Assessment of Mitochondrial Function Following Long-Term Treatment of Human Bronchial Epithelial Cells with Total Particulate Matter from a candidate Modified Risk Tobacco Product versus Cigarettes

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Introduction

Mitochondrial dysfunction caused by cigarette smoking is involved in driving the oxidative stress-induced physiology in airway diseases. Reduction of harmful and potentially harmful constituents (HPHCs) by heating rather than combusting tobacco could reduce the mitochondrial changes that contribute to oxidative stress and cell damage. We evaluated mitochondrial function in human bronchial epithelial cells (BEAS-2B) following a 1 or 12-week exposure to total particulate matter (TPM) from the aerosol of a candidate modified-risk tobacco product, the Tobacco Heating System 2.2 (THS2.2)[1,2], in comparison with TPM from the smoke of a 3R4F reference cigarette. The results show that alterations in oxidative phosphorylation are accompanied by increased oxidative stress and protein damage in cells exposed for 1 week to 3R4F smoke TPM or a 20-fold higher concentration of THS2.2 aerosol TPM, while a 12-week exposure resulted in decreased oxidative stress due to an increased efficiency of the antioxidant defense system.

Methods

Generation of TPM - Cigarette smoke from 3R4F cigarettes was generated on a 20-port Borgwaldt smoking machine and aerosol from THS2.2 was generated on a 30-port SM2000/P1 smoking machine according to the Health Canada Intense protocol[3]. Mainstream smoke from 3R4F cigarettes or aerosol from THS2.2 sticks were trapped on Cambridge glass fiber filters followed by extraction.
Cell Culture and Treatment - The human bronchial epithelial cell line BEAS-2B (LGC Standards) was grown in complete BEGM™ (Lonza) on collagen A-coated plates. Cells were exposed to 3R4F smoke TPM at a final concentration of 7.5 µg/mL, with THS2.2 aerosol TPM at final concentrations of 7.5, 37.5 and 150 µg/mL or vehicle control for a total treatment duration of 1 or 12 weeks.
Measurement of ATP level - BEAS-2B cells were evenly seeded on 96-well collagen I-coated plates. ATP level was measured with CellTiter Glo 2.0 Assay (Promega) according to kit manual using an Infinity multiplate reader (Tecan).
Measurement of oxygen consumption - Mitochondrial oxygen consumption rate in intact cells was measured with the use of a Clark electrode Model YSI 5300 (Yellow Springs Instrument Co). An equal amount of cells was transferred to the measurement chamber where the basal respiration rate was recorded in the presence of pyruvate (5 mM) (Sigma-Aldrich). Oligomycin (1 µM) (Sigma-Aldrich) was added to inhibit oxygen consumption related to the ATP synthesis and to measure proton leakage. Maximal respiration rate was started by adding 0.5 µM FCCP (Sigma-Aldrich).
Immunoblotting – BEAS-2B cells were resuspended in RIPA lysis buffer (Sigma-Aldrich) and incubated for 20 minutes on ice. The samples were supplemented with reducing Laemmli loading buffer (Sigma-Aldrich) and denatured at 95°C for 5 min. Equal amount of protein lysates were separated by SDS-PAGE, transferred to nitrocellulose or PVDF membrane and blocked in Odyssey Blocking Buffer (Li-Cor) for 1 h. Blots were incubated overnight with primary antibodies (NRF2 (Abcam), GPx1 (Cell Signaling Technology) and β-actin (Sigma-Aldrich)) at appropriate concentrations, washed with PBS-0.1 % Tween 20 (Sigma-Aldrich) and incubated with fluorescently-labeled secondary antibodies for 1 h. Membranes were visualized using an Odyssey infrared Imaging System (Li-Cor).
Detection of oxidatively modified proteins – The level of carbonylated proteins was estimated using the OxyBlot Protein Oxidation Detection Kit (Merck Millipore) according to the manufacturer's protocol and separated by a 10% SDS-PAGE. The DNP-derivatized proteins were detected by incubation with rabbit anti-DNP (1:150) and anti-rabbit antibody IRDye 800CW (1:5000; LiCor). Carbonylated proteins were visualized using an Odyssey infrared Imaging System (Li-Cor).
Measurement of ROS levels – ROS generation was determined using the MitoSox Red fluorescent probe (Invitrogen) according to the kit manual. The level of $O_2^{\bullet-}$ were measured on an iCys Laser Scanning Cytometer (CompuCyte Corporation).
Gene Expression Analysis - Total RNA was extracted from BEAS-2B cells using the miRNeasy Mini Kit with the QIAcube robot (QIAGEN, Hilden, Germany). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent), and high-quality RNA (RIN avg=9.9) was processed following a fully automated GeneChip® 3′ HT IVT Plus procedure prior to hybridization to HuG133.0 Plus 2.0 GeneChips (Affymetrix). Differential gene expression comparing 3R4F smoke or THS2.2 aerosol TPM-treated cells vs DMSO controls was computed with the limma R package employing a false-discovery rate (FDR) cut-off value of 0.05.

References

(1) Smith, Maurice R., et al. "Evaluation of the Tobacco Heating System 2.2. Part 1: description of the system and the scientific assessment program." *Regulatory Toxicology and Pharmacology* 81 (2016): S17-S26.
(2) Schaller, Jean-Pierre, et al. "Evaluation of the Tobacco Heating System 2.2. Part 2: Chemical composition, genotoxicity, cytotoxicity, and physical properties of the aerosol." *Regulatory Toxicology and Pharmacology* 81 (2016): S27-S47.
(3) Health Canada Test Method T-115, Determination of ''Tar'' and Nicotine in Sidestream Tobacco Smoke. 1999.

Results

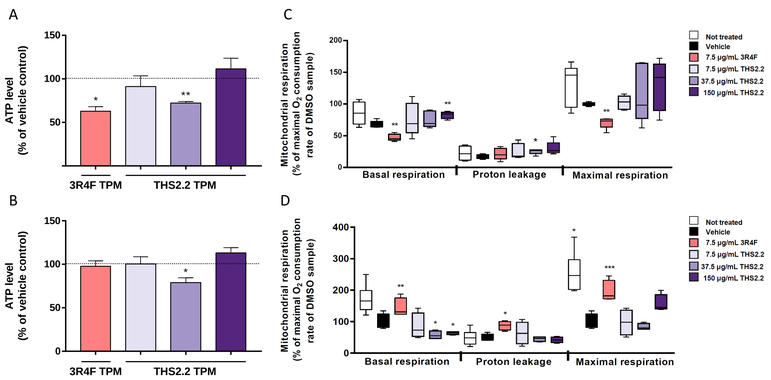

*Figure 1: The effect of TPM from 3R4F cigarette smoke and THS2.2 aerosol on cellular ATP level and oxygen consumption in the BEAS-2B cells. Cellular ATP levels following (A) 1 week or (B) 12 weeks of exposure to TPM from 3R4F cigarette smoke (7.5 µg/mL) and three doses of TPM from THS2.2 aerosol (7.5, 37.5 or 150 µg/mL). Mitochondrial oxygen consumption expressed as basal respiration, proton leak and maximal respiration following (C) 1 week or (D) 12 weeks of exposure to TPM from 3R4F smoke (7.5 µg/mL) and three doses of TPM from THS2.2 aerosol (7.5, 37.5 or 150 µg/mL). Data shown are means ± SEM of four independent experiments, *p< 0.05, **p< 0.01, ***p< 0.001.*

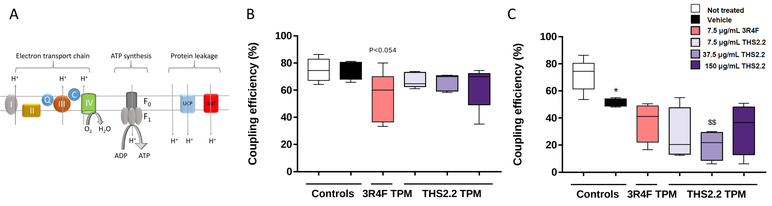

*Figure 2: The effect of TPM from 3R4F cigarette smoke and THS2.2 aerosol on coupling efficiency in BEAS-2B cells. (A) Schematic overview of the electron transfer chain. Coupling efficiency following (B) 1 week or (C) 12 weeks of exposure to TPM from 3R4F cigarette smoke (7.5 µg/mL) and three doses of TPM from THS2.2 aerosol (7.5, 37.5 or 150 µg/mL). Data shown are means ± SEM of four independent experiments, *p< 0.05 versus NT, $$ p< 0.01 versus vehicle. NT: not treated.*

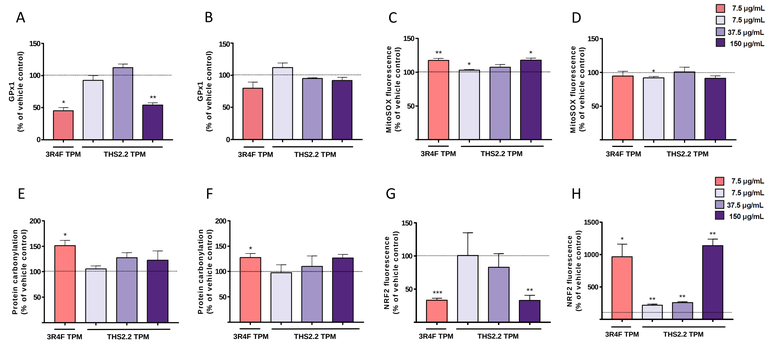

*Figure 3: The effect of TPM from 3R4F cigarette smoke and THS2.2 aerosol on oxidative stress in BEAS-2B cells. (A, B) Glutathione peroxidase, (C, D) mitochondrial ROS, (E, F) protein carbonylation levels and (G, H) NRF2 levels in BEAS-2B cells following 1 week (A, C, E, G) or 12 weeks (B, D, F, H) of exposure to TPM from 3R4F cigarette smoke (7.5 µg/mL) and three doses of TPM from THS2.2 aerosol (7.5, 37.5 or 150 µg/mL). Data shown are means ± SEM of four independent experiments, *p< 0.05, ** p< 0.01.*

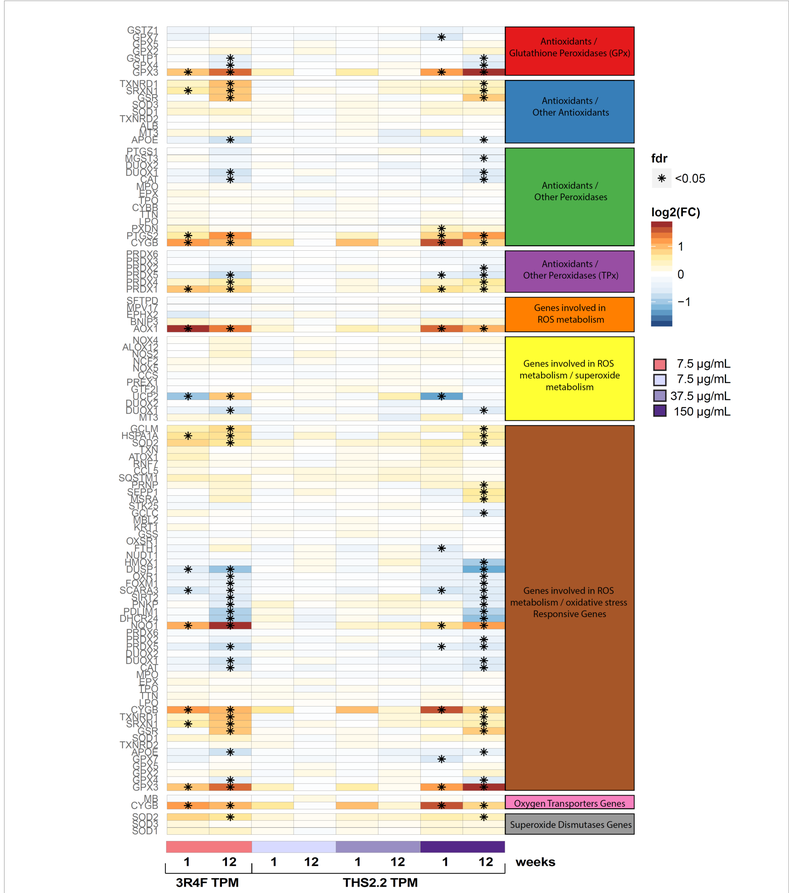

Figure 4. Altered expression levels of genes linked to the oxidative stress response in BEAS-2B cells following exposure to 3R4F cigarette smoke or THS2.2 aerosol, compared with untreated controls, for 1 or 12 weeks. The heatmap depicts changes in gene expression for oxidative stress-related genes (gene symbols listed on the left) relative to those in corresponding non-exposed controls expressed as log2 (fold change (fc)). Up- and downregulation of gene expression levels are represented by orange and blue shades, respectively, with darker colors indicating greater absolute fold changes. Selected "oxidative stress" gene panel was obtained from Qiagen. *Statistically significant fold changes are highlighted by * (adjusted p < 0.05).*

Conclusions

This study demonstrated that exposure of BEAS-2B cells to TPM from smoke of 3R4F reference cigarettes induced alterations in oxidative phosphorylation with concomitant increase of mitochondrial ROS and regulation of genes involved in ROS metabolism. These effects subsided by week 12, indicating adaptation to the chronic stress. Similar effects were also noted in BEAS-2B cells treated with TPM from THS2.2 aerosol at a 20 times higher concentration than 3R4F smoke TPM, but not at a comparable or 5 times higher concentration.

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Competing Financial Interest
The research described in this poster was sponsored by Philip Morris International