Nrf2 Responses in a 3D Human Airway Model Exposed to Whole Aerosol from Combustible Cigarettes or Heated Tobacco Products

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Abstract

The nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway, activated in human lung cells by cigarette smoke, regulates genes involved in the antioxidative stress response. Here, we evaluated whole smoke/aerosol from two marketed combustible cigarettes (CC), 1R6F reference cigarette, four HTP (glo™) styles, and a marketed HTP comparator on cell viability and Nrf2 response in a 3D human airway model (EpiAirway™) transfected with a luciferase Nrf2 promoter.

EpiAirway™ tissues were exposed at the air liquid interface to whole smoke or aerosol. Eighteen hours post-exposure, luciferase activity and cell viability were measured. Relative luciferase activity was expressed as fold change over the air exposed control. Post-exposure, whole smoke/aerosol deposition was quantified using chemical analysis (e.g., glycerol, nicotine, carbonyls).

Differential Nrf2 activation was observed following exposure to CC whole smoke compared to the glo™ and the marketed comparator HTP aerosols. Moreover, the minimum exposure-correlated nicotine concentration required to induce a >2-fold increase (threshold response) in Nrf2 activation was >30x lower for CC than the HTPs.

These data show that the 3D Nrf2 EpiAirway™ in vitro model can be used to assess and discriminate responses for a biomarker (oxidative stress) from disease pathways associated with smoking (e.g., respiratory and cardiovascular disease).

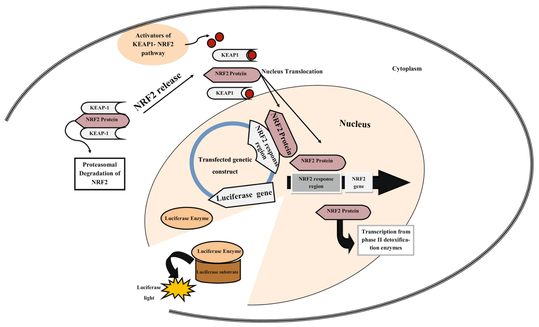

Figure 1: Schematic of the Nrf2 pathway with luciferase linked Nrf2 gene expression. Adapted from Mozaheb et al., 2019

References

- ISO 3204:1999. Tobacco and tobacco products – Atmosphere for conditioning and testing (4th edition)
- ISO 20778:2018. Cigarettes – Routine analytical cigarette smoking machine – Definitions and standard conditions with an intense smoking regime (1st edition)
- Mozaheb et al., Scientific Reports, 9: 3248, 2019

Results

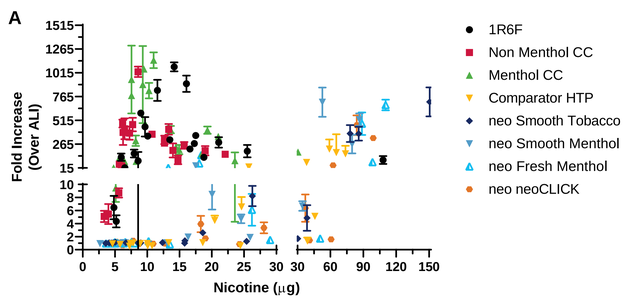

Figure 2. **Nicotine concentration to induce >2-fold Nrf2 activation >30x lower for combustible cigarettes than HTPs.** (A) EpiAirway™ Nrf2 tissues were exposed to whole smoke or aerosol for 24-27 minutes, then allowed to recover for 18 hours. Following recovery cells were lysed for the determination of Nrf2 linked luciferase activity. (B) EpiAirway™ Nrf2 tissues were exposed to assay positive controls for each exposure: 0.5% triton X, water ($CoCl_2$ vehicle), $CoCl_2$, 0.5% DMSO/PBS (t-BHQ vehicle), or *tert*-butylhydroquinone (t-BHQ) for 18 hours. Cells were then lysed for the determination of Nrf2 linked luciferase activity. Data are presented as mean ± SD, triplicate tissues, n=3 (A) or n=24 (B). ALI; air liquid interface

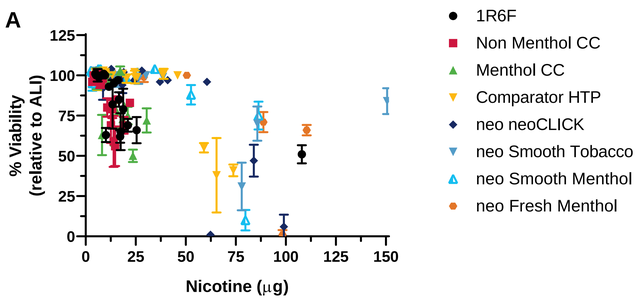

Figure 3. **Viability of HTPs tissues higher than combustible cigarette tissues at similar nicotine concentrations.** (A) EpiAirway™ Nrf2 tissues were exposed to whole smoke or aerosol for 24-27 minutes, then allowed to recover for 18 hours. Following recovery, LDH release into the basolateral media was measured. (B) EpiAirway™ Nrf2 tissues were exposed to assay positive controls for each exposure: 0.5% triton X, water ($CoCl_2$ vehicle), $CoCl_2$, 0.5% DMSO/PBS (t-BHQ vehicle), or *tert*-butylhydroquinone (t-BHQ) for 18 hours. LDH release into the basolateral media was measured following the exposure. Data are represented as mean ± SD, triplicate tissues, n=3 (A) or n=24 (B). ALI; air liquid interface

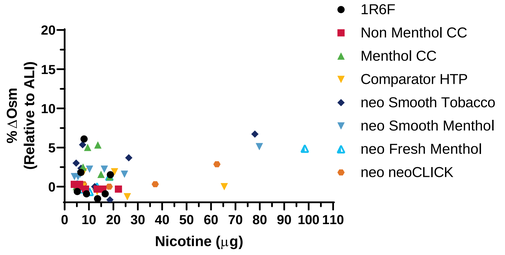

Figure 4. **Minimal changes in osmolality following whole smoke or aerosol exposure.** Osmolality was measured from basolateral media in the exposure well following whole smoke or aerosol exposure (24-27 min). Percent change in osmolality vs. nicotine exposure concentration measured in dosimetry well was calculated. Data are represented as mean. ALI; air liquid interface

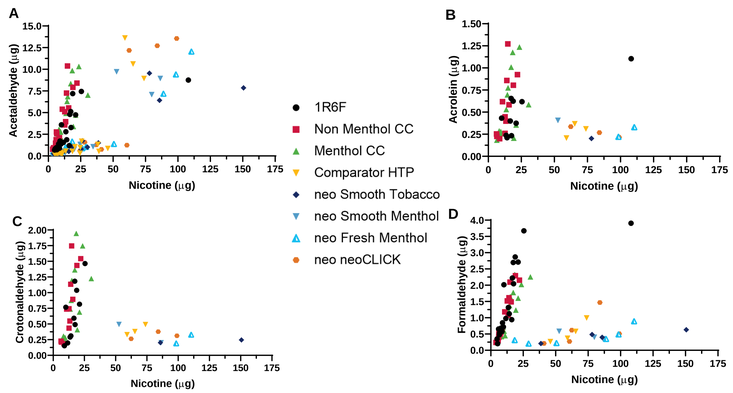

Figure 5. Higher concentrations of carbonyls measured following combustible cigarette exposure than HTP exposure. Levels of four carbonyls (µg) versus delivered nicotine (µg) in whole smoke and aerosol exposures. Acetaldehyde (A), Acrolein (B), Crotonaldehyde (C) and Formaldehyde (D) trapped in CMF-PBS were quantified to confirm delivery of gas phase constituents at the ALI. Carbonyls in CMF-PBS were DNPH-derivatized and quantified by HPLC/MS.

Materials and Methods

3D Tissue Model: EpiAirway™ tissues comprised of normal, human-derived tracheal/bronchial cells that have been cultured to form a highly differentiated model and transfected with a Nrf2 lentiviral luciferase reporter were obtained from MatTek, Inc. Tissues were maintained at the air-liquid interface according to the manufacturer's guidelines.
Test Articles: Market combustibles, HTP, and glo™ products were obtained by RAI Services Company. 1R6F cigarettes were obtained by Labcorp. Test articles were conditioned in accordance with ISO 3402:1999 prior to each experiment.
Whole Smoke/Aerosol Generation: Whole smoke and aerosol were generated using a Vitrocell® VC10® Smoke Exposure System. The tissues were exposed to whole smoke from market combustibles and 1R6F generated under ISO 20778 (2018) regime (55 mL volume, 2 sec duration, 30 sec puff interval, 100% vent blocking) with 20 mL/min vacuum for 24 or 27 minutes (48 or 54 total puffs). Tissues exposed to whole aerosol from glo™ and HTP products whole aerosol generated under an ISO or modified ISO 20778 (2018) (55 mL volume, 3 sec duration, 30 sec puff interval, no vent blocking) for a total of 24.5 or 26 minutes (49 or 52 puffs), respectively. Three exposure wells in the module contained tissues and the fourth, a dosimetry trap, contained 0.9 mL PBS.
Chemical Controls: Airway irritants 0.05% $CoCl_2$, *tert*-butylhydroquinone (t-BHQ; 250 or 500 µM), water (vehicle), or PBS/0.5% DMSO (t-BHQ vehicle) were added apically to EpiAirway™ tissues for 18 hours.
Luciferase Activity: The amount of luciferase activity was measured 18 hours post-exposure using the ONE Glo™ Luciferase Report Assay System according to the manufacturer's instructions (Promega, UK).
LDH Release: The lactate dehydrogenase (LDH) assay was performed according to the manufacturer's instructions (Takara Bio). LDH activity was determined by measuring the optical density of the sample at 490 nm.
Osmolality Determination: Duplicate osmolality measurements were obtained by sampling from the PBS dosimetry trap following each exposure using a Fiske 2020 osmometer which was calibrated prior to each use.
Nicotine Determination: Samples from the PBS dosimetry trap were analyzed using a LC-MS/MS. The linear range of the method was 0.08 to 50 µg/mL. The limit of detection and limit of quantification were 0.026 and 0.08 µg/mL, respectively.
Statistical Analysis: Linear interpolation was used to determine the lowest nicotine concentration to induce a 2-fold increase (SAS).

Summary and Conclusions

- **Apical application of chemicals known to induce Nrf2 luciferase-linked expression in the lung ($CoCl_2$, t-BHQ) elicited a 5 to 200-fold increase, with a dose dependent increase seen for t-BHQ (Figure 2B). No impact on cell viability was observed following any of the chemical exposures (Figure 3B).**
- **Whole smoke from the market CCs caused an increase in Nrf2 luciferase-linked expression with a peak response of 1150 or 1030-fold at 0.55 or 1.36 µg nicotine, respectively (Figure 2A).**
- **A minimum of a 30x difference in nicotine concentration was required to induce a 2-fold increase in Nrf2 luciferase-linked expression between the market CCs and the HTP products (Figure 3A).**
- **A statistically ($p<0.02$) higher nicotine concentration was required from glo™ to induce a 2-fold increase in Nrf2 when compared to their respective market combustible.**
- **The results suggest that glo™ with less oxidative stress induction could lead to reduced lung disease compared to combustible cigarettes.**
- **The Tobacco Harm Reduction paradigm for Next Generation Tobacco Products places combustible cigarettes as the most harmful. Results from this study add to the weight of evidence that would place HTPs downstream of combustible cigarettes along this spectrum of potential harm.**