoke extract for 48 hours followed by RSV (MOI 2) and cigarette smoke extract for an additional 24 hours. (*A*) Supernatants were collected, and adherent cells were trypsinized. All cells were added together to determine total cells counts using Coulter counter. (*B*) Cells were again collected from the supernatant, and adherent cells were trypsinized. Cells were quantified using a Coulter counter, but the percent of adherent cells is shown. These experiments (*A* and *B*) are composites of three experiments performed on cells from three separate donors. (*C*) hTBE cells were plated in an adherent monolayer and exposed to control media for 72 hours, 2% cigarette smoke extract for 72 hours, RSV (MOI 2) for 24 hours after exposure to control media for 48 hours, or 2% cigarette smoke extract for 48 hours followed by RSV (MOI 2) and cigarette smoke extract for 24 hours. Cells were mobilized and stained with propidium iodide. Flow cytometry was performed to detect the percent of cells with $<$ 2n DNA content, indicating cell death. One-way ANOVA with a Bonferroni's test for multiple comparisons shows a statistically significant difference between cells exposed to cigarette smoke extract and RSV as compared cells only exposed to RSV ($P < 0.01$). Graph shown is representative of three experiments performed in triplicate using cells from three separate donors. (*D*) hTBE cells were plated in an adherent monolayer and exposed to control media for 72 hours, 2% cigarette smoke extract for 72 hours, RSV (MOI 2) for 24 hours after exposure to control media for 48 hours, or 2% cigarette smoke extract for 48 hours followed by RSV (MOI 2) and cigarette smoke extract for 24 hours. ATP reagent was added, and relative intensity was quantified using a luminometer. One-way ANOVA with a Bonferroni's test for multiple comparisons shows a statistically significant difference between cells exposed to cigarette smoke extract and RSV as compared cells only exposed to RSV ($P < 0.01$). Graph shown is representative of three experiments performed in triplicate using cells from three separate donors.

increased when cells were exposed to cigarette smoke before RSV, we performed a plaque assay. hTBE cells were plated in an adherent monolayer and treated with control media alone for 48 hours, followed by exposure to RSV (MOI 2) for 24 hours or 2% cigarette smoke extract for 48 hours, followed by both 2% cigarette smoke extract and RSV (MOI 2) for 24 hours. Cells and supernatant were collected and placed on a monolayer of Vero cells to perform plaque assay by serial dilutions. As shown in Figure 7C, a composite figure of plaque assays on hTBE cells from four different donors, there are a greater number of viral plaques when the cells are exposed to cigarette smoke before RSV compared with RSV-infected cells that were not exposed to cigarette smoke. The cigarette smoke extract–exposed cells had more virus than the cells not exposed to cigarette smoke extract, even though there were 15 to 20% less cells in the group exposed to cigarette smoke. This suggests that exposure to cigarette smoke extract leads to greater RSV replication in airway epithelium.

## DISCUSSION

In this study, we demonstrate that cigarette smoke commits cells to necrotic cell death rather than apoptotic cell death, and this is associated with increased viral replication. Although these studies are novel, studies in other systems have suggested that cigarette smoke switches apoptosis to necrosis. Multiple mechanisms have been suggested (15–20). In particular, in airway epithelial cells, van der Toorn and coworkers showed that cigarette smoke extract disturbs mitochondrial function, decreasing the

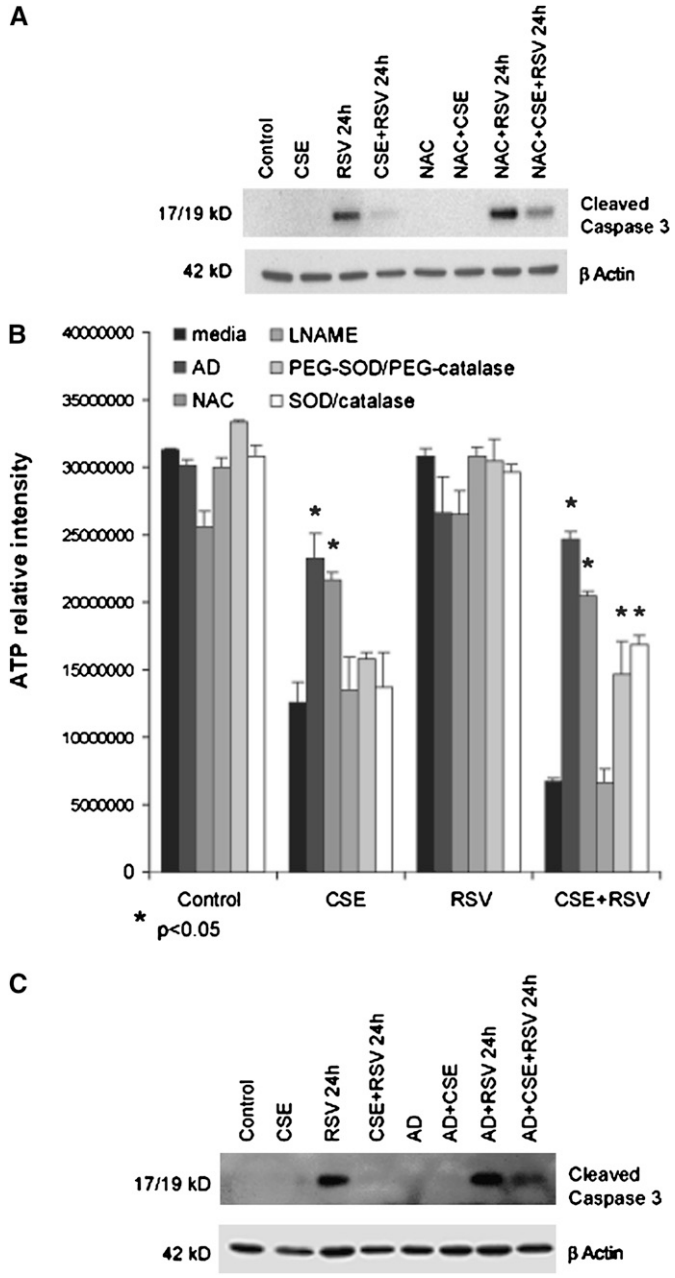


*Figure 6.* (*A*) N-acetylcysteine (NAC) and aldehyde dehydrogenase partially prevent the alteration in cleaved caspases seen with cigarette smoke extract pretreatment in RSV-infected cells. hTBE cells were plated in an adherent monolayer and exposed to control media for 72 hours, 2% cigarette smoke extract (CSE) for 72 hours, RSV (MOI 2) for 24 hours after exposure to control media for 48 hours, or 2% cigarette smoke extract for 48 hours followed by RSV (MOI 2) for 24 hours. The cells under these four conditions were pretreated with 2 mM NAC for 30 minutes and were then harvested for whole cell protein, and Western blot for cleaved caspase 3 was performed. Results shown are representative of three experiments performed on cells from three separate donors. (*B*) hTBE cells were plated in an adherent monolayer and then treated with inhibitors of various components of cigarette smoke, including aldehyde dehydrogenase (1 U/ml), NAC (2 mM), LNAME (1 μl/ml), PEG-superoxide dismutase (50 units/ml) with PEG-catalase (50 units/ml), superoxide dismutase (20 μg/ml) with catalase (5 μg/ml). They were then exposed to control media for 72 hours, 2% cigarette smoke extract (CSE) for 72 hours, RSV (MOI 2) for 24 hours after exposure to control media for 48 hours, or 2% cigarette smoke extract for 48 hours followed by RSV (MOI 2) for 24 hours. ATP reagent was added, and relative intensity was quantified using a luminometer. One-way ANOVA with a Bonferroni's test for multiple comparisons shows a statistically significant difference between cells exposed to cigarette smoke extract alone and those pretreated with aldehyde dehydrogenase or NAC before cigarette smoke extract. A statistically significant difference was also demonstrated between cells exposed to cigarette smoke extract and RSV and those groups pretreated with aldehyde dehydrogenase, NAC, PEG-superoxide dismutase/PEG-catalase, and superoxide dismutase/catalase ($P < 0.05$) before cigarette smoke and RSV. Graph shown is representative of three experiments, each performed in triplicate using cells from three separate donors. (*C*) hTBE cells were plated in an adherent monolayer and exposed to control media for 72 hours, 2% cigarette smoke extract (CSE) for 72 hours, RSV (MOI 2) for 24 hours after exposure to control media for 48 hours, or 2% cigarette smoke extract for 48 hours followed by RSV (MOI 2) for 24 hours. The cells under these four conditions were pretreated with aldehyde dehydrogenase (AD) for 30 minutes and were then harvested for whole cell protein, and Western blot for cleaved caspase 3 was performed. Results shown are representative of three experiments performed on cells from three separate donors.

capacity of mitochondria for ATP synthesis (20). Our studies support these findings, while also demonstrating an effect on viral replication in airway epithelium. We propose that cigarette smoke–induced ATP depletion precedes necrosis. Apoptosis is an ATP-requiring event, and depletion of ATP should decrease caspase activation and lead to necrosis (38). We see an earlier and more marked depletion of ATP before the subsequent necrosis.

Seventy-two hours of exposure to cigarette smoke extract does cause necrotic cell death (15–20%), as is seen in Figure 5C; however, we interpret the decrease in cleaved caspase activation seen in cigarette smoke extract–pretreated, RSV-infected cells to be due inhibition of caspase activation and not due to cigarette smoke–induced cell death. Figure 5C shows that 80 to 85% of the cigarette smoke extract–exposed cells were still viable after 72 h. This is further illustrated in Figure 7B, in which cells were exposed for 96 hours to cigarette smoke extract. These cells show an altered morphology but are clearly viable and adherent to the plate. There are still plenty of cells alive to detect apoptosis in the cigarette smoke extract–exposed cells, if this process were occurring. We feel that apoptosis did not occur because cigarette smoke extract depletes ATP, which is required for apoptosis.

Cigarette smoke is a complex mixture of agents that could trigger necrosis of cells. In our study, N-acetylcysteine, an inhibitor of reactive oxygen species, nitric oxide, and reactive aldehydes, prevented the effects of cigarette smoke on epithelial cells. We evaluated the individual effect of inhibitors of reactive oxygen species, nitric oxide, and reactive aldehydes on the effects of cigarette smoke. These data suggest that the effect is due to reactive aldehydes, since pretreatment with aldehyde dehydrogenase prevented ATP depletion under cigarette smoke extract alone and cigarette smoke extract with RSV conditions. The nonspecific inhibitor, N-acetylcysteine, also showed a protective effect. There were also protective effects demonstrated by inhibitors of reactive oxygen species in cells exposed to first cigarette smoke extract and then virus, demonstrating that the system is complex, and there may be multiple components of cigarette smoke contributing to ATP depletion and subsequent necrosis in this setting. These findings are consistent with the studies of Hoshino and colleagues, who showed that the effects of cigarette smoke on airway epithelium could be inhibited both by N-acetylcysteine and aldehyde dehydrogenase and not by $HbO_2$ (nitric oxide scavenger), catalase ($H_2O_2$ scavenger), uric

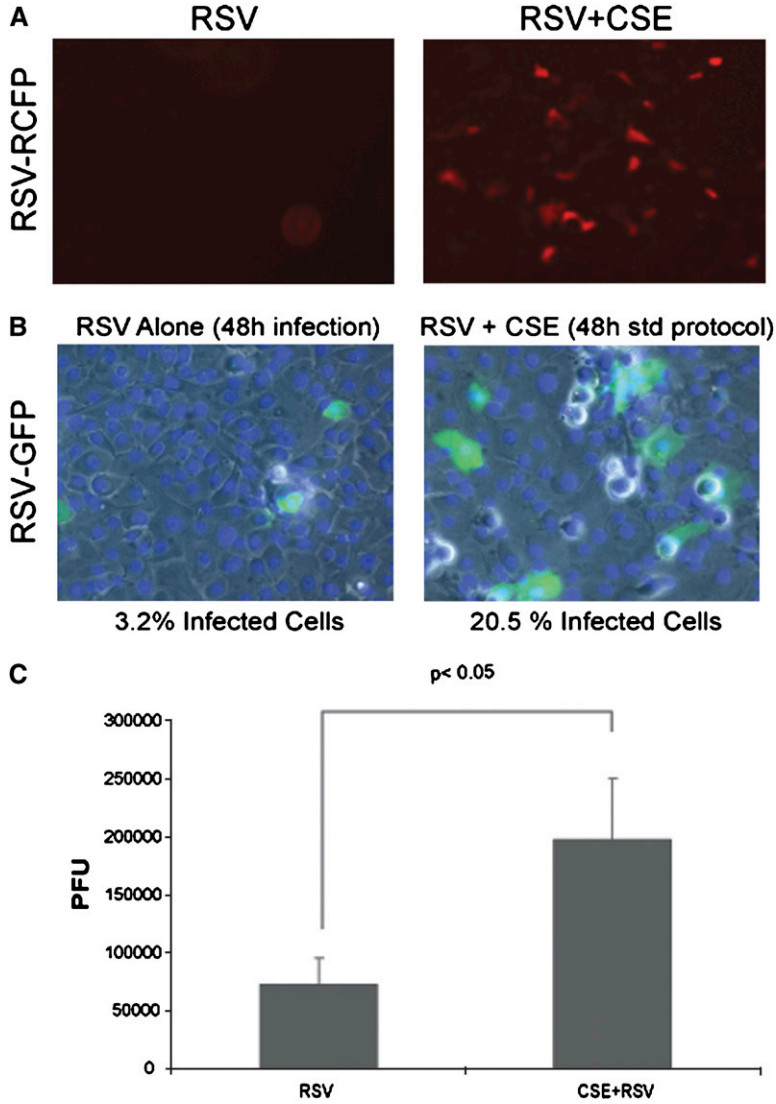


*Figure 7.* Cigarette smoke extract (CSE) increases viral load. hTBE cells were plated in an adherent monolayer and exposed to control media for 48 hours or 2% cigarette smoke extract for 48 hours. (*A*) Red fluorescently-labeled RSV (MOI 2) was added to the cells for another 48 hours, media was removed and replaced with PBS, and fluorescent micrographs were taken. (*B*) Green fluorescently-labeled RSV (MOI 2) was added to the cells for another 48 hours, cells were fixed with zinc formalin, Vectashield with DAPI stain for nuclei was added, and fluorescent and light micrographs were obtained and overlayed. (*C*) RSV (MOI 2) was added for another 24 hours. Both cells in the supernatant and adherent cells were collected and sonicated, and 10-fold dilutions were performed. Dilutions were added to 90% confluent Vero cells, overlayed, and incubated for 5 days. Neutral red was added and viral plaques counted. The experiment was performed four times with hTBE cells from four separate donors and the plaque-forming units (PFU) averaged.

acid (peroxynitrite scavenger), mannitol (hydroxyl radical scavenger), or superoxide dismutase (superoxide scavenger), suggesting that reactive aldehydes mediate these effects (31). It is also consistent with our observation that acrolein, a reactive aldehyde in cigarette smoke, mimics the effect of cigarette smoke (data not shown).

It is likely that the enhanced viral replication in cigarette smoke extract–exposed cells is due to increased infection of surrounding cells. As shown in our fluorescent RSV data in Figure 7A, at 48 hours of cigarette smoke and RSV exposure (MOI 2), only about 20% of cells are infected. We have previously shown that even with an MOI of 25 and 24 hours of RSV infection, cells are not expressing significant RSV protein and are not 100% infected (39). So we conclude that cigarette smoke exposure facilitates greater infection of airway epithelial cells. The mechanism for this is unclear. It may be due to necrosis itself or another effect of cigarette smoke extract.

We investigated mechanisms for the increased viral replication after exposure of cells to cigarette smoke extract. Others have recently shown in a neonatal mouse model that cigarette smoke can limit expression of interferon-inducible genes (40). We investigated whether cigarette smoke was limiting other antiviral responses after infection, but cigarette smoke extract had no effect on type I interferon and interferon-inducible genes such as retinoic acid–inducible gene I (RIG-I), Toll-like receptor 3 (TLR3), protein kinase R (PKR), and interferon regulatory factor 7 (IRF7) mRNA and protein (data not shown). It is possible, however, that cigarette smoke extract inhibits other interferon-mediated effects on the cells.

We demonstrated that cigarette smoke extract exposure results in increased viral titers in airway epithelial cells. The likely explanation is that this observation is due to increased viral spread to surrounding cells, but whether it is the result of necrosis, increased viral binding, increased internalization of virus, or increased viral replication once inside the cell cannot be definitively determined on the basis of this data.

RSV is a clinically important pathogen, particularly for infants, patients with obstructive lung disease, and the immunosuppressed. Its clinical importance in smokers and patients with COPD is being increasingly recognized (41–44). Pretreatment with N-acetylcysteine counteracts the effects of cigarette smoke on caspase activation and cellular apoptosis in RSV infection. This is an encouraging observation and offers the possibility of therapeutic intervention. For many smokers who suffer repeated viral infections and are unable to quit, treatment with N-acetylcysteine might offer a way to limit the severity of their viral infection.

***Conflict of Interest Statement*:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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