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Pod-based menthol and tobacco flavored e-cigarettes cause mitochondrial dysfunction in lung epithelial cells

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Abstract

Current FDA regulations have resulted in a ban of flavored e-cigarette pods, with only menthol and tobacco flavored pods being exempted. Previous work using menthol and tobacco-flavored e-cigarettes have been shown to induce mitochondrial reactive oxygen species. We hypothesized that exposure to pod-based JUUL Menthol and Virginia Tobacco aerosols will alter mitochondrial respiration and electron transport chain protein levels. We determined mitochondrial respiration by using a Seahorse technique and electron transport chain complexes by total OXPHOS antibodies after exposing lung epithelial cells, Beas-2b, to pod-based Menthol and Virginia Tobacco favored aerosols. Menthol pod exposure resulted in an immediate increase in proton leak and decrease coupling efficiency, as well as a decrease in complex I, II, and IV. Menthol pod exposure twenty-four hour post exposure resulted in a decrease in basal respiration, maximal respiration, and spare capacity, as well as a decrease in complex I. Tobacco pod exposure resulted in no significant alterations to mitochondrial respiration, but immediately post final exposure resulted in a significant increase in complex I, IV, and V. Our results indicate that exposure to Menthol flavored e-cigarette pods causes mitochondrial respiration dysfunction in lung epithelial cells.

Graphical Abstract

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Author Contributions

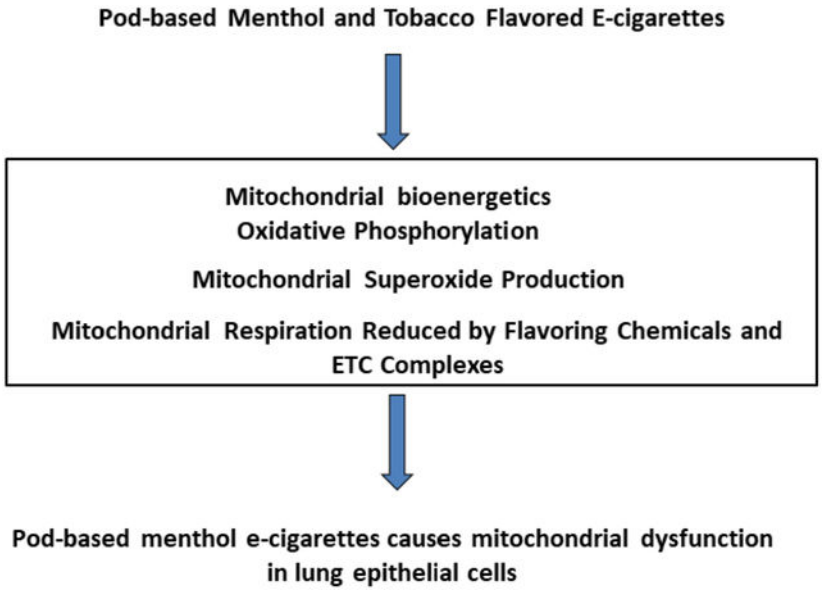
TL: Conducted the experiments. Wrote and edited the manuscript.

TM: Conducted vapor phase sampling and edited the manuscript.

IR: Conceived and designed the experiments. Wrote and edited the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



Keywords

Mitochondrial Bioenergetics; E-cigarettes; Pod-based; Menthol; Tobacco

1. Introduction

Electronic cigarettes (e-cigarettes) are devices that generate aerosols from a liquid typically composed of propylene glycol, vegetable glycerin, nicotine, and flavoring chemicals (Bals et al., 2019). As of 2018, there were more than 250 e-cigarette brands and over 8,000 different flavorings in the United States (Kaur et al., 2018). These flavorings composed of flavoring chemicals are classified as 'Generally Recognized As Safe' for ingestion, but the effects of inhalation of these chemicals are still relatively unknown (Kaur et al., 2018).

Current e-cigarette use has remained stable from 2014 to 2018 at roughly 3%. Despite this, use in young adults (18–24 years old) has increased from 5.1% to 7.6%, along with a significant increase in use among never smokers (Dai and Leventhal, 2019). A recent epidemiological study also found that current e-cigarette use in high school students was 27% and 10% in middle school students (Cullen et al., 2019). Within these current users, 59 % of high school students and 54% of middle school students reported that JUUL was the usual e-cigarette device used (Cullen et al., 2019). In 2018, JUUL had a 70% market share of US convenient store vapor product sales (Ramamurthi et al., 2018). JUUL e-liquids are packaged in pods, composed of a mixture of propylene glycol, vegetable glycerin, nicotine, benzoic acid, and flavoring chemicals (Ramamurthi et al., 2018). These pods were initially sold in one of eight flavors with 5% nicotine (Omaiye et al., 2019).

In the recent Population Assessment of Tobacco and Health Study, the most common flavor used by either adults or youth participants in the past 30 days was fruit flavor (Schneller et al., 2019). Meanwhile, menthol/mint flavors had a 17% preference and tobacco flavors had a 24% preference in adult participants (Schneller et al., 2019). While menthol/mint flavors had

a 10.8% preference and tobacco flavors have a 5% preference in youth participants (Schneller et al., 2019). Another epidemiology study observing the flavor preference in a cohort over time showed menthol/mint flavor preference remained stable with the preference of 11% at baseline to 9% at follow up (Du et al., 2020).

Studies have begun to observe the effects of both e-cigarettes and flavoring chemicals on mitochondrial function. Cinnamaldehyde, a flavoring chemical used in e-cigarette liquids, effects on mitochondrial respiration was measured using seahorse technique (Clapp et al., 2019). Beas-2b lung epithelial treated with cinnamaldehyde resulted in a dose-dependent decrease in mitochondrial respiration and glycolysis (Clapp et al., 2019). Other studies observed a dose-dependent increase in mitochondrial superoxide (mitosox) production in cells that were treated with tobacco and menthol e-liquid (Zahedi et al., 2019). Similarly, another study showed an increase in mitosox in 16-HBE epithelial cells due to aerosol exposure of e-cigarette pod-based devices (Muthumalage et al., 2019).

Due to recent FDA regulation, flavored e-cigarette pods except for menthol and tobacco flavors have been banned from sale in the United States. From recent reports, it has been shown that flavoring chemicals and exposure to e-liquids have resulted in mitochondrial release of reactive oxygen species (Lerner et al., 2016). Therefore, we hypothesize that exposure to pod-based Menthol and JUUL Virginia Tobacco flavored e-cigarette exposures will result in alteration of mitochondrial respiration and electron transport chain complex protein levels in lung epithelial cells.

2. Material and Methods

2.1 Scientific Rigor and Reproducibility

We used a rigorous and unbiased approach in experimental planning and analyzing the data to produce reproducible data along with a full and detailed report of methods and analyzed data. All biological and chemical resources used in this study were validated and authenticated.

2.2 Ethical approval: Institutional biosafety approvals

All experiments performed in this study were approved and in accordance with the University of Rochester Institutional Biosafety Committee.

2.3 Procurement of JUUL Pods

JUUL pod flavor, “Menthol” and “Virginia Tobacco” with 5% nicotine were purchased from online store as well as local retail store.

2.4 Determining liquid and vapor phase constituents

A previous study from this lab has been conducted on the chemical composition of JUUL pods e-liquids (Muthumalage et al., 2019). To quantify vapor phase constituents, JUUL Menthol and Virginia Tobacco pods were aerosolized and collected in 1L vacuum bottles (3 puffs per minute over 10 minutes). These samples were sent to ALS Environmental, CA, for analysis. Vapor phase constituents quantified by EPA method TO-15 and mass spectral

library search for tentatively identified compounds. Determined chemical constituents were then categorized by functional group.

2.5 Cell Culture

Lung epithelial cells, Beas2b cells, (ATTC, Virginia) were cultured in complete media in DMEM: F12 complete media (Corning, ref #16–405-CL, Arizona) with 5% FBS, 1% pen/strep, and 15 mM HEPES. Prior to exposure, cells were cultured in a 5% CO_2 incubator to 80% confluency in T-75 flasks

2.6 *In Vitro* Exposure

JUUL device was connected to one end of the Scireq inExpose e-cig exposure system pump (Scireq, Canada). The other end of the pump was then connected to the Enzyscreen chamber (Enzyscreen, Netherlands). Cell culture plates were then exposed to either air or Menthol or Virginia Tobacco pods with 5% nicotine for 22 minutes using a puffing profile with 3 puffs per minute for a total of 66 puffs with 55ml/min puff volume. Cells then kept in vapors for 8 minutes in order to be exposed for a total of 30 minutes before being returned to the incubator. Two more exposure sessions occurred afterward at 12-hour intervals.

2.7 Mitochondrial Bioenergetics using Seahorse XFp Analyzer

To determine mitochondrial respiration, approximately 20,000 cells per well were plated in seahorse mini-plate (Agilent Technologies, Cat #103022–100, California), with well A and well H left blank for background control, and cells were allowed to grow to roughly 90% confluency. Immediately prior to exposure, cells were serum-deprived to DMEM: F12 complete media with 0% FBS. At either immediate or 24-hours post final exposure, media in the plate was removed and 180μl seahorse media, composed of 10 mM glucose, 1mM pyruvate, and 2 mM glutamine in Agilent Seahorse XF DMEM Media, was added per well and incubated for 1 hour in a non-CO_2 37°C incubator. The night prior to running the assay, the Seahorse XFp analyzer was turned on and the XF cartridge was hydrated by adding seahorse XF calibrant. On the day of the assay, three of the four ports were loaded with mitochondrial inhibitor drugs to obtain a concentration of 2μM oligomycin, 1.5μM FCCP, and 0.5 μM Rotenone/Antimycin A after injection. MitoStress test kit (Agilent Technologies, cat#103010–100, California) was run on Seahorse xFP analyzer. Afterward, cell counts were performed using acridine orange/propidium iodide stain to normalize data. Data were analyzed using Wave software.

2.8 Immunoblot Analysis of Electron Transport Chain (ETC) Protein Levels

To determine ETC protein levels, 300,000 cells per well were plated in a 6-well plate and allowed to grow to roughly 80% confluency. Immediately prior to first exposure, cells were serum-deprived to DMEM: F12 media with 0% FBS. After final exposure, cell pellets were collected at an immediate and 24 hour time point and stored at −80°C. Whole-cell lysate from air, Menthol, and Virginia Tobacco exposed cells protein levels were determined using a BCA protein assay. Equal concentrations of sample protein were added to a 12% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were probed with total OXPHOS human antibody cocktail (Abcam, ab110411, 1:1000, United Kingdom) and anti-

GAPDH (Santa Cruz, Sc365062, 1:2000, Texas) overnight at 4˚C. Following probing, anti-mouse (1:5000) secondary antibody was incubated for 1 hour at room temperature. Membranes were then detected using enhanced chemiluminescence imaging reagent (ThermoFisher, ref #32106, Massachusetts) and detected by Bio-Rad ChemiDoc MP imaging system (Bio-Rad, Hercules, CA). Band intensities were analyzed using ImageJ software.

2.9 Statistical Analysis

Statistical analysis of the data was conducted by performing unpaired two-tailed t-test to analyze comparisons between the two groups using GraphPad 8.1.1. Data are represented as mean ± SD. Statistical significance was reported as *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

3. Results

3.1 Menthol and Virginia Tobacco flavor pod-based vapor phase constituents

Quantitative chemical composition of JUUL Menthol and Virginia Tobacco flavor aerosols showed slight differences with unique chemical constituents to these Menthol and Virginia Tobacco pods. There were sixteen overlapping chemical constituents between both Menthol and Virginia Tobacco pods. The unique chemical constituents of Menthol pod were found to be 1,4-Dioxane, 1-Propanol, 2-Ethyl-1-hexanol, and 2-(2-Ethoxyethoxy) Ethanol (Table 1). The unique Virginia Tobacco pod chemical constituents were found to be Propene, 2-Propanol, 1-Hydroxy-2propanone, 2,2,4-Trimethyl-1,3-dioxolane, 2-Hydroxy-, Enthylpropanoic Acid, and 2,2-Dimethly-1,3-dioxolane-4-methanol (Table 2).

3.2 Menthol pod exposure alters mitochondrial bioenergetics

To determine JUUL Menthol pod exposure effects on mitochondrial oxidative phosphorylation, by performing cell mitochondria stress test using Seahorse XFp analyzer. At immediate and 24-hour time points, Menthol flavor pod exposure resulted in an increase in the extracellular acidification rate, potentially indicating a shift to glycolysis (Figure 1A & 1C). Immediately post-final exposure, non-mitochondrial oxygen consumption and proton leak was significantly increased and coupling efficiency was significantly decreased compared to air-exposed cells (Figure 1B). Twenty-four hours post-final exposure, non-mitochondrial oxygen consumption was significantly increased, and basal respiration, maximal respiration, and spare capacity was significantly decrease compared to air exposed cells (Figure 1D).

3.3 Virginia Tobacco pod exposure does alter mitochondrial bioenergetics

To determine JUUL Virginia Tobacco flavor exposure effects on mitochondrial oxidative phosphorylation by performing cell mitochondria stress test using Seahorse XFp analyzer. At both the immediate and 24-hour time points, Virginia Tobacco pod resulted in a significant increase in non-mitochondrial oxygen consumption compared to air-exposed cells (Figure 2B & 2D). Immediately post-final exposure resulted in a significant decrease in coupling efficiency compared to air-exposed cells (Figure 2B). Twenty-four hours post-final exposure, did not result in any alteration in mitochondrial bioenergetics (Figure 2D).

3.4 Menthol exposure alters protein levels of the ETC

To determine JUUL Menthol flavor pod exposure effects on ETC protein level, western blot analysis was performed on whole cell lysate. Immediately post final exposure resulted in a significant decrease in complex I, complex II, and complex IV, and a non-significant decrease in complex V (Figure 3B). Twenty-four hours post final exposure resulted in a significant decrease in complex I, and a non-significant decrease in complex II, complex IV, and complex V (Figure 3C). Complex III was not able to be quantified due to running over of bands from other ETC subunits.

3.5 Virginia Tobacco exposure alters protein levels of the ETC

To determine JUUL Virginia Tobacco pod exposure effects on ETC protein level, western blot analysis was performed on whole cell lysate. Immediately post-final exposure resulted in a significant increase in complex V, complex IV, and Complex I (Figure 4B). 24 hours post final exposure did not result in a significant alteration of ETC protein levels (Figure 4C). Complex III was not able to be quantified for either time point.

4. Discussion

This study was attempted to determine whether e-cigarette pod-based JUUL Menthol and Virginia Tobacco flavor exposure alters the mitochondrial respiration in lung epithelial cells. Our study measured chemical constituents found in the vapor phase of Menthol and Virginia Tobacco pods, and found that a majority of the chemicals were shared between both flavors. This is similar to previous GC-MS data collected on JUUL Classic Menthol and JUUL Virginia Tobacco flavored e-liquids, with the majority of the chemicals found in Classic Menthol were shared by Virginia Tobacco pods (Muthumalage et al., 2019).

Our study also showed that Menthol flavor pod exposure resulted in a decrease in coupling efficiency and increase in proton leak at the immediate time point, and this decrease in coupling efficiency was also seen in Virginia Tobacco flavor pod at the immediate time point. Menthol pod exposure also resulted in mitochondrial dysfunction with a significant decrease in basal and maximal respiration and spare capacity at the 24-hour time points. Meanwhile, Virginia Tobacco pod exposure did not alter mitochondrial bioenergetics at the 24-hour time point. Both Menthol and Virginia Tobacco flavored pod exposures resulted in an increase in non-mitochondrial oxygen consumption. Menthol pod exposure also resulted in a potential increase in extracellular acidification rate, a measure of glycolysis, at both time points which would indicate a shift towards glycolysis. This alteration in extracellular acidification rate has also been seen in a previous study conducted on heat not burn cigarettes, i.e. IQOS exposure on Beas2b which resulted in a significant increase in extracellular acidification rate (Sohal et al., 2019). Along with this, a significant increase in proton leak was seen in Beas2b exposed to IQOS (Sohal et al., 2019), similar to the proton leak seen at the immediate time point of cells exposed to JUUL Menthol pod. We also showed that ETC subunit levels were significantly reduced, with complex I, II, and IV reduced at immediate time point and complex I reduced at 24-hour time point. Unlike Menthol flavor exposure, Virginia Tobacco flavor exposure resulted in a significant increase in complex I, II, and V at the immediate time point, but at the 24-hour time point no effect

on ETC complexes were seen. A study conducted on the effect of Blu Classic Tobacco exposure on primary lung fibroblast showed that exposure to e-cigarettes exhibited complex IV sensitivity (Lerner et al., 2016). In another study, human lung fibroblasts were treated with e-cigarette condensate showed a reduction in complex I, II, and IV (Lei et al., 2017), similar to the results seen from our Menthol pod results.

One component of e-cigarettes that may result in mitochondrial dysfunction may be due to flavoring chemicals found in pod-based e-cigarettes since a previous study has shown that a flavoring chemical, such as cinnamaldehyde, can alter mitochondrial respiration/ bioenergetics (Clapp et al., 2019). In this study, Beas2b cells were treated with various concentrations of cinnamaldehyde, which resulted in a dose-dependent decrease in basal respiration, ATP production, reserve capacity, proton leak, and maximal respiration (Clapp et al., 2019). Some of the unique chemical constituents of JUUL Menthol aerosols have been shown in previous studies to have some adverse health effects, e.g., inhalation of 1,4-dioxane has been found to induce nuclear enlargement in nasal respiratory epithelial cells from a 13-week exposure (Kasai et al., 2008). While in a two-year 1,4-dioxane inhalation study, preneoplastic lesions were found in the nasal cavity (Kasai et al., 2009). Nonneoplastic lesions in the nasal cavity showed a significant increase in nuclear enlargement, atrophy, and respiratory metaplasia, indicating 1,4-dioxane as a potential carcinogen (Kasai et al., 2009). A six-hour inhalation exposure of 2-ethyl-1-hexanol in Swiss mice, Wistar rats, and English Short Hair guinea pigs resulted in local irritation of the respiratory tract. Although the irritation was only temporary and all animals recovered with an hour (Wakayama et al., 2019). In this study, we were able to quantify many vapor phase compounds. However, there may be other responsible chemicals for mitochondrial dysfunction that we were unable to capture due to reasons, such as the volatility of compounds (temperature changes during sampling) and the auto-shutoff mechanism of JUUL devices.

The mitochondrial dysfunction seen in this study may potentially be due to an increase in mitophagy. A previous study conducted on the effects of menthol and tobacco e-liquids on neuronal stem cells have shown alterations in mitophagy, e.g. neuronal stem cells treated with 1% menthol for 4 hours resulted in an increase in mitophagy while treatment with 1% tobacco did not result in a significant increase in mitophagy (Zahedi et al., 2019). These results are similar to the observed results in this study with Menthol pod exposure and not Virginia Tobacco resulting in dysfunction of mitochondrial bioenergetics. Previous research in our lab has shown that CSE treatment leads to impaired mitophagy resulting in mitochondrial dysfunction by cigarette smoke extract (CSE) treatment to lung epithelial cells (Sundar et al., 2019). CSE treatment of lung epithelial cells resulted in an increase in DRP1 and a decrease in Mfn2 levels, along with a decrease in Miro1 and Pink1 (Sundar et al., 2019). CSE treatments also resulted in decreases in complex 1, II, III, and IV, along with alteration in mitochondrial respiration (Sundar et al., 2019). Since the mitophagy seen in cells treated with menthol e-liquids are similar to CSE treatments, which are associated with a decrease in mitochondrial respiration and ETC complex protein levels, there is a potential for an increase in mitophagy to be a cause of alteration in mitochondrial respiration. This highlights the need for future studies looking at the potential of mitophagy induced by e-

cigarette exposure, particularly using the pod-based flavors, leading to alteration in mitochondrial respiration.

Despite e-cigarettes being marketed as a safer alternative to traditional cigarettes, similar results seen in our study have also been observed in cells treated with CSE (Sundar et al, 2019). CSE treatment of isolated mitochondria resulted in a decrease in mitochondrial oxygen consumption along with a reduction in complex I and II activity (van der Toorn et al., 2007). Other studies have indicated a reduction in complex IV activity in smokers compared to non-smokers (Miro et al., 1999). One study comparing 3R4F reference cigarette and tobacco heating system showed that both products resulted in a reduction in basal respiration and ATP production in a one-week exposure (Malinska et al., 2018). This result indicates that effects on mitochondria due to tobacco heating system are lower than traditional cigarette smoke (Malinska et al., 2018).

Mitochondrial dysfunction due to exposure from cigarette smoke has been known to cause disease in users (Ahmad et al, 2015). Cigarette smoke has been known to induce cardiomyopathy in part due to mitochondrial dysfunction (Yang et al., 2007). Studies have shown that cigarette smoke can impair myocardial OXPHOS function in reperfusion injury and also increase sensitivity to heart ischemia/reperfusion injury (Yang et al., 2007). Other mitochondrial dysfunction such as alteration of mitochondrial membrane potential, mitochondrial respiration, and ATP content caused by traditional cigarette smoke has been implicated in pulmonary diseases like COPD (Fetterman et al., 2017). CSE treatment has also been shown to induce impairment in mitophagy, which leads to cellular senescence (Ahmad, et al 2015). Similar to the impact of CSE treatment, COPD patients small airway epithelial cells show impaired mitophagy and increased cellular senescence, potentially indicating the potential of CSE induced mitophagy impairment to induce chronic airway diseases (Ahmad et al., 2015). Since our JUUL pod exposure caused mitochondrial dysfunction comparable to traditional cigarette smoke, in the reduction of ETC complex protein level and decrease in mitochondrial respiration, there is a potential risk of e-cigarette chronic use to induce pulmonary and cardiovascular diseases. A recent longitudinal study showed that either current or former e-cigarette use was significantly associated with having a higher risk of developing respiratory diseases (Bhatta and Glantz, 2020).

Although mitochondrial dysfunction was seen in Beas-2b cell line in this study, it has been shown that different cell types react differently to oxidative stress (Thangboonijt, 2015). A previous study has observed that treatment with hydrogen peroxide resulted in Beas-2b cells having a lower production of intracellular reactive oxygen species than normal human bronchial epithelial cells, as well as, concluding that baseline mitochondrial function was different in different cell types (Thangboonijt, 2015). Based on this study, future studies will need to confirm the results seen in this study are also observed in primary lung epithelial cells.

In conclusion, our study has shown that exposure to currently available JUUL Menthol flavor pod exposure results in an alteration in mitochondrial respiration with a reduction in basal respiration and maximal respiration based upon the time post-exposure. Along with this, reduction in ETC subunits is seen based upon exposure to JUUL Menthol. Meanwhile,

exposure to currently available JUUL Virginia Tobacco pods did not result in an alteration in mitochondrial bioenergetics. This study implicates the need for more stringent regulation on e-cigarette flavoring products with new emerging products like bar-based disposable e-cigarettes still not regulated by federal or state governments.

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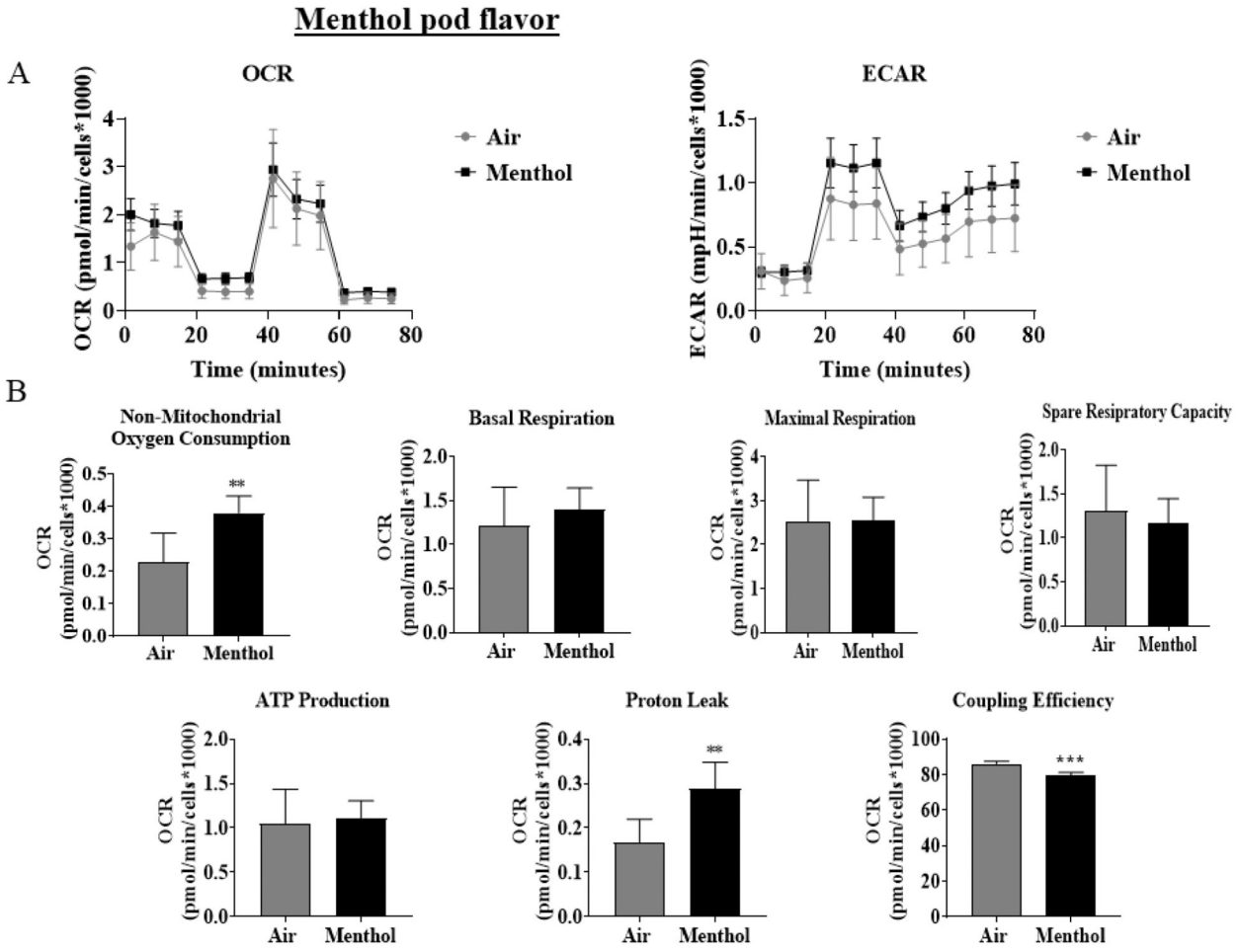
Highlights

- JUUL Menthol and tobacco flavored pod aerosols were tested on mitochondrial energetics
- Menthol flavored e-cigarette pods cause mitochondrial dysfunction
- Menthol pod exposure reduces basal and maximal respiration in mitochondria
- Virginia Tobacco pods exposure did not cause alteration to mitochondrial bioenergetics
- Menthol pod exposure reduces OXPHOS complexes



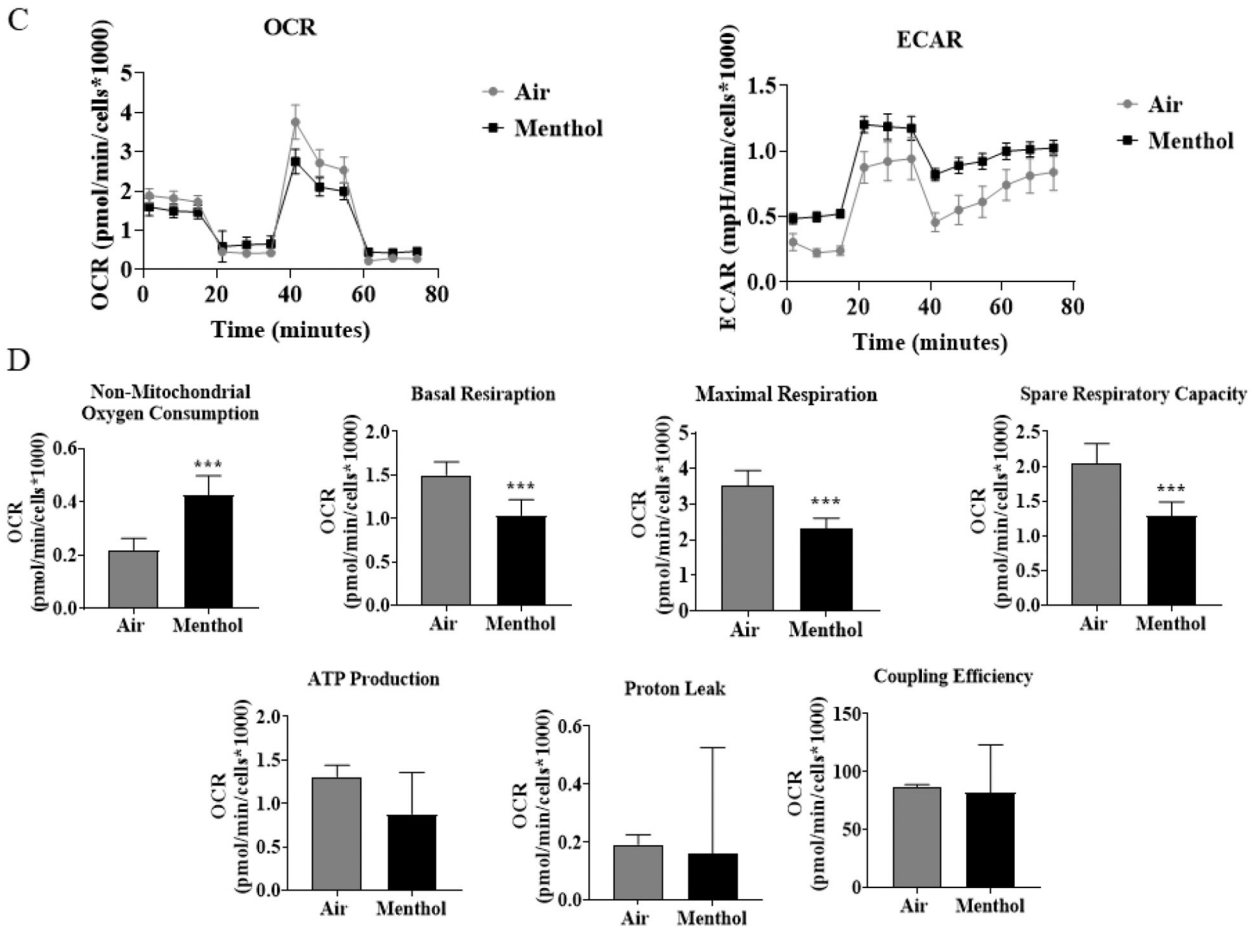
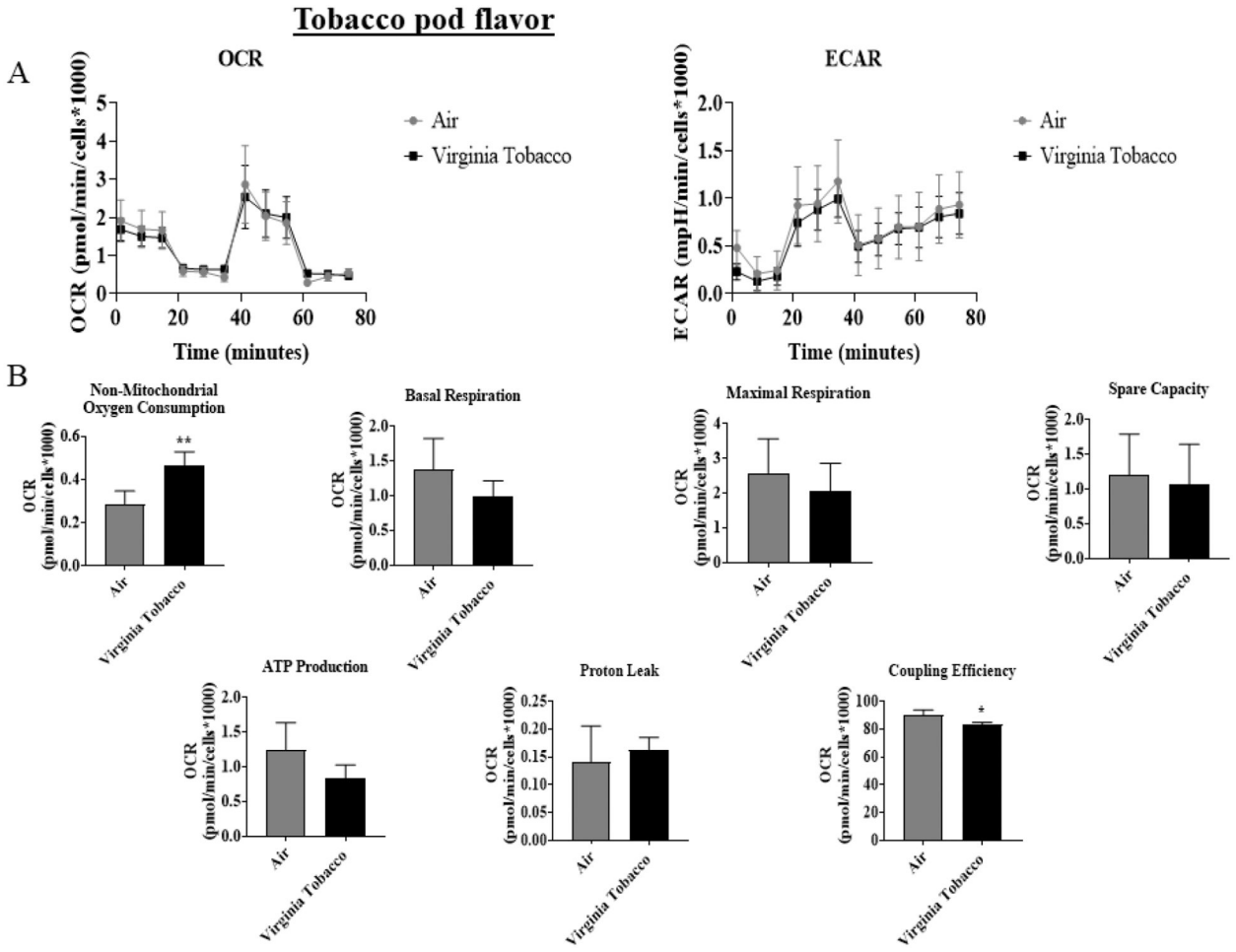


Figure 1: Menthol flavor pod exposure alter mitochondrial respiration in lung epithelial cells immediately and 24-hours post-exposure

Beas2b cells with 20,000 cells per seahorse well were grown to 90% confluency. Immediately after serum deprivation, the wells were exposed to a three-session exposure to JUUL Menthol pods with 12-hour intervals between sessions. (A) Immediate post final exposure graphs of oxygen consumption rate and extracellular acidification rate. (B) Immediate post final exposure values for mitochondrial respiration. ** $p < 0.01$, or *** $p < 0.001$ vs air exposed, unpaired t-test N = 6 wells per group. (C) 24 hours post final exposure graphs of oxygen consumption rate and extracellular acidification rate. (D) 24 hours post final exposure values for mitochondrial respiration. *** $p < 0.001$ vs air exposed, unpaired t-test N = 6 wells per group.



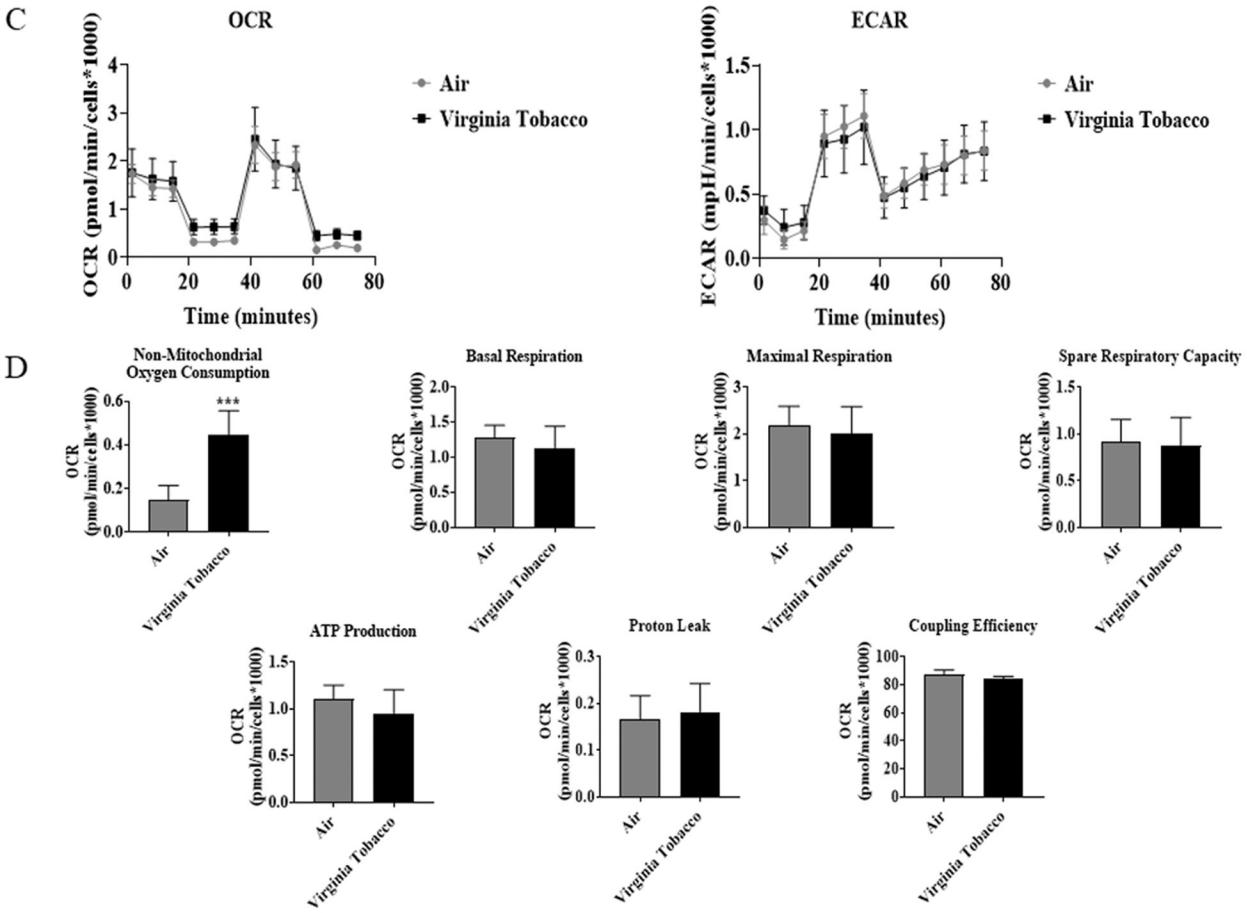


Figure 2: Virginia Tobacco pod exposure do not alter mitochondrial respiration in lung epithelial cells immediately or 24-hours post-exposure

Beas2b cells with 20,000 cells per seahorse well were grown to 90% confluency. Immediately after serum deprivation, the wells were exposed to a three-session exposure to JUUL Virginia Tobacco pods with 12-hour intervals between sessions. (A) Immediate post final exposure graphs of oxygen consumption rate and extracellular acidification rate. (B) Immediate post final exposure values for mitochondrial respiration. * $p < 0.05$, or ** $p < 0.01$ vs air exposed, unpaired t-test N = 6 wells per group. (C) 24 hours post final exposure graphs of oxygen consumption rate and extracellular acidification rate. (D) 24 hours post final exposure values for mitochondrial respiration. *** $p < 0.001$ vs air exposed, unpaired t-test N = 4–6 wells per group.

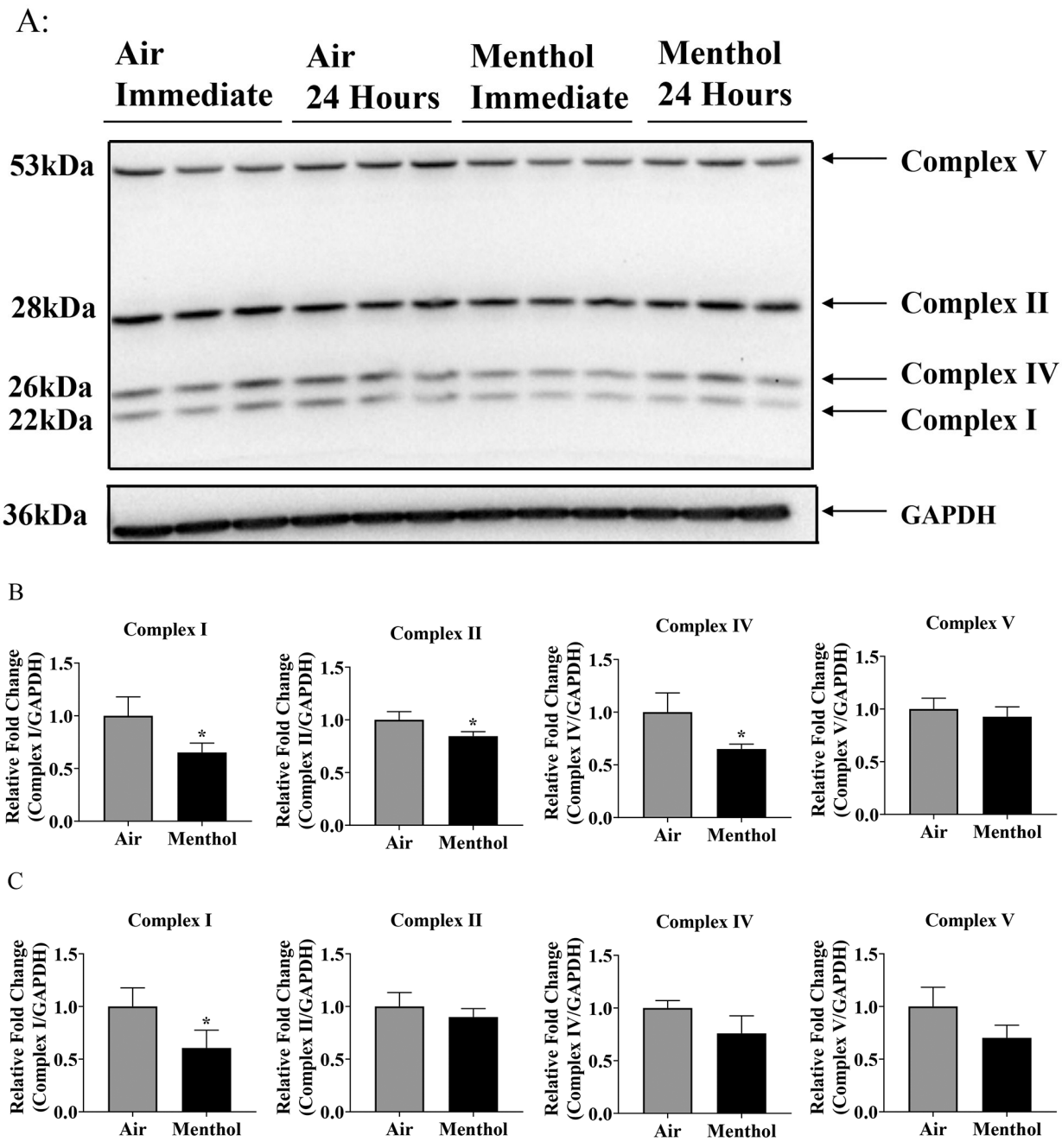


Figure 3: Menthol pod exposure alter ETC protein levels

Beas2b cells with 300,000 cells per well were grown to 80% confluency. Immediately after serum deprivation the wells were exposed to a three-session exposure to JUUL Menthol pods with 12-hour intervals between sessions (A) Image of the western blot for human total OXPHOS protein and GAPDH. (B) Immediate post final exposure fold change of ETC protein levels. * $p < 0.05$ vs air exposed, unpaired t-test N = 3 wells per group. (C) 24 hours post final exposure fold change of ETC protein levels. * $p < 0.05$ vs air exposed, N = 3 unpaired t-test wells per group.

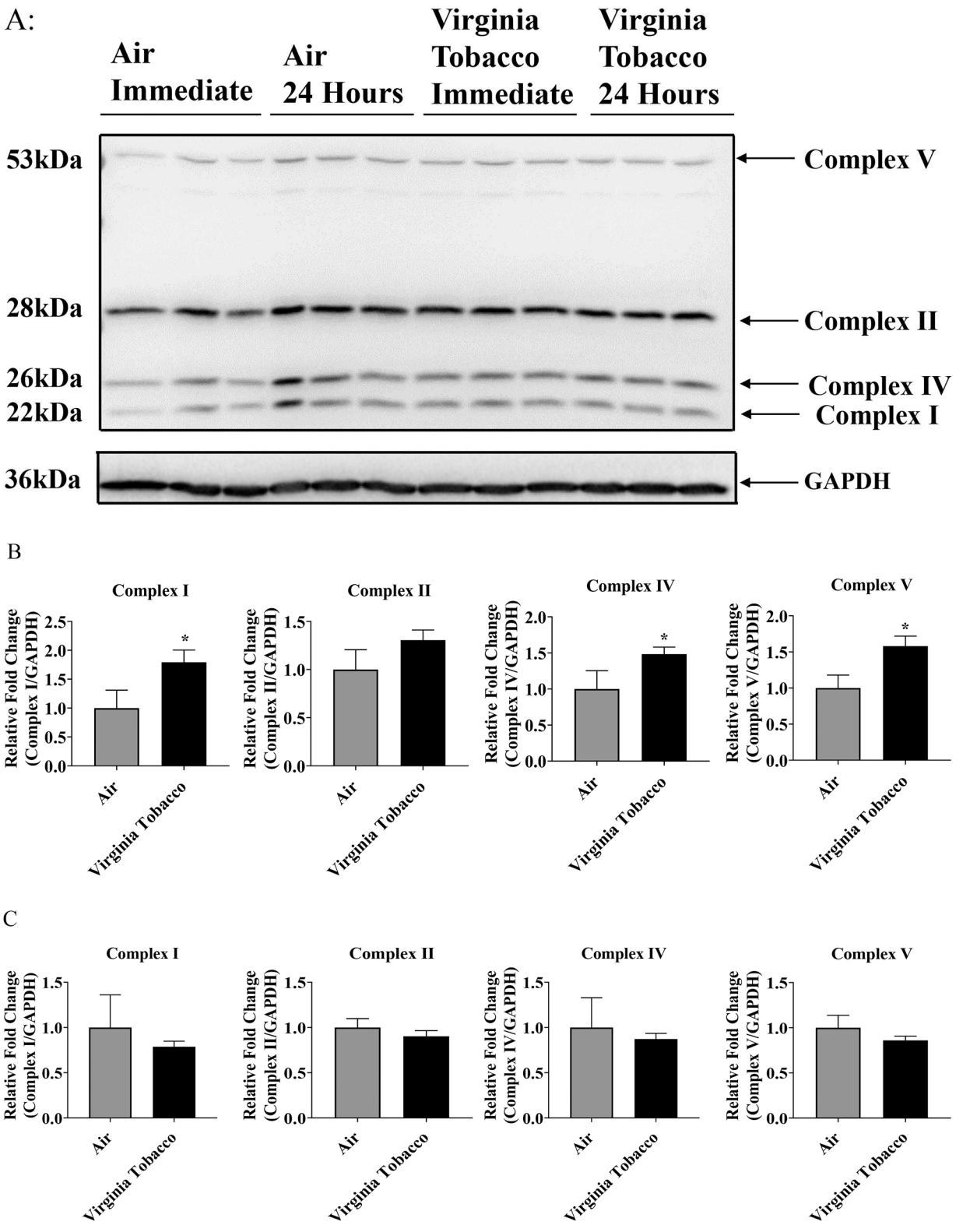


Figure 4: Virginia Tobacco pod exposure alter ETC protein levels

Beas2b cells with 300,000 cells per well were grown to 80% confluency. Immediately after serum deprivation the wells were exposed to a three-session exposure to JUUL Virginia Tobacco pods with 12-hour intervals between sessions (A) Image of the western blot for human total OXPHOS protein and GAPDH. (B) Immediate post final exposure fold change of ETC protein levels. * $p < 0.05$ vs air exposed, unpaired t-test N = 3 wells per group. (C) 24 hours post final exposure fold change of ETC protein levels. Unpaired t-test N = 3 wells per group.

Table 1:

Chemical Composition of Menthol flavor pod-based Aerosol

Chemical Compounds	Functional Group	Average Concentration (μg/m^3)
Dichlorodifluoromethane	Alkyl Halide	2.45
Ethanol	Alcohol	3300
1,4-Dioxane	Ether	8.5
Toluene	Benzene Ring	23
Ethylbenzene	Benzene Ring	25.5
Styrene	Alkene/Benzene Ring	56.5
Sulfur Dioxide	Sulfide	>34.5
Acetaldehyde	Aldehyde	18
2-Methylbutane	Alkane	420
n-Pentane	Alkane	1250
tert-Butanol	Alcohol	7.8
1-Propanol	Alcohol	6
Cyclopentane	Alkane	140
Trimethylsilanol	Alcohol	70.5
Dimethyl ester carbonic acid	Ester	16
Propylene Glycol	Alcohol	2400
2-Ethyl-1-hexanol	Alcohol	3.45
2-(2-Ethoxyethoxy)Ethanol	Ether/Alcohol	17
Unknown Siloxane	Siloxane	12.5
Levomenthol	Alcohol	3 60

Aerosol collection occurred from 30 puffs of Menthol flavor pods in a 1L vacuum bottle and analyzed by ALS environmental. Average concentration of chemical compounds was obtained from two separate aerosol collections.

Table 2:

Chemical Composition of Virginia Tobacco flavor pod-based Aerosol

Chemical Compounds	Functional Group	Average Concentration (μg/m^3)
Propene	Alkene	3.35
Dichlorodifluoromethane	Alkyl Halide	2.25
Ethanol	Alcohol	9850
2-Propanol	Alcohol	6.5
Toluene	Benzene Ring	23
Ethylbenzene	Benzene Ring	33.5
Styrene	Alkene/Benzene Ring	99
Sulfur Dioxide	Sulfide	>38.5
Acetaldehyde	Aldehyde	23.5
2-Methylbutane	Alkane	220
n-Pentane	Alkane	675
tert-Butanol	Alcohol	6
Cyclopentane	Alkane	81
Trimethylsilanol	Alcohol	16
Dimethyl ester carbonic acid	Ester	16
1-Hydroxy-2-propanone	Ketone/Alcohol	7
2,2,4-Trimethyl-1,3-dioxolane	Ether	10.35
Propylene Glycol	Alcohol	3350
2-Hydroxy-, Ethylpropanoic Acid	Ester/Alcohol	30.5
2,2-Dimethyl-1,3-dioxolane-4-methanol	Alcohol/Ether	10.3
unknown Siloxane	Siloxane	56
Levomenthol	Alcohol	285

Aerosol collection occurred from 30 puffs of Virginia Tobacco flavor pods in a 1L vacuum bottle and analyzed by ALS environmental. Average concentration of chemical compounds was obtained from two separate aerosol collections.