Cinnamaldehyde in flavored e-cigarette liquids temporarily suppresses bronchial epithelial cell ciliary motility by dysregulation of mitochondrial function

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[1]Curriculum in Toxicology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina; [2]Center for Environmental Medicine, Asthma, and Lung Biology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina; [3]Department of Pediatrics, University of North Carolina, Chapel Hill, North Carolina; and [4]Department of Medicine, University of North Carolina, Chapel Hill, North Carolina

Clapp PW, Lavrich KS, van Heusden CA, Lazarowski ER, Carson JL, Jaspers I. Cinnamaldehyde in flavored e-cigarette liquids temporarily suppresses bronchial epithelial cell ciliary motility by dysregulation of mitochondrial function. *Am J Physiol Lung Cell Mol Physiol* 316: L470–L486, 2019. First published January 3, 2019; doi:10.1152/ajplung.00304.2018.—Aldehydes in cigarette smoke (CS) impair mitochondrial function and reduce ciliary beat frequency (CBF), leading to diminished mucociliary clearance (MCC). However, the effects of aldehyde e-cigarette flavorings on CBF are unknown. The purpose of this study was to investigate whether cinnamaldehyde, a flavoring agent commonly used in e-cigarettes, disrupts mitochondrial function and impairs CBF on well-differentiated human bronchial epithelial (hBE) cells. To this end, hBE cells were exposed to diluted cinnamon-flavored e-liquids and vaped aerosol and assessed for changes in CBF. hBE cells were subsequently exposed to various concentrations of cinnamaldehyde to establish a dose-response relationship for effects on CBF. Changes in mitochondrial oxidative phosphorylation and glycolysis were evaluated by Seahorse Extracellular Flux Analyzer, and adenine nucleotide levels were quantified by HPLC. Both cinnamaldehyde-containing e-liquid and vaped aerosol rapidly yet transiently suppressed CBF, and exposure to cinnamaldehyde alone recapitulated this effect. Cinnamaldehyde impaired mitochondrial respiration and glycolysis in a dose-dependent manner, and intracellular ATP levels were significantly but temporarily reduced following exposure. Addition of nicotine had no effect on the cinnamaldehyde-induced suppression of CBF or mitochondrial function. These data indicate that cinnamaldehyde rapidly disrupts mitochondrial function, inhibits bioenergetic processes, and reduces ATP levels, which correlates with impaired CBF. Because normal ciliary motility and MCC are essential respiratory defenses, inhalation of cinnamaldehyde may increase the risk of respiratory infections in e-cigarette users.

aldehyde; e-liquid; electronic cigarette; flavoring; toxicity; vaping

INTRODUCTION

Mucociliary clearance is the primary physical airway defense that prevents inhaled pathogens and debris from injuring the airway epithelium and underlying tissues. Particles deposited on the epithelial surface during inspiration become trapped within airway mucus and motile airway cilia beat in coordinated metachronal waves to propel mucus particle aggregates toward the larynx (54). Airway cilia are complex, microtubule-based organelles that are essential to effective mucus transport. Chemicals that interfere with normal ciliary motility can impede mucociliary clearance and increase the residence time of inhaled pathogens, thereby enhancing susceptibility to respiratory infection. Reduced mucociliary clearance is a functional abnormality in smokers that often precedes or occurs with the development of chronic bronchitis and obstructive lung disease (57). Cigarette smoke (CS) alters mucus composition, airway surface hydration, cilia structure, and intercellular cilia coordination and reduces ciliary beat frequency (CBF) (3–9). Many of the direct effects of CS on cilia structure and function are believed to be due to highly reactive aldehydes inhaled when smoking.

As the use of traditional tobacco products has fallen in recent years, the popularity of new and emerging tobacco products, such as e-cigarettes, has surged. There are currently thousands of flavored e-cigarette products on the market (63). These products are not only popular with current smokers attempting to quit but have rapidly gained popularity with adolescents and young adults who have never smoked (8, 27, 62). E-cigarettes have been heavily marketed as safer alternatives to traditional cigarettes and effective cessation aids to reduce smoking (20). This representation has led to the perception by many that e-cigarettes are harmless (45). This perception is supported by numerous scientific studies reporting that e-cigarettes produce substantially lower levels of the known toxicants and carcinogens commonly found in cigarette smoke, including reactive aldehydes (19, 21, 26). However, recent publications have highlighted the potential risk for exposure to toxicants (18), metals (26, 60), and reactive chemical compounds, such as flavoring agents (3), that are not present in traditional cigarette smoke.

Flavorings agents used in e-cigarettes are "generally recognized as safe" (GRAS) by the US Food and Drug Administration (FDA). However, this recognition of safety only pertains to their use in foods and not exposures by inhalation. Indeed, prolonged inhalation of some GRAS flavorings, including

diacetyl, 2,3-pentanedione, and acetoin, is well documented to cause irreversible lung disease (31, 34, 37). The Flavor and Extract Manufacturers Association of the USA (FEMA), a trade association of flavor ingredient manufacturers that evaluates the safety of food flavorings, has identified 1,037 GRAS flavoring agents as potential respiratory hazards due to possible volatility and respiratory irritant properties; however, most of these compounds still lack any toxicological evaluation for inhalation exposures (17). Recent work by Gorukanti et al. (27) found that most current e-cigarette users perceive flavoring agents in e-cigarettes as safe to inhale, and the majority of college students they surveyed believed the FDA's GRAS designation extended to safety of inhalation exposures. These findings are cause for concern, as chemical flavoring agents with reactive functional groups, such as aldehydes, are commonly used to flavor e-cigarettes, and there is currently no regulation governing the quantities used in these products.

The effects of reactive aldehydes in cigarette smoke on airway cilia and mucociliary clearance have been well studied. However, it is unclear whether reactive aldehyde flavoring agents in e-cigarettes will cause a similar response. Several recent studies have reported exceedingly high concentrations of aldehyde flavoring agents in commercially available e-liquids. Behar et al. (5) identified cinnamaldehyde in 20 of 39 e-liquids tested with concentrations ranging from 1.7×10^{-5} to 1.1 M. Our group recently evaluated a panel of seven e-liquids and identified numerous aldehyde flavorings, including vanillin, ethyl vanillin, benzaldehyde, 4-anisaldehyde (4-methoxybenzaldehyde), cocoa hexenal (5-methyl-2-phenyl-2-hexenal), and cinnamaldehyde (14). The concentration of cinnamaldehyde in the e-liquids we tested ranged from 8.9×10^{-4} to 1.13 M (14). Many of the aldehydes listed here have structural similarity to toxic aldehydes in cigarette smoke. We have shown previously that cinnamon e-liquids and cinnamaldehyde suppress respiratory immune cell function at concentrations significantly lower than those resulting in cell death (14). However, whether cinnamon-flavored e-liquids and/or cinnamaldehyde disrupt normal hBE cell function, such as airway ciliary motility or bioenergetic pathways, is completely unknown. To address this knowledge gap, we exposed hBE cells to cinnamaldehyde-containing e-liquids, vaped e-liquid aerosol, and cinnamaldehyde alone, quantified changes in cellular cilia motility, investigated molecular drivers of impaired cilia function, and examined how the addition of nicotine modifies these responses.

MATERIALS AND METHODS

Primary hBE Cell Culture

Human lung tissue was procured under an Institutional Review Board-approved protocol, and hBE cell harvest and culture were performed using established procedures previously described in detail (23). Briefly, hBE cells were enzymatically dissociated with 0.01% protease XIV (Sigma-Aldrich, St. Louis, MO) and 0.001% DNase (Sigma-Aldrich) in minimum essential medium (Sigma-Aldrich) from the trachea, main stem, and lobar bronchi of six nonsmoker lung donors described in Table 1. Primary hBE cells were plated at a density of $2–6 \times 10^6$/100 mm collagen-coated (Advanced BioMatrix, San Diego, CA) cell culture plate (Corning Life Sciences, Tewksbury, MA) in BEGM medium (prepared in house). At 70–90% confluence, cells were passaged by trypsin (Sigma-Aldrich) dissociation, counted, and plated on human placental type IV collagen-coated (Sigma-

Table 1. *Demographic data for lung donors*

Donor	Age, yr	Sex	Race	Smoking	Cause of Death
1	57	W	Cau	NS	Intracranial hemorrhage
2	46	W	Cau	NS	Head trauma
3	14	M	Cau	NS	Head trauma
4	27	M	Blk/AA	NS	Head trauma
5	26	M	Hisp	NS	Head trauma
6	23	M	Cau	NS	Head trauma

Blk/AA, Black/African American; Cau, Caucasian; Hisp, Hispanic; M, man; NS, nonsmoker; W, woman. Non-cystic fibrosis lungs were obtained from organ donors whose lungs were unsuitable for transplant due to acute injury or lack of a matching recipient.

Aldrich) Transwell permeable supports (Corning Life Sciences) at a density of 3×10^5 cells/Transwell. Upon confluence, apical growth medium was removed, and cultures were differentiated at an air-liquid interface (ALI) for >40 days. Basolateral ALI growth medium (prepared in house) was replaced three times/wk, and cultures were washed with PBS (Sigma-Aldrich) once/wk to prevent accumulation of apical mucus and debris. Cells were cultured at 37°C with 95% humidity and 5% carbon dioxide.

Beas-2B Bronchial Epithelial Cell Culture

Beas-2B cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in serum-free keratinocyte growth medium (KGM; Lonza, Walkersville, MD) under standard airway epithelial cell growth conditions (37°C, 95% humidity, and 5% carbon dioxide). All studies were performed using Beas-2B cells between passage 40 and passage 60, with passage 1 defined as the thawed cells from the supplier. All experiments using Beas-2B cells were repeated four times ($n = 4$) with three technical replicates for each experimental condition.

Flavored E-cigarette Liquids, Cinnamaldehyde, and Nicotine

Three cinnamaldehyde-containing, nicotine-free e-liquids ("Kola," "Hot Cinnamon Candies," and "Sinicide") were purchased from a local vape shop (The Vapor Girl, Chapel Hill, NC). E-liquids were stored in opaque glass bottles at room temperature and diluted for use at the time of each experiment. All e-liquids used in this study were reported by the manufacturer to contain the humectants PG and VG at relative proportions of 55 and 45%, respectively. A mixture of 55% PG (Thermo Fisher Scientific, Waltham, MA) and 45% VG (Sigma-Aldrich) was used as a vehicle control for all e-liquid and vape experiments. Food grade trans-cinnamaldehyde (≥98% pure) and GC-grade nicotine (≥99% pure) were purchased from Sigma Aldrich and stored at room temperature in the opaque glass bottles provided by the manufacturer. Cinnamaldehyde and nicotine were diluted for use at the time of each experiment.

CBF and Percent Active Area

Twenty-four hours before CBF analysis, 150 μl of PBS was applied to the apical surface of well-differentiated (>40 days old) hBE cell cultures for 20 min to loosen mucus and debris. Apical PBS and basolateral medium were removed by vacuum aspiration, and 2.5 ml of warm (37°C) ALI medium was added to the basolateral compartment. Twenty minutes before CBF analysis, the apical compartment of ALI cultures was briefly rinsed again with 150 μl of PBS. For each CBF experiment, a well-differentiated hBE cell culture was placed on Nikon Diaphot inverted microscope equipped with a ×20 phase contrast objective (Nikon, Melville, NY), and CBF for a single ×20 field of view was observed. Cilia motion was recorded with a high-speed digital camera (Basler AG, Ahrensburg, Germany) and analyzed with software designed to quantify beat frequency and the percent of motion in the field of view (SAVA; Ammons Engineering,

Ciliary Beat Frequency

Percent of 20x Field in Motion
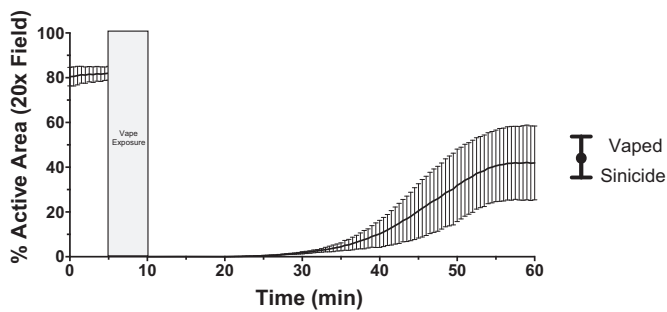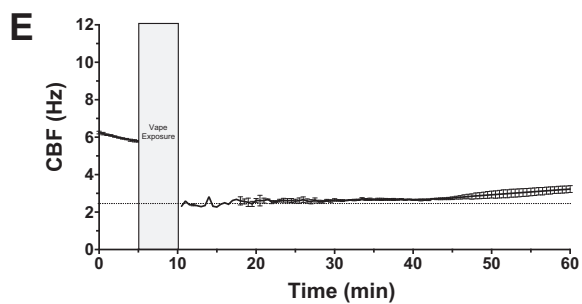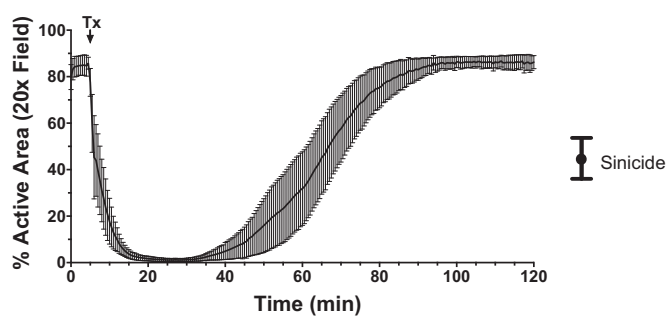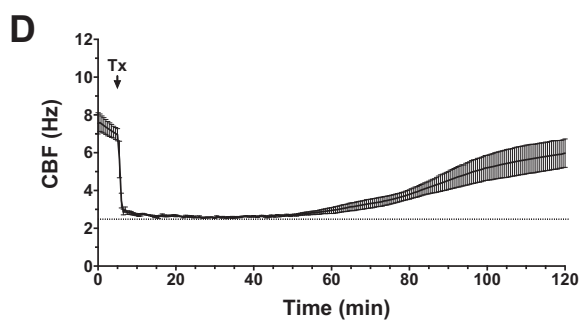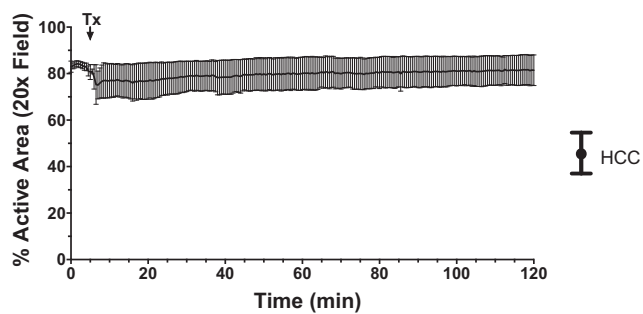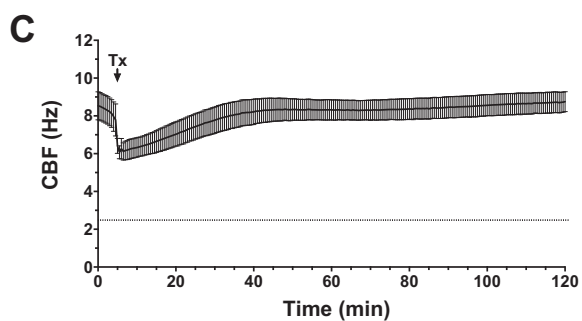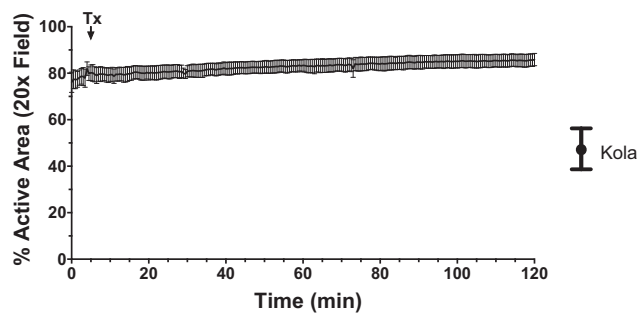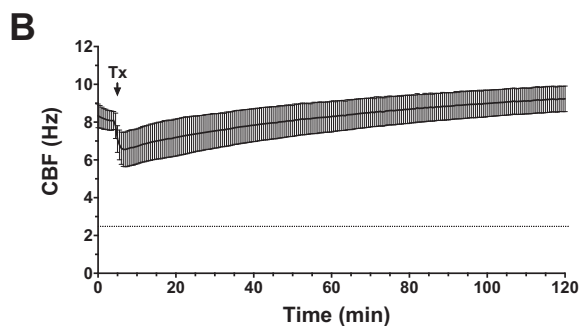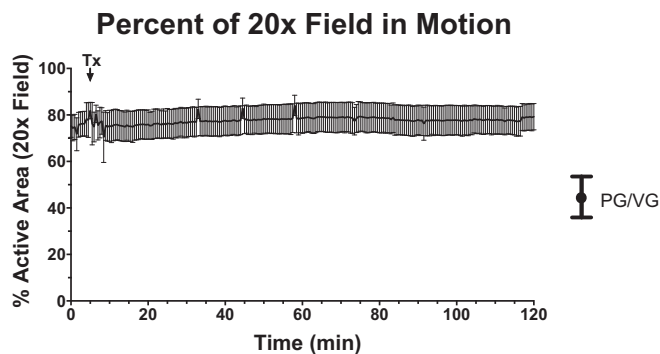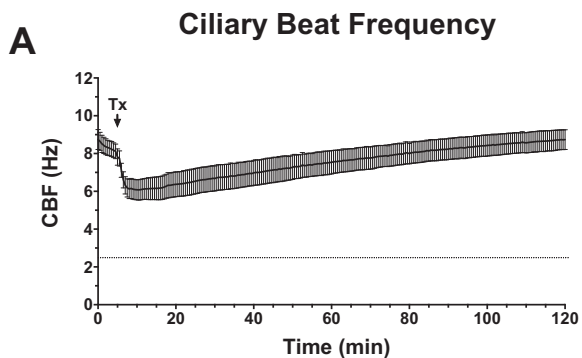
Mt. Morris, MI) (51). Baseline whole-field CBF and percent active areas (%AA) were evaluated at 30-s intervals for 5 min. Following baseline CBF and %AA assessment, cultures were challenged apically with 150 μl of 1% (vol/vol) flavored e-liquid, 1% PG/VG vehicle, cinnamaldehyde (1, 5, 10, and 15 mM), nicotine (0.5mg/ml), or ALI growth medium alone (media control). Whole-field CBF and %AA were quantified at 30-s intervals for 115 min following the apical addition of treatments. Apical challenges were not removed for the duration of the experiment. All ciliary beat measurements were conducted at room temperature (~24°C).

The threshold of detection for CBF was 2.5 Hz, and whole-field analysis data with <100 points of information collected were omitted from analysis. CBF and %AA data for each experiment were plotted as a function of time using GraphPad Prism version 7. Cell cultures from four donors ($n = 4$) were used for CBF and %AA analysis. To determine whether any e-liquid or cinnamaldehyde treatment significantly altered CBF and %AA, the area under the curve (AUC) for each CBF and %AA plot was calculated and analyzed using a one-way ANOVA and Holm-Sidak multiple-comparisons test. All statistical analyses were carried out using GraphPad Prism version 7. Differences with a P value of ≤0.05 were considered significant.

Vaped E-liquid Aerosol Exposures

Baseline whole-field CBF and %AA were evaluated in well-differentiated hBE cell cultures at 30-s intervals for 5 min as described above. Following baseline CBF and %AA assessment, cultures were placed into the center of a 3-liter acrylic exposure chamber and exposed to Sinicide or PG/VG aerosol, similar to previous studies conducted by Carson et al. (11). E-cigarette aerosol was generated by connecting a LAVABOX DNA 200 Box Mod e-cigarette (Volcano e-Cigs, Honolulu, HI) with a SMOK TFV4 Mini Tank and sub-ohm (0.37 Ω) TF-CLP2 Clapton Coil (SMOKtech, Shenzhen, China) to the exposure chamber with a 10-cm length of tubing (4.8 mm ID; Masterflex L/S 15, Vernon Hills, IL). Similar to previous studies, a vacuum pump connected to a flow meter was used to pull aerosol from the e-cigarette device at a flow rate of 3 l/min (11). One 5-s puff of aerosol was generated every 30 s for 5 min (10 puffs total) using an e-cigarette device setting of 70 W. E-cigarette tank vents were fully opened to supply maximal airflow to the heating coil. Mouthpiece vents were completely closed to prevent dilution of aerosol. Thirty seconds after the final puff, the cell culture was removed from the exposure chamber and immediately placed on the microscope for CBF analysis.

To assess particle deposition at the airway surface during vape exposures, 12-mm-glass coverslips were inserted into empty transwells and exposed to e-cigarette aerosols at the same time as hBE cultures. Immediately following vape exposures, glass coverslips were removed and weighed on a microbalance. Initial coverslip weights were subtracted from post-exposure weights do determine the mass of aerosol deposition. Whole-field CBF and %AA of hBE cultures were measured at 30-s intervals for 55 min following the aerosol exposure. CBF and %AA data was plotted and AUC values for each experiment were calculated as described above. Cell cultures from three donors ($n = 3$) were used for vape experiments. An unpaired t-test was used to determine whether the effect of Sinicide was significantly different from PG/VG. All statistical analyses were carried out using GraphPad Prism version 7. Differences with a P value ≤0.05 were considered significant.

Evaluation of Mitochondrial Membrane Potential

The cationic carbocyanine dye JC-1 (Thermo Fisher Scientific, Waltham, MA), which exhibits potential-dependent accumulation in mitochondria, was used to evaluate the effects of cinnamaldehyde on mitochondrial membrane polarization. Beas-2B cells were plated at 5×10^4 cells/well in black-wall, clear-bottom, 96-well cell culture plates (Corning Life Sciences). Cell culture medium was aspirated 24 h after plating, and cells were incubated for 1 h in 37°C KGM medium containing 2.0 μg/ml JC-1. Following incubation, cell culture medium containing JC-1 was aspirated, wells were washed briefly with 1x PBS, and cells were exposed to 37°C KGM medium containing cinnamaldehyde for 15 min. The J-aggregate (red)-to-JC-1 monomer (green) fluorescence intensity ratio was determined using a CLARIO-star multimode microplate reader (BMG Labtech, Offenburg, Germany) with an excitation wavelength of 488 nm and detection at 590 and 525 nm, respectively.

JC-1 dye was also used to evaluate mitochondrial membrane polarization in differentiated primary hBE cells exposed to cinnamaldehyde or nicotine. ALI growth medium containing 2.0 μg/ml JC-1 was added to both the apical and basolateral compartments, and cell cultures were incubated at 37°C with 95% humidity and 5% carbon dioxide for 24 h. Following incubation, cell culture medium containing JC-1 was aspirated, transwells were washed briefly with $1\times$ PBS, basolateral ALI growth medium was replaced, and cell cultures were apically exposed to 150 μl of ALI medium containing cinnamaldehyde or nicotine for 120 min. Mitochondrial depolarization was assessed by quantifying the fluorescence emission shift from red to green, as described above. A Nikon Microphot-SA microscope with a $\times20$ objective, a Nikon DS-Fi2 digital camera, and a Nikon DS-L3 camera controller were used to visualize J-aggregate and JC-1 monomer fluorescence in Beas-2B cells.

Data were collected from four independent experiments ($n = 4$). A one-way ANOVA and Dunnett's multiple-comparisons test were used to determine whether the effects of cinnamaldehyde or nicotine were significantly different from the media control or the baseline J-aggregate-to-JC-1 monomer ratio. All statistical analyses were carried out using GraphPad Prism version 7. Differences with a P value of ≤0.05 were considered significant.

Analysis of Mitochondrial Respiration and Glycolysis

Live-cell metabolic assays were conducted using a Seahorse XFe24 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA). Beas-2B cells were plated at 3×10^4 cells/well in XF24 cell culture plates. Twenty-four hours after plating, Beas-2B growth medium was aspirated and replaced with keratinocyte basal medium (KBM) without supplements or growth factors (Lonza). Cells were analyzed 24 h after plating in KBM. All cultures were >90% confluent at the time of analysis. XFe24 sensor cartridges were hydrated overnight with XF Calibrant at 37°C before analysis. XF Cell Mito Assay Media (XF Base Media with 10 mM glucose, 1 mM sodium pyruvate, and 2 mM L-glutamine) was prepared fresh on the day of assay and adjusted to pH 7.4. KBM was aspirated from Beas-2B cells and replaced with warm (37°C) XF Cell Mito Assay Medium. Cells were incubated at 37°C without CO_2 for 1 h before Seahorse analysis. Stock concentrations of oligomycin (Sigma-Aldrich), FCCP (Sigma-Aldrich), rotenone (Sigma-Aldrich), and antimycin A (Sigma-Aldrich) were prepared in DMSO (Sigma-Aldrich) and stored at −20°C. Stock solu-

Fig. 1. Effects of cinnamaldehyde-containing e-liquids on airway cilia motility. *A–D*: following 5-min baseline measurements, well-differentiated primary human bronchial epithelial (hBE) cells were exposed to 1% dilutions of e-liquids (vol/vol) in cell culture medium and ciliary beat frequency (CBF; *left*), and percent active area (%AA) of the $\times20$ field of view in motion (*right*) was recorded for 120 min ($n = 4$). *E*: 5-min baseline measurements were recorded before well-differentiated primary hBE cells were exposed to vaped Sinicide e-liquid aerosols for 5 min. CBF and %AA were recorded for 50 min following exposures ($n = 3$). Data are presented as means< SE. HCC, Hot Cinnamon Candies; PG/VG, propylene glycol/vegetable glycerin.

tions were diluted to working concentrations in XF Cell Mito Assay Media with final DMSO concentrations of <0.1%. Assay injections were ordered as follows: 1) vehicle or cinnamaldehyde (0.05–5.0 mM adjusted to pH 7.4), 2) 1 μM oligomycin, 3) 1 μM FCCP, and 4) 0.5 μM rotenone and 0.5μM antimycin A. Sequential mix, wait, and measurement times were set at 3, 2, and 3 min, respectively. Parameters for mitochondrial and glycolytic function were calculated as recommended by the instrument manufacturer (Agilent Technologies). Data was collected from four independent experiments ($n = 4$). A one-way ANOVA and Dunnett's multiple comparisons test was used to determine whether the effects of cinnamaldehyde were significantly different from the vehicle control. All statistical analyses were carried out using GraphPad Prism version 7. Differences with a P value of ≤0.05 were considered significant.

Adenine Nucleotide Quantification

Nucleotide extraction from hBE cells. Well-differentiated hBE cells cultures were gently washed with PBS, and 150 μl of 10 mM cinnamaldehyde (diluted in ALI growth medium) was added to the apical surface. Cultures were incubated with cinnamaldehyde for 15 min, 120 min, or 24 h, followed by a brief PBS wash of both the apical and basolateral surfaces to remove excess cinnamaldehyde. To assess intracellular nucleotide content, Transwell membranes were immediately excised with a no. 11 scalpel and transferred into a 24-well plate containing 0.5 ml of ice-cold trichloroacetic acid (TCA; 5% in HPLC-grade water). After 30 min, the TCA solution was transferred into a 13 × 100 mm borosilicate glass tube, and TCA was extracted with 2.0 ml ethyl ether (this procedure was repeated 5 times). Residual ether was removed by evaporation with nitrogen gas.

Derivatization of adenosine and adenine nucleotides. Adenosine and adenine nucleotides were derivatized using a modification of the protocol described by Lazarowski et al. (39). Briefly, 200-μl aliquots of the TCA-extracted samples (diluted 1:10 in PBS) were added to microcentrifuge tubes containing 100 μl of derivatization buffer (62 mM citric acid and 38 mM Na_2POH_4, pH 4.0), followed by the addition of 10 μl of 50% chloroacetaldehyde (vol/vol in HPLC grade water; Sigma-Aldrich). Samples were heated at 80°C for 40 min, cooled on ice, and analyzed by HPLC within 72 h of derivatization.

HPLC analysis. Identification and quantification of ethenylated species were performed with an automated Waters HPLC apparatus equipped with a fluorescence detector (excitation, 307 nm; emission, 410 nm). Derivatized samples were transferred to 0.7-ml plastic vials (Sun-SRi, Duluth, GA) and kept at 4°C in the sample injector rack. A 100-μl sample aliquot was injected into a Chromolith reversed-phase column (RP-18e, 100-3 mm; Merck, Darmstadt, Germany). The mobile phase (1.0 ml/min, isocratic) was composed of 200 mM NaH_2PO_4-H_2O, 0.2 M Na_2HPO_4-$2H_2O$, and 5 mM tetrabutyl NH_4 bromide (pH 6.1). Typical elution times (in min) of authentic etheno (ε) standards were as follows: ε-AMP, 2.09; ε-Ado, 2.61; ε-ADP, 3.10; and ε-ATP, 4.96. Individual peak areas were calculated using Waters Empower Pro software build 1154. Data were collected from four independent experiments ($n = 4$). A one-way ANOVA and Dunnett's multiple-comparisons test were used to determine whether cinnamaldehyde-induced changes in nucleotide content at each time point were significantly different from untreated (0 min) control. All statistical analyses were carried out using GraphPad Prism version 7. Differences with a P value of ≤0.05 were considered significant.

Chemical Inhibition of Glycolysis and Mitochondrial Respiration in hBE Cells for CBF and %AA Analyses

The glycolytic function of well-differentiated hBE cells was inhibited by replacing growth medium with a modified Ringer solution consisting of 115 mM NaCl, 20 mM HEPES buffer, 5 mM K_2HPO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 2 mM glutamine, and 10 mM 2-deoxyglucose (7). hBE cell cultures were washed with PBS and baseline CBF, and %AA were determined by taking 10 random SAVA measurements (×20 magnification) throughout each culture. Following baseline measurements, 500 μl of the modified Ringer solution was added to the basolateral compartment, and 150 μl was added apically. CBF and %AA measurements were taken again at 15 min and 30 min after the addition of modified Ringer solution by randomly sampling 10 random fields. After the 30-min measurements were recorded, the apical and basolateral modified Ringer solution was aspirated and replaced with 500 μl of ALI growth medium in the basolateral compartment and 150 μl of ALI medium apically. CBF and %AA was

Fig. 2. Quantification of e-liquid-induced effects on cilia motility. Area under the curve (AUC) was calculated for both ciliary beat frequency (CBF) and percent active area (%AA) data collected from e-liquid exposure experiments ($N = 4$; *A*) and vaped Sinicide e-liquid experiments ($n = 3$; *B*). Significant differences from the propylene glycol/vegetable glycerin (PG/VG) control were determined by 1-way ANOVA and Holm-Sidak multiple-comparisons test. For vaped Sinicide experiments, significant differences from the PG/VG control were determined by unpaired *t*-test. Data presented as means ± SE. Significance represented as **$P < 0.01$; ●, individual data points derived from the different human bronchial epithelial cell cultures. HCC, Hot Cinnamon Candies.
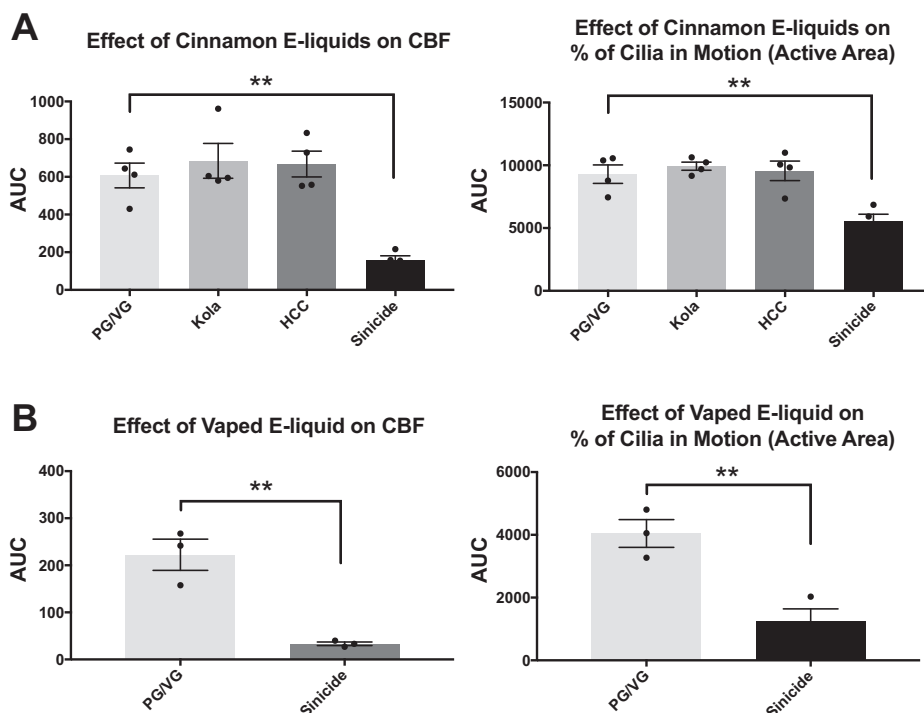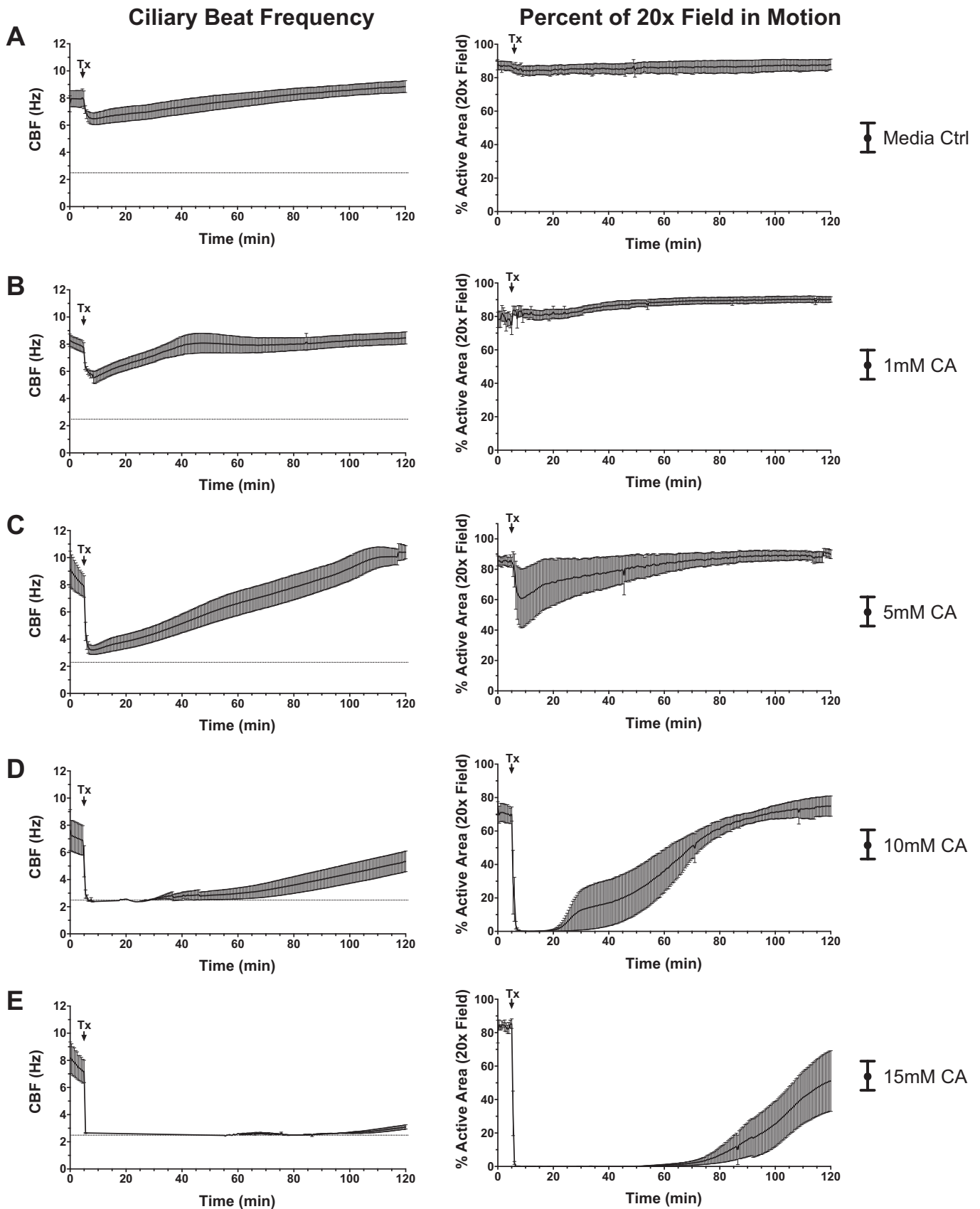
Fig. 3. Effects of cinnamaldehyde (CA) on airway cilia motility. Following 5-min baseline measurements, well-differentiated primary human bronchial epithelial (hBE) cells were exposed to either vehicle (cell culture medium) control (*A*) or 1, 5, 10, or 15 mM cinnamaldehyde (*B–E*). Ciliary beat frequency (CBF; *left*) and percent of the ×20 field of view in motion [percent active area (%AA); *right*] were recorded for 120 min (*n* = 4). Data presented as means< SE.

assessed 15, 30, and 90 min after the addition of normal growth medium by sampling 10 random fields, as described above.

Inhibition of hBE cell mitochondrial respiration (but not glycolysis) was accomplished by replacing the 10 mM 2-deoxyglucose in the modified Ringer solution with 10 mM glucose and supplementing the Ringer solution with 10 µM antimycin A, a potent mitochondrial complex III inhibitor. hBE cell cultures were washed with PBS and CBF, and %AA measurements were collected as described above.

Inhibition of both glycolysis and mitochondrial respiration was achieved by using the modified Ringer solution (glucose free) with 10 mM 2-deoxyglucose and 10 µM antimycin A. Measurements of CBF and %AA were collected as described above. CBF and %AA data for each experiment were plotted as a function of time using GraphPad Prism version 7. Well-differentiated hBE cell cultures from four donors ($n = 4$) were used. Differences in CBF and %AA from baseline were determined by one-way ANOVA and Holm-Sidak multiple comparisons test. Differences with a P value of ≤0.05 were considered significant.

RESULTS

Temporal Analysis of CBF and %AA During Flavored E-liquid and Vape Exposures

To better understand the biological effects of flavored e-liquids on respiratory ciliary motility, we exposed well-differentiated hBE cells to PG/VG vehicle or e-liquids containing different concentrations of cinnamaldehyde. The effects of exposure on CBF and %AA were recorded over 120 min (Fig. 1). Apical addition of the Kola and Hot Cinnamon Candies (HCC) e-liquids did not significantly alter CBF or %AA compared with the PG/VG vehicle (Fig. 1, A–C). However, exposure to the Sinicide e-liquid rapidly reduced both CBF and %AA (Fig. 1D). Interestingly, ciliostasis was transient with %AA, being fully recovered by 90 min. CBF began to recover by 50 min but did not reach baseline levels by 120 min. Exposure of well-differentiated hBE cells to 10 puffs of vaped Sinicide aerosol produced an inhibition CBF and %AA similar to what was observed with apical e-liquid exposures (Fig. 1E). Both the Sinicide e-liquid and vaped Sinicide aerosol exposures resulted in a significant reduction in CBF and %AA as compared with the PG/VG vehicle (Fig. 2). The mean deposition of aerosol from 10 puffs was determined to be 828.7 ± 78.5 µg/well. A recent study by Behar et al. (6) reports that cinnamaldehyde is efficiently transferred from unheated e-liquid to e-cigarette aerosol when vaping. Based on these data, we estimate that 10 puffs (828.7 µg of deposited aerosol) delivers 123.8 µg/well cinnamaldehyde, which is the

same amount of cinnamaldehyde that would be delivered in 150 µl of a 6.25 mM solution applied apically.

Temporal Analysis of CBF and %AA Following Exposure to Cinnamaldehyde and Nicotine

Previously published work from our group quantified the cinnamaldehyde content of the e-liquids used for this study: "Kola," 0.896 ± 0.360 mM; "Hot Cinnamon Candies," 39.32 ± 3.41 mM; and "Sinicide," 1,131 ± 19.9 mM (14). Our finding that exposure of hBE cells to a 1% dilution of the Sinicide e-liquid (11.31 mM cinnamaldehyde content based on quantification of neat Sinicide e-liquid) resulted in rapid but transient ciliostasis led us to investigate whether cinnamaldehyde alone, at concentrations approximate to those in the 1% Sinicide e-liquid, would produce a similar response.
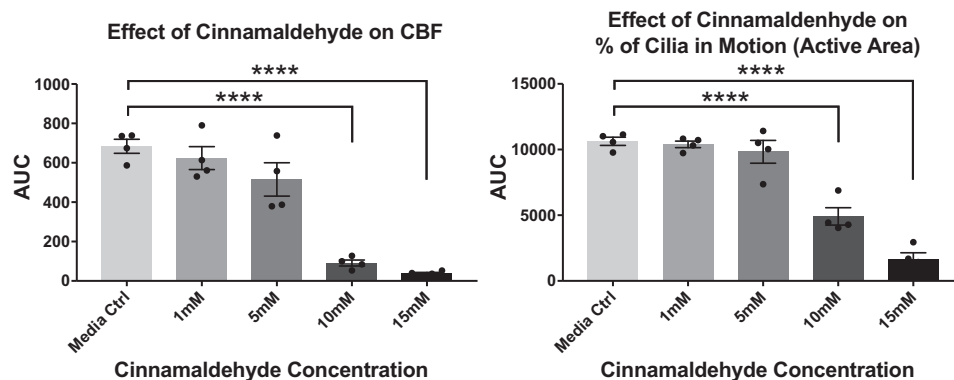
Apical exposures to 1, 5, 10, and 15 mM cinnamaldehyde produced dose-dependent reductions in both CBF and %AA (Fig. 3), with 10 and 15 mM exposures significantly reducing CBF and %AA compared with vehicle control [Fig. 4 and Supplemental Video S1 (DOI: 10.17632/nr6cm9bhvh.1); Supplemental Material for this article is available on the *AJP-Lung Cellular and Molecular Physiology* website]. Similar to the effects of Sinicide on cilia motility, 10 and 15 mM cinnamaldehyde caused a rapid reduction in cilia function that recovered over time. The time to recover was also dose dependent, with the higher-exposure doses requiring more time for recovery. As observed with the Sinicide exposures, CBF recovery after 10 and 15 mM cinnamaldehyde exposures did not recover to the baseline frequency by 120 min. However, normal ciliary beat was apparent 24 h after exposure (data not shown).
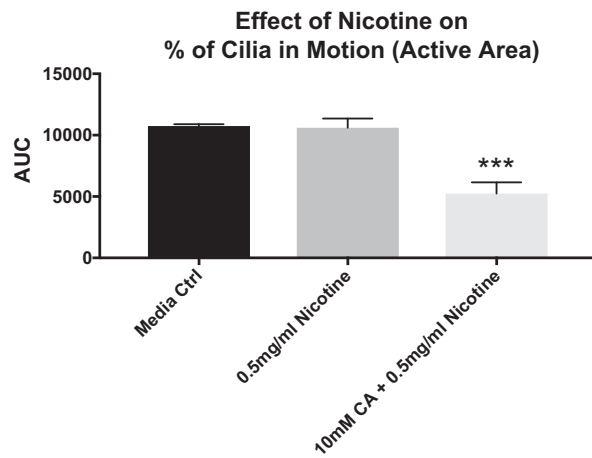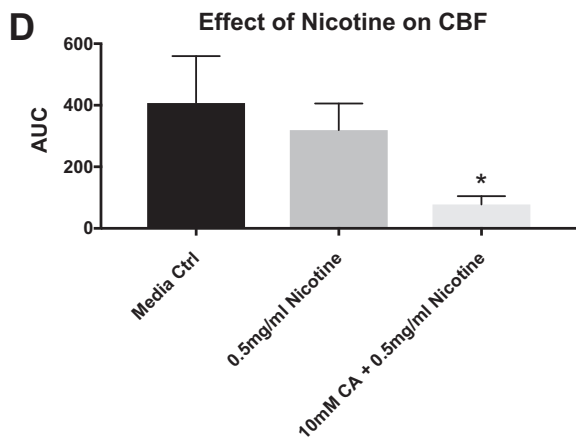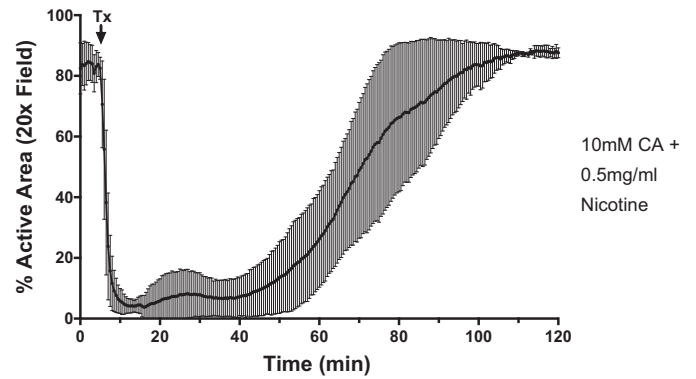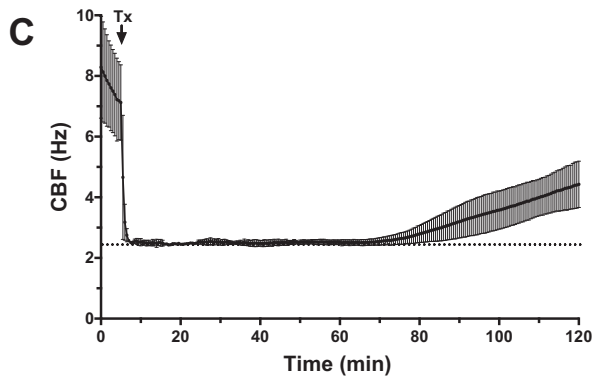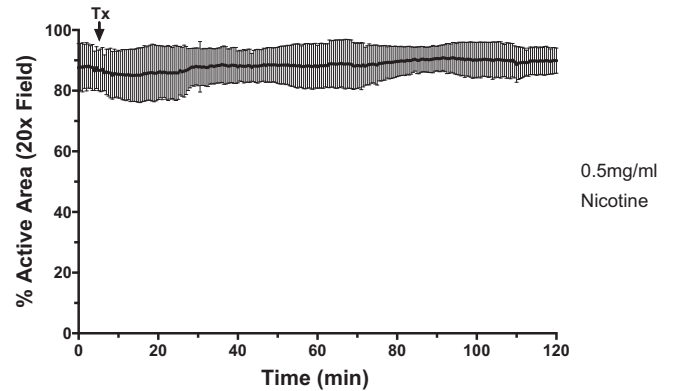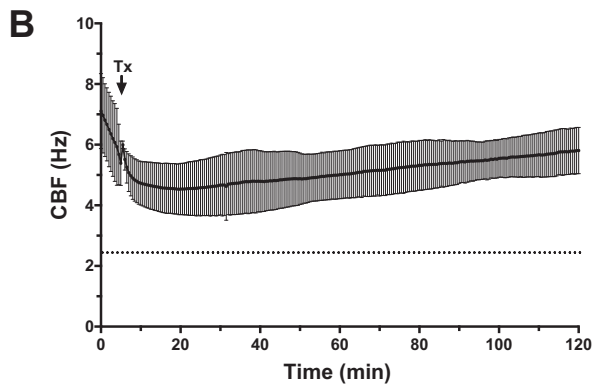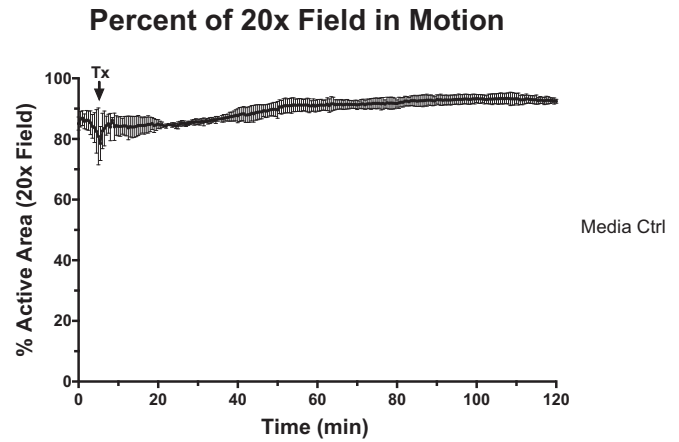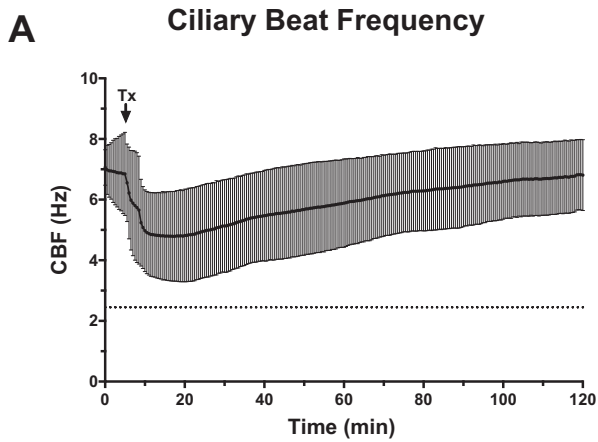
To assess whether nicotine significantly modifies ciliary beat on differentiated hBE cells, we exposed cells to either nicotine alone (0.5 mg/ml) or a mixture of 0.5 mg/ml nicotine and 10 mM cinnamaldehyde. Apical exposure of nicotine did not significantly alter either CBF or %AA (Fig. 5, A, B, and D). Moreover, the addition of nicotine to cinnamaldehyde did not alter cinnamaldehyde-induced ciliostasis (Fig. 5, C and D).

Cinnamaldehyde Disrupts Mitochondrial Membrane Potential in Beas-2B Bronchial Epithelial Cells

Cinnamaldehyde has previously been reported to decrease mitochondrial membrane potential (36) and disrupt the normal transfer of protons, the driving force behind ATP synthesis (55). To investigate whether cinnamaldehyde impairs mitochondrial membrane potential in bronchial epithelial cells, we

Fig. 4. Quantification of cinnamaldehyde-induced effects on cilia motility. Area under the curve (AUC) was calculated for both ciliary beat frequency (CBF) and percent active area (%AA) data collected from each independent experiment ($n = 4$). Significant differences from the vehicle (cell culture medium) control were determined by 1-way ANOVA and Holm-Sidak multiple-comparisons test. Data presented as means ± SE. Significance represented as ****$P < 0.0001$; ●, individual data points derived from the different human bronchial epithelial cell cultures.

A

Ciliary Beat Frequency

Percent of 20x Field in Motion

Media Ctrl

B

0.5mg/ml
Nicotine

C

10mM CA +
0.5mg/ml
Nicotine

D

Effect of Nicotine on CBF

**Effect of Nicotine on
% of Cilia in Motion (Active Area)**

labeled Beas-2B cells with JC-1 dye, exposed cells to cinnamaldehyde, and measured the fluorescence shift from red to green, which is indicative of mitochondrial membrane depolarization. As shown in Fig. 6, *A* and *B*, cinnamaldehyde elicited a concentration-dependent reduction in mitochondrial membrane permeability. Exposures to 1.0 mM and 5.0 mM cinnamaldehyde resulted in a significant loss of membrane permeability as compared with cells exposed to media alone.

Effects of Cinnamaldehyde on Beas-2B Bronchial Epithelial Cell Mitochondrial Respiration and Glycolysis

Motile cilia require ATP to meet the energy demands of ciliary proteins, including the molecular motors dynein and kinesin. Thus, reductions in intracellular ATP levels can result in reduced ciliary motility (49). To determine whether cinnamaldehyde alters ATP production in bronchial epithelial cells, we used a Seahorse XFe24 Analyzer to assess bioenergetic functions following exposure to various concentrations of cinnamaldehyde.

Exposure of Beas-2B cells to various concentrations of cinnamaldehyde resulted in a dose-dependent reduction in mitochondrial oxygen consumption (Fig. 7, *A* and *B*). Exposures of 0.25, 0.5, and 5.0 mM cinnamaldehyde significantly reduced ATP production, reserve capacity, proton leak, and maximal respiration (Fig. 7, *C*, *D*, *E*, and *F*, respectively). Interestingly, exposures of 0.25, 0.5, and 5.0 mM cinnamaldehyde also significantly reduced the extracellular acidification rate (ECAR; Fig. 8, *A* and *B*), indicating an effect on glycolytic function. Stimulation of glycolysis by addition of oligomycin revealed that 0.5 and 5.0 mM exposures of cinnamaldehyde significantly reduced glycolytic capacity (Fig. 8*C*). Taken together, these data indicate that cinnamaldehyde exposure causes a dose-dependent reduction in both mitochondrial respiration and glycolytic function.

Cinnamaldehyde Reduces Mitochondrial Membrane Potential in Differentiated Primary hBE Cells

To investigate whether cinnamaldehyde disrupts mitochondrial membrane potential in differentiated primary hBE cell cultures, we labeled differentiated hBE cells with JC-1 dye and quantified the fluorescence shift from red to green following exposure to cinnamaldehyde. Both 10 and 15 mM cinnamaldehyde exposures produced a significant decrease in the J-aggregate (red) to JC-1 monomer (green) ratio after 15 min, indicative of depolarization of the mitochondrial membrane (Fig. 9, *A* and *B*), which was restored after 120 min. The apical addition of nicotine alone (0.5 and 5.0 mg/ml) had no effect on mitochondrial membrane potential at 15 or 120 min postexposure (Fig. 9, *C* and *D*).

Reduction of Intracellular ATP Levels in Differentiated Primary hBE Cells Exposed to Cinnamaldehyde

The above observations suggest that cells experience a concomitant reduction in intracellular ATP levels following exposure to cinnamaldehyde. To confirm cinnamaldehyde-induced reductions in intracellular ATP levels, well-differentiated hBE cells were exposed to 10 mM cinnamaldehyde for various times, and adenosine nucleotide (ATP, ADP, and AMP) levels were quantified. Apical addition of 10 mM cinnamaldehyde significantly reduced hBE cell ATP levels after 15 min (Fig. 10*A*). One-hundred twenty-minute and 24-h exposures did not significantly alter ATP levels compared with the untreated controls, indicating that ATP levels were restored by the later time points, although the cinnamaldehyde challenge is not removed. Notably, the rapid reduction of ATP levels after 15-min cinnamaldehyde exposure correlated with increased ADP and AMP levels, providing evidence that the reduction in ATP was due to hydrolysis by cellular activity and not exocytotic release or leakage at the cell membrane. Adenosine (Ado) was not detected in the extracted samples (Fig. 10, *B* and *C*).

Inhibition of Cellular Bioenergetic Activity and Temporal Analysis of hBE Cell Cilia Motility

Various aldehydes are reported to suppress airway ciliary motility and impede mucociliary transport, including formaldehyde, acetaldehyde, acrolein, methacrolein, and crotonaldehyde (15, 28, 32, 47, 52, 58). Many of these aldehydes have been identified as mitochondrial toxins and inhibitors of glycolytic activity (44, 46, 53, 56). However, with the exception of some bacterial studies, there are very few reports that link toxin-induced impairments of bioenergetic pathways with altered ciliary motility (7, 25). To better understand how targeted inhibition of glycolysis and mitochondrial respiration effects human airway cilia motility, we exposed well-differentiated hBE cells to the glycolytic pathway inhibitor 2-deoxy-D-glucose, the mitochondrial complex III inhibitor antimycin A, and a combination of both to assess changes in CBF and %AA. Interestingly, hBE cells exposed to 2-deoxy-D-glucose experienced a significant increase in CBF but not %AA as compared with baseline levels (Fig. 11*A*). Repletion of glucose at 30 min further increased CBF without affecting %AA. Exposures to antimycin A caused a significant reduction in both CBF and %AA that was partially restored when the antimycin A-containing medium was replaced with recovery medium (Fig. 11*B*). Cells treated with glucose-free medium containing both 2-deoxy- D-glucose and antimycin A experienced a significant and rapid reduction in CBF and %AA with complete ciliostasis occurring 30 min postexposure (Fig. 11*C*). Replacing the inhibitors with recovery medium restored CBF to baseline

Fig. 5. Ciliary beat frequency (CBF) in response to nicotine alone and cinnamaldehyde (CA) containing nicotine. Following 5-min baseline measurements, differentiated primary human bronchial epithelial (hBE) cells were exposed to vehicle (cell culture medium) control (*A*), 0.5 mg/ml nicotine (*B*), or a mixture of 10 mM cinnamaldehyde and 0.5 mg/ml nicotine (*C*). CBF (*left*) and percent of the ×20 field of view in motion [percent active area (%AA); *right*] were recorded for 120 min (*n* = 4). Data presented as means< SE. *D*: area under the curve (AUC) was calculated for both CBF and %AA data collected from each independent experiment (*n* = 4). Significant differences from the vehicle (cell culture medium) control were determined by 1-way ANOVA and Holm-Sidak multiple comparisons test. Data presented as means< SE. Significance represented as *P< 0.05 and ***P< 0.001.
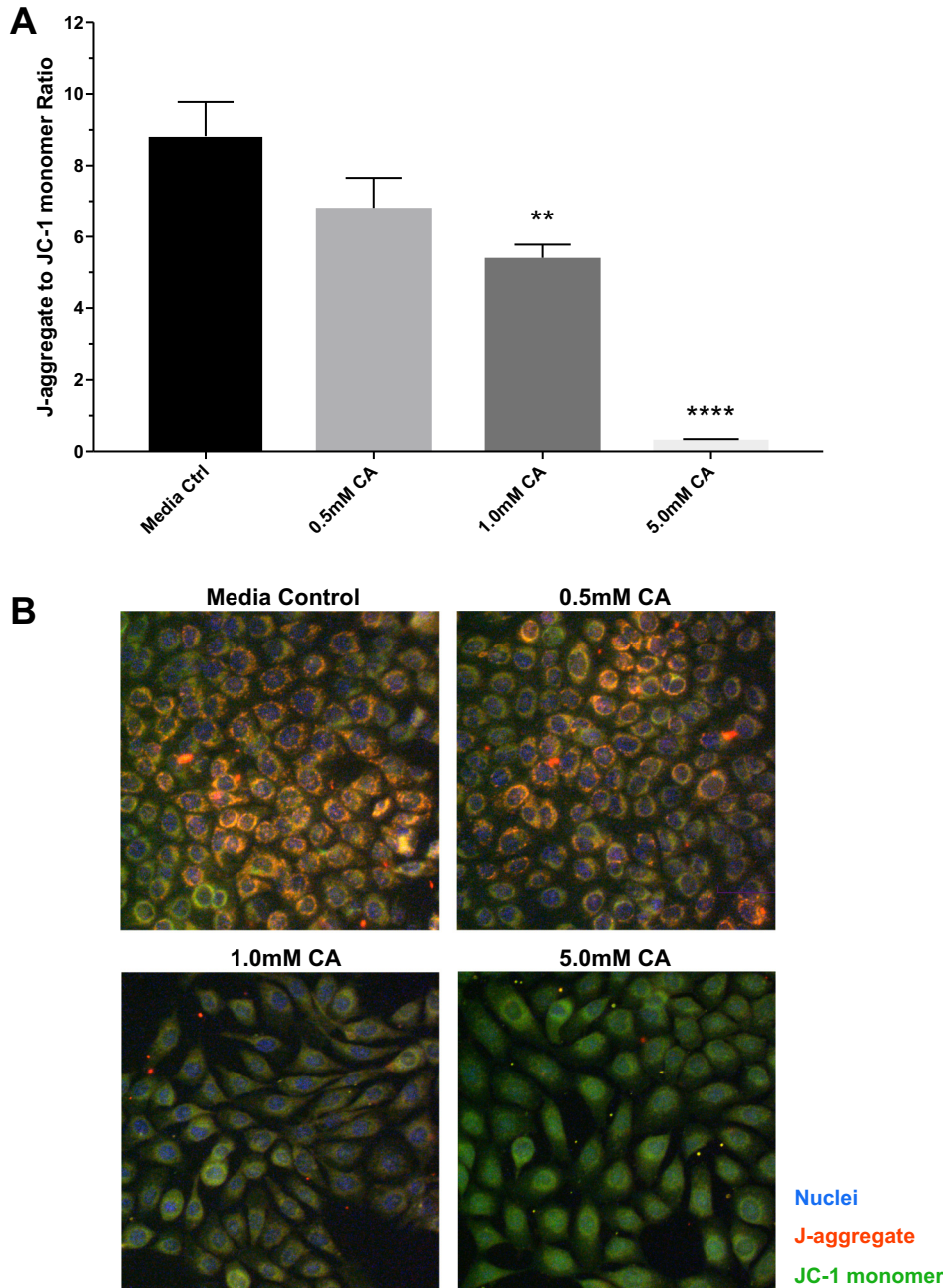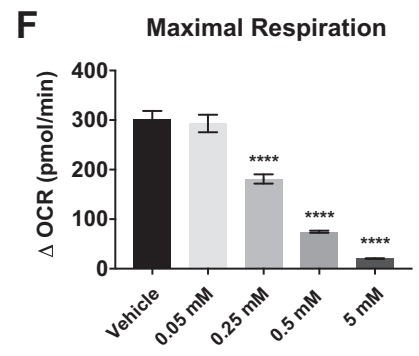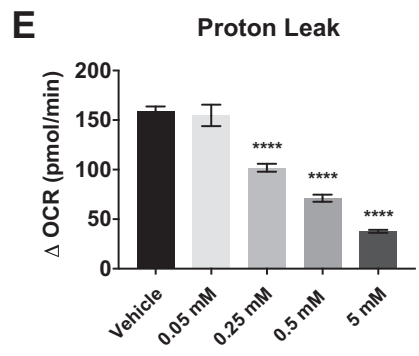
A

B

Media Control | 0.5mM CA

1.0mM CA | 5.0mM CA

Nuclei
J-aggregate
JC-1 monomer

Fig. 6. Cinnamaldehyde (CA) dysregulates mitochondrial membrane potential in bronchial epithelial cells. *A*: Beas-2B cells (SV40 virus-transformed bronchial epithelial cell line) labeled with 2.0 μg/ml JC-1 were exposed to vehicle (cell culture medium) and 0.5, 1.0, and 5.0 mM CA for 15 min. Mitochondrial depolarization was determined by a reduction in the J-aggregate (red) to JC-1 monomer (green) ratio using a fluorescence plate reader ($n = 4$). *B*: CA-induced changes in the fluorescence signals were validated using microscopy (representative images shown). Significant differences from the vehicle control were determined by 1-way ANOVA an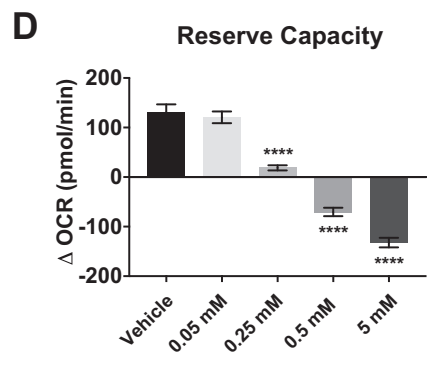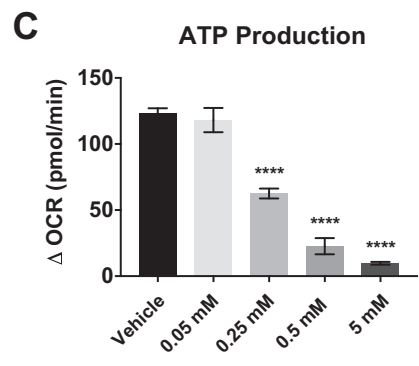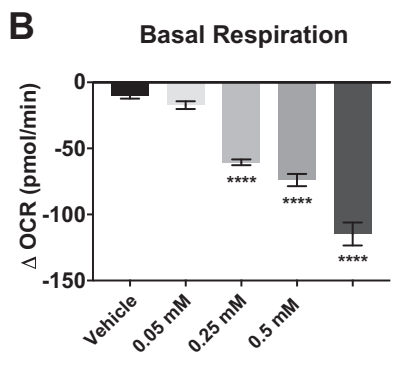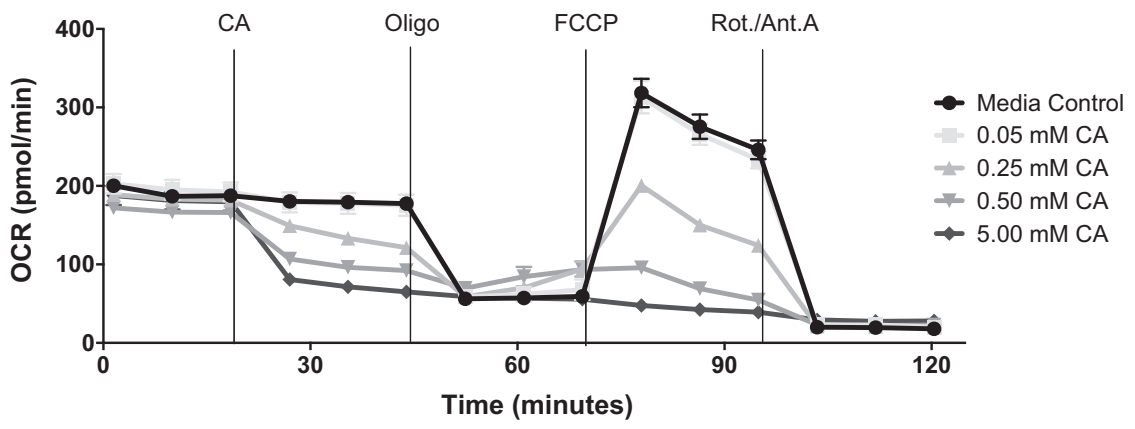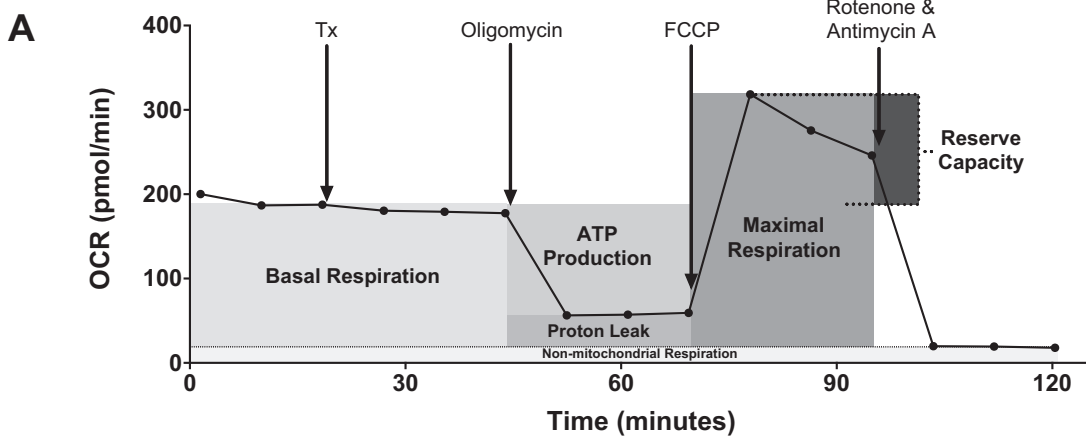d Dunnett's multiple-comparisons test. Data presented as means ± SE. Significance represented as **$P < 0.01$ and ****$P < 0.0001$.

levels by 60 min; however, %AA did not fully recover by 120 min.

DISCUSSION

In the present study, primary cultures of hBE cells were collected from healthy nonsmoker donors, differentiated to a mucociliary phenotype, and used to evaluate temporal changes in ciliary motility subsequent to a single acute exposure to cinnamon-flavored e-liquid, vaped cinnamon e-liquid, or cinnamaldehyde flavoring. The data presented here demonstrate that cinnamaldehyde at concentrations relevant to e-cigarette exposures is capable of causing rapid ciliostasis and that nicotine does not affect these responses. Reductions in ciliary motility correlated with a robust but temporary reduction in intracellular ATP levels, and cinna-

maldehyde exposures of Beas-2B cells resulted in a dose-dependent reduction in mitochondrial function and glycolysis, which was not affected by nicotine. Although cinnamaldehyde suppressed bioenergetic activity, reduced ATP levels, and impaired cilia motility, exposures did not significantly reduce cell viability or have long-lasting effects of ciliary motility. Together, these data indicate that cinnamaldehyde, in the context of e-cigarette use, can temporarily dysregulate airway cilia motility, a critical component of effective mucociliary clearance and essential respiratory innate defense mechanism.

Cinnamaldehyde-induced loss of ciliary motility and subsequent impairments of mucociliary transport may lead to increased susceptibility to respiratory infections and negative health outcomes. This is demonstrated by the typical

A

Tx — Oligomycin — FCCP — Rotenone & Antimycin A

Basal Respiration — ATP Production — Proton Leak — Maximal Respiration — Reserve Capacity — Non-mitochondrial Respiration

CA — Oligo — FCCP — Rot./Ant.A

- Media Control
- 0.05 mM CA
- 0.25 mM CA
- 0.50 mM CA
- 5.00 mM CA

B Basal Respiration

C ATP Production

D Reserve Capacity

E Proton Leak

F Maximal Respiration

pathologies of respiratory diseases with ciliary dysfunction. For example, primary ciliary dyskinesia (PCD), an autosomal recessive disorder of motile cilia, causes abnormalities in ciliary dynein structure and function, which results in immotile cilia or impaired CBF (10). The clinical respiratory manifestations of PCD are directly linked to the failure of mucociliary transport and include nasal congestion, chronic sinusitis, recurrent respiratory tract infections, and bronchiectasis (54). In addition, cigarette smoke significantly reduces cilia length, and this phenomenon is more pronounced in COPD (33, 40). These structural defects impair mucociliary clearance and contribute to frequent respiratory infections, bacterial colonization of the lower respiratory tracts, chronic inflammation, oxidative stress, and severe tissue damage (2). Our finding that acute exposure to vaped, cinnamon-flavored e-liquid and cinnamaldehyde induces rapid stasis of airway epithelial cell cilia is cause for concern, as it demonstrates a potential risk for e-cigarette users inhaling cinnamaldehyde. Interestingly, previous reports have shown both stimulatory and inhibitory effects of nicotine on CBF. Hahn et al. (30) observed that nicotine transiently stimulated CBF in ferret tracheal epithelial explants. However, work from Salathe et al. (48) indicates that nicotine does not alter CBF or mobilize Ca^{2+} in sheep tracheal epithelial cells. In this study, we did not observe significant changes in CBF after the addition of nicotine, which is in agreement with the findings reported by Salathe et al. (48). Although further studies are needed to address e-cigarette aerosol deposition in the lungs to ultimately understand exposure doses associated with vaping, our findings add to the growing body of evidence that inhalational exposures of food-safe flavorings may have unintended and potentially harmful respiratory effects.

The sustained beating of airway cilia demands a tremendous amount of energy/ATP, which is supplied by mitochondria amassed at the base of each cilium (22). Inhibition of mitochondrial respiration disrupts ATP production, reduces the amount of available ATP for ciliary dynein, and, in turn, impairs ciliary motility (7). Reactive aldehydes present in cigarette smoke have been shown to impair mitochondrial function by various mechanisms (13, 53, 56). Additionally, aromatic aldehydes similar in structure to cinnamaldehyde have been reported to impair mitochondrial function. For example, anisaldehyde, benzaldehyde, and p-tolualdehyde, which have all been identified in e-liquids and e-cigarette aerosols, are reported to inhibit pyruvate/malate- and succinate-mediated mitochondrial respiration in isolated rat liver mitochondria at concentrations ranging from 0.5 to 1.0 mM (61). Hence, many of the aldehydes commonly used to flavor e-cigarettes have known significant effects on mitochondrial function. Cinnamaldehyde has also been reported to impair mitochondria isolated from rat liver by uncoupling the electron transport chain and signif-

icantly inhibiting NAD(P)H oxidase (complex I) activity (55). Our data show that exposing bronchial epithelial cells to various concentrations of cinnamaldehyde impairs mitochondrial membrane permeability and reduces bronchial epithelial cell mitochondrial respiration. Exposure of bronchial epithelial cells to nicotine alone did not significantly alter mitochondrial function. However, we did not investigate whether nicotine in combination with cinnamaldehyde has either a synergistic or antagonistic effect on cinnamaldehyde-mediated changes in mitochondrial function.

In contrast to the previous report describing cinnamaldehyde as an uncoupling agent, we did not observe increases in basal oxygen consumption following the addition of cinnamaldehyde, indicating that cinnamaldehyde did not uncouple the electron transport chain. Interestingly, addition of cinnamaldehyde rapidly reduced the rate of extracellular acidification, which is an indicator of cellular glycolytic function. The biochemical mechanisms mediating these effects are still unclear but likely involve modification of essential mitochondrial proteins. Acrolein, an electrophilic α,β-unsaturated aldehyde, is reported to readily react with thiolate groups on redox-active cysteine residues to form covalent additions (9, 43). However, covalent bonds between acrolein and cysteine are somewhat unstable at physiological pH and temperature, which results in a transition to Schiff base adducts with the ϵ-NH_2 group of lysine and free NH_2-terminal α-NH_2 groups (9). Cinnamaldehyde, also an α,β-unsaturated aldehyde, would likely generate covalent and Schiff base additions to reactive cysteine residues, similar to acrolein. Hence, cinnamaldehyde-induced modification of mitochondrial and glycolytic pathway proteins may be involved in the loss of bioenergetic functions and reduction of intracellular ATP.

ATP is the canonical energy carrier that powers cellular machinery, fuels metabolic reactions, and serves as a building block for nucleic acids. Depletion of ATP has widespread effects on many essential cellular systems. Molecular motor proteins, including dynein, kinesin, and myosin ATPases, convert chemical energy into mechanical motion by conferring a small conformational change on the globular motor domain of the protein (50). Reductions in intracellular ATP levels beyond the minimal requirements for normal activity dysregulate motor protein functions, including ciliary motility and intracellular transport (7, 35). Additionally, the alveolar epithelium relies on basolateral Na^+/K^+-ATPases to actively transport Na^+ out of epithelial cells and generate a transepithelial osmotic gradient that causes the movement of fluid out of the alveolar airspace (17). The active transport of Na^+ and K^+ across the cell membrane to maintaining an ionic gradient consumes ~40% of the ATP in mammalian cells (42). Studies on the respiratory effects of hypoxia have shown that hypoxia-induced ATP depletion inhibits Na^+/K^+-ATPase function by downregulation of active Na^+ pumps at the membrane, which impairs fluid clearance from the alveolar space but conserves

Fig. 7. Cinnamaldehyde (CA) induces a concentration-dependent reduction in bronchial epithelial cell mitochondrial respiration. A: Beas-2B cells (SV40 virus-transformed bronchial epithelial cell line) were exposed to 0.05, 0.25, 0.5, and 5.0 mM concentrations of CA, and changes in mitochondrial oxygen consumption rate (OCR) were evaluated using a Seahorse bioanalyzer ($n = 4$). B–F: exposures of 0.25, 0.5, and 5 mM CA significantly suppressed basal OCR (B), ATP production (C), reserve capacity (D), proton leak (E), and maximal respiration (F) in a concentration-dependent manner. Significant differences from the vehicle control were determined by 1-way ANOVA and Dunnett's multiple comparisons test. Data presented as means $\pm$ SE. Significance represented as ****$P \le 0.0001$.
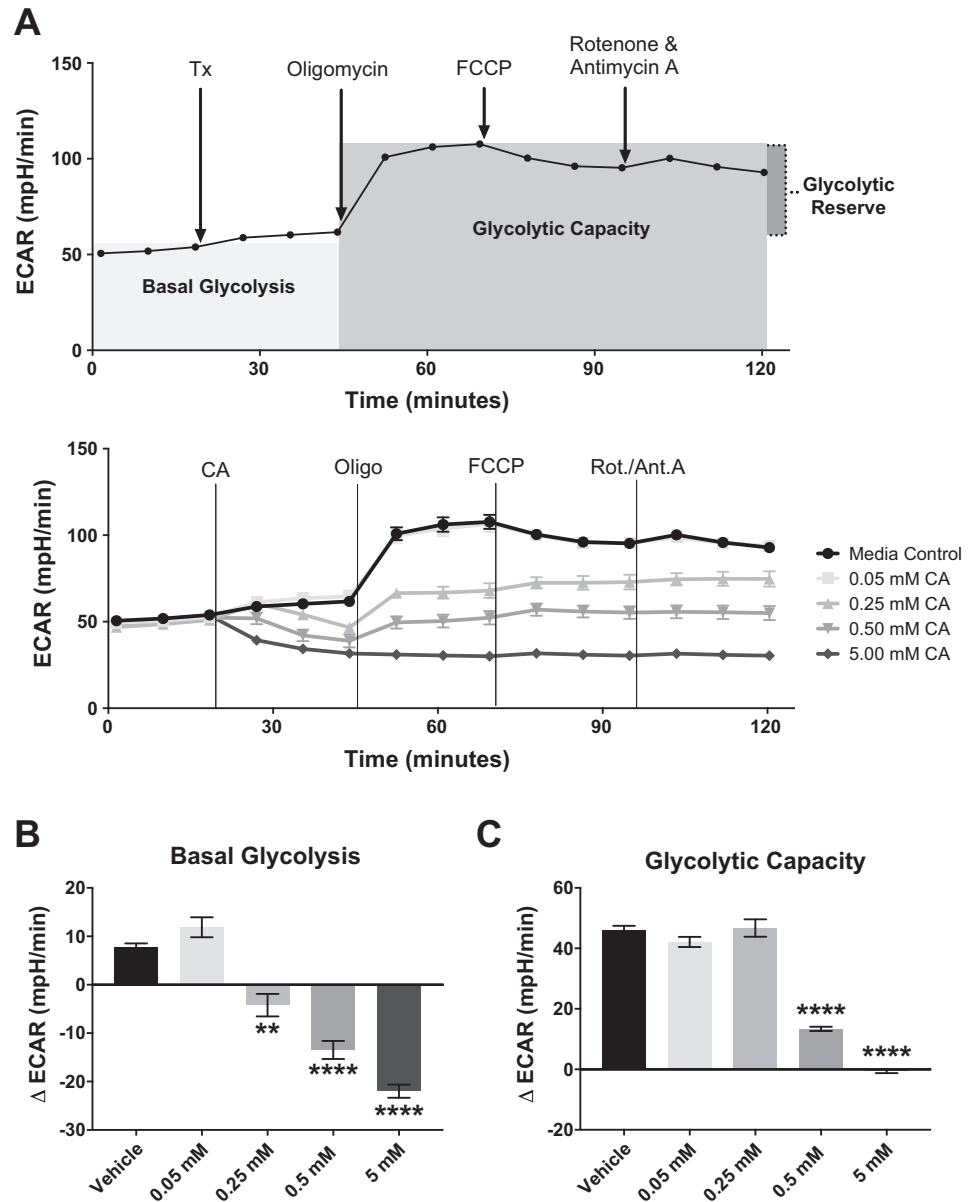
Fig. 8. Cinnamaldehyde (CA) reduces bronchial epithelial cell glycolytic function. *A*: Beas-2B cells (SV40 virus-transformed bronchial epithelial cell line) were exposed to 0.05, 0.25, 0.5, and 5.0 mM concentrations of CA, and the rate of extracellular acidification (ECAR) was measured using a Seahorse bioanalyzer ($n = 4$). *B* and *C*: exposures of 0.25, 0.5, and 5 mM CA significantly suppressed basal ECAR (*B*), whereas 0.5 and 5 mM CA significantly reduced Beas-2B cell glycolytic capacity (*C*). Significant differences from the vehicle control were determined by 1-way ANOVA and Dunnett's multiple-comparisons test. Data presented as means ± SE. Significance represented as **$P < 0.01$ and ****$P < 0.0001$. Tx, treatment/challenge; Rot/Ant A, rotenone/antimycin A.

ATP levels (29). Recent work has shown that alveolar epithelial/endothelial barrier function, which is tightly regulated by intracellular ATP levels (12), was disrupted by exposure to cinnamon-flavored e-cigarette liquid (26). Hence, these data suggest that cinnamaldehyde-induced reductions in ATP could have significant impact on multiple functions of alveolar epithelial cells.

In addition to being an essential energy source, ATP is also released by airway epithelial cells, where it and its metabolite adenosine serve as secreted signaling molecules that regulate multiple cellular processes via autocrine/paracrine activation of purinergic receptors (38). These processes include stimulation of CFTR-dependent and CFTR-independent (CaCC-mediated) chloride channel activity, regulation of Na^+ absorption, regulation of mucin secretion, and regulation of CBF (38). More recently, ATP-mediated activation of purinergic receptors and subsequent activation of Duox1 has been shown to play an important role in airway epithelial cell migration and repair

following injury (59). Although there is very little data on the effect of depletion of intracellular ATP on purinergic receptor-dependent responses, it is plausible that cinnamaldehyde-induced ATP depletion would disrupt purinergic receptor-mediated functions.

Much of the current e-cigarette research has focused on identifying and quantifying known toxic aldehydes generated by e-cigarettes. However, fewer studies have investigated whether common aldehyde flavoring agents may also be toxic to respiratory tissues. Bahl et al. (1) evaluated the effects of 29 e-liquids on human embryonic stem cells (hESCs), mouse neural stem cells (mNSEs), and human pulmonary fibroblasts (hPFs) and observed that a cinnamon-flavored e-liquid exhibited the most cytotoxicity across all cell types. Follow-up studies by Behar and colleagues (4, 6) attributed the cytotoxic effect to cinnamaldehyde and 2-methoxycinnamaldehyde. Moreover, subcytotoxic cinnamaldehyde exposure resulted in depolymerization of microtubules, reduced proliferation, im-

A 10mM Cinnamaldehyde

B 15mM Cinnamaldehyde
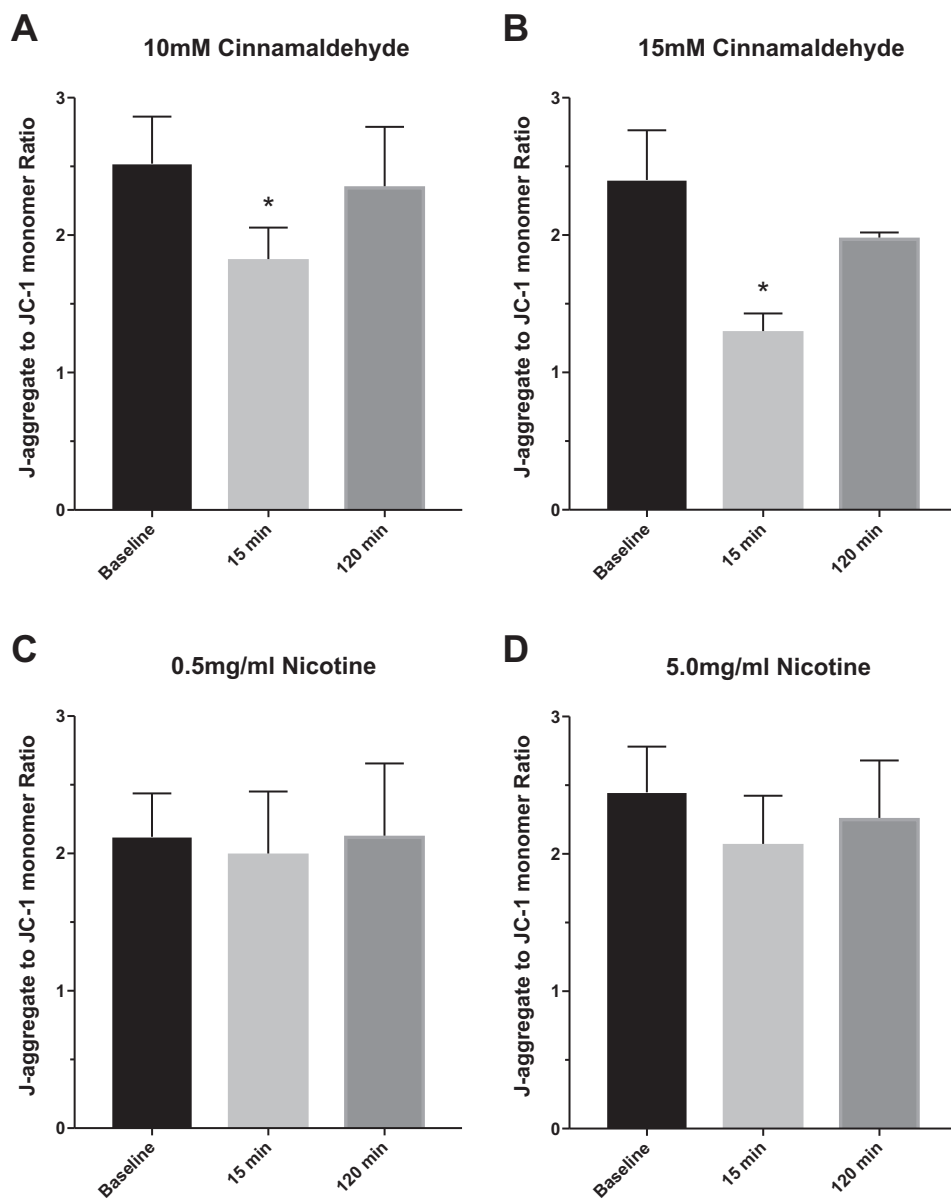
C 0.5mg/ml Nicotine

D 5.0mg/ml Nicotine

Fig. 9. Effects of cinnamaldehyde and nicotine on mitochondrial membrane potential in differentiated primary human bronchial epithelial (Hbe) cells. Differentiated hBE cells labeled with 2.0 µg/ml JC-1 were exposed to 10.0 mM cinnamaldehyde (*A*), 15 mM cinnamaldehyde (*B*), 0.5 mg/ml nicotine (*C*), or 5.0 mg/ml nicotine (*D*) for 120 min. Mitochondrial depolarization was determined at baseline, 15 min postexposure, and 120 min postexposure by a reduction in the J-aggregate (red) to JC-1 monomer (green) ratio using a fluorescence plate reader ($n = 4$). Significant differences from the vehicle control were determined by 1-way ANOVA and Dunnett's multiple comparisons test. Data presented as means ± SE. Significance represented as *$P < 0.05$.

paired cell migration, and increased DNA double-strand breaks (5). Gerloff et al. (24) recently reported that cinnamaldehyde impairs epithelial barrier function in 16-HBE human bronchial epithelial cells. Additionally, they observed that ortho-vanillin, but not cinnamaldehyde, stimulated IL-8 release by Beas-2B bronchial epithelial cells and HLF-1 human lung fibroblasts. However, earlier work from Lerner et al. reported (41) that exposure of HFL-1 cells to diluted cinnamon-flavored e-liquid produced a strong IL-8 response that was greater than exposure to cigarette smoke extract. We have previously shown that three cinnamaldehyde-containing e-liquids impaired human neutrophil and alveolar macrophage phagocytosis, altered PMA-induced neutrophil extracellular trap (NET) formation, and suppressed the ability of natural killer (NK) cells to eliminate leukemia target cells (14). Exposures of these cells types to cinnamaldehyde alone recapitulated the effects of the cinnamon-e-liquids, indicating that the altered immune cell function directly resulted from the bioactivity of this aldehyde

flavoring agent. These studies strongly indicate that inhalation of cinnamaldehyde will likely induce adverse health effects at several levels.

The data presented here clearly demonstrate the capability of cinnamaldehyde to alter normal bronchial epithelial cell function by suppressing airway cilia motility, which is an essential respiratory mucosal defense mechanism. The cellular responses we observed following cinnamaldehyde exposure are similar to those described in previous studies using toxic aliphatic aldehydes found in cigarette smoke. This raises concern for inhalational exposures to aromatic aldehyde flavoring agents, such as cinnamaldehyde, in the context of vaping. Recent evidence suggests that aldehydic flavorings such as cinnamaldehyde can react with base compounds of e-liquids such as propylene glycol, forming acetals with modified or enhanced biological reactivity (16). Thus, reactions of chemical flavorings with other e-liquid compounds will likely generate secondary and tertiary compounds with unknown biolog-
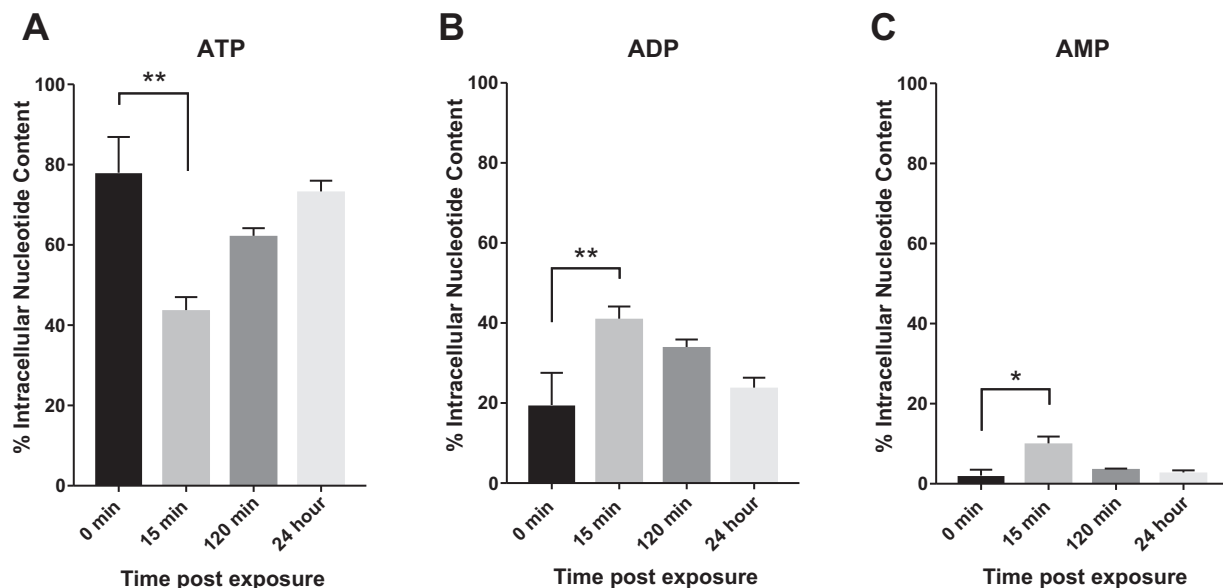
Fig. 10. Exposure of well-differentiated human bronchial epithelial (hBE) cells to cinnamaldehyde causes a rapid but temporary reduction in intracellular ATP levels. Well-differentiated hBE cells were exposed to 10 mM cinnamaldehyde for 15 min, 120 min, and 24 h, and the effect on intracellular adenosine nucleotides was quantified by high-performance liquid chromatography ($n = 4$). Exposure caused a significant reduction in ATP levels after 15 min (*A*) that correlated with increased levels of ADP (*B*) and AMP (*C*). Significant differences from the vehicle control were determined by 1-way ANOVA and Dunnett's multiple-comparisons test. Data presented as means ± SE. Significance represented as *$P < 0.05$ and **$P < 0.01$.

ical activity and toxicity. Based on our findings, we conclude that cinnamaldehyde, at sufficient exposure concentrations, disrupts bronchial epithelial cell mitochondrial respiration and glycolytic function, reduces intracellular ATP levels, and impairs cilia motility. Further work is necessary to determine how inhalation of cinnamaldehyde aerosols generated by e-cigarettes recapitulates our in vitro findings in e-cigarette users in vivo and whether exposures could lead to increased susceptibility to respiratory infections and enhanced risk of obstructive lung disease. Ultimately, understanding the biological effects of exposures to flavoring agents and concentrations at which these effects occur will be an essential component to evaluating the safety of e-cigarettes.
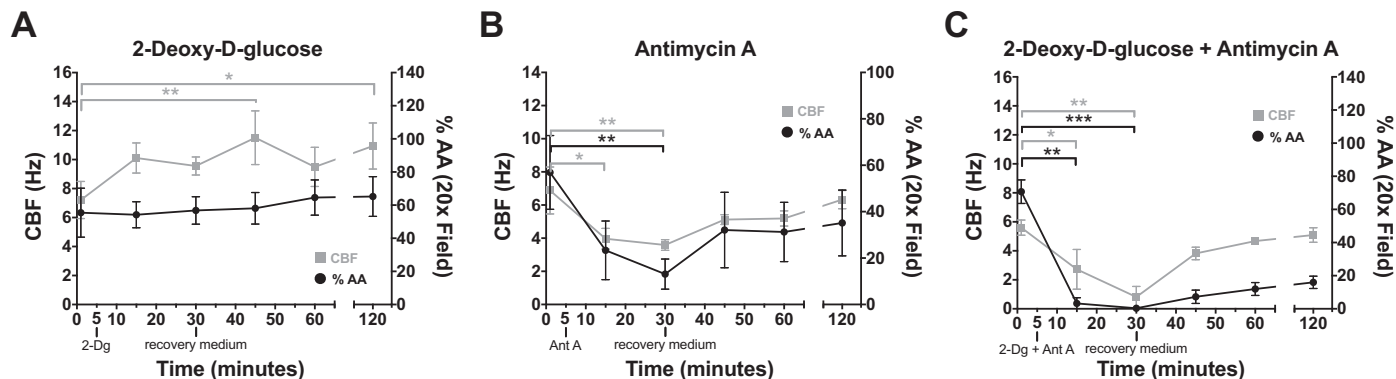
Fig. 11. Chemical inhibition of well-differentiated human bronchial epithelial (hBE) cell glycolytic function and mitochondrial respiration significantly alters cilia motility. *A* and *B*: inhibition of glycolytic function with 2-deoxy-D-glucose (2-DG) significantly increased ciliary beat frequency (CBF; *A*), whereas inhibition of mitochondrial respiration with antimycin A caused a significant reduction in CBF and percent active areas (%AA) (*B*). *C*: addition of both chemical inhibitors produced a greater reduction in CBF and %AA. However, ciliary motility was restored by removing inhibitors and supplementing cultures with fresh growth medium ($n = 4$). Significant differences from baseline CBF and %AA were determined by 1-way ANOVA and Holm-Sidak multiple-comparisons test. Data presented as means ± SE. Significance represented as *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

sibility of the authors and does not necessarily represent the official views of the NIH or the FDA.

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