Transcriptomic Response of Primary Human Bronchial Cells to Repeated Exposures of Cigarette and ENDS Preparations

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Abstract

Cigarette smoke deregulates several biological pathways by modulating gene expression in airway epithelial cells and altering the physiology of the airway epithelium. The effects of repeated exposures to the aerosol of electronic nicotine delivery systems (ENDS) on gene expression in airway epithelium are relatively unknown. In order to assess the effect of repeated exposures of ENDS, primary normal human bronchial epithelial (NHBE) cells grown at the air-liquid interface (ALI) were exposed to cigarette and ENDS preparations daily for 10 days. Whole-smoke conditioned media from 3R4F reference cigarettes and aerosol conditioned media from commercially available e-liquid (1.8% nicotine; weight/volume) were prepared by bubbling mainstream smoke or aerosol through RPMI 1640 cell culture medium, respectively. Transcriptomic analysis was performed using next-generation sequencing. Exposure to cigarette smoke preparations significantly altered gene expression in a dose-dependent manner compared to vehicle control, including genes linked to oxidative stress, xenobiotic metabolism, cancer pathways, epithelial-mesenchymal transition, fatty acid metabolism, degradation of collagen and extracellular matrix, O-glycosylation, and chemokines/ cytokines, all of which are known pathways found to be altered in smokers. Conversely, ENDS preparations had minimal effect on transcriptional pathways. This study revealed that a sub-chronic exposure of primary NHBE cultures to cigarette and ENDS preparations differentially regulated genes and canonical pathways, with the minimal effect observed with ENDS preparations compared to cigarette preparations. This study also demonstrates the versatility of primary NHBE cultures at ALI to evaluate repeat-dose exposures of tobacco products.

Materials and Methods

Cell Culture: Primary NHBE cells (three donors; without identifiers and therefore exempt status from the Institutional Review Board) were provided by Nationwide Children's Hospital Epithelial Cell Core (Columbus, OH) (S1 Table for donor information). Passage 1 primary NHBE cells were seeded on collagen type IV (0.3mg/mL; Sigma Aldrich, Saint Louis MO) coated Corning™ 6.5mm 24-well Transwells (Fisher Scientific, Waltham MA) and grown at the air-liquid interface (ALI) with PneumaCult™ ALI medium (StemCell Technologies Inc., Tukwila WA).

Cigarette and ENDS Preparations: Whole-smoke conditioned-media (WS-CM) from 3R4F cigarettes was prepared by bubbling mainstream smoke through RPMI 1640 media and stored as aliquots at -80°C. ENDS preparations (aerosol conditioned media; ACM) was prepared by bubbling aerosol generated from commercially available e-liquid (1.8% nicotine; weight/volume) into RPMI 1640 cell culture medium. The final nicotine content of the WS-CM and ACM was used to normalize exposure of cells and is expressed as µg/mL Equi-Nicotine (Eq-Nic.) units.

Exposure Design: WS-CM and ACM preparations were diluted in Hank's Balanced Salt Solution (HBSS) to various concentrations of nicotine (Eq-Nic). Exposure concentrations were chosen based on our previously reported investigation into cytotoxicity of WS-CM and ACM on primary NHBE cultures [1]. Vehicle control treatments included diluted RPMI 1640 medium in HBSS. All treatments of cells were performed on separate plates to minimize cross-exposure. Primary NHBE cultures were fully-differentiated after 4 weeks at ALI, as determined by optimal trans-epithelial electrical resistance (TEER) and the presence of ciliated cells and mucus production. Fully differentiated primary NHBE cultures at 4 weeks ALI (28 days) were exposed apically to either WS-CM or ACM preparations or vehicle control (100µL volume) for 1 hour per day 8 times over a 10-day period (Fig. 1). Doses were chosen based on non-cytotoxic levels after sub-chronic exposures that we observed from previously published data [2]. Two concentrations, low and high (3.5 and 7.0µg/mL Eq-Nic, respectively), were designated for WS-CM and ACM. All exposures were performed in duplicates, with cultures from three donors.

Schematic diagram of the experimental design used in this study

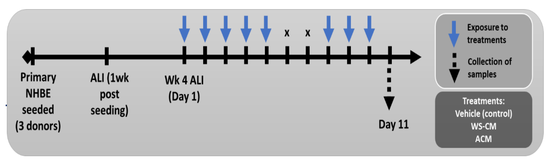

Figure 1. Fully differentiated primary NHBE cultures grown at ALI were exposed to low (3.5µg/mL Eq-Nic) and high (7.0µg/mL Eq-Nic) doses of WS-CM, ACM, or vehicle (control) for 1 hour per day. NHBE cultures were exposed over 10 days (total of 8 exposures) and RNA was extracted from the cultures 24 hours after the last exposure. "x" indicates no exposure.

Materials and Methods

RNA isolation and gene expression: At the end of experiments, total RNA was isolated using the RNeasy Micro Kit (#74004 Qiagen, Germantown MD). Input RNA quality and quantity were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA) and Qubit Fluorometer (Thermo Fisher), respectively. Samples with RNA integrity number (RIN) values greater than 7 and RNA concentration greater than 100ng/µL were sent for sequencing. Messenger RNA (mRNA) sequence libraries were generated with NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (NEB #E7760L) and NEBNext® Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) with an input amount of 200ng total RNA per sample. Libraries were pooled and sequenced on an Illumina NovaSeq SP flowcell in paired-end 150bp format (Illumina, San Diego CA) to read yield between 35 – 40 million reads.

Next Generation Sequence data analysis: All RNAseq datasets (fastq.gz format) were aligned and quantified.

- Co-expression analysis was performed using QUBIC R package. IRIS-EDA webserver was used to perform a cell-gene metric quality check and Principal Component Analysis (PCA). PCA was applied to the sample set to visualize the existence of a potential batch effect. The first two Principal Components were plotted with samples colored by the donor information, treatment type (WS-CM, ACM, and nicotine), and treatment doses (low and high), and the grouping of samples by color was considered as evidence of a batch effect. Count outliers were examined by Cook's Distance in DESeq2 [3].
- Hierarchical clustering of the normalized count data was applied to identify the group samples based on their gene expression values. The sample level grouping results were further compared to the true metadata to find patterns between samples' gene expression values and the corresponding metadata.
- Differential expression (DE) analysis was performed to identify Differentially Expressed Genes (DEGs) among different comparisons (e.g., WS-CM vs Vehicle control, ACM vs Vehicle control, WS-CM vs ACM) using DESeq2 [3]. DEGs were defined as genes whose Benjamini–Hochberg adjusted p-value was less than 0.05, and absolute log2 fold-change greater than 1.5.
- Enrichment analysis was performed to analyze the identified DEGs using Enrichr v2.1 [4]. The databases for pathway mapping were KEGG_2019_Human, GO_Molecular_Function_2018, GO_Biological_Process_2018 and GO_Cellular_Component_2018 provided by Enrichr package. Further enrichment analysis of identified DEGs (log2 fold-change >0.58 and Benjamini-Hochberg adjusted p-values <0.05) was performed using Reactome [5].

Results

Repeated exposures of WS-CM and ACM exert a dose-dependent effect on gene expression

A

	WS-CM		ACM	
	Low	High	Low	High
	3.5µg/mL	7.0µg/mL	3.5µg/mL	7.0µg/mL
Upregulated	29	165	15	154
Downregulated	3	37	1	13

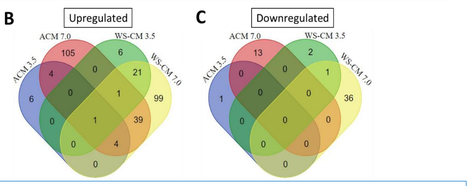

Figure 2: The number of differentially expressed genes (DEGs) in low and high concentrations of WS-CM and ACM. A) **Table 1.** Number of DEGs after treatment with WS-CM or ACM (Eq-Nic) compared to vehicle. B&C) Venn diagrams showing regulation of genes by low and high doses of WS-CM and ACM, as shown by B) upregulated and C) downregulated genes. DEGs were identified using p-value < 0.05 and log2 fold change > 1 or < -1 (fold change > 2 or < 0.25).

Common DEGs are shared between low and high concentrations of WS-CM and ACM

Treatment (µg/mL Eq-Nic)	Total	Genes
ACM (3.5, 7.0), WS-CM (3.5, 7.0)	1	ABCB6
ACM (3.5, 7.0), WS-CM (7.0)	4	LINC00339, AARSD1, MASP2, TEN1-CDK3
ACM (7.0), WS-CM (3.5, 7.0)	1	MSLNL
ACM (3.5), ACM (7.0)	4	CCDC150, INE1, SPACA4, YJEFN3
ACM (7.0), WS-CM (7.0)	39	STRC, CLMP, MLC1, TIAF1, MIRLET7D, EGFL8, LINC02449, GNRH1, LEAP2, RPS27AP5, SLC9A5, SIGLEC16, ZGLP1, MSH5, HYPK, HSF4, INHA, MALAT1, MLXIPL, PABPC1L, FOSB, FGF17, LMNTD2-AS1, MIR429, MAGEA6, OGFOD2, TNNI3, LOC100129175, PILRA, FKBP6, BBS1, NEAT1, NPR2, PDXDC2P-NPIPB14P, FLNB-AS1, LOC101927825, ARHGAP8, TBC1D3I, CHKB
WS-CM (3.5, 7.0)	22	SFRP2, PADI1, B3GNT6, SLC1A2, TMEM59L, BPIFA2, LUCAT1, LOC102724852, AKR1B10, H19, TNNT3, ALDH3A1, CEACAM5, GPX2, CYP1A1, AKR1B15, ME1, CYP1B1-AS1, FLNC, CYP1B1, LOC101927136, FN1*

Table 2. Up- and down-regulated (bold*) DEGs were identified using p-value < 0.05 and log2 fold change > 1 or < -1 (fold change > 2 or < 0.5). The doses used for WS-CM and ACM treatments are indicated in parentheses (3.5 and 7.0 µg/mL Eq-Nic).

Identification of significant DEGs by low and high concentrations of WS-CM and ACM

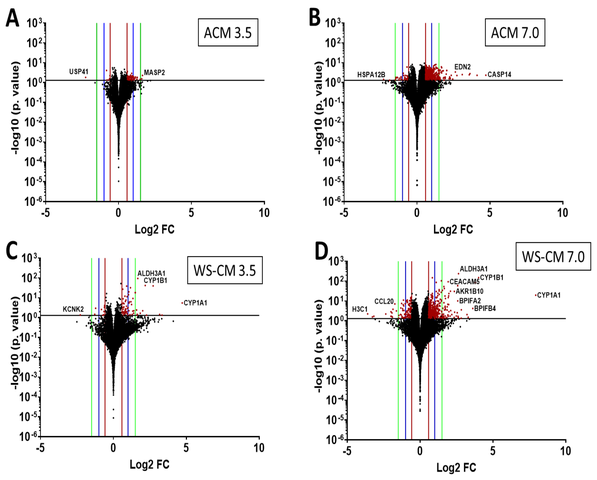

Figure 3: DEGs were regulated by A) ACM 3.5µg/mL Eq-Nic, B) ACM 7.0µg/mL Eq-Nic, C) WS-CM 3.5µg/mL Eq-Nic, and D) WS-CM 7.0µg/mL Eq-Nic. Significant DEGs were identified using p-value < 0.05 and log2 fold change > 0.58 or < -0.58 (red line), log2 fold change > 1 or < -1 (blue line), or log2 fold change > 1.5 or < -1.5 (green line).

DEGs identified with repeated exposures to WS-CM and ACM

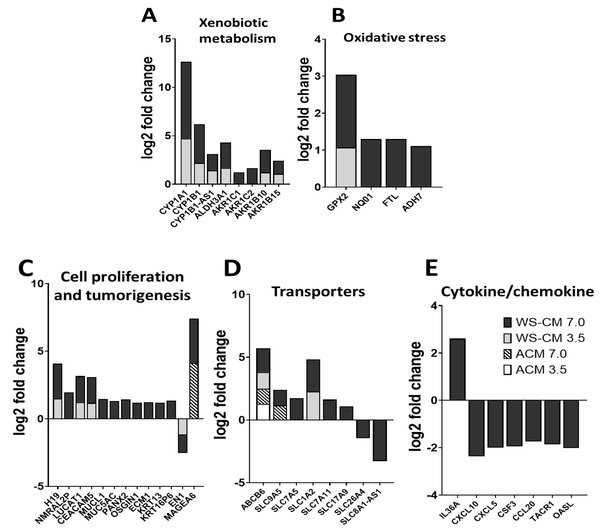

Figure 4: Differentially expressed genes representing specific biological pathways after repeated exposures to low and high concentrations of WS-CM and ACM. Selected DEGs are associated with A) xenobiotic metabolism, B) oxidative stress, C) cell proliferation and tumorigenesis, D) transporter proteins, and E) cytokine/chemokine signaling. Significant DEGs identified using p-value < 0.05 and log2 fold change >1 and <-1 (fold change > 2 or < 0.5).

Results

WS-CM and ACM exert a dose-dependent effect on gene expression

- Repeated exposures to WS-CM and ACM exerted changes in gene expression in a dose-dependent manner (**Figs. 2 and 3**).
- At both low and high doses, WS-CM altered expression of 32 and 202 total genes compared to Vehicle control, respectively, after 10 days exposure (**Fig. 2A**).
- Of the 32 genes regulated by a low dose of WS-CM, 23 genes were also regulated by a high dose of WS-CM (**Fig. 2B**).
- In comparison, 16 and 167 genes were significantly regulated by the low and high dose of ACM, respectively (**Fig. 2**).
- Only 9 genes were common between low and high doses of ACM.
- Exposure to WS-CM resulted in more upregulated than downregulated genes.
- Several of the same genes were identified between ACM and WS-CM exposures.
- ABCB6, which encodes a member of the ATP-binding cassette (ABC) transporter superfamily, was upregulated by all treatment exposures (WS-CM and ACM at both low and high doses) (**Table 2**).
- Thirty-nine genes were similarly regulated by high doses of ACM and WS-CM. The volcano plots further show the DEGs depending on the exposures using different fold change cut-offs (**Fig. 3**).

DEGs identified with repeated exposures to WS-CM and ACM

- Amongst the 202 genes significantly regulated by the high dose of WS-CM, and the 32 genes regulated by the low dose of WS-CM, several genes were of notable importance.
- Several genes associated with xenobiotic metabolism were altered by low and high doses of WS-CM with all the genes being upregulated (**Figs. 3D and 4A**).
- Amongst these were cytochrome P450 genes *CYP1A1, CYP1B1,* and AKR genes (high dose), none of which were regulated to the same extent by ACM (only *CYP1B1* detected).
- Oxidative stress markers were also significantly upregulated by a high dose of WS-CM, but not ACM, including *GPX2* (glutathione peroxidase 2), and *NQO1* (NAD(P)H quinone dehydrogenase 1) (**Fig. 4B**).
- Interestingly, a number of genes associated with cell proliferation and tumorigenesis were also significantly altered by the high dose of WS-CM, including *CEACAM5* (CEA cell adhesion molecule 5), *MUCL1* (mucin-like 1), *MUC5AC* (mucin 5AC) *KRT13* (keratin 13), *LUCAT1* (lung cancer-associated transcript 1), and *OSGIN1* (oxidative stress-induced growth inhibitor 1) (**Fig. 4C**).
- None of these genes were differentially regulated by either dose of ACM, except for *MAGEA6* (melanoma-associated antigen 6) which was upregulated by both high dose ACM and WS-CM.
- Several genes coding for transporter proteins were also significantly regulated by WS-CM, with *ABCB6* (ATP Binding cassette subfamily B member 6) and *SLC9A5* (Solute Carrier Family 9 member A5) being regulated by ACM also (**Fig. 4D**).
- Finally, several cytokine and chemokine genes were downregulated by WS-CM, but not ACM (**Fig. 4E**).

Conclusions

The transcriptional alterations induced by cigarette preparations reflect some of the physiological changes recently reported by our group after a 10-day exposure in primary NHBE cultures [5]. Conversely, ENDS preparations appear to regulate far fewer pathways compared to cigarette preparations, reflecting the minimal physiological changes previously reported [5]. This study also underlines the use of primary NHBE cultures at ALI as a suitable *in vitro* model for tobacco product evaluation, which reflects similar responses to those observed in humans.

References

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