Electronic cigarette vapor with nicotine causes airway mucociliary dysfunction preferentially via TRPA1 receptors

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**AT A GLANCE COMMENTARY:**

**Scientific Knowledge on the Subject**

E-cigarettes are marketed as safer alternatives to conventional cigarettes due to their defined composition and noncombustible nature. However, it is unclear how exposure to e-cigarette vapor, colloquially referred to as “vape”, affects naïve airway epithelia. It is largely unknown to what extent individual constituents of vape, such as nicotine and flavoring agents, influence pulmonary function, if at all. The transient receptor potential ankyrin 1 (TRPA1) is a molecular target for vape effects due to its expression in airway epithelia and its reported gating by nicotine, reactive oxidants, and flavors, especially cinnamaldehyde.
What This Study Adds to the Field

This study implicates nicotine as a key “vape” constituent that acutely impairs airway mucociliary functions in vitro and in vivo (sheep). A functional, nicotine-sensitive TRPA1 receptor is natively expressed in human and sheep bronchial epithelial cells and mediates the effects of nicotine and e-cigarette vapors. Importantly, its inhibition prevents mucociliary dysfunction in vitro and in vivo. These findings implicate TRPA1 as a driver of mucociliary dysfunction induced by nicotine-containing e-cigarette vapor.

This article has an online data supplement, which is accessible from this issue’s table of content online at www.atsjournals.org
ABSTRACT

RATIONALE: Electronic cigarette (e-cig) use has been widely adopted under the perception of safety. However, possibly adverse effects of e-cig vapor in never-smokers are not well understood.

OBJECTIVES: Effects of nicotine-containing e-cig vapors on airway mucociliary function were tested in differentiated human bronchial epithelial cells (HBECs) isolated from never-smokers and in the airways of a novel, ovine large animal model.

METHODS: Mucociliary parameters were measured in HBECs and in sheep. Systemic nicotine delivery to sheep was quantified using plasma cotinine levels, measured by ELISA.

MEASUREMENTS AND MAIN RESULTS: In vitro, exposure to e-cig vapor reduced airway surface liquid hydration and increased mucus viscosity of HBECs in a nicotine-dependent manner. Acute nicotine exposure increased intracellular calcium levels, an effect primarily dependent on transient receptor potential ankyrin 1 (TRPA1). TRPA1 inhibition with A967079 restored nicotine-mediated impairment of mucociliary parameters including mucus transport in vitro. Sheep tracheal mucus velocity (TMV), an in vivo measure of mucociliary clearance, was also reduced by e-cig vapor. Nebulized e-cig liquid containing nicotine also reduced TMV in a dose-dependent manner and elevated plasma cotinine levels. Importantly, nebulized A967079 reversed the effects of e-cig liquid on sheep TMV.

CONCLUSIONS: Our findings show that inhalation of e-cig vapor causes airway mucociliary dysfunction in vitro and in vivo. Furthermore, they suggest that the main
nicotine effect on mucociliary function is mediated by TRPA1 and not nicotinic acetylcholine receptors.

**ABSTRACT WORD COUNT:** 227

**MeSH KEY WORDS:** Electronic Nicotine Delivery Systems; mucus; TRPA1; Mucociliary Transport; Airway Epithelium
INTRODUCTION

Since their introduction, electronic cigarettes (e-cigs) have continuously evolved in design, in part to compensate for their relatively poor ability to deliver nicotine compared to tobacco cigarettes (1). E-cigs originally rose in popularity as a potentially ‘safe’ and ‘cleaner’ method for inhaling nicotine, leading to their consideration as a cessation tool (2). However, unsubstantiated safety claims and availability of countless palatable flavors have led to an upsurge in e-cig use amongst never-smoking adolescents (3) and young adults (4). Despite this emerging public health issue, especially the recent report of chronic bronchitis in young e-cig users (5), informative e-cig studies on pulmonary physiology remain sparse. Ongoing debates for public policy, such as whether e-cigs are helpful or harmful or the ethics of e-cig dissemination (6), require more scientifically based investigations.

Vapor generated from e-cig liquids, colloquially known as “vape”, consists of droplets, 5-500 nm in diameter [Gaussian distributed particle size; (7)] of i) e-cig liquid vehicle components propylene glycol (PG) and/or vegetable glycerin (VG), which contain chemical additives such as ii) nicotine and iii) various flavoring agents. Between factors such as wattage settings for the heating element, PG/VG composition, and a user’s experience with a device, a single session of inhaled vape can deliver nicotine doses that exceed those in smoke from 1 cigarette (8). Furthermore, recent efforts have partially demonstrated how PG/VG and various e-cig flavorings cause negative outcomes in vitro and in vivo such as cell death, airway inflammation, and impaired airway hydration (9-14).
Early nicotine-related pulmonary research focused on airway smooth muscle since nicotine was discovered to elicit extracellular calcium (Ca\(^{2+}\)) influx via nicotinic acetylcholine receptors (nAChRs). In the lung, nicotine induces both bronchoconstriction and bronchodilation in a dose-dependent manner. However, formative studies were often confounded by the effects of other cigarette smoke constituents (e.g. acrolein). Many e-cigs were designed for nicotine delivery and manufacturers have rapidly improved this function. However, it is currently unknown how inhaling e-cig vapor containing nicotine impacts functions of the ciliated airway epithelium in the bronchi, which is subjected to a large surface dose (15).

In a previous study, we found that e-cig vapor containing nicotine impairs mucociliary parameters such as airway surface liquid (ASL) volume, ciliary beat frequency, and transepithelial ion transport in differentiated primary HBECs (14). To address the mechanism of how e-cig vapor induces mucociliary dysfunction, we again utilized the primary HBEC culture system as well as a novel, large ovine animal model of e-cig vapor exposure. Sheep are particularly suited to probe effects of inhaled aerosols due to their airway responses to stimuli being similar to human beings, in asthmatic and cystic fibrosis models for example (16-18). Indeed, nicotine deposited by e-cig vapor impaired various parameters of airway mucociliary function in HBECs and in sheep. However, we found that nicotine preferentially stimulates transient receptor potential ankyrin 1 (TRPA1) channels. Importantly, TRPA1 inhibition prevented nicotine’s deleterious effects on mucociliary clearance \textit{in vitro} and in sheep airways \textit{in vivo}. Some of these results have been previously reported in abstracts (19, 20).
METHODS

Materials and methods used are described in greater detail in the online supplement.

Tissue Culture

Lungs were provided by organ procurement organizations with IRB approval. Primary human bronchial epithelial cells were isolated with informed consent from de-identified donor lungs of never-smoking individuals rejected for transplant; 18 males and 13 females, ages 26 ± 2.3 and 25.5 ± 3.7 years respectively (see Table E1 in online supplement). Donors did not suffer from any documented airway disease. The study was not powered to dissect gender differences. Primary HBECs were cultured and differentiated at the air-liquid interface (ALI) for 4 weeks as described (14). Viability of cultures was assessed by the presence of active ciliary beating before and after any measurements.

In vitro Aerosol and E-cig Exposures of HBECs

E-cig vapor was generated using a Joyetech eVic™ supreme e-cigarette coupled to a Joyetech Delta atomizer and connected to a Vitrocell® VC-1 smoking robot (14). E-cig exposure parameters were modified from ISO standards for cigarette-smoking machines (21) and described in the online supplement. Cultures were nebulized with defined solutions supplemented with NaCl using Vitrocell® CLOUD (14, 22). Final DMSO concentrations did not exceed 0.1%.

Airway Surface Liquid (ASL) Volume

Meniscus scanning of cultures was utilized to estimate ASL volume as described (23).
**In vitro Mucociliary Transport (MCT)**

HBECs were cultured on modified Transwell inserts to encourage MCT development for 6-8 weeks (24). Movement of fluorescent microbeads (ThermoFisher Scientific) was measured to estimate MCT speed (µm/s) using the *Manual Tracking* ImageJ plugin.

**mRNA and protein expression**

mRNA and protein expression were measured by qPCR and Western blotting, respectively, according to standard techniques.

**Calcium Imaging using GCaMP6s sensor**

HBEC cultures were infected in an undifferentiated state with lentiviruses to deliver pEF1-Puromycin-expressing GCaMP6s cDNA (25). This construct was designed using pGP-CMV-GCaMP6s (Addgene plasmid #40753) gifted by Dr. Douglas Kim (26). GCaMP6s-expressing cultures were perfused at room temperature with HEPES-buffered HBSS pH 7.4 at 250 µL min\(^{-1}\) (27). GCaMP6s emissions were recorded every 3 s using MetaFluor (Molecular Devices) and recorded data reported as relative calcium levels \(F_x/F_0\). Approximate Ca\(^{2+}\) sensitivity was confirmed with UTP perfusion (10 µM) after a 10 min recovery period. Data were analyzed using IGOR software (WaveMetrics).

**Fluorescence Recovery After Photobleaching (FRAP)**

Relative mucus viscosity was indirectly assessed by measuring diffusion rate of FITC-dextran using fluorescence recovery after photobleaching (FRAP). Greater FRAP half-life \(t_{1/2}\) correlates with an increased mucus viscosity. Cultures were layered overnight with FITC-dextran (70 kDa; Sigma-Aldrich). After treatments, emission from FITC-labeled mucus layer was recorded as previously described (28, 29). Photobleaching was done at
10\% of full laser power for 2 iterations (~500 ms). FRAP was recorded at least three times per filter and averaged. A one-phase association non-linear regression was then fit to estimate FRAP half-life.

**Mucus Concentration (percent solids)**

‘Initial weight’ of absorptive paper was measured before use. After placement on cultures, it was gently lifted and measured immediately for ‘wet weight’. ‘Dry weight’ was assessed after overnight exposure to 60°C. Mucus concentration in sheep tracheal secretions were measured using a fixed volume rather than absorptive paper. Percent mucus solids was calculated as previously described (30).

**Animal Study**

Conscious, adult female sheep (ewes) were nasally intubated (17). Male sheep (rams) were not used since they are naturally aggressive and are not amenable for experimentation without the use of general anesthesia. All procedures were approved by Mount Sinai Medical Center Animal Research Committee. Exposures are described in greater detail in the online supplement.

Tracheal Mucus Velocity (TMV) was measured by tracking radiopaque Teflon trioxide insufflated into the trachea using videotaped fluoroscopy (17). Sheep plasma cotinine was determined in plasma, diluted 10-fold in 1% BSA, 0.05% Tween-20 PBS, using Cotinine ELISA Kit (Abnova) following manufacturer’s protocol.

**Statistical Analyses**

Data are presented as mean ± SEM and were analyzed by PRISM software (GraphPad, San Diego, CA). Each sample (n) represents a single biological replicate.
Normal distribution of data was confirmed using the Shapiro-Wilk normality test. Data were considered significant if $p < 0.05$ for compared means with both parametric and non-parametric tests. Statistical differences were tested using paired t-tests, one-way, and two-way ANOVA with post-hoc tests as deemed appropriate for collected data. Additional information on statistical analyses are described in the figure legends.
RESULTS

Nicotine in e-cigarette vapor impairs parameters of airway mucociliary function in vitro

E-cig vapor containing 36 mg/mL nicotine (PG/VG + nicotine), delivered according to the methods in the supplement, deposits 12.5 ± 1.1 µM (n = 6) nicotine in our exposure system (see Fig. E1A in online supplement), which is about half the nicotine deposited in the ASL after smoke exposure from 1 cigarette (31). Cultures exposed to nicotine-containing e-cig vapor exhibited larger decreases in ASL (Fig. 1A,B) after 24 hrs. ASL volume after exposure to e-cig vapor containing no nicotine (PG/VG) recovered after 24 hrs like the air control. Cultures exposed to PG/VG + nicotine had greater FRAP t1/2 than either PG/VG or air exposed cultures, indicative of increased mucus viscosity (Fig. 1C). These outcomes revealed that airway hydration and mucus viscosity were impaired following nicotine deposition during e-cig vapor exposure.

To investigate whether the majority of the vape-induced mucociliary dysfunction could be attributed to the effects of nicotine, solutions were deposited onto the apical surface of cultures by a mesh nebulizer in the CLOUD exposure system (Fig. 1D). Mass deposition of a single dose of various nicotine tartrate salt solutions (100, 300, 600, or 900 µM) yielded 41.3±9.4, 79.4±14.4, 124.7±26.5, and 176.5±29.7 ng/cm² respectively (see supplemental Fig. E1). The lowest deposition (~40 ng/cm²) equates to approximately 1 µM nicotine in the apical surface liquid.

Nicotine deposition affects ASL volume in a dose-dependent manner reaching a plateau at concentrations ≥ 4 µM (see supplemental Fig. E1). Specifically, 4 µM deposited nicotine (by nebulizing 600 µM nicotine solution) lowered ASL volume more than saline...
control (0.1% NaCl in distilled water) at 4 and 24 hrs post-exposure (Fig. 1E). Nicotine also increased mucus viscosity (Fig. 1F). These data indicate nicotine as an active ingredient of e-cig vapor that impairs parameters of mucociliary function in ciliated airway epithelial cultures.

**TRPA1 is expressed in airway epithelial cells and functions as a nicotine receptor**

Nicotinic signaling in the airway is theoretically possible via several receptors expressed in various lung tissues (32, 33). TRPA1 is a Ca\(^{2+}\)-selective ion channel that was first reported to be nicotine-sensitive in chemosensory neurons (34). TRPA1’s relevance to airway epithelial cell responses to agonists is poorly understood, however, possibly because of its low abundance in these cells.

Consistent with previous studies (35, 36), we found that TRPA1 is expressed in primary HBECs (see supplemental Fig. E2). We therefore assessed TRPA1 function in cultures by probing effects of cinnamaldehyde, a TRPA1 agonist and common e-cig flavor, on intracellular calcium levels ([Ca\(^{2+}\)]\(_i\)) using the fluorescent Ca\(^{2+}\) sensor GCaMP6s (26). These experiments were done in the presence or absence of the TRPA1 antagonists A967079 (10 µM) and HC030031 (10 µM) at doses previously shown to selectively inhibit TRPA1 under submerged conditions (35-37). A967079 (10 µM) had no effect on activation of TRPV4, another TRP channel, in our cultures (see supplemental Fig. E3). Cinnamaldehyde perfusion rapidly increased [Ca\(^{2+}\)]\(_i\), a response significantly reduced by A967079 (Fig. 2A). This [Ca\(^{2+}\)]\(_i\) response occurred on the apical membrane of the cells (see Video E1 in online supplement). Nicotine perfusion (100 µM), approximate to the experimentally-determined EC\(_{50}\) in our cultures, also increased [Ca\(^{2+}\)]\(_i\) (Fig. 2B). This response was again significantly reduced by A967079 and HC030031, as well as
mecamylamine, a dual inhibitor for TRPA1 and nAChRs (34, 38). The nAChR-selective antagonist hexamethonium did not significantly reduce the nicotine-induced Ca\(^{2+}\) response, suggesting nicotine elicited Ca\(^{2+}\) responses preferentially via TRPA1. Experiments with the sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) inhibitor thapsigargin suggested that nicotine triggered an extracellular influx of Ca\(^{2+}\) in a TRPA1-dependent manner (see Fig. E4 in online supplement), consistent with apically-located increases in [Ca\(^{2+}\)]\(_i\) caused by purinergic stimulation (see Video E2 in online supplement). These data suggest that TRPA1 is a major contributor to nicotine-induced [Ca\(^{2+}\)] changes in airway epithelial cell cultures.

**Inhibiting TRPA1 prevents ASL volume loss and increased mucus density caused by nicotine-containing e-cig vapor**

Exposure of HBECs to a selective TRPA1 agonist (JT010; ~1 µM) mimicked the effects of nicotine on ASL volume (see supplemental Fig. E5). We therefore tested the ability of TRPA1 inhibition to prevent e-cig vapor- and nicotine-induced mucociliary dysfunction. Cultures were nebulized to apically deposit ~2 µM A967079 and then exposed to e-cig vapor-containing nicotine or nebulized nicotine. The significant decrease in ASL in cultures exposed to PG/VG + nicotine was prevented by nebulized A967079 (Fig. 3A). Cultures exposed to PG/VG + nicotine also exhibited mucus dehydration, measured by percent mucus solids (3.53±0.2%), which was close to the reported value in mucus samples of chronic bronchitis patients (30). A967079 reduced percent mucus solids under these conditions (2.78±0.4%; Fig. 3B), suggesting TRPA1 inhibition improved mucus hydration during nicotine exposure and predicting a significant improvement in mucociliary clearance (30). Indeed, A967079 protected against nicotine’s adverse effect
on mucus viscosity (Fig. 3C). Similar findings were observed when 10 μM A967079 was added to the basolateral media (see supplemental Fig. E5). Nebulized nicotine also reduced ASL at 4 and 24 hrs and A967079 partially prevented this decrease (Fig. 3D). Lastly, cultures infected with TRPA1 shRNA were protected from nicotine-induced ASL volume loss, providing further evidence that these effects are specific for TRPA1 (see supplemental Fig. E5).

Next, pairs of cultures from the same donor were differentiated at the ALI on Transwell inserts (Fig. 4A) modified to encourage development of directed mucociliary transport (MCT, Fig. 4B). Paired cultures with similar baseline MCT were chosen for experiments. Consistent with ΔASL and mucus viscosity, nicotine significantly reduced MCT 1 hr after nebulization (Fig. 4C). However, pre-exposure to nebulized A967079 prevented nicotine’s deleterious effect on MCT (Fig. 4D). Technical restrictions did not allow us to repeat these in vitro experiments with e-cig vapor. Nevertheless, these results suggest that TRPA1 inhibition prevents nicotine-induced mucociliary dysfunction in vitro.

**Nicotine in e-cigarette vapor impairs sheep tracheal mucociliary clearance in a dose-dependent manner and is prevented by TRPA1 inhibition**

To confirm the effects of e-cig vapor on mucociliary function in vivo, a large animal exposure model was developed using sheep. Ewes have been previously used by us to measure tracheal mucus velocity (TMV), a surrogate for mucociliary clearance (MCC), because of their airway similarities to human beings including the predictability of pharmacological testing for human airway diseases such as asthma and cystic fibrosis (16, 18). Average baseline sheep TMV was 10.2 ± 0.169 mm/min (n = 24).
First, vapor fluid (PG/VG) with different concentrations of nicotine was aerosolized into the airways in a controlled fashion. After recording baseline TMV, TMV values were recorded for a total of 12 hrs. PG/VG alone decreased TMV to approximately 80% of baseline TMV for about 6 hrs (Fig. 5A). Nicotine-containing (10 mg) e-cig liquid had a similar effect as PG/VG alone but 20 mg nicotine caused TMV to reduce to approximately 50%. To see whether repeated exposures caused similar reductions in TMV, aerosols were re-administered 6 hrs after the initial application. The repeated exposures showed similar reductions in TMV regarding decrease and duration (Fig. 5B). Plasma cotinine levels after 15 mg nicotine exposures were elevated to levels estimated in human beings after smoking 2 cigarettes (Fig. 5C). No obvious toxic nicotine effects were observed in the sheep. These data show that aerosolized e-cig liquid reduce TMV and that nicotine adds to the effect in a concentration-dependent manner. Next, sheep were directly exposed to e-cig vapor generated from PG/VG + nicotine (36 mg/mL; 40 inhalations). Sheep TMV was halved following exposure (Fig. 5D) despite a low but detectable level of plasma cotinine (Fig. 5E).

The ability of the TRPA1 inhibitor A967079 to prevent nicotine-dependent TMV decreases was tested next. First, TRPA1 expression was confirmed in freshly isolated ovine airway epithelial cells (see supplemental Fig. E2). Sheep were pretreated with nebulized A967079 (0, 5, 10, or 20 mg) or vehicle control (diluted ethanol) before exposure to 15 mg nicotine-containing e-liquid (in PG/VG). Inhalation of 0, 5, or 10 mg A967079 did not prevent the TMV decrease upon exposure to 15 mg nicotine (Fig. 6A). However, inhalation of 20 mg A967079 prevented most of the TMV reduction caused by
15 mg nicotine. Plasma cotinine levels were not affected by A967079 (Fig. 6B), confirming that nicotine uptake and metabolism were unaltered.

Furthermore, mucus hyper-concentration was found in collected sheep tracheal secretions following inhalation of aerosolized 15 mg nicotine-containing e-cig liquid, consistent with the decrease in TMV. In addition, sheep pretreated with aerosolized A967079 (20 mg; Fig. 6C) revealed a lower mucus concentration upon inhalation of 15 mg nicotine, consistent with the in vitro observations.
DISCUSSION

In this study, we investigated effects of e-cig vapor on airway mucociliary function. *In vitro*, ASL hydration and mucus viscosity were negatively impacted by nicotine-containing e-cig vapor. The TRPA1 agonists cinnamaldehyde and nicotine elicited Ca\(^{2+}\) responses in HBEC cultures but not in the presence of TRPA1 antagonists. The TRPA1 antagonist A967079 protected against both ASL volume loss and increased mucus viscosity during nicotine delivery by e-cig vapor. Sheep TMV was impaired with PG/VG but more with nicotine in a dose-dependent manner. This was again prevented by A967079 pre-treatment. Our working theory is that nicotine delivered by e-cig vapor mediates its effects mostly via TRPA1 to affect proper ASL volume and mucus viscosity, leading to a decrease in MCC (Fig. 7).

To test whether nicotine had effects independent of other e-cig vapor constituents, we utilized the Vitrocell® CLOUD exposure system to nebulize fixed nicotine doses onto the apical surface of ALI cultures (22, 39). Notably, the nicotine used differed between the e-cig vapor and nebulized exposures. Nicotine suspended in e-cig liquid is shifted towards the free-base (uncharged) form due to the basic pH as previously reported (40). E-cig liquids used in this study had pH ranging between 8.8 – 9.3. However, we measured that ~0.02 µL e-cig liquid is deposited into the ASL of cultures following vape exposures (not shown), suggesting that pH of the ASL itself is likely unaffected by vape deposition. Even so, ciliary beating is not significantly influenced by changes in pH between 7.5 and 10.5 in isolated human bronchial cells, suggesting a degree of buffering in the ASL (41). Nicotine tartrate salt, which was used for nebulized exposures, is negatively-charged and is more acidic in solution (pH < 7). However, it induces receptor-mediated Ca\(^{2+}\) influx in
our cultures (Fig. 2) and both nicotine in e-cig vapor and in salt form induced similar mucociliary dysfunction outcomes.

Nicotine itself reduced ASL hydration and MCT in vitro, mirroring our previous findings (14). In that previous study, e-cig vapor down-regulated apical ion transport through cystic fibrosis transmembrane conductance regulator (CFTR) and large conductance, Ca$^{2+}$-activated and voltage-dependent K$^+$ (BK) channels. Impaired CFTR-driven Cl$^-$ efflux has also been reported in rats chronically ingesting nicotine (42). Since dynamic ion transport by CFTR and BK channels help to maintain a ∼7-µm ASL height necessary for adequate ciliary beating (43), sustained down-regulation of apical ion flux can reduce ASL height and lead to impaired ciliary movement and reduced MCC (44).

Nicotine stimulation of TRPA1 channels caused an apical influx of Ca$^{2+}$. In the short-term, this would be expected to increase CFTR conductance and ciliary beating (27) as well as activate Ca$^{2+}$-sensitive BK channels. However, we previously observed reduced CFTR and BK channel conductance following e-cig vapor exposure. We speculate that sustained TRPA1 activation will disrupt short-term Ca$^{2+}$ signaling possibly by diminishing ER Ca$^{2+}$ stores. The initial [Ca$^{2+}$], elevation can also induce mucus secretion (45). This together with reduced ion transport will lead to ASL volume loss and MCC slowing.

Previous research on nicotine effects on the airway focused on the role of nAChR subunits (46). Most airway nAChR studies implicated a role for the α7 subunit since its expression is elevated in smokers (47, 48) and since it plays a role in smoking-related lung cancer pathogenesis (32, 49). α7-nAChR is a pentameric Ca$^{2+}$ channel. Its function is limited by desensitization (46). In contrast, TRPA1 is a tetrameric Ca$^{2+}$ channel that is activated by reactive electrophiles (e.g. cinnamaldehyde and acrolein), prostaglandins,
bradykinin, hypoxia and as more recently shown, also by nicotine (50). Sensory neurons in trachea of TRPA1 knockout mice had a significant reduction in calcitonin gene-related peptide release in response to nicotine-containing particulate matter (38). Consistent with this notion of nicotine activating TRPA1 channels, we observed that TRPA1 antagonists reduced nicotine-induced Ca\(^{2+}\) response to a larger extent than hexamethonium in airway epithelial cell cultures, even though only low TRPA1 expression was seen in airway epithelia (35). On the other hand, it has been shown that smoke exposure up-regulates TRPA1 expression in A549 cells (51), suggesting that airway tissue of chronic smokers or e-cig users might have greater TRPA1 expression. Although it is possible that our observations were confounded by off-target A967079 effects, A967079 is known to be weak (ED\(_{50}\) > 5 \(\mu\)M) or not active against various G-protein coupled receptors, enzymes, transporters, and ion channels, including other TRP channels (52). Indeed, we observed no effect of A967079 (10 \(\mu\)M) on TRPV4-induced calcium influx (see supplemental Fig. E3). Furthermore, concentrations of TRPA1 antagonists used in this study were consistent with other \textit{in vitro} studies (35-37).

To better validate our \textit{in vitro} findings, we adapted inhalation of e-cig vapor to a large animal model with sheep to measure whole animal TMV as a marker of MCC. As stated before, sheep are particularly suited to probe effects of inhaled aerosols due to their airway responses to stimuli being similar to human beings (16, 17). While e-cig vapor containing nicotine drastically reduced TMV when vaped into sheep, it resulted in low amounts of systemic nicotine uptake, indicated by plasma cotinine levels. This mirrored our recent clinical observations for smoking cessation in veterans using e-cigs: the major
reason for failing to switch from tobacco smoking to e-cig vaping was insufficient systemic nicotine uptake if vaping didn’t follow a certain inhalation topography (53).

Nebulization of e-cig liquid into sheep had an effect similar to vapor on TMV, but also a much better systemic nicotine delivery. This indicated that nebulized liquid is a more effective nicotine delivery method than vapor. TMV reductions after a single dose lasted for ~6 hrs, upon which a repeat dose decreased TMV again. Similar findings of vapor containing nicotine on MCC were reported in a murine model (11). In contrast, that study did not find that e-cig liquid had an immediate effect on murine MCC, suggesting that the large animal is more suited to predict human responses. Reductions in ovine TMV upon inhalation of nicotine-containing liquid were markedly protected by pretreatments with nebulized A967079. Notably, sheep required higher concentrations of A967079 because of the low deposition efficiency (~10%) of nebulized treatments (54). Furthermore, previous studies showed that doses ~20-fold higher than effective in vitro doses are optimal for nebulized treatments in sheep (55, 56). A small remaining TMV decrease may be related to direct effects of PG/VG due to its physiochemical properties that can hinder ciliary beating as recently reported (57, 58).

Lastly, TRPA1 may not only be stimulated by nicotine, but also by free radicals generated during the vaporization process of e-liquid (59), either directly or by downstream lipid peroxidation products through its redox-sensitive cysteine residues. The latter is the primary mechanism-of-activation for the TRPA1 agonists cinnamaldehyde and allyl isothiocyanate (60). Coincidentally, cinnamaldehyde is the main e-cig liquid additive for cinnamon flavoring and several widely-used e-cig flavoring additives such as
vanillin and menthol are known effectors of other TRP Ca\textsuperscript{2+} channels (61). It is therefore plausible that many e-cig flavors influence airway MCC via TRP family members.

CONCLUSION

This study demonstrates that nebulized and vaporized e-cig liquids with nicotine have significant and deleterious effects on airway mucociliary function of naïve airway epithelial cells from never-smoking individuals and of the sheep trachea. This effect was mainly mediated via TRPA1 receptors. How other e-cig vapor constituents, such as certain types of flavoring agents, affect these processes are areas of future investigation.

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FIGURE LEGENDS

Figure 1. E-cig vapor containing nicotine impairs various parameters of mucociliary function in primary human ALI cultures. (A) General schema illustrating exposure of ALI cultures to e-cig vapor. (B) Treatment effects were assessed by comparing absolute changes in ASL volume from pre-exposure (ΔASL) at each time-point. Cultures exposed to e-cig vapor containing nicotine (PG/ VG + Nicotine) exhibited reduced ASL after 24 hrs compared to e-cig vapor generated from vehicle (PG/ VG) or air controls (n = 8). (C) Mucus viscosity estimated by FRAP: Cultures exposed to e-cig vapor containing nicotine (PG/ VG + nicotine) had greater FRAP half-life (t1/2), indicating higher mucus viscosity, compared to PG/ VG or air only controls (n = 8). (D) General schema illustrating deposition of nebulized nicotine (or drugs) onto ALI cultures. (E) Cultures nebulized with nicotine had lower ASL compared to control-exposed cultures at 4 and 24 hrs post-exposure (n = 8). (F) Cultures nebulized with nicotine also exhibited greater FRAP t1/2 compared to saline control (0.1% NaCl) exposed cultures (n = 8). All data sets passed normality (Shapiro-Wilk). Each n represents a unique lung donor. *, p < 0.05 between treatments, determined by Sidak’s post hoc test after two-way ANOVA (B/E), by Sidak’s post hoc test after one-way ANOVA (C), or by two-tailed paired t-test (F).

Figure 2. Primary human ALI cultures exhibit TRPA1 function. (A) Cinnamaldehyde (500 µM), a TRPA1 agonist, increased intracellular Ca2+ (GCaMP6s fluorescence). This response was partially blocked by the TRPA1 antagonist A967079 (10 µM), as measured by maximum, rate, and cumulative Ca2+ responses (n = 8). (B) Nicotine (100 µM) also
increased intracellular Ca\(^{2+}\) and this response was again prevented by TRPA1 antagonists, including A967079 or HC030031 (10 µM), or the dual TRPA1/nAChR antagonist mecamylamine (10 µM). Inhibition by the nAChR antagonist hexamethonium (10 µM) was not statistically significant. All n ≥ 8 for each treatment. “Maximal Change” and “Rate of Change” data for nicotine (B) did not pass the Shapiro-Wilk normality test and nonparametric tests were used. *, p < 0.05 between treatments, determined by two-tailed paired t-tests. #, p < 0.05 between treatments with n > 2 treatments groups, determined by Sidak’s (parametric) or Kruskal-Wallis (nonparametric) post hoc test after one-way ANOVA.

**Figure 3.** TRPA1 inhibition prevents ASL volume loss and increased mucus viscosity following nicotine delivery by e-cig vapor or nebulization. (A) A967079 was deposited onto ALI cultures by nebulization 1 hr before exposure to e-cig vapor containing nicotine (PG/VG + Nicotine). A967079 improved ΔASL compared to 0.1% DMSO-exposed controls (n = 9). (B/C) Nebulized A967079 also (B) reduced the percent mucus solids (n = 8) and (C) lowered FRAP t\(_{1/2}\) (n = 8) following PG/VG + Nicotine exposure. Of note: the percent mucus solids with PG/VG + Nicotine exposure is close to the values of patients with chronic bronchitis. (D) ASL volume of cultures pre-treated with A967079 were protected from nebulized nicotine at 4 and 24 hrs post-exposure (n = 8). All data sets passed normality (Shapiro-Wilk). *, p < 0.05 between treatments, determined by two-tailed paired t-tests, or Sidak’s post hoc test after two-way ANOVA (ASL change over time).
**Figure 4.** Nebulized nicotine impairs mucociliary transport (MCT) *in vitro.* (A) Adaptation of the original "Mucociliary Transport Device (MCTD)" design by Sears et al (24) to 12-mm Transwell inserts. Marine-safe silicone sealant was applied to the center of 12-mm Transwell inserts to form a cone (outlined in blue) with an approximate 1 to 1.5-mm diameter at its base. (B) Primary HBEC cultures were differentiated at the ALI for 6-8 weeks until continuous MCT developed. MCT was estimated by recording the movement of 1-μm diameter fluorescent beads placed on the apical surface of cultures the prior evening. MCT velocity was calculated from distance traveled by each bead (i.e., displacement) over time (blue arrow). (C) Baseline MCT was similar between cultures exposed to nicotine (67.9 ± 9.5 μm/s; n = 8) and controls (63.0 ± 13.3 μm/s; n = 8). Nebulized nicotine significantly reduced MCT after 1 hr compared to nebulized saline control. (D) Baseline MCT was similar between cultures exposed to DMSO (46.8 ± 5.1 μm/s; n = 8) and A967079 (48.7 ± 9.4 μm/s; n = 8). A967079-exposed cultures had significantly improved MCT compared to DMSO-treated cultures following nicotine exposure. Baseline MCT differences in C/D are possibly due to difference in donor lungs with no overlap. All data sets passed normality (Shapiro-Wilk). *, p < 0.05 between treatments, determined by Sidak’s *post hoc* test after one-way ANOVA.

**Figure 5.** Sheep tracheal mucus clearance (TMV) is inhibited by e-cigarette vapor mainly in a nicotine-dependent manner (PG/VG had a small but initially significant effect). Sheep TMV was measured by recording movement of small radiopaque Teflon trioxide disks before and after exposures. E-cig liquids were dissolved in 100% ethanol since the e-cig liquid is otherwise too viscous, then administered by nebulization based on total delivered
mass of dissolved nicotine. E-cig vapor was generated using a Joyetech e-cigarette device at settings used for in vitro experiments as described in the Online Supplement Methods. Arrows indicate exposure times. (A) TMV was reduced following PG/VG ± nicotine inhalation by nebulized e-cig liquid (n = 3 per treatment) in a nicotine dose-dependent manner. Ethanol inhalation alone had no influence on TMV (n = 2). (B) Reduction of TMV was again seen after a second exposure at 6 hrs after the initial dose (n = 3). (C) Plasma levels of cotinine, the primary metabolite of nicotine, showing nicotine uptake following inhalation of nebulized e-cig liquid containing 15 mg nicotine. (D) TMV was also reduced following vaping sheep with PG/VG + nicotine (36 mg/mL, 40 inhalations). (E) As expected from our published human study in veterans (53), e-cigarette vapor is a poor nicotine delivery medium showing low but detectable plasma cotinine levels. Dark grey box is p < 0.05 compared to baseline TMV using Sidak’s post hoc test for two-way ANOVA; light grey box is p < 0.05 compared to baseline, PG/VG, and 10 mg nicotine using the same analysis.

Figure 6. Inhaled A967079 prevents impaired sheep tracheal MCC caused by nicotine inhalation. (A) Sheep were nebulized with varying doses of A967079 prior to PG/VG + 15 mg of nicotine (n = 3 per treatment). TMVs for 5 and 10 mg A967079-exposed sheep were not significantly different from PG/VG + 15 mg of nicotine only. However, 20 mg A967079-exposed sheep exhibited TMV values that were significantly improved compared to PG/VG + 15 mg of nicotine. (B) Plasma cotinine levels were not significantly different between sheep exposed to PG/VG + 15 mg of nicotine with or without 20 mg A967079 pre-treatment, confirming nicotine delivery. (C) Percent (%) mucus solids were
assessed in sheep mucus collected before and at the end of TMV measurements from two sheep per treatment (DMSO or A967079). Mucus solids were measured in triplicates for two separate mucus collections per sheep (n = 12). Nicotine-exposed sheep had significantly increased % mucus solids that was reduced with 20 mg A967079 pre-treatment. Light grey box is p < 0.05 compared to lower doses of A967079 (0, 5, 10 mg) using Sidak’s post hoc test for two-way ANOVA. *, p < 0.05 between treatments, determined by Sidak’s post hoc test of one-way ANOVA.

**Figure 7.** Working model for impaired airway mucociliary function by nicotine delivery from e-cig vapor. Generalized schema outlining components of the ciliated airway epithelium. At the apical surface of ciliated airway epithelial tissue, TRPA1 is expressed and functional. Upon exposure to inhaled nicotine, TRPA1 is activated, leading to Ca\(^{2+}\) influx with subsequent signaling that, over time, results in net loss of ASL hydration and increased mucus solids and viscosity, leading to impaired MCC.
Figure 1

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Figure 2

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Figure 3

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Figure 4

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Figure 5

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Figure 6

187x123mm (300 x 300 DPI)
Figure 7

242x136mm (300 x 300 DPI)
Tissue Culture

Primary human bronchial epithelial cells (HBECs) were cultured as previously described (1). HBECs were isolated from appropriately consented donors whose lungs were found unsuitable for transplantation. Donor lungs used in this study were from young non-smoking individuals with no documented airway disease (see Table E1). After expansion, cells were re-differentiated at the air-liquid interface (ALI) on collagen-coated 12-mm Transwell inserts (Corning) for 4 weeks. After ALI was established, cultures were apically washed with 1X Dulbecco’s PBS (DPBS; Corning) and basolateral media was replaced every 2-3 days. Prior to experiments, cultures were apically washed with warm 1x DPBS (37 °C) and allowed to equilibrate overnight.

Lentiviruses were prepared by co-transfecting Human Embryonic Kidney cells (HEK293T) with packaging DNA plasmids and a plasmid-of-interest. A pEF1-puro-expressing GCaMP6s cDNA, which was designed using the original pGP-CMV-GCaMP6s construct gifted by Dr. Douglas Kim (Addgene plasmid #40753), was used to deliver GCaMP6s (2). For TRPA1 knockdown experiments, TRPA1 shRNA-encoding pLKO.1-puro plasmids were purchased from Sigma Aldrich Mission shRNA library (Catalogue #: TRCN0000044798, TRCN0000044799, TRCN0000044800, TRCN0000044801, TRCN0000044802). Virus-containing media were collected, concentrated, titered, and stored at -80°C until use. De-differentiated basal cells were
infected with viruses in growth media containing polybrene. shRNA-encoding viruses were pooled for HBEC infection. Cells were then differentiated at the ALI under puromycin selection (3).

A human orthologue of TRPA1 that was recently resolved by cryo-electron microscopy was kindly gifted by Dr. David Julius (UCSF, CA) for our studies. This orthologue (or its empty vector control) was transfected into HEK293T cells for 24 hrs for use as a positive control for HBEC TRPA1 expression studies.

**Animal Study**

Adult ewes (female sheep) were used for this study. All procedures were approved by Mount Sinai Medical Center Animal Research Committee. Sheep were conscious and nasally intubated during the course of study (4). Aerosols were generated by an Airlife nebulizer with a dosimetry system and a respirator was used. Aerosols were delivered directly into the trachea only during inspiration at a frequency of 20 breaths min\(^{-1}\) at a tidal volume of 500 mL. For nebulized e-cig liquid studies, nicotine (36 mg/mL) dissolved in propylene glycol and vegetable glycerin (50%/50% w/v PG/VG) stock solution was diluted with 100% ethanol to achieve the desired total nicotine content then nebulized into the sheep airway. In the sheep model, the addition of ethanol had no influence on TMV (see “Ethanol Control” in Fig. 5A) and was therefore used for dilution since they helped aerosolization of the e-cig fluid. These procedures are estimated to deposit ~10% of compounds based on past documented nebulizer efficiencies (5). For direct vaping studies, nicotine solution (36 mg/mL nicotine in 50%/50% w/v PG/VG) was used to generate e-cig vapor using a Joyetech™ Delta atomizer coupled to an eVic
Supreme® (Joyetech). Each puff was generated at ~6.5W for 4.5 seconds and manually collected using a 60 mL syringe. Each puff was then manually administered into the inspiratory tube of the ventilator during inspiration for delivery into the sheep airway.

**In vitro E-cigarette Vapor Exposure (VC-1)**

12-mm Transwell cultures were mounted into insert holders inside a 12-well metal block for VC-1 smoke exposure robot (Vitrocell, Waldkirch, Germany) at room temperature. A Joyetech™ Delta atomizer coupled to an eVic Supreme® was used to generate a puff of e-cig vapor from e-cigarette liquid (50%/50% w/v PG/VG) containing nicotine (0 or 36 mg/mL). For our study, the atomizer was set to 3.2 – 3.6 V for a power setting of ~6.5 W. Each puff was generated and collected for 4.5 seconds for 70 mL total volume. The puff was then immediately administered and propelled over 4 seconds through a distribution system with a filtered air flow at a constant 0.25 L min\(^{-1}\). Each puff was applied once every 30 seconds. Cultures were exposed to either filtered air alone or 40 total e-cigarette vapor puffs over a 20 min period. Vacuum was applied through the individual cell culture delivery at a total of 5 L min\(^{-1}\) to generate a constant flow above the cultures.

**In vitro Apical Deposition via Nebulization (CLOUD)**

12-mm Transwell cultures were mounted into insert holders inside pre-warmed (37.5°C) 12-well metal block for the CLOUD 12 exposure chamber (Vitrocell, Waldkirch, Germany). Cultures were then nebulized with nicotine tartrate salt solution (0 or 600 μM;
Sigma-Aldrich), which was supplemented with 0.9% NaCl (2.5 μL) to provide sufficient ionic strength for droplet formation. Cultures were incubated for 5 minutes, which was chosen after identifying the minimum time necessary for apical deposition of nebulized solutions.

**Volume Deposition by Nebulization**

Cell-free 12-mm Transwell inserts containing 100 μL deionized water were mounted into insert holders of a 12-well metal block and warmed to 37.5°C. Fluorescein solution (200 μL) dissolved in 1X PBS (15 μg/mL; Sigma-Aldrich) was nebulized and incubated for 5 minutes. Samples (50 μL) from each insert was transferred into a 96-well plate, diluted with 50 μL deionized water, and fluorescein emission (510 nm) of samples were compared to fluorescein standards using a plate reader (6).

**Mass Deposition by Quartz Crystal Microbalance (QCM)**

Up to two QCMs were mounted into inserts holders on the outer wells of a 12-well metal block and warmed to 37.5°C. QCM outputs were connected to a desktop computer and frequency readings were recorded using Vitrocell software. QCM-reported masses were zeroed then observed for at least 1 min to confirm stable frequency readings or repeated until stable. Defined solutions or solvent controls (200 μL) were nebulized into the chamber and QCM readings were recorded for up to 15 min until readings stabilized. Deposited mass of nebulized solution was calculated as final mass subtracted by initial mass, in ng/cm². Deposited mass of specific chemicals was
calculated by subtracting mass of chemical solution by mass of solvent control. At least 3 readings were recorded per treatment.

**Measuring Airway Surface Liquid (ASL) Volume by Meniscus Scanning**

Ultra-high-resolution images (≥10 megabytes per acquisition) of cultures were acquired with an Epson flatbed scanner. Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) to estimate ASL volume using a modified plug-in kindly provided by Dr. Michael Myerburg (University of Pittsburgh) as previously published (7). Baseline ASL volume of cultures were measured prior to exposure for each culture and were not significantly different between treatments.

**XY Calcium Imaging using GCaMP6s sensor**

GCaMP6s-infected HBEC cultures were mounted in a chamber and perfused with various agonists [cinnamaldehyde (500 μM; Sigma-Aldrich), (-)-nicotine hydrogen tartrate salt (100 μM; Sigma-Aldrich), and GSK1016790A (10 nM; Cayman Chemicals)] and antagonists [A967079 (10 μM; Tocris), HC030031 (10 μM; Tocris), hexamethonium (10 μM; Tocris), mecamylamine (10 μM; Tocris)] in HEPES-buffered HBSS pH 7.4 at 250 μL min⁻¹ using a syringe pump (Harvard Apparatus, Holliston, MA). The perfusion chamber was placed onto an upright Nikon Eclipse E600fn microscope (Nikon Instruments, Tokyo, Japan) and visualized with a 60x objective. Images were acquired every 3 seconds with a CoolSnap CCD camera (Photometrics, Tucson, AZ) after GCaMP6s excitation (495 nm) with a DG4 rapid wavelength switcher (Sutter Instrument, Novato, CA) operated by MetaFluor software (Molecular Devices, San Jose, CA).
Confocal Calcium Imaging using GCaMP6s sensor

GCaMP6s-infected HBEC cultures were mounted in a chamber and perfused with either cinnamaldehyde (500 μM) or UTP (100 μM) dissolved in HEPES-buffered HBSS pH 7.4 at 250 μL min\(^{-1}\). The perfusion chamber was placed onto a Piezo Flexure Stage for image acquisition on a Leica TCS SP5 Confocal Microscope (Leica Microsystems, Wetzlar, Germany). GCaMP6s emissions in a multi-line xz plane were captured every 500 ms (cinnamaldehyde) and 800 ms (UTP) at 510 nm.

Measuring Mucociliary Transport (MCT) in cultures grown in MCT Devices (MCTDs)

12-mm Transwell inserts were modified to become MCT devices. HBECs were differentiated at the air-liquid interface for 6-8 weeks on MCTDs to develop continuous MCT as previously described (8). To record MCT, 2.5 μL of 1-μm green fluorescent microbeads (ThermoFisherScientific), diluted to 100 ng/μL in 1x Hank’s buffered saline solution (HBSS), were placed on the apical surface and allowed to equilibrate overnight. Next day, MCTDs were nebulized with 800 μL of 1x HBSS at 37.5°C for 10 min to gently hydrate the apical surface (deposit ~5 μL of solution). Thirty minutes after apical hydration, MCT was recorded every 4-5 seconds for 30 to 60 seconds at 495 nm. MCT for each culture was recorded again after treatments (i.e., nebulized nicotine). MCT was calculated as the distance traveled by each unique microbead per frame using the Manual Tracking ImageJ plugin. Alternatively, single long exposures (1-1.5 s) were used to generate trails of moving beads and MCT speed was estimated from the length of trails divided by exposure duration.
Assessing mRNA expression via qPCR

mRNA and protein expression were measured by qPCR and Western blotting respectively, according to standard biochemistry techniques. Total HBEC RNA was collected & isolated using E.Z.N.A. Total RNA Kit (OMEGA Bio-tek) and the concentration was determined using a NanoDrop1000 (ThermoFisherScientific). Message RNA was isolated using PolyATtract® System 1000 (Promega). After a cDNA library was synthesized using iScript cDNA synthesis kit (Biorad). TaqMan qPCR was performed using a PCR master mix (TaqMan) with primers for target genes TRPA1 (Hs00929057_m1; ThermoFisherScientific) and GAPDH (4352665; Applied Biosystems). SYBR green primer pair was designed to selectively span TRPA1 exon 25-26 (C-terminus) and SYBR Green-based qPCR was used. Expression data was generated by normalizing threshold cycle (C_T) value of target gene to GAPDH.

Assessing protein expression via Western blot

HBECs were lysed in RIPA buffer containing inhibitor cocktail, collected, centrifuged at 4°C at 10,000 RPM for 10 min, and the supernatant isolated and labeled as “whole cell lysate”. Protein concentrations were calculated using BCA assay (Biorad). Samples of 20 μg of total protein were supplemented with 4x loading dye, boiled at 95°C, loaded onto precast gels (10%), separated for 1.5 hrs, then transferred onto Immobilon-P membranes. Membranes were blocked with 5% milk in Tris-buffered PBS with 0.05% Tween-20 (TBS-T, pH 7.4) for 1 hr at room temperature. Primary antibodies (Abs) used for this publication were: anti-TRPA1 (1:1000; Aviva Systems Biology) and anti-β-actin
Primary Abs were incubated overnight at 4°C. Appropriate HRP-linked secondary Abs (α-rabbit or α-mouse) were incubated for 1h at room temperature. 15 min washes with TBS-T occurred between steps. Expression signals were visualized using chemiluminescence. Protein expression was calculated from the signal of target protein, normalized to signal of β-actin protein expression. Antibodies for TRPA1 were tested with and confirmed reaction with a known TRPA1 orthologue.

**Tracheal Mucus Velocity (TMV)**

Sheep TMV was measured as recently described (4). Radiopaque Teflon trioxide disks (5-10) were introduced into the sheep’s trachea through the endotracheal tube. Inspired air through the trachea was pre-warmed and humidified using a Bennett humidifier (Puritan Bennett), to prevent mucosal dehydration during extended intubation necessary for hourly TMV value approximations (and cuff was deflated). Disk movements were recorded using videotaped fluoroscopy. Velocities of individual disks were used as surrogates for calculating TMV and were approximated by measuring distance traveled by each disk over one minute using radiopaque reference markers on a collar worn around the sheep’s neck. Each value of a time point represents the average velocities of several individual disks. TMV measures were obtained every hour for a total of 12 hrs and at later time points as indicated in figures. Although different solvents were used for various inhaled agents, they did not significantly influence TMV.
REFERENCES


**Figure E1.** Deposition parameters for Vitrocell VC-1® smoke robot and CLOUD® exposure chamber. (A) Aerosols deposited following e-cig vapor exposure (6 adjacent wells) or air (3 adjacent wells) were collected to quantify concentrations of delivered nicotine by LC-MS/MS. Transwell inserts received 12.5 ± 1.1 µM nicotine with our e-cig vapor exposure paradigm (n = 6). (B) Volume deposition of fluorescein (15 μg/mL) for one (1x) or four (4x) sequential exposures in three adjacent inserts (n ≥ 5). (C) Mass deposition of increasing concentrations of nicotine tartrate salt in solution, measured by quartz crystal microbalance. Using such measurements, a single aerosolized dose of 600 µM nicotine (200 µL total volume) is estimated to result in approximately 4 µM in the ASL of ALI cultures (n = 6). All experiments were done with the holder block warmed to 37.5°C. (D) Nebulized nicotine reduced ASL in a dose-dependent manner at 24 hrs post-exposure (n = 8). All data sets passed normality (Shapiro-Wilk). *, p < 0.05 between treatments and saline control (0 µM nicotine), determined by Sidak’s *post hoc* test of one-way ANOVA.

**Figure E2.** TRPA1 mRNA and protein are expressed in human ALI cultures and freshly-isolated sheep tracheal epithelial cells. TRPA1 expression of 4 weeks, ALI-differentiated HBEC cultures and freshly isolated sheep (ovine) bronchial epithelial cells was assessed. (A) cDNA generated from total mRNA was probed with SYBR green primers designed to selectively span TRPA1 exon 25-26 (C-terminus). SYBR green primers and TaqMan primers were validated with mRNA from over-expressed human TRPA1 orthologue in HEK293T cells. HBEC TRPA1 mRNA was expressed at 2.26 x 10^{-6} (n = 3) while ovine TRPA1 mRNA was expressed ~10-fold higher (2.89 x 10^{-5}; n = 3). (B)
Whole cell lysate (20 μg) from HBEC cultures were assessed for TRPA1 protein expression. Whole cell lysate (4 μg) from HEK293T cells transfected with the human TRPA1 cDNA was used as a positive control.

**Figure E3.** TRPA1 antagonist A967079 does not affect TRPV4 activation in human ALI cultures. GCaMP6s-expressing cultures were excised and mounted into a perfusion chamber. A967079 (10 μM) failed to inhibit TRPV4 activation by GSK1016790A (10 nM; n = 3). GSK, GSK1016790A – TRPV4 agonist

**Figure E4.** Nicotine induces extracellular Ca\(^{2+}\) influx through TRPA1 in human ALI cultures. GCaMP6s-expressing cultures were excised and mounted into a perfusion chamber. Perfusion of 100 μM nicotine elicits Ca\(^{2+}\) influx in the presence of SERCA inhibitor thapsigargin (1 μM, 250 μL min\(^{-1}\), 20 min) but not with addition of A967079 (n = 5). Data sets passed normality (Shapiro-Wilk) except “Slope” analysis. *, p < 0.05 between treatments, determined by two-tailed paired t-test (parametric) or by Wilcoxon test (nonparametric).

**Figure E5.** TRPA1 mediates nicotine-induced ASL volume loss in HBEC cultures. (A) ALI cultures nebulized with the TRPA1-selective agonist JT010 (~1 μM in the ASL) showed reduced ASL at 4 hrs (n = 6). (B) Cultures were incubated for 1 hr with A967079 (10 μM in basolateral media) prior to exposure to e-cig vapor containing nicotine (PG/VG + Nicotine). A967079 improved ΔASL (n = 9) and (C) percent mucus solids (n = 6) compared to 0.1% DMSO-treated controls. (D) TRPA1 mRNA expression
was measured using TaqMan primers. Knockdown effect was determined from $\Delta\Delta C_T$ values that was log-transformed. TRPA1 expression was knocked-down during differentiation (6 weeks) via TRPA1 shRNA induced by a pLKO.1-puro vector-expressing lentivirus. Empty vector served as control. (E) Nicotine exposure significantly reduced ASL of empty vector-infected cultures but not TRPA1 shRNA-infected cultures ($n = 6$), suggesting a protective effect. (F) Exposure to 0.1% NaCl (saline control) had no effect on $\Delta$ASL for both empty vector- and TRPA1-shRNA-infected cultures. All data sets passed normality (Shapiro-Wilk). *, $p < 0.05$ between treatments, determined by two-tailed paired t-test. #, $p < 0.05$ between “empty vector” cultures treated with saline control and nicotine; $\$, $p < 0.05$ between nicotine-treated “TRPA1-shRNA” and nicotine-treated “empty vector” cultures, determined by Sidak’s post hoc test after one-way ANOVA.

**Video E1.** Apical perfusion of TRPA1 agonist cinnamaldehyde (500 μM) increases $Ca^{2+}$ levels in human ALI cultures. GCaMP6s-expressing cultures were excised and mounted into a perfusion chamber. GCaMP6s emissions were imaged in the xz-axis every 500 msec for 1 minute. Cinnamaldehyde increases apical intracellular $Ca^{2+}$ levels, as represented by increased GCaMP6s emission (in green) upon by binding of cytosolic $Ca^{2+}$ to GCaMP6s. Delayed response (starting at ~21 sec) was attributed to distance traveled from solution origin.

**Video E2.** Apical perfusion of UTP (10 μM) increases apical $Ca^{2+}$ levels in human ALI cultures. GCaMP6s emissions (in green) were imaged in the xz-axis every 800 msec for
1 minute. Delayed response (starting at ~35 sec) was attributed to distance traveled from solution origin.

**Table E1.** Individual Lung Donor Information
Supplemental Figure E1

91x165mm (300 x 300 DPI)
Supplemental Figure E2

113x106mm (300 x 300 DPI)
Supplemental Figure E3

Relative Intracellular $[Ca^{2+}]_i$
Average trace of 3 different lungs

GCaMP6s Emission ($F_x/F_0$)

GSK

15 min recovery

10 μM A967079

10 μM UTP

4 min
Supplemental Figure E4

120x119mm (300 x 300 DPI)
Supplemental Figure E5

140x179mm (300 x 300 DPI)
**Table E1. Individual Lung Donor Information**

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