

## Cardiovascular, Pulmonary, and Renal Pathology

# Co-Exposure to Cigarette Smoke and Alcohol Decreases Airway Epithelial Cell Cilia Beating in a Protein Kinase C $\epsilon$ -Dependent Manner

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**Alcohol use disorders are associated with increased lung infections and exacerbations of chronic lung diseases. Whereas the effects of cigarette smoke are well recognized, the interplay of smoke and alcohol in modulating lung diseases is not clear. Because innate lung defense is mechanically maintained by airway cilia action and protein kinase C (PKC)-activating agents slow ciliary beat frequency (CBF), we hypothesized that the combination of smoke and alcohol would decrease CBF in a PKC-dependent manner. Primary ciliated bronchial epithelial cells were exposed to 5% cigarette smoke extract plus 100 mmol/L ethanol for up to 24 hours and assayed for CBF and PKC $\epsilon$ . Smoke and alcohol co-exposure activated PKC $\epsilon$  by 1 hour and decreased both CBF and total number of beating cilia by 6 hours. A specific activator of PKC $\epsilon$ , DCP-LA, slowed CBF after maximal PKC $\epsilon$  activation. Interestingly, activation of PKC $\epsilon$  by smoke and alcohol was only observed in ciliated cells, not basal bronchial epithelium. In precision-cut mouse lung slices treated with smoke and alcohol, PKC $\epsilon$  activation preceded CBF slowing. Correspondingly, increased PKC $\epsilon$  activity and cilia slowing were only observed in mice co-exposed to smoke and alcohol, regardless of the sequence of the combination exposure. No decreases in CBF were observed in PKC $\epsilon$  knockout mice co-exposed to smoke and alcohol. These data identify PKC $\epsilon$  as a key regulator of cilia slowing in response to combined smoke and alcohol-induced lung injury.**

**hol-induced lung injury. (Am J Pathol 2012, 181: 431–440; <http://dx.doi.org/10.1016/j.ajpath.2012.04.022>)**

Chronic inflammatory lung disease represents the third leading cause of death in the United States,<sup>1</sup> primarily because of cigarette smoking. Although a large percentage of cigarette smokers consume alcohol, relatively few studies have examined the combination effects of cigarette smoke plus alcohol on the various functions of the lung. Understanding the interplay of these two important agents may reveal novel pathway targets that might treat or prevent adverse health consequences. Although innate lung defenses against inhalation injury are often attributed to the action of immune effector cells, earlier mechanical defenses in the lung consist of exhalation, cough, and mucociliary clearance, in which inhaled particles, toxins, and pathogens are trapped in the mucus layer that covers the airways and are propelled from the lungs via the unidirectional motion of the beating cilia.

The effect of cigarette smoke on ciliary beat frequency (CBF) is not clearly defined or well characterized. Depending on the model system, there are reports of both decreased and increased cilia beating after cigarette smoke exposure.<sup>2,3</sup> Alcohol also has a biphasic effect on CBF:

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transient modest alcohol exposure rapidly stimulates ciliary beat, whereas sustained higher dose alcohol exposure leads to a desensitization of the ciliostimulatory machinery, resulting in impaired mucociliary clearance.<sup>4</sup> Despite these advances in our understanding of individual effects of cigarette smoke or alcohol on cilia, little is known about co-exposure effects. Previously, we reported that cigarette smoke and alcohol co-exposure resulted in a significant decrease in bacterial clearance from the lung in a rodent model.<sup>5</sup> Unique to this co-exposure was the observation that ciliary beating not only failed to stimulate in response to an otherwise routine stimulatory challenge, but also CBF actually decreased below baseline values.<sup>6</sup> The mechanism of this active cilia-slowness response has not been defined.

Regulatory mechanisms that control decreases in cilia beating are not as well described as cilia stimulatory mechanisms. Cilia stimulation involves the second messengers, calcium, cAMP, nitric oxide, and cGMP, which have all been shown to activate target kinases (PKA, PKG) to produce an increase in ciliary beating.<sup>7</sup> However, decreases in CBF have been associated with the action of protein kinase C (PKC). PKC-activating agents, such as phorbol esters, have been reported to slow cilia, and PKC-mediated phosphorylation of ciliary substrates also slows ciliary beating.<sup>8</sup> Likewise, numerous agents have been reported to decrease ciliary beating such as respiratory syncytial virus,<sup>9</sup> sodium metabisulphite,<sup>10</sup> organic dusts from animal confinement,<sup>11</sup> tumor necrosis factor  $\alpha$ ,<sup>12</sup> and acetaldehyde.<sup>13</sup> Importantly, all of these agents activate PKC in the lung. Of the various PKC isoforms, Wong et al<sup>14</sup> first proposed that the action of calcium-independent novel isoform, PKC $\epsilon$ , was responsible for cilia slowing in response to neuropeptide Y. We previously reported that PKC $\epsilon$  is a novel isoenzyme contained in ciliated airway epithelium.<sup>15</sup>

On the basis of those studies, we hypothesized that the combination of cigarette smoke and alcohol would slow ciliary motility in a PKC-dependent manner. To investigate this hypothesis, we examined cigarette smoke and alcohol effects on cilia beat and PKC activity with the use of *in vitro* models of intact ciliated primary bovine cells and isolated bovine ciliary axonemes, in culture, *in situ*-exposed precision-cut mouse lung slices, and *in vivo* smoke- and alcohol-treated normal and homozygous Prkce knockout mice. Our findings suggest that only the combination of cigarette smoke and alcohol, but not either individually, leads to a rapid slowing of CBF. This smoke and alcohol combination exposure leads to the activation of the PKC isoform  $\epsilon$  (PKC $\epsilon$ ), which immediately precedes cilia slowing.<sup>16</sup> This unique effect of combined cigarette smoke and alcohol may be an important component of the increased lung infections and chronic lung disease exacerbations observed in smoking persons who also consume alcohol.

## Materials and Methods

### Preparation of Bovine Bronchial Epithelial Cells

Grossly healthy bovine lungs were obtained from a local slaughterhouse (ConAgra, Omaha, NE), and bronchi were

isolated. Explants of ciliated bronchial epithelial cells were cultured after enzymatic digestion of the bronchi as previously described.<sup>7</sup> Basal, nonciliated cells were collected through a mesh filter from the same preparation as previously described.<sup>15</sup>

### Preparation of Ciliary Axonemes

Bovine ciliary axonemes were isolated with the use of the method of Hastie et al,<sup>17</sup> processed, and characterized as previously described.<sup>18</sup>

### Preparation of CSE

Bronchial epithelial cells and lung tissue slices were exposed in submerged cultures to liquid media extracts of cigarette smoke as previously described.<sup>19</sup> In that previous study, it was determined that a 5% dilution of this cigarette smoke extract (CSE) was optimal for stimulating maximal PKC activity in the absence of any cell death. Likewise, we have shown that 100 mmol/L ethanol (a blood alcohol concentration clinically observed under conditions of alcohol abuse) is an optimal alcohol dose that affects cilia without cell toxicity.<sup>20</sup>

### Mouse Smoke and Alcohol Co-Exposure Model

Healthy wild-type (C57Bl/6J) mice and PKC $\epsilon$  knockout (PKC $\epsilon$ KO) mice (B6.129S4-Prkce<sup>tm1Msg/J</sup>; The Jackson Laboratory, Bar Harbor, ME; stock number 004189) were group-housed (five mice per cage) and maintained in microisolator units at the animal facility of the University of Nebraska Medical Center (UNMC), accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Mice were allowed food and water *ad libitum* and were used experimentally at approximately 8 to 10 weeks of age. Animal handling was in accordance with guidelines set by the US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training; the US Department of Agriculture implementing regulations, (9CFR), of the Animal Welfare Act; US Public Health Service Policy Assurance for the Humane Care and Use of Laboratory Animals negotiated with the Office of Laboratory Animal Welfare; The Guide for the Care and Use of Laboratory Animals; and the UNMC/University of Nebraska Omaha Institutional Animal Care and Use Committee Guidelines for the Care and Use of Live Vertebrate Animals. The UNMC Institutional Animal Care and Use Committee approved all protocols. Mice were exposed to cigarette smoke via whole-body chamber, fed alcohol in their drinking water, or co-exposed to both with the use of a model previously characterized.<sup>6</sup> In some cases, the order of smoke or alcohol as a first exposure was reversed with no change in the duration of exposure.

Health status is assessed on a quarterly basis at UNMC with the use of dirty-bedding exposure of sentinel mice. The following specific pathogens of laboratory mice are excluded from the animal facility: Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, Theiler's mouse encephalomyelitis virus/

GDVII strain, respiratory enteric orphan virus, *Mycoplasma pulmonis*, mouse parvovirus, epizootic diarrhea virus of infant mice, parvovirus, lymphocytic choriomeningitis virus, Hantavirus, mouse adenovirus, Ectromelia virus, mouse pneumonitis virus, polyoma virus, mouse thymic virus, mouse cytomegalovirus, *Encephalitozoon cuniculi*, and cilia-associated respiratory bacillus. In addition, sentinel mice are routinely tested for the presence of endo- and ecto-parasites (pinworms and fur mites), and these are excluded. Sporadic positive results for mouse norovirus have been detected in sentinel animals housed within this facility, but there is no direct evidence that colony mice were contaminated with the agent at the time of this study (data not shown). The facility does not test for lactate dehydrogenase elevating virus.

Tracheae from PKC $\delta$  knockout mice were obtained from the University of California Los Angeles as a gift from Dr. Steven Pandol.<sup>21</sup> Wild-type controls were obtained concurrently from the same facility.

### Preparation of Mouse Lung Slices

Precision-cut mouse lung slices were prepared as previously described<sup>22</sup> with the use of the method of Delmotte and Sanderson.<sup>23</sup>

### Preparation of Tracheal Rings

Tracheal rings were prepared as previously described.<sup>6</sup>

### Quantitation of Ciliary Beating

Explants of ciliated bovine bronchial epithelial cells, tracheal ring epithelium, and precision-cut lung slices were measured for changes in cilia motility. CBF analysis and measurement of the total number of motile points during whole-field analysis were accomplished with the use of the Sisson-Ammons Video Analysis method as previously validated and characterized.<sup>24</sup>

### PKC Activity Assay

Determination of PKC isoform catalytic activity was accomplished in fractionates from axonemes, cells, and tissues by direct isoform-specific substrate peptide phosphorylation assays as previously described.<sup>25</sup>

### Translocation Inhibitor Peptide Production

PKC $\epsilon$  translocation inhibitor peptide ( $\epsilon$  V1-2) was obtained commercially (AnaSpec, Fremont, CA). In addition, two other carrier protein variants of this inhibitor were constructed at UNMC (gift of Dr. Sam Sanderson) to ensure cell permeability of the peptide. One contained a myristolated group and the other contained a *Drosophila* antennapedia peptide (R-Q-I-K-I-W-F-Q-N-R-R-M-K-W-K-K) to make it cell permeable as previously described.<sup>26</sup> Cells were preincubated with the translocation inhibitors from 0.5 to 24 hours before experimental assay in the presence or absence of 50  $\mu$ mol/L digitonin to maximize cell penetration.

### Immunostaining

PKC was immunolocalized on bovine ciliary axonemes with the use of the method of Stout et al,<sup>27</sup> using rabbit anti-PKC $\epsilon$  obtained commercially from Santa Cruz Biotechnology (Santa Cruz, CA).

### Viability Assays

Cell and tissue viability was determined by lactate dehydrogenase activity assay (Sigma-Aldrich, St. Louis, MO). No significant lactate dehydrogenase release was detected in cells under any treatment condition. Experimental treatment and assays that used precision-cut mouse lung slices were not conducted until 1 week after slicing at which time lactate dehydrogenase levels had returned to baseline levels.

### Statistical Analysis

All quantitative experiments were performed in triplicate. All data were analyzed with GraphPad Prism (version 4.00 for Windows; GraphPad Software, San Diego CA) and represented as mean  $\pm$  SE. Data were analyzed for statistical significance with the use of one-way analysis of variance followed by Bonferroni post hoc testing between each condition group. Significance was accepted at the 95% confidence interval.

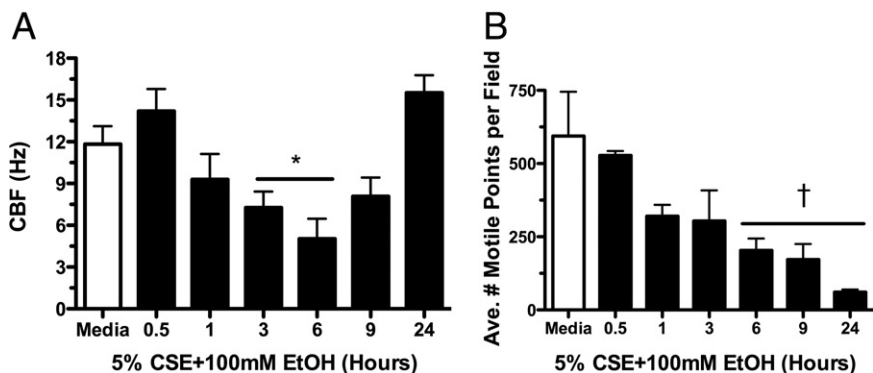
### Materials

Reference cigarettes (3R4F) were obtained from the University of Kentucky (Tobacco Health Research Institute, University of Kentucky, Lexington, KY). Absolute ethanol was obtained from Pharmco-AAPER (Shelbyville, KY). Translocation inhibitor peptide was a gift from Dr. Steven Pandol (University of California Los Angeles). All other materials were obtained from Sigma-Aldrich.

## Results

### Smoke and Alcohol Co-Exposure Significantly Slow Cilia Beating in Vitro

To determine the effect of combined smoke and alcohol exposure on cilia beating in intact cells, primary cultures of ciliated bovine bronchial epithelial cells were exposed to 5% CSE and 100 mmol/L ethanol for 30 minutes to 24 hours, and CBF was determined over time. CBF began to decrease by 1 hour with a significant ( $P < 0.05$  versus time-matched media controls) decrease in CBF observed from 3 to 6 hours compared with media control (Figure 1A). CBF slowing continued such that there was a significant ( $P < 0.01$ ) decrease in total number of motile points at 6 hours of treatment with smoke and alcohol (Figure 1B). A remaining small subset of ciliated cells could still be detected beating at 24 hours at a CBF no different from control. Co-exposure with CSE concentrations up to 20% and ethanol concentrations as low as 20 mmol/L showed similar effects (data not shown). Neither cigarette



**Figure 1.** Smoke and alcohol slow cilia beat frequency (CBF). Primary ciliated bovine bronchial epithelial cells were treated with 5% cigarette smoke extract (CSE) and 100 mmol/L ethanol (EtOH) in liquid submerged *in vitro* cultures. CBF (A) and the average number of motile points per field of cells (B) were determined by Sisson-Ammons Video Analysis. A representative media control (M199) is indicated by the white bar. Data are shown as means  $\pm$  SEs ( $n = 9$ ). \* $P < 0.05$  versus control media at matched time points of 3 to 6 hours for CBF; † $P < 0.01$  versus control media at matched time points of 6 to 24 hours for average motile points.

smoke alone (5% CSE) nor ethanol alone (100 mmol/L) decreased cilia beating (see Supplemental Figure S1, A-D, at <http://ajp.amjpathol.org>). Brief ethanol exposure (1 hour) transiently stimulated CBF. These data suggest that most ciliated cells slow and stop beating in response to the combination of smoke and alcohol.

### Smoke and Alcohol Co-Exposure Activates PKC $\epsilon$ Activity in Epithelial Cells

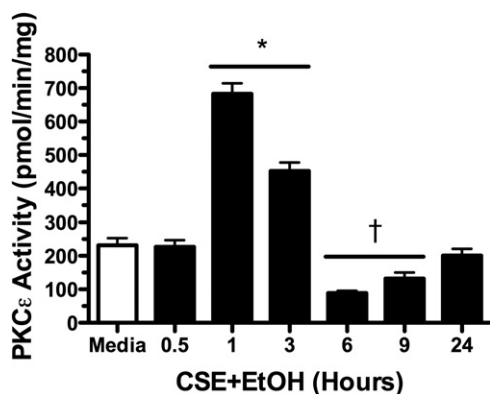
Because PKC has been implicated in cilia slowing, we assayed PKC $\epsilon$  activity after smoke and alcohol exposures in ciliated bovine bronchial epithelial cells and found that the combination of 5% CSE plus 100 mmol/L ethanol significantly ( $P < 0.001$  versus time-matched media controls) stimulated PKC $\epsilon$  activity (Figure 2). PKC $\epsilon$  activity was significantly ( $P < 0.05$ ) decreased from 6 to 9 hours compared with media controls and returned to baseline levels by 24 hours. No activation of PKC $\epsilon$  was observed under conditions of smoke alone or alcohol alone (see Supplemental Figure S2, A and B, at <http://ajp.amjpathol.org>). No activity changes were observed in the other novel isoform found in bronchial epithelium,

PKC $\delta$ , under treatment conditions of smoke, alcohol, or both (data not shown). Collectively, these data indicate that the smoke and alcohol stimulation of PKC $\epsilon$  occurs before cilia slowing and that a decrease in PKC $\epsilon$  precedes ciliostasis.

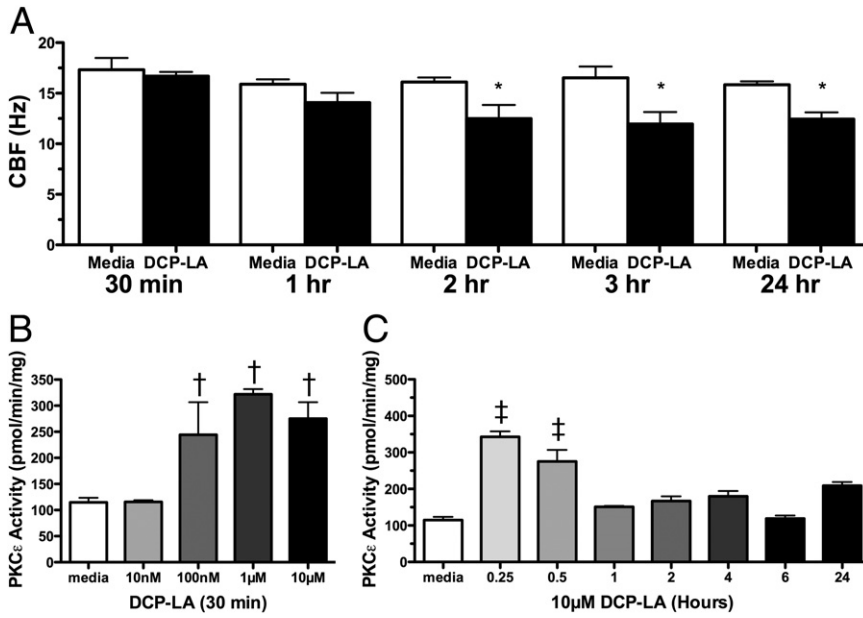
To further define a sequential effect for PKC $\epsilon$  in regulating CBF, bovine bronchial epithelial cells were treated with a linoleic acid derivative, 8-[2-(2-pentylcyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA), a specific activator of PKC $\epsilon$ . DCP-LA (10  $\mu$ mol/L) decreased CBF at 2, 3, and 24 hours compared with baseline media controls at the same time points (Figure 3A). DCP-LA was capable of dose- and time-dependently activating PKC $\epsilon$  before it slowed cilia (Figure 3, B and C). These findings implicate PKC $\epsilon$  activation as an important upstream regulator of cilia slowing in the bronchial epithelial cell after combined smoke and alcohol exposure.

### Combined Smoke and Alcohol Stimulation of PKC $\epsilon$ Is Specific to Ciliated Epithelial Cells

The cellular specificity of the combination of cigarette smoke and alcohol effect on PKC $\epsilon$  was examined in both ciliated and nonciliated bronchial epithelial cells to determine whether combined smoke- and alcohol-stimulated PKC activity was localized to the ciliated cells. When basal, nonciliated primary bovine bronchial epithelial cells were stimulated with the combination of 5% CSE and 100 mmol/L ethanol for 1 hour, no activation of PKC $\epsilon$  was observed (Figure 4A). However, the nonselective classical and novel PKC isoform activating phorbol ester, phorbol-12-myristate-13-acetate (PMA; 100 ng/mL), significantly activated PKC $\epsilon$  in the basal cell. Smoke and alcohol failed to activate PKC $\epsilon$  at any concentration (CSE 5% to 20% and ethanol 10 to 100 mmol/L) or time (1 to 24 hours) (data not shown). Conversely, ciliated cells from the same primary bovine preparations showed significant PKC $\epsilon$  activation in response to smoke and alcohol (Figure 4B). Similar to PMA, 10  $\mu$ mol/L DCP-LA stimulated PKC $\epsilon$  activity in both basal and ciliated cells (data not shown). These data indicate that the action of smoke and alcohol on PKC $\epsilon$  is specifically targeted to the kinase localized in the ciliated cell.



**Figure 2.** Effect of combination smoke and alcohol on protein kinase C (PKC) $\epsilon$  activity. Primary ciliated bovine bronchial epithelial cells were treated with 5% cigarette smoke extract (CSE) and 100 mmol/L ethanol (EtOH) in submerged *in vitro* cultures. PKC $\epsilon$  activity was assayed at various time points from 30 minutes to 24 hours. A representative media control (M199) is indicated by the white bar. Data are shown as means  $\pm$  SEs ( $n = 9$ ). \* $P < 0.001$  versus control media at matched time points of 1 to 3 hours; † $P < 0.05$  versus control media at matched time points of 6 to 9 hours.



**Figure 3.** Effect of a protein kinase C (PKC) $\epsilon$  activator on cilia beat. Primary ciliated bovine bronchial epithelial cells were treated with 10  $\mu$ mol/L 8-[2-(2-pentylcyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) for 30 minutes to 24 hours in submerged *in vitro* cultures. Ciliary beat frequency (CBF; **A**) was determined by Sisson-Ammons Video Analysis, and PKC $\epsilon$  activity was determined for various concentrations (10 nmol/L to 10  $\mu$ mol/L) of DCP-LA (**B**) and at various times (15 minutes to 24 hours) of 10  $\mu$ mol/L DCP-LA (**C**). Media controls (M199) are indicated by white bars. Data are shown as means  $\pm$  SEs ( $n = 9$ ). \* $P < 0.01$  versus control media at matched time points of 2 to 24 hours for CBF; <sup>†</sup> $P < 0.05$  versus control media at 30 minutes for 100 nmol/L to 10  $\mu$ mol/L DCP-LA for PKC $\epsilon$  activation; <sup>‡</sup> $P < 0.05$  versus control media at matched time points of 15 to 30 minutes for 10  $\mu$ mol/L DCP-LA for PKC $\epsilon$  activation.

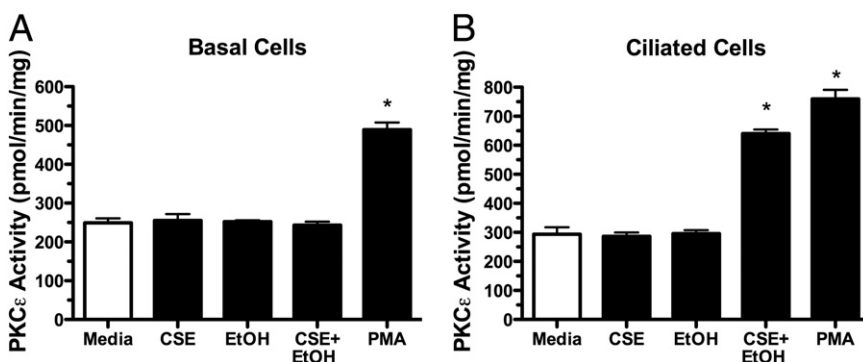
### PKC $\epsilon$ Activation-Induced Cilia Beating Is Not Translocation Dependent

To further characterize the presence of subcellular localized PKC $\epsilon$  action in the ciliated cell, ciliated bovine bronchial epithelial cells were pretreated with direct catalytic site inhibitors and translocation inhibitors of PKC $\epsilon$ . In the ciliated cell, the novel PKC $\epsilon$  inhibitor, Ro 31-8220 (10  $\mu$ mol/L), decreases PKC $\epsilon$  catalytic activity rates below that of baseline and leads to cilia slowing and ciliostasis.<sup>28</sup> In contrast, the PKC $\epsilon$  translocation inhibitor, ( $\epsilon$  V1-2; 10  $\mu$ mol/L), failed to significantly decrease PKC $\epsilon$  activity (Figure 5A), slow CBF, and did not alter the number of motile points (Figure 5B) in the ciliated cell both in the presence and absence of a cell permeabilizing agent (digitonin). Two different soluble conjugates of the PKC $\epsilon$  V1-2 translocation inhibitor peptide (myristolated peptide and *Drosophila* antennapedia peptide) were used without observing changes in kinase activity to control for cell permeability (data not shown). The use of 50  $\mu$ mol/L digitonin to enhance peptide solubility did not alter these results (Figure 5A). These results show that the effect of PKC $\epsilon$  activation on cilia is not regulated by translocation

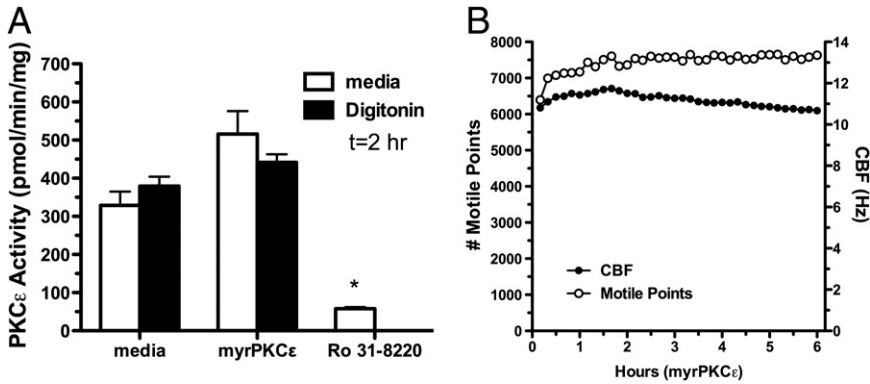
control, suggesting a directly localized action of PKC $\epsilon$  on the cilium.

### Catalytically Active PKC $\epsilon$ Is Localized Directly on the Ciliary Axoneme

To confirm the localization of PKC $\epsilon$  on the cilia, subcellular organelle extracts of ciliary axonemes were prepared and probed for the presence and activity of PKC $\epsilon$ . Isolated bovine axonemes stained with antibodies to PKC $\epsilon$  showed the presence of the isoenzyme throughout the hair-like structure of the axoneme (Figure 6, A and B). Isotype controls that used a nonspecific IgG in place of the anti-PKC $\epsilon$  showed no nonspecific staining (Figure 6C). In addition to localizing PKC $\epsilon$  protein in the isolated axonemes, we were able to measure PKC $\epsilon$  activity in the isolated axonemes. Kinase activity assays of isolated axonemes extracted from unstimulated bovine tracheae showed the presence of a calcium-independent and lipid-dependent basal-level phosphorylation of a PKC $\epsilon$ -specific substrate peptide (Figure 6D). These data indi-



**Figure 4.** Differential effects of smoke and alcohol on basal versus ciliated cells. Both nonciliated basal (**A**) and ciliated (**B**) primary bovine bronchial epithelial cells were treated in submerged culture with M199 media (white bars), 5% cigarette smoke extract (CSE), 100 mmol/L ethanol (EtOH), individually and in combination for 1 hour, and protein kinase C (PKC) $\epsilon$  activity assayed. As a positive control, cells were treated with 100 ng/mL phorbol-12-myristate-13-acetate (PMA) for 15 minutes, and PKC $\epsilon$  activity was assayed. Data are shown as means  $\pm$  SEs ( $n = 9$ ). \* $P < 0.001$  versus control media for PMA treatment in both cell types and smoke+EtOH in ciliated cells.



**Figure 5.** Differential effects of translocation inhibitor versus catalytic site inhibitor on ciliated cell protein kinase C (PKC $\epsilon$ ) and motility. Ciliated primary bovine bronchial epithelial cells were treated with 10  $\mu$ mol/L myristolated PKC $\epsilon$  translocation inhibitor peptide in the presence or absence of 50  $\mu$ mol/L digitonin (black bars) or 10  $\mu$ mol/L active site inhibitor Ro 31-8220 and PKC $\epsilon$  activity at 2 hours (A) or number of motile points from 1 to 6 hours (B) assayed. Data are shown as means  $\pm$  SEs ( $n = 9$ ). \* $P < 0.001$  versus control media for Ro 31-8220 treatment. CBF, ciliary beat frequency.

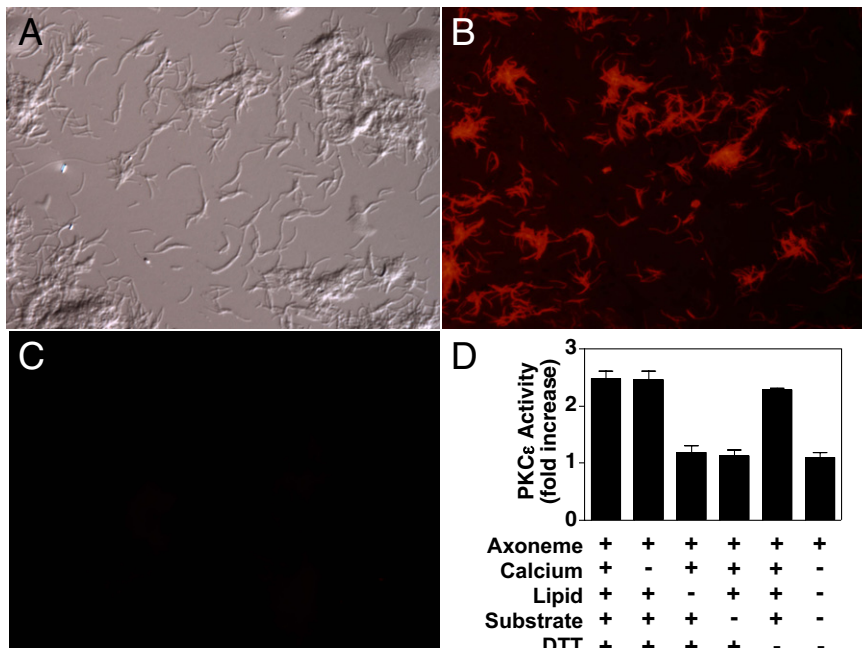
cate that catalytically active PKC $\epsilon$  is localized directly on the ciliary axoneme.

### Smoke and Alcohol Effects on PKC $\epsilon$ -Mediated Cilia Action in Lung

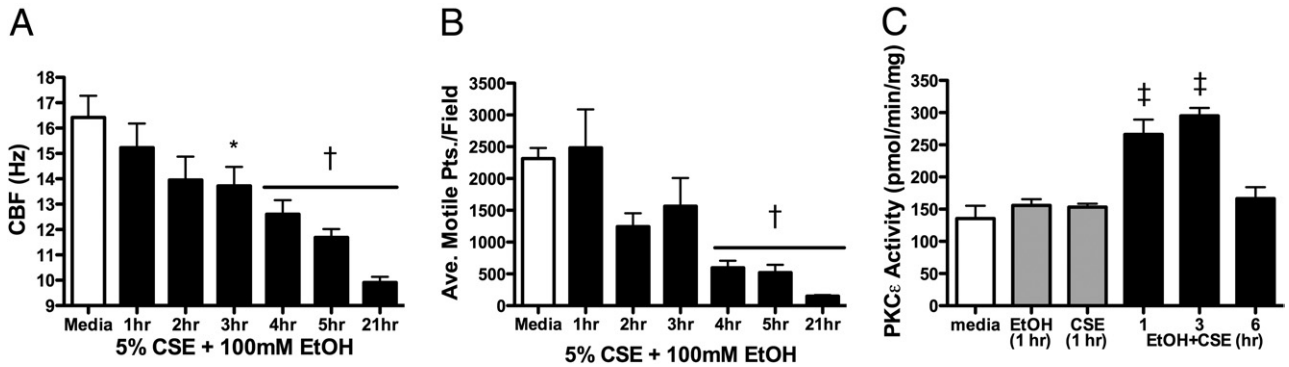
To show a lung-specific action of smoke and alcohol on cilia, precision-cut untreated C57Bl/6 naive mouse lung slices were treated with CSE, alcohol, or both in combination. A time-dependent decrease in CBF was observed in the large airways of mouse lung slices after smoke and alcohol co-exposure ( $P < 0.05$ ) at 3 hours and continuing overnight (Figure 7A). In smoke-only exposure, no changes in CBF were observed at all time points compared with control (see Supplemental Figure S3A at <http://ajp.amjpathol.org>). Compared with media control (untreated slices), at 4 to 24 hours the number of motile points were markedly diminished in the larger airways after smoke and alcohol (Figure 7B;  $P < 0.01$ ). In slices treated with alcohol alone, the number of motile points

increased at 1 hour ( $P < 0.05$ ) but was unchanged at all other time points compared with control (see Supplemental Figure S3B at <http://ajp.amjpathol.org>). Brief ethanol exposure (1 hour) stimulated CBF. Significant ( $P < 0.001$ ) increases in PKC $\epsilon$  activity were detected in mouse lung slices treated with smoke and alcohol before cilia slowing (Figure 7C), consistent with those observations made in isolated ciliated cells.

To establish *in vivo* relevance, we exposed wild-type C57Bl/6 mice to whole-body cigarette smoke chambers and added alcohol to their drinking water with the use of a previously established co-exposure model.<sup>6</sup> Mice that were co-exposed to both cigarette smoke and alcohol showed a significant ( $P < 0.01$ ) reduction in tracheal ring CBF (Figure 8A). No significant changes in CBF were observed in the tracheal cilia of mice exposed to only cigarette smoke or only fed alcohol in their drinking water. In those tracheal epithelial cells extracted from mice co-exposed to smoke and alcohol, PKC $\epsilon$  was increased approximately threefold over the sham (air)-exposed



**Figure 6.** Localization of protein kinase C (PKC $\epsilon$ ) directly on the isolated bovine trachea ciliary axoneme. Axonemes were visualized by differential interference contrast microscopy (A), stained with rabbit anti-PKC $\epsilon$  antibodies (B) or nonspecific IgG (C), and visualized by confocal laser scanning microscopy. Axonemes were also assayed for PKC $\epsilon$  activity in the presence or absence of calcium, lipid, substrate, or dithiothreitol (DTT) (D).



**Figure 7.** Effect of combination smoke and alcohol on cilia in lung slices. Precision-cut mouse lung slices were treated with 5% cigarette smoke extract (CSE) and 100 mmol/L ethanol (EtOH) in submerged *in vitro* culture. Ciliary beat frequency (CBF; **A**) and the average number of motile points per field of cells (**B**) were determined by Sisson-Ammons Video Analysis from 1 to 21 hours. Protein kinase C (PKC) $\epsilon$  activity was assayed from 1 to 6 hours (**C**). Representative media controls (M199) are indicated by the white bars. Data are shown as means  $\pm$  SEs ( $n = 9$ ). \* $P < 0.05$  versus control media at matched time points of 3 to 21 hours for CBF; † $P < 0.01$  versus control media at matched time points of 4 to 21 hours for average motile points; ‡ $P < 0.001$  versus control media at matched time points of 1 to 3 hours.

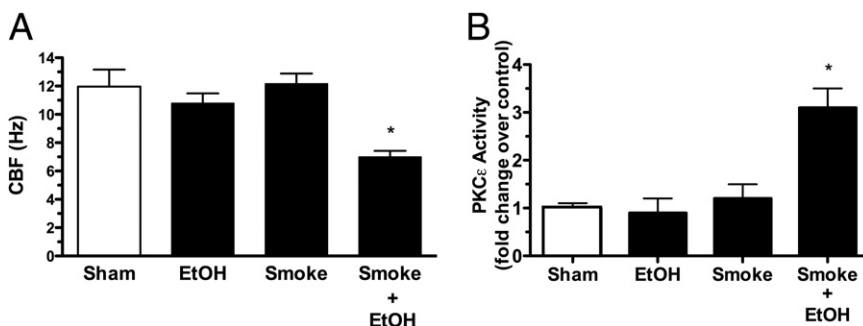
mouse group (Figure 8B). This elevation in kinase activity was not observed in the tracheal epithelium of the alcohol-alone or smoke-alone exposure groups (Figure 8B) and was observed regardless of whether the mice were initially exposed to cigarette smoke followed by alcohol feeding, or if the animals began an alcohol feeding regimen followed by subsequent cigarette smoke exposure (Figure 9, A and B). A similar pattern of results for PKC activity (Figure 9) and CBF (data not shown) was also observed in mouse trachea and whole-lung slices regardless of the combination exposure sequence.

To confirm the pivotal role of PKC $\epsilon$  in smoke and alcohol-induced ciliary slowing, we investigated the effect on CBF in PKC $\epsilon$ KO mice. As expected, CBF significantly ( $P < 0.05$ ) decreased in response to the novel PKC inhibitor, Ro 31-8220 (Figure 10A), in the tracheal rings cut from wild-type mice. However, no slowing in cilia beating or ciliostasis was observed from similarly treated PKC $\epsilon$ KO mice. As an additional control, Ro 31-8220 effectively induced ciliostasis before ciliated cell detachment in the tracheal epithelium of PKC $\delta$  knockout mice but not in PKC $\epsilon$ KO mice. In precision-cut mouse lung slices from PKC $\epsilon$ KO mice, DCP-LA *in situ*-treated tissue did not stimulate PKC $\epsilon$  activity (Figure 10B) or cause cilia slowing (not shown), validating the absence of PKC $\epsilon$  activity in the PKC $\epsilon$ KO mice. In keeping with these observations, the CBF decreases observed in response to the combination of *in situ* treatment of smoke and alcohol

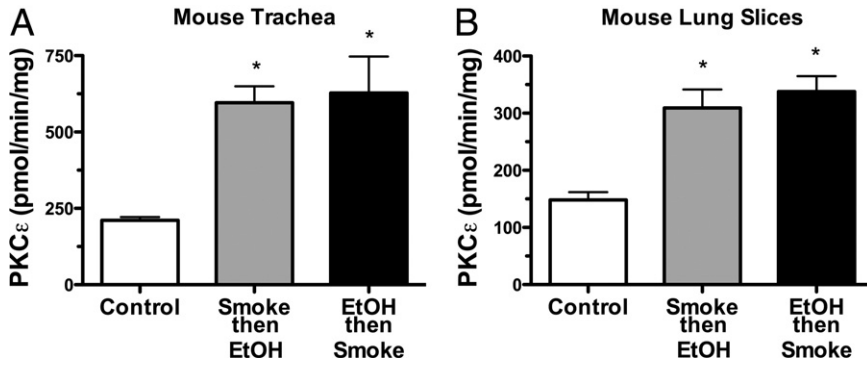
in the wild-type mouse lung slice is not detected in the PKC $\epsilon$ KO mouse slices (Figure 10C). Neither 5% CSE nor alcohol alone slowed CBF from PKC $\epsilon$ KO mouse slices (see Supplemental Figure S4 at <http://ajp.amjpathol.org>). Collectively, these data indicate the requirement for PKC $\epsilon$  in the regulation of cilia slowing induced by the combined action of cigarette smoke and alcohol exposure.

### Discussion

In this study, the co-exposure of cigarette smoke and alcohol, but neither alone, resulted in a significant and rapid slowing in CBF, which in part depended on PKC $\epsilon$  activation in the axoneme of the ciliated airway epithelial cell. Our results extend the studies by Wong et al<sup>14</sup> in which a novel isoform of PKC was suggested to be responsible for the cilia slowing induced by neuropeptide Y. Our data establish that the calcium-independent action of the novel PKC isoform, PKC $\epsilon$ , controls cilia beat slowing. PKC $\delta$  is the other novel PKC isoform in lung epithelium. It is implicated in the regulation of cytokine production in the lung.<sup>29</sup> However, it does not appear to be involved in cilia slowing. We did not observe any changes in PKC $\delta$  activity in response to cigarette smoke and alcohol co-exposure (data not shown). Likewise, the cilia-slowing response remained normal in the PKC $\delta$



**Figure 8.** Effect of *in vivo* smoke and alcohol exposure on cilia. Mice were either sham-treated (white bars) with air and water, whole body exposed to cigarette smoke (smoke), fed 20% alcohol (EtOH), or exposed to both alcohol and cigarette smoke in combination (smoke+EtOH) for 8 weeks. Ciliary beat frequency (CBF; **A**) and protein kinase C (PKC) $\epsilon$  activity (**B**) were assayed from tracheal epithelium. Data are shown as means  $\pm$  SEs ( $n =$  six mice per group). \* $P < 0.01$  for smoke+EtOH-treated versus sham-treated mice.



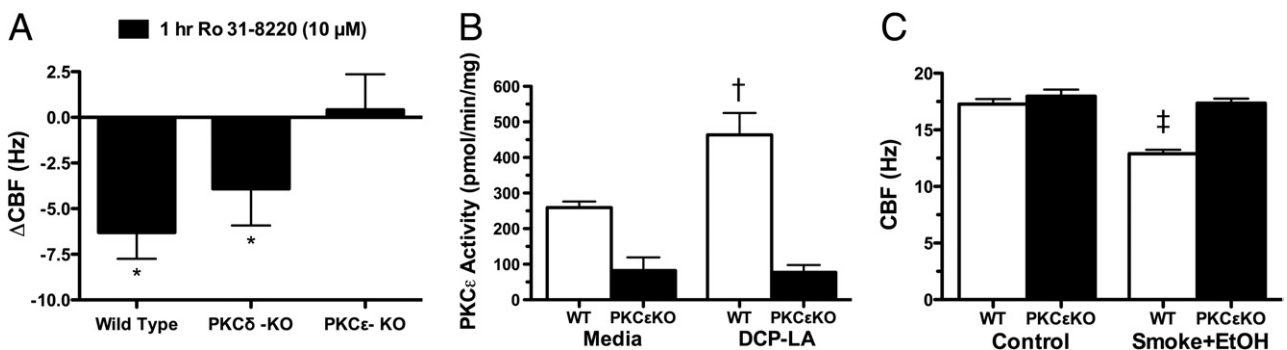
**Figure 9.** Sequence of *in vivo* smoke and alcohol co-exposure does not alter the effects on protein kinase C (PKC) $\epsilon$ . Mice were either whole-body smoke-exposed first followed by alcohol feeding (gray bars) or alcohol-fed first followed by whole-body smoke exposure (black bars) *in vivo*, and PKC $\epsilon$  activity was measured in both tracheal epithelium (A) and precision-cut lung slices (B). Data are shown as means  $\pm$  SEs ( $n = 6$  mice/group). \* $P < 0.01$  for either smoke then EtOH-treated or EtOH then smoke-treated versus sham-treated mice.

knockout mouse (Figure 10A). The next step is to identify the cilia substrate(s) for PKC $\epsilon$  in response to smoke and alcohol and to determine the mechanism behind how such a phosphorylation event slows cilia beating. A 37-kDa membrane-associated substrate for PKC has been isolated in ovine cilium that is involved with PKC-mediated cilia slowing,<sup>8</sup> but no such homologues have been shown in mice, bovines, or humans. Coupled with our previous finding that the ciliated cell lacks the PKC $\epsilon$ -targeting protein RACK1, which facilitates translocation-activated kinase activity to its substrate,<sup>30</sup> our present finding that PKC $\epsilon$  activity can be identified directly on the ciliated axoneme suggests that cilia-localized substrate targets for PKC $\epsilon$  are likely for the regulation of CBF. Cilia structural components regulating the mechanics of functional cilia slowing are not currently defined.

A novel finding that has emerged from this work is that a small subpopulation of cultured ciliated cells continue to beat at normal baseline frequency levels even after 24 hours of exposure to smoke and alcohol (Figure 1). This finding underscores the importance of examining large populations of motile cells with the use of a whole-field analysis approach to control for the number of motile points over time. Otherwise, analysis of the small subpopulation of motile cells after 24 hours of treatment would have masked our findings. This subpopulation of cells

resistant to the cilia-slowing effects of smoke and alcohol may be attributed to tissue culture artifact. The cell explants were i) enzyme digested and re-attached to matrix and subsequently lost any directional organization and control, ii) submerged in liquid cultures, or iii) of bovine origin. In contrast to the cultured cells, mouse precision-cut slices do not show unresponsive subpopulation of cells because the intact ciliated epithelia are attached to native tissue with a directional architecture. Ciliated cells from trachea or large airways were observed to eventually detach, similarly to the detachment injury reported in long-term smoke-exposed mouse tissues.<sup>2</sup>

In a previous study of long-term smoke exposure, we had shown in an *in vivo* mouse model of cigarette smoke exposure that cilia slowing and detachment of ciliated cells takes place over a chronic exposure to cigarette smoke of 6 to 9 months.<sup>2</sup> However, others have reported that shorter times of cigarette smoke exposure may enhance mucociliary transport.<sup>3</sup> This discrepancy may be because of an early mechanical stimulation effect in response to particles in cigarette smoke. Similarly, we have observed that filterable cigarette smoke particles  $> 0.2 \mu\text{m}$  in size can stimulate ciliary axoneme bending in isolated cilia.<sup>31</sup> However, in this study, short-term cigarette smoke exposure alone did not activate PKC $\epsilon$  or alter CBF. Moreover, the combination of cigarette smoke and



**Figure 10.** Effect of smoke and alcohol exposure on cilia from protein kinase C (PKC) $\epsilon$  knockout mice. Tracheal rings and lung slices were cut from mice that lacked PKC $\epsilon$  expression. Change in ciliary beat frequency (CBF) in response to *ex vivo* 10  $\mu\text{mol/L}$  Ro 31-8220 treatment (versus baseline CBF) in the trachea of wild-type, PKC $\epsilon$  knockout (PKC $\epsilon$ KO), and PKC $\delta$  knockout (PKC $\delta$ -KO) mice were assayed by Sisson-Ammons Video Analysis (A). Lung slice PKC $\epsilon$  activity in response to *ex vivo* treatment with 10  $\mu\text{mol/L}$  8-[2-(2-pentylcyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) was assayed in wild-type (WT) and PKC $\epsilon$ KO mice (B). Lung slice PKC $\epsilon$  activity in response to *in situ* treatment with smoke and alcohol was assayed in WT and PKC $\epsilon$ KO mice (C). Data are shown as means  $\pm$  SEs ( $n = 6$ ). \* $P < 0.01$  for changes in CBF in WT and PKC $\delta$ KO mice in the presence versus absence of Ro 31-8220; † $P < 0.001$  for PKC $\epsilon$  activation in WT versus PKC $\epsilon$ KO mice in response to DCP-LA; ‡ $P < 0.001$  for PKC $\epsilon$  activation in WT versus PKC $\epsilon$ KO mice in response to the combination of smoke+alcohol. EtOH, ethanol.



alcohol does not enhance cilia beating, but rather produces the unique and rapid effect of cilia slowing that otherwise would require a much longer exposure to just cigarette smoke alone.

The loss of total motile points is the result of both detached cells and/or complete ciliostasis. This is consistent with the PKC $\epsilon$  inhibition-mediated detachment because of Ro 31-8220<sup>28</sup> because auto-downregulation of PKC $\epsilon$  is observed temporally after smoke and alcohol activation of PKC $\epsilon$  (Figure 2). Such an auto-downregulation response would be functionally equivalent to direct inhibition of the PKC $\epsilon$  catalytic active site as accomplished with Ro 31-8220 treatment. Our current and previously published data<sup>28</sup> support the conclusion that Ro 31-8220 induces the detachment of ciliated cells via the direct chemical inhibition of PKC $\epsilon$ . Detaching and unattached ciliated cells beat slower because of the physical mechanics of cilia beat. Thus, the cilia slowing that precedes the detachment of a ciliated cell in response to PKC $\epsilon$  inhibition would be indistinguishable from the active cilia slowing initiated by PKC $\epsilon$  activation in the absence of ciliated cell detachment as that observed with smoke and alcohol treatment. Interestingly, no such detachment is observed when cells were treated with the PKC $\epsilon$  translocation inhibitor as was observed with a catalytic site inhibitor. Phorbol esters and lipid activators (Figure 3) do not appear to stimulate ciliated cell detachment even though they both lead to CBF slowing. These observations suggest that a translocatable form of PKC $\epsilon$  not regulating CBF and a cilia-localized form of PKC $\epsilon$  susceptible to activation/inhibition regulation of cilia beat exist in airway epithelium. Clearly, a differential regulation of PKC $\epsilon$  exists in basal versus ciliated epithelial cells. This is likely because the PKC $\epsilon$ -targeting protein, RACK1, is expressed in basal nonciliated bronchial epithelial cells, but not in ciliated epithelial cells from the same tissue.<sup>30</sup> Such compartmentalized regulation of kinase action may provide the specificity of cilia responses to external stimuli such as smoke and alcohol, leaving no such PKC $\epsilon$  effect on basal airway epithelium.

A potential mechanism for cilia slowing due to smoke and alcohol-mediated cilia slowing observed *in vivo* may be related to the action of reactive aldehyde accumulation in the lung. Both ethanol metabolism and tobacco pyrolysis result in the generation of malondialdehyde and acetaldehyde. Indeed, we have previously detected these reactive aldehydes in the lungs of mice co-exposed to cigarette smoke and alcohol.<sup>32</sup> Of importance is the fact that neither cigarette smoke nor alcohol in high concentrations alone is sufficient to produce both the levels of malondialdehyde and acetaldehyde observed under co-exposure conditions. In addition, we have demonstrated significant concentrations of malondialdehyde-acetaldehyde (MAA) protein adduct formation in lungs, but only under conditions of smoke plus alcohol co-exposure.<sup>32</sup> *In vitro*, MAA-adducted proteins bind to scavenger receptor A, resulting in the activation of PKC.<sup>33</sup> In addition, direct lung instillation of MAA-adducted lung surfactant protein results in the activation of airway epithelial PKC $\epsilon$  activation.<sup>22</sup> Current studies are under way to characterize MAA-adducted proteins as ligands for

scavenger receptor in airway epithelium and the *in vivo* effects of MAA-adducted protein on cilia beating to explore stable hybrid adduct formation as a mechanism for the cilia-slowng effects observed in response to combined smoke and alcohol exposure.

Distinct differences exist between the actions of combined smoke and alcohol versus ethanol only on cilia. Brief modest exposure to alcohol alone actually stimulates a rapid increase in CBF in both *in vitro* cell models<sup>34</sup> and *in vivo* rodent models<sup>6</sup> because of a transient elevation of nitric oxide. However, continued chronic alcohol exposure leads to a desensitization of cilia to further CBF stimulation by any cAMP-elevating agent such as  $\beta$  agonists.<sup>4,35</sup> Although no significant slowing of cilia below baseline beating is observed with alcohol only treatment at any time point, combination smoke and alcohol exposure *in vivo* led to discernible and biologically relevant cilia slowing, particularly after a subsequent *in vitro* exposure of *in vivo*-treated tissues with a  $\beta$  agonist.<sup>6</sup> Whether  $\beta$  agonists are capable of potentiating cilia slowing induced by smoke and alcohol has yet to be determined.

In summary, we found that co-exposure to cigarette smoke and alcohol resulted in a rapid slowing of CBF via a PKC $\epsilon$ -dependent manner. These observations are consistent with our preclinical rodent models that established that mucociliary clearance is significantly diminished under conditions of alcohol and cigarette smoke.<sup>5,6</sup> These results may have clinical importance because most persons with alcohol use disorders smoke cigarettes.<sup>36,37</sup> Persons with alcohol use disorders have an increased risk in both the occurrence and severity of lung infections,<sup>38</sup> which could potentially affect infection-mediated exacerbations of chronic inflammatory lung diseases such as bronchitis, pneumonia, and chronic obstructive pulmonary disease. Future research should examine lung infections and chronic lung disease exacerbations within the context of smoke and alcohol co-exposure. Given that lung defense involves both innate and adaptive immune defenses to inhaled pathogens, the effect of combined cigarette smoke and alcohol effects should likely be considered for those lung defenses downstream of mucociliary clearance as well.

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