Nrf2 Response to Whole Smoke in Three Dimensional (3D) Airway Cultures

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Abstract #24

Abstract

Lung diseases are frequently accompanied by molecular changes, including those associated with the Nrf2 signaling pathway. Cigarette smoke has been shown to activate this pathway in lung tissue. The goals of this study were to assess the effect of cigarette smoke on the Nrf2 promoter and on genes associated with oxidative stress, inflammation, and metabolism in human 3D EpiAirway™ tissue models (MatTek, Inc.). Whole smoke (WS) exposures (8 – 64 minutes; 8 minutes/cigarette) with Kentucky Reference 3R4F cigarettes were conducted under ISO conditions using the VITROCELL VC1® smoke exposure system (VC1). Viability and tissue integrity were assessed with the lactase dehydrogenase and transepithelial electrical resistance (TEER) assays, respectively. Nrf2 promoter activation was determined by a luciferase assay, while gene expression changes were assessed via QRT/PCR at 6, 12, 18, or 24 hours post-exposure.

Dose-dependent decreases in viability and tissue integrity were observed. Cell viability was ≥ 90% for exposures up to 32 minutes (4 cigarettes), while a maximum response of 38% viability and diminished TEER were observed for the 64 minute (8 cigarettes) treatment group. Therefore, luciferase and gene expression studies were conducted between 8 and 32 minutes. Time- and dose-related increases in Nrf2 promoter activation were observed with levels exceeding 200-fold at the 12 and 18 hours post-exposure time-points, and the Nrf2 promoter was also differentially regulated in WS as compared to gas vapor phase exposures. Statistically significant increases ($p<0.05$) ranging from 2 to >100-fold were observed across the time-course for genes associated with oxidative stress, inflammation, and metabolism.

Collectively, the data indicate that putative biomarkers of effect in the Nrf2 signaling pathway were responsive to cigarette smoke exposures in human 3D EpiAirway™ tissue models. These models may be useful in evaluating tobacco and aerosol exposures and may further understanding of the biological relevance of the responses.

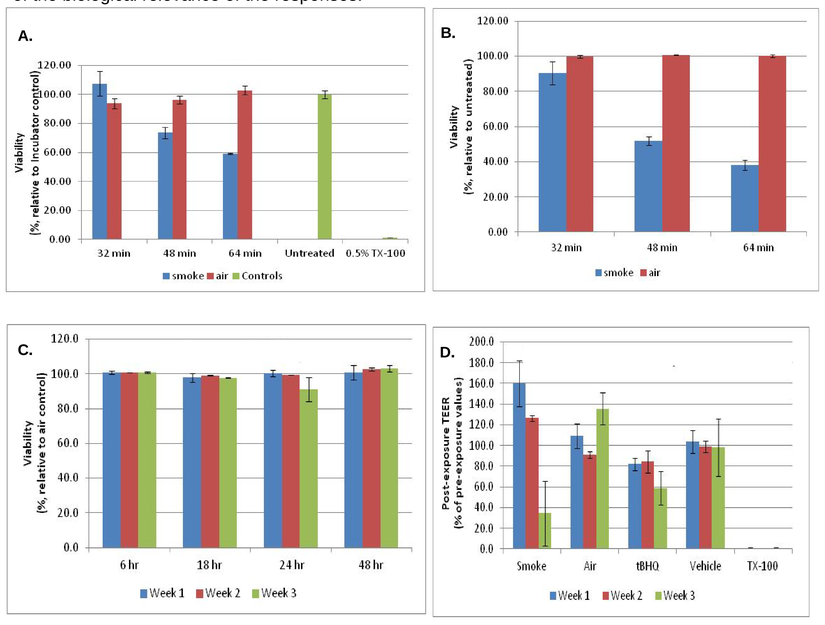

Figure 1 (A-B). Viability and Membrane Integrity Testing via MTT (Panel A), LDH (Panels B - C) and TEER (Panel D). Tissues were exposed to smoke or clean air for 32, 48, or 64 Minutes (Panels A - B) and for 32 min only (Panels C - D) then harvested after 24 hr (Panels A, B and D) and at the indicated times post-exposure of 6, 18, 24, 32, or 48 hr (Panel C). Viability (+/- standard deviation) is presented relative to air or incubator controls for smoke and air samples (N=3). Studies were conducted over three independent weeks. Positive and negative controls were 0.5 % Triton X-100 and PBS, respectively. MTT: 3-(4,5 Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide; LDH: lactase dehydrogenase; TEER: transepithelial electrical resistance

Results

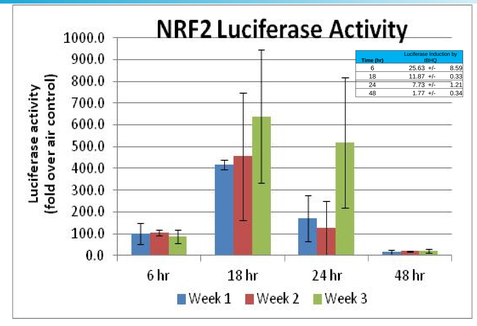

Figure 2. Luciferase Testing. Tissues were exposed to smoke or clean air for 32 minutes and harvested after the indicated post-exposure times (6, 18, 24, or 48 hr). Luciferase activity (+/- standard deviation) is presented relative to air control for smoke and air samples and vehicle control for the positive control (N=3). Studies were conducted over three independent weeks. Positive control assessment (inset) of Nrf2 luciferase activity was induced with 500 µM tert-butylhydroquinone (tBHQ) dissolved in 0.5% DMSO. Controls were maintained on the tissues throughout the exposure period including time to post-exposure harvest.

Table 1: Assessment of Whole Smoke, Gas Phase Only, and Vacuum Rate on Nrf2 Activation

Vacuum (ml/min)	Condition	Mean Luciferase Fold Change Compared to Air Control[1]
5	Whole Smoke	5.1 +/- 0.9
5	Gas Phase	1.2 +/- 0.06
20	Whole Smoke	337 +/- 44.7
20	Gas Phase	9.1 +/- 1.4

[1]Data is representative of 32 minute 3R4F exposures at 0.5 L/min dilution. Luciferase activity was assessed 18 hr post-exposure, and the mean fold change +/- standard error of mean (N=3) is represented. Viability of whole smoke or gas phase exposed tissues, relative to matched clean air controls, remained above 95% for all exposures.

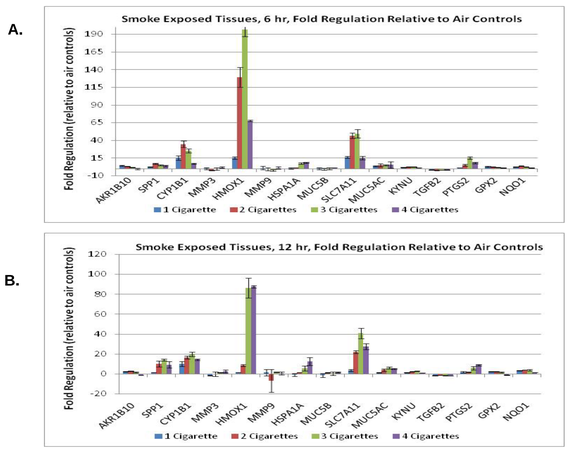

Figure 3 (A-B). Gene expression regulation 6 and 12 hours post exposure to cigarette smoke, relative to clean air controls. Average fold regulation +/- standard deviation (N=3) is depicted for each target gene analyzed. Expression values of each gene were normalized to GAPDH expression for the respective sample and fold regulation was subsequently calculated relative to air controls.

Materials and Methods

Cigarette Smoke Generation: Whole smoke from Kentucky Reference 3R4F cigarettes was generated with a VITROCELL VC1® Smoke Exposure System (1) . The tissues were exposed to 3R4F cigarette smoke using the ISO regime with 20 ml/min vacuum and 0.5 L/min dilution for 32, 48, or 64 minutes. In each exposure, 3 tissues were exposed to smoke or gas vapor phase and 3 were exposed to clean air only (negative controls). For WS versus gas phase assessments, tissues were exposed to 3R4F cigarette smoke using the ISO regime with 20 or 5 ml/min vacuum and 0.5 L/min dilution for 32 minutes, and a Cambridge filter pad was placed inline for gas phase exposures.

3D Cell Models: EpiAirway™ and stably transduced Nrf2 EpiAirway™ cells were obtained from MatTek, Inc. Three–dimensional cultures were prepared from primary human tracheal-bronchial epithelial cells, grown on a collagen coated microporous membrane, cultured at air-liquid interface to generate a pseudostratified morphology. The reporter model was generated by stable transduction of the primary cells with a lentiviral vector, antibiotic selection, and subsequent characterization before differentiation into the 3D culture/tissue.

Cytotoxicity and Membrane Integrity: The tissues were exposed to 3R4F cigarette smoke as described above. In each exposure, 3 tissues were exposed to smoke and 3 were exposed to clean air only (negative controls). The apical surfaces of EpiAirway™ tissues were rinsed with PBS and TEER was measured. The TEER was read immediately after exposure and then after a 24 hour post-exposure period. Additional controls included tissues treated apically with 100 µl 0.5% Triton X-100 (positive control for tissue death) and incubator control tissues that were not exposed to the Vitrocell exposure conditions. Viability was determined by LDH release at 6 and 24 hours post exposure as well as by **3-**(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) after the 24 hour post-exposure period. The smoke exposures were repeated 3 weeks in a row with N=3 for each treatment group per week to determine the reproducibility of the results.

Luciferase: Tissues were exposed to 3R4F cigarette smoke using the ISO regime for 32 min as described above. Negative control tissues were exposed to clean air only, and positive control tissues were exposed to tert-butylhydroquinone (tBHQ) to assess control-induction of NRF2 activity. Additional controls included tissues treated with vehicle (0.5% DMSO for t-BHQ). Tissues were harvested and luciferase activity measured using ONE-Glo (Promega, Madison, WI) tissue lysis buffer and luciferase reagent at 6, 18, 24, or 48 hours post exposure.

Gene Expression: RNA was isolated, quantified, and qualified using BioRad's Experion automated electrophoresis unit, and reverse transcribed to cDNA prior to qPCR analysis. Custom PCR arrays from SA Biosciences (Qiagen) and the SA Biosciences web based RT2 Profiler PCR Array Data Analysis software version 3.5 was used for gene expression analysis with the raw data (Ct values). Results containing average Ct, Average Delta(Ct), and 2^(-Avg.(Delta(Ct)) were calculated.

Summary

- The EpiAirway model responds to whole smoke in a dose- and time-dependent manner (Figure 1A - B).
- Cell viability (Figure 1C) was ≥ 90% for exposures up to 32 minutes (4 cigarettes), while a maximum response of 38% viability (Figure 1A-B) and diminished TEER were observed for the 64 minute (8 cigarettes) treatment group.
  - Therefore, whole smoke exposures for luciferase and gene expression studies were conducted between 8 and 32 minutes. Data presented herein for luciferase and gene expression analyses represent results at the designated post-exposure times following the maximum whole smoke exposure of 32 minutes.
- Time- and dose-related increases in Nrf2 promoter activation were observed with levels exceeding 200-fold at the 12 and 18 hours post-exposure time-points (Figure 2). The decline after 18 hours is evident of typical temporal regulation of the promoter and not toxicity as evidenced by the data in Figure 1C.
- The Nrf2 promoter was differentially regulated in WS as compared to gas vapor phase exposures (Table 1)
- Statistically significant increases ($p<0.05$) ranging from 2 to >100-fold were observed across the time-course for genes associated with oxidative stress, inflammation, and metabolism (Figure 3).
  - Consistent with the activation of the Nrf2 promoter, the Nrf2 responsive genes (i.e. HO-1, SLC7A11, NQO1, CYP1B1) in this assessment were highly induced.

References and Acknowledgements

1)Vitrocell® VC1 Smoking Machine Overview (http://www.vitrocell.com)

❖ **The authors acknowledge MatTek Corporation , Inc. for conducting the contracted research.**