Acetaldehyde-Stimulated PKC Activity in Airway Epithelial Cells Treated with Smoke Extract from Normal and Smokeless Cigarettes (44556)

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Abstract. **Previously, we have found that acetaldehyde, a volatile component of cigarette smoke, stimulates the protein kinase C (PKC) pathway and inhibits ciliary motility. A "smokeless" cigarette (Eclipse) now exists in which most of the tobacco is not burned, reducing the pyrolyzed components in the extract. We hypothesized that acetaldehyde is a component of cigarette smoke that activates PKC in the airway epithelial cell, and therefore the Eclipse cigarette would not activate epithelial cell PKC. In this study, bovine bronchial epithelial cells (BBEC) were incubated with cigarette smoke extract (CSE) or Eclipse smoke extract (ESE). We found that PKC activity was significantly higher in cells exposed to 5% CSE than cells exposed to 5% ESE or media. When acetaldehyde levels of both extracts were measured by gas chromatography, CSE was found to have 15–20 times greater concentration (μ*M*) of acetaldehyde than ESE. When BBEC were treated with 5% CSE, ciliary beating was further decreased from baseline levels. This decrease in ciliary beating was not observed in cells treated with ESE, suggesting that acetaldehyde contained in CSE slows cilia. These results suggest that volatile components such as acetaldehyde in cigarette smoke may inhibit ciliary motility *via* a PKC-dependent mechanism.**

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Cigarette smoking represents a significant source of toxic compounds that can ultimately contribute to airway dysfunction. Chronic exposure to cigarette smoke promotes pneumonia, upper respiratory tract infections, chronic obstructive pulmonary disease, and cancer. Airway epithelial cells are particularly affected by cigarette smoke. It has been shown previously that a volatile component of liquid cigarette smoke extract (CSE) inhibits ciliary beat frequency (CBF) of bronchial airway epithelial cells (1, 2). It is well established that one of the volatile components of cigarette smoke is acetaldehyde (3), and that acetaldehyde can inhibit CBF directly (4–6). We have recently reported that both CSE and acetaldehyde activate protein kinase C (PKC) in bronchial epithelial cells (7). In certain cell types, agents that are known to activate PKC have been associated with the lowering of CBF (8, 9). The purpose of the present study is to investigate the role of cigarette smoke and acetaldehyde in the activation of PKC in airway epithelium.

Acetaldehyde, which is present in significant concentrations in cigarette smoke and is produced during alcohol metabolism, has been demonstrated to impair mucociliary clearance of the lung (1, 10). Acetaldehyde is also known to impair protein function through the formation of acetaldehyde-protein adducts (11, 12). Acetaldehyde directly impairs bronchial ciliary function causing slowing of ciliary beating, inhibits ciliary dynein ATPase activity, and binds to cilia proteins critical for motion including dynein and tubulin. These data suggest that acetaldehyde-induced cilia dysfunction may be related to direct cilia ATPase inactiva-

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tion and adduct formation with cilia dynein and tubulin (10). Because acetaldehyde is very volatile, the production and release of acetaldehyde vapor into the airway can directly result in ciliary slowing or ciliastasis as shown by a model of vapor-phase generation of acetaldehyde from ethanol (6).

Eclipse, a "smokeless cigarette" that has recently been test-marketed, differs from a regular cigarette in that the nicotine is extracted by heating the tobacco with air passed through a burning charcoal heat source. Because Eclipse has simpler smoke chemistry, consisting of 80% glycerol and water, it would be expected to contain fewer toxic components. Smoke condensate from the Eclipse cigarette produces markedly fewer DNA adducts (13) and is less mutagenic (14) than smoke condensate from traditional tobacco-burning cigarettes. Eclipse smoke extract should contain reduced amounts of volatile substances, such as acetaldehyde, compared with smoke from traditional cigarettes. Part of this study was to determine whether the Eclipse cigarette has a reduced amount of acetaldehyde in its smoke extract. Reduced acetaldehyde should result in lowered PKC activity, and ciliary motility would not be inhibited. Therefore, we hypothesize that acetaldehyde is a major component of cigarette smoke responsible for activating PKC in airway epithelium and that an extract of cigarette smoke that contains less acetaldehyde should not activate PKC to the same extent as smoke extracts from traditional cigarettes.

Materials and Methods

Cell Preparation. Cells were prepared from bovine lung obtained fresh from a local abattoir. Bronchi were necropsied from the lung, cleaned of adjoining lung tissue, and incubated overnight at 4°C in 0.1% bacterial protease (Sigma Type IV, St. Louis, MO) in Minimum Essential Media. Following the overnight incubation, the bronchi were rinsed in Dulbecco's Modified Eagles Medium (DMEM) with 10% fetal calf serum repeatedly to collect the cells lining the lumen. These cells were then filtered through a 250-μm nylon mesh and washed again in DMEM. This technique typically produces a high viability cell preparation of 95% epithelial cells (15). The cells were then plated in 1% type I collagen-coated 100-mm polystyrene Petri dishes at a density of 1×10^5 cells/cm^2 in a 2:1 media mixture of LHC-9 and RPMI (16). Cell incubations were performed at 37°C in humidified 95% air/5% CO_2. Each dish contained ≈ 2 mg total cellular protein. Cell viability after treatment with smoke extracts and acetaldehyde was determined by both trypan blue exclusion assay and media release of cellular LDH (Sigma, Procedure no. 228-UV). No significant cell death was observed in BBEC treated with <20% smoke extract.

PKC Activity Assay. PKC activity was determined in crude whole-cell fractions of bronchial epithelial cells. The assay employed was a modification of procedures previously described (17) using 900 μ*M* PKC substrate peptide (Peninsula), 12 m*M* $Ca(C_2H_3O_2)_2$, 8 μ*M* phosphatidyl-L-serine, 24 μg/ml PMA, 30 m*M* dithiothreitol, 150 μ*M* ATP, 45 m*M* $Mg(C_2H_3O_2)_2$, and 10 μCi/ml [γ-^{32}P] ATP in a Tris-HCl buffer (pH 7.5). Samples (20 μl) were added to 40 μl of the above reaction mixture and incubated for 15 min at 30°C. Incubations were halted by spotting 50 μl of each sample onto P-81 phosphocellulose papers (Whatman). Papers were then washed five times for 5 min each in phosphoric acid (75 m*M*), washed once in ethanol, dried, and counted in nonaqueous scintillant as previously described (18). Kinase activity was expressed in relationship to total cellular protein assayed as measured by the method of Bradford (19) and calculated in pmol/min/mg. All samples were assayed in triplicate, and no less than three separate experiments were performed per unique parameter. Effects of smoke compared with media treatment on PKC activity were analyzed for statistical significance using Student's paired *t* test.

Smoke Extract Preparation. The cigarettes used were obtained from the University of Kentucky, Tobacco-Health Research division (unfiltered, Code 2R1). The cigarette was connected to a peristaltic pump apparatus, lit, and bubbled through 25 ml of sterile RPMI media. One cigarette per 25 ml volume was used. The pump was equilibrated at a rate of one cigarette per 10 mins, or 160 cubic cm/min. The cigarettes were 85 mm in length, and ≈ 60 mm were consumed. The Eclipse was lit and bubbled through the same media volume for the same period under identical conditions. The resultant media were then filtered through a 0.22-μm filter to remove particles and bacteria before dilution into LHC9-RPMI as a percentage of the total volume. CSE was made fresh immediately prior to use (except where indicated for control studies), and the stock solution sealed at all times to prevent loss of volatile components prior to cell treatment.

Acetaldehyde Concentration Assay. The concentrations of acetaldehyde were measured in the extracellular milieu by transferring the media from exposed cells to 1.8 ml microcentrifuge tubes that were filled to the top, capped, and sealed with parafilm and stored at –80°C until analysis. At this time, the samples were thawed at 4°C and transferred rapidly in a cold room to headspace vials and capped. Acetaldehyde concentrations were determined by headspace gas chromatography using the method of Eriksson *et al.* (20). Recovery by this method was greater than 95% with a sensitivity of ≈ 1 μ*M*. The percentage of acetaldehyde that forms irreversible stable adducts with proteins is less than 1% of the acetaldehyde present under these experimental conditions (11, 21). The effects of smoke extract dose and time on media acetaldehyde accumulation were analyzed for statistical significance using two-way analysis of variance (ANOVA).

Ciliary Beat Frequency Assay. The motion of actively beating ciliated cells was quantitated by measuring ciliary beat frequency (CBF) using phase contrast microscopy, videotape analysis, and computerized frequency spectrum analysis. The cells were maintained at room temperature (24°C) during the time course of the CBF measure-

ments, as the temperature gradient is known to affect CBF (22). All observations were recorded for analysis using a Panasonic WV-D5000 video camera and a Panasonic AG-1950 videotape recorder. Beat frequency analysis was performed on videotaped experiments using customized software written in LabView (National Instruments, Austin, TX) running on a Macintosh G3 computer. The predominant frequency of a cilium or small group of cilia was determined by collecting data sampled at 40 Hz from 512 samples (12.8 sec) and performing frequency spectrum analysis. The CBF determined in this manner was deemed acceptable when a single dominant frequency was obtained using this technique. All frequencies represent the mean ± one standard error of the mean from six separate cell groups or fields. Effects of smoke compared with media treatment on CBF were analyzed for statistical significance using Student's paired *t* test.

Results

Acetaldehyde Inhibits CBF in BBEC. To determine if acetaldehyde alone was capable of decreasing baseline CBF, confluent monolayers of BBEC were treated with various concentrations (0–1 m*M*) of acetaldehyde added directly to the liquid tissue culture medium. Cells were incubated for 1 hr at room temperature and CBF measured. The addition of 10–250 μ*M* acetaldehyde markedly decreased BBEC CBF dose-dependently with the maximal inhibition of CBF occurring from 0.25–1 m*M* (Fig. 1). No significant cell death was measured due to acetaldehyde at these concentrations for up to 1 hr. This supports that acetaldehyde inhibits cilia beating in BBEC rapidly (10), and establishes this inhibition to be concentration-dependent.

Cigarette Smoke, Not Eclipse, Activates PKC in BBEC. To determine if cigarette smoke extracts can activate PKC in BBEC, cells were exposed from 30 min to 3 hr with either 5% CSE or 5% ESE, and PKC activity was measured. CSE significantly elevated BBEC PKC activity from 0.5–1 hr, but PKC activity returned to baseline media control levels by 3 hr (Fig. 2). Unlike CSE, 5% ESE only slightly increased PKC activity by 1 hr, and no significant changes in PKC activity were observed beyond 3 hr. This suggests that cigarette smoke extract is capable of activating PKC rapidly, but Eclipse smoke extract is not. These data also suggest that the component of CSE responsible for PKC activation only has a transient effect on maintaining maximal PKC activity in the BBEC.

Increasing the exposure time of BBEC to Eclipse smoke extract did not significantly activate PKC. BBEC were treated for up to 24 hr with 5%–10% ESE with no observed activation of PKC (Fig. 3). CSE-treated cells transiently activated PKC from 0.5–3 hr, but no PKC activity was observed beyond 5–6 hr. Cell viability was reduced in 5% CSE-treated cells at 24 hr, but not in ESE-treated cells. These data suggest that the inability of ESE to elevate PKC activity was not simply due to a delayed response as compared with CSE.

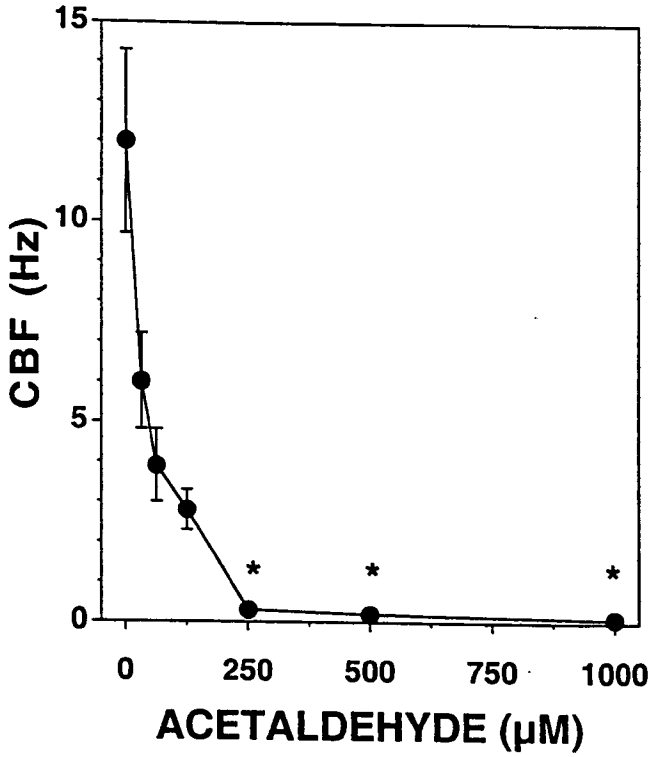

Figure 1. Acetaldehyde inhibits CBF in BBEC. BBEC were treated with 0–1 m*M* acetaldehyde in liquid media and CBF measured for 1 hr at room temperature. Bars represent SEM of six individual measurements performed on different cells per unique treatment (*$P <$ 0.001 for cells treated with 0.25–1 m*M* acetaldehyde compared with media control cells).

Increased concentrations of Eclipse smoke extract do not significantly activate PKC. BBEC were treated for up to 4 hr with 0%–100% ESE with no observed activation of PKC (data not shown). No significant reduction in cell viability was observed at any concentration of ESE used. As a control, the addition of 10 m*M* acetaldehyde to 5%–20% ESE resulted in the stimulation of BBEC PKC from 1–3 hr (data not shown). Conversely, when CSE was not prepared fresh and exposed to the air overnight, neither PKC nor CBF was activated (data not shown). These data suggest that an increased concentration of ESE was not sufficient to elevate PKC activity, regardless of the exposure time and that no component of ESE inhibits PKC activity when the BBEC are co-incubated with acetaldehyde.

Acetaldehyde Levels Decline in Cigarette Smoke Media Over Time. The increase in BBEC PKC activity due to CSE treatment diminished over time and eventually returned to baseline unstimulated levels (Fig. 2). To determine if the decrease in PKC activity over time is related to the volatilized loss of acetaldehyde concentration in the CSE, the acetaldehyde concentration was assayed directly in the media of cells treated with various concentrations of CSE for various times. Cells treated with 5% CSE contained up to 15 μ*M* acetaldehyde in the media at 30 mins (Fig. 4). This acetaldehyde concentration decreased by 3 hr to less than 1 μ*M*.

When tissue culture dishes were sealed with parafilm to

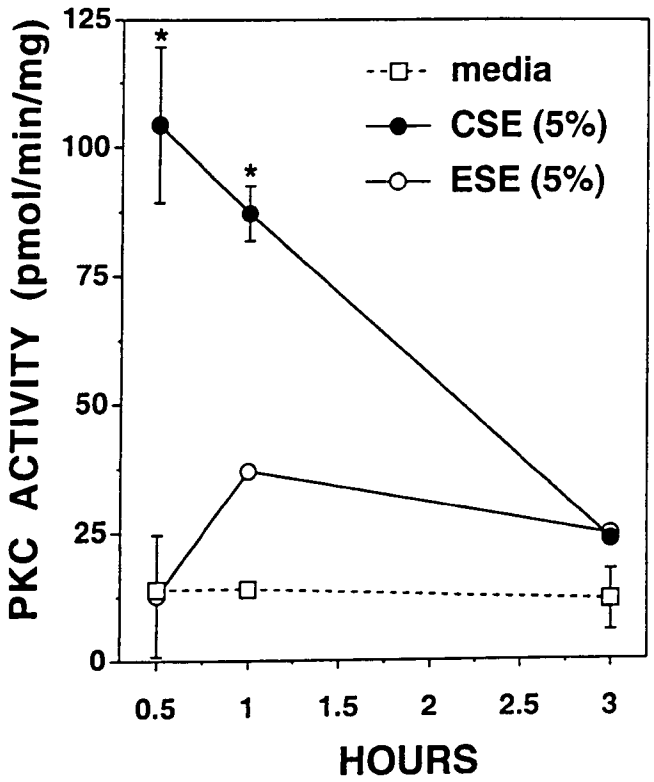

Figure 2. Cigarette smoke, but not Eclipse smoke, activates PKC in BBEC. Cells were treated 30 min–3 hr with a 5% dilution of either cigarette smoke (CSE) or Eclipse smoke extract (ESE) and PKC activity determined. Bars represent SEM of three different samples measured in triplicate (*$P < 0.001$ for cells treated with CSE from 0.5–1 hr compared with media control).

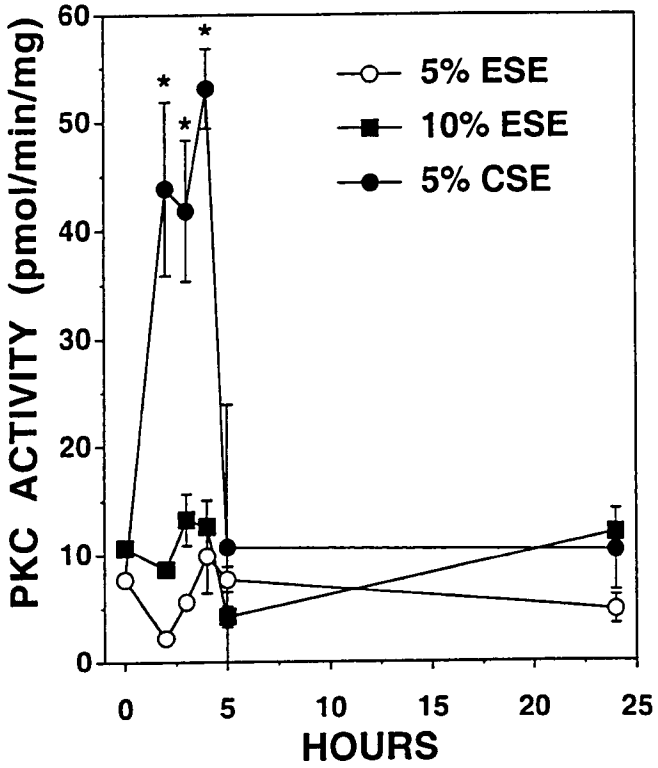

Figure 3. CSE, but not ESE, activates PKC in a time-dependent manner. Cells were treated 0–24 hr with a 5%–10% dilution of Eclipse smoke extract (ESE) or 5% cigarette smoke extract (CSE) and PKC activity determined. Bars represent SEM of three different samples measured in triplicate (*$P < 0.001$ for cells treated with CSE from 0.5–3 hr compared with media control).

retard the volatilized loss of acetaldehyde, a significantly greater concentration of acetaldehyde was detected in CSE-treated media as compared with unsealed dishes (Fig. 5). However, no significant acetaldehyde levels were detected in sealed tissue culture dishes that contained up to 20% Eclipse smoke extract. This suggests that the lower concentrations of acetaldehyde contained in Eclipse smoke extract are not due to the volatilized loss of acetaldehyde in these samples.

Cigarette Smoke Extract Inhibits CBF. Acetaldehyde inhibits stimulated increases in CBF (10). To determine if Eclipse smoke extract has the same effect on CBF as CSE, we treated BBEC with various concentrations of ESE and CSE and measured CBF. Baseline CBF decreased after 1 hr when BBEC were treated with 5% CSE (Fig. 6). This decrease corresponded to the activation of PKC in the BBEC. Conversely, there was no significant decrease in baseline CBF in cells treated with 5% ESE. If BBEC are pretreated with 5 μM HBDDE, a specific inhibitor of PKC α and γ isoforms, the decrease in baseline CBF due to CSE is inhibited (Fig. 7). HBDDE alone had no effect on CBF (data not shown). This suggests that the presence of acetaldehyde contained in CSE plays a role in PKC activation, and the subsequent activation of PKC results in a reduction on baseline CBF in the BBEC.

Discussion

In this study, we determined that PKC activity in bovine bronchial epithelial cells is greater following CSE-treatment than following ESE-treatment. We also found that CSE contains significantly more acetaldehyde than ESE. Because ESE contains less acetaldehyde, baseline CBF levels are not decreased when cells are treated with ESE as compared with CSE stimulation. PKC activation may be responsible for the acetaldehyde-stimulated decrease in CBF observed in cigarette smoke–treated ciliated airway epithelial cells as earlier studies have linked acetaldehyde to PKC regulation (23–26).

It is not the objective of this study to establish any parameters for a "safer" cigarette. Indeed, certain lower tar cigarettes have been associated with equal risks as smokers attempt to compensate for nicotine by extracting greater amounts of smoke from these cigarettes (27). Additional hazards related to the production of these smokeless cigarettes, such as glass fiber contamination, have also been reported (28). However, because the Eclipse cigarette delivers levels of nicotine equivalent to that of the traditional cigarette (29), it may represent an alternative to smoking cessation (30) that will also impact other parameters of lung function and therefore merit detailed biochemical and molecular study. For the purposes of this study, we have used

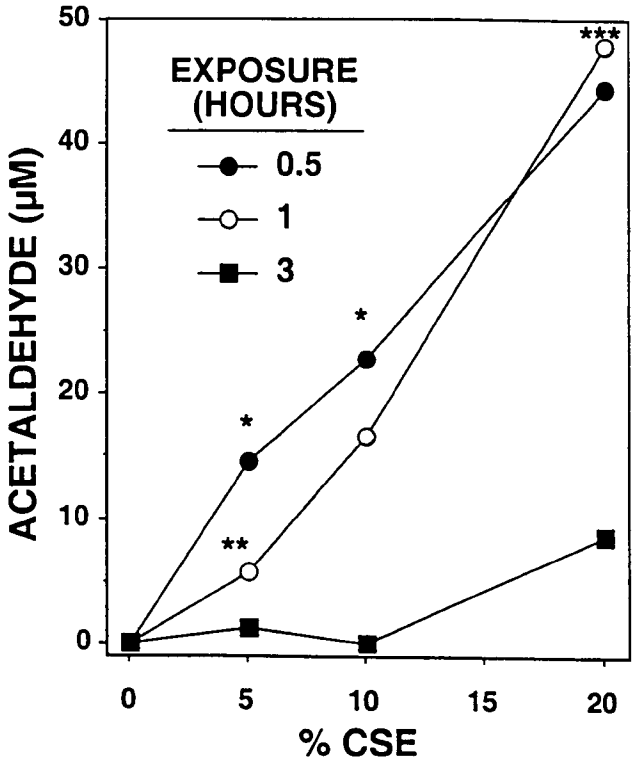

Figure 4. Acetaldehyde concentration in BBEC media treated with various concentrations of CSE. Cells were treated 30 min–3 hr with 0%–20% dilutions of cigarette smoke extract (CSE) and media acetaldehyde concentration determined. Each data point represents three different samples measured in triplicate (**$P < 0.001$ for cells treated with 5% CSE for 1 hr vs 3 hr, *$P < 0.0001$ for cells treated with 5% CSE at 0.5 hr and 10% CSE for 0.5 and 1 hr vs 3 hr, and ***$P < 0.0005$ for cells treated with 20% CSE for 0.5–1 hr vs 3 hr).

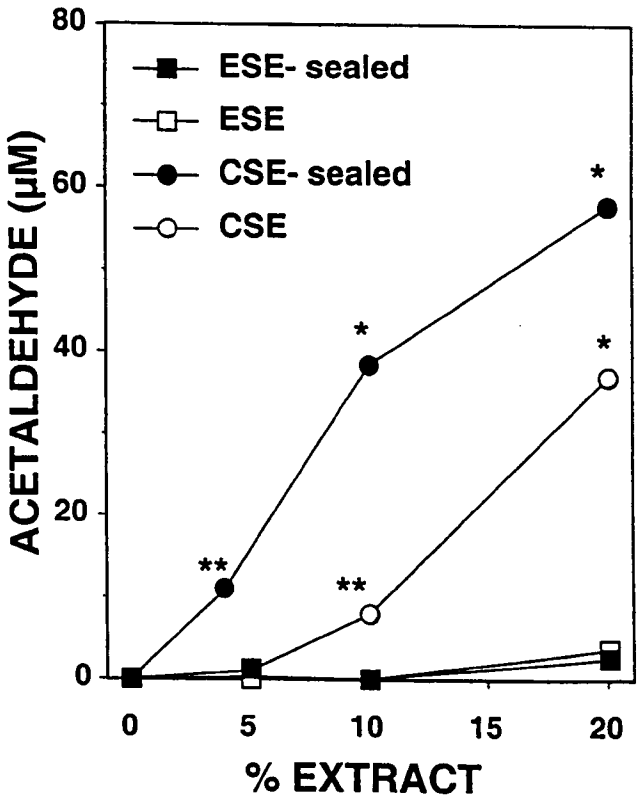

Figure 5. Acetaldehyde loss in sealed and unsealed dishes of cigarette and Eclipse smoke media extracts. Cells were treated 1 hr with 0%–20% dilutions of cigarette smoke extract (CSE) or Eclipse smoke extract (ESE) in either parafilm-sealed or unsealed dishes and media acetaldehyde concentration determined. Each data point represents three different samples measured in triplicate (*$P < 0.0001$ for cells treated with 10% and 20% CSE in sealed dishes and 20% CSE in unsealed dishes vs 10% and 20% ESE in sealed dishes, and **$P < 0.001$ for cells treated 5% CSE in sealed dishes and 10% CSE in unsealed dishes vs 5% and 10% ESE in sealed dishes).

the Eclipse cigarette as a mechanism to minimize the acetaldehyde concentration contained in cigarette smoke extract.

Various effects of acetaldehyde on airway tissue have been reported. CSE has important time-dependent effects on bronchial epithelial cell migration and attachment, but these effects appear to be independent of acetaldehyde (31). CSE augmented IL-8 release from bronchial epithelial cells in a concentration- and time-dependent manner. Most of the augmenting activity of CSE on IL-8 release from bronchial epithelial cells was lost after volatilization or lyophilization treatment. Two major volatile factors in cigarette smoke, acrolein and acetaldehyde, augmented IL-8 release (32). Recently, our laboratory showed that cigarette smoke coupled with C5a activation of the airway epithelial cells produces a substantially larger release of the pro-inflammatory cytokine IL-8 than CSE alone (7). This process requires PKC activation in the bronchial epithelial cell. Only in the presence of C5a can PKC activity cause the release of IL-8. Because Eclipse smoke extract fails to activate PKC in bronchial epithelial cells, less IL-8 is released upon exposure to ESE and C5a (data not shown) than with CSE and C5a. This suggests that the Eclipse cigarette smoke may contribute less to smoking-induced pro-inflammatory cytokine production in the lung.

Numerous studies have explored the mechanism of cigarette smoke–induced inhibition of ciliary beating. Cigarette smoke can alter vascular permeability of the lung endothelium through effects on cytoskeletal elements, particularly the association of extracellular divalent cations and the polymerization of cellular actin (33). Cigarette smoke is known to generate active oxygen species such as O_2- and H_2O_2, and contains a variety of irritants capable of reducing CBF. Active oxygen compounds in cigarette smoke have been suggested to inhibit cilia as CSE is significantly less toxic when superoxide dismutase and catalase are added to CSE (34). The concentrations of the purified volatile components of CSE that were required to inhibit CBF in hamster oviduct were at least 3–50 times higher than their corresponding concentrations in smoke solutions (35). Although the reason for this discrepancy remains unknown, we have also observed in our studies that the concentration of acetaldehyde in CSE is somewhat less than the pure acetaldehyde concentration required for maximal PKC activation.

PKC activation was observed to be transient due to CSE and acetaldehyde exposure with maximal PKC activity returning to baseline levels by 3–6 hr. Several PKC iso-

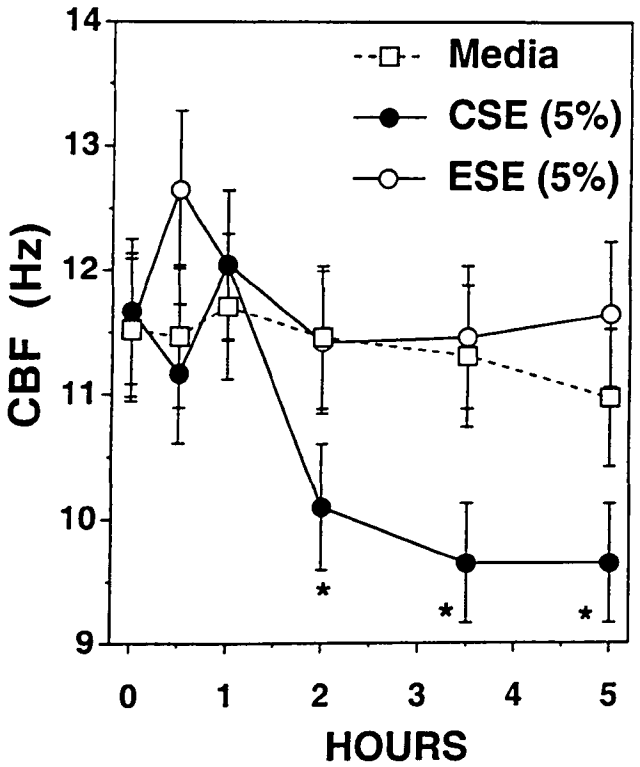

Figure 6. Effects of ESE and CSE on CBF in BBEC. Cells were treated 0–5 hr with 5% dilutions of cigarette smoke (CSE) or Eclipse smoke extract (ESE) and ciliary beat frequency (CBF) determined. Bars represent SEM of six individual measurements performed on different cells per unique treatment (*$P < 0.01$ for cells treated with 5% CSE compared with media control cells).

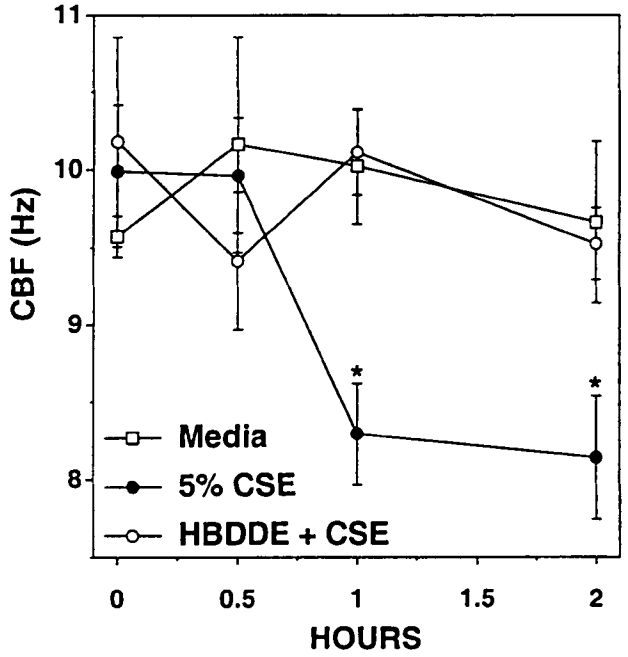

Figure 7. Reversal of CSE effects on CBF by PKC inhibition. Cells were pretreated with 5 μ*M* HBDDE for 1 hr prior to the addition of 5% CSE and ciliary beat frequency (CBF) determined. Bars represent SEM of six individual measurements performed on different cells per unique treatment (*$P < 0.01$ for cells treated with 5% CSE compared with media control cells).

forms have been reported to downregulate after stimulation, particularly after treatment with phorbol esters (36). These findings have also been extended to lung epithelial cells (37). Our observed time course of cigarette smoke exposure suggests a similar phenomenon of PKC auto-inhibition or desensitization. We have found that repeated doses of CSE or acetaldehyde do not reactivate the downregulated PKC in BBEC.

Acetaldehyde may not be activating PKC directly, but rather signaling *via* surface membrane interactions. Nonenzymatic alkylation of human serum albumin by acetaldehyde resulted in the abnormalities of binding sites for fatty acids and prostaglandins. Modification of the plasma membrane proteins with acetaldehyde sharply diminished the density of PGE_2 binding sites without changing the association constants (38). It has been suggested that the protective effect of prostaglandins in alcoholic liver injury occurs by their competition with acetaldehyde for binding sites on specific prostaglandin-binding receptors of liver plasma membranes (39). Indeed, acetaldehyde has been shown to bind to rat hepatocyte membranes and alter endocytosis (40). These interactions may be accomplished *via* the formation of acetaldehyde-induced protein adducts at the membrane extracellular surface. These surface adducted proteins may bind to and transduce intracellular signaling *via* scavenger receptor proteins that preferentially bind adducted proteins (41–43). The exact mechanisms of PKC activation by acetaldehyde remain the focus of future studies.

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