Mitochondrial dysfunction caused by cigarette smoking is involved in the driving oxidative stress–induced physiology in airway diseases. Reduction of harmful and potentially harmful constituents (HHCs) by heating rather than combusting tobacco would 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). 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 SMO2000/P1 smoking machine according to the Health Canada Intense protocol. Mainstream smoke from 3R4F cigarettes or aerosol from THS2.2 sticks were trapped on Cambridge glass filter fibers followed by extraction.

**Cell Culture and Treatment** - The human bronchial epithelial cell line BEAS-2B (BGSC Standards) was grown in complete BEGM™ (Lonza) on collagen-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 3 or 12 weeks.

**Measurement of ATP level** - BEAS-2B cells were evenly seeded on well-bottom-coated plates. ATP level was measured with CellTiter-Glo 3.0 Assay (Promega) according to the kit manual using an Infinity multidetector (Thermo).

**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). Dipyridamole (1 µM) (Sigma-Aldrich) was added to inhibit oxygen consumption related to the ATP synthase and to measure proton leakage.

**Maximal respiration rate** was started by adding 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 or non-reducing loading buffer (Sigma-Aldrich) and heated at 95°C for 5 min. Equal amount of protein lysates were separated by SDS-PAGE, transferred to nitrocellulose or PVDF membranes and blocked in Odyssey Blocking Buffer (Li-Cor) for 1 h. Blots were incubated overnight with primary antibodies (NFκB (Abcam), GAPDH (Cell Signaling Technology) and α-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 (LiCor).

**Detection of oxidatively modified proteins** - The level of carbonylated proteins was estimated using the OxyBlot Protein Oxidation Detection Kit (Millipore) according to the manufacturer’s protocol and separated by a 10% SDS-PAGE. The OxyBlot detection proteins were identified by incubation with rabbit anti-DNP (1:150) and anti-rabbit antibody HRPO BODC (1:5000) (LeClerc). Carbonylated proteins were visualized using an Odyssey infrared Imaging System (Li-Cor).

**Measurement of ROS levels** - ROS generation was determined using the Mitotracker Red fluorescent probe (Invitrogen) according to the kit manual. The level of O₂⁻ was measured on an iCy 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). Quality was assessed using the Agilent 2100 Bioanalyzer (Agilent), and high-quality RNA (RIN >9) was processed following a fully automated GeneChip® 3’ HVT Plus protocol prior to hybridization to HuGene 1.0 Plus 2.0 GeneChip® Arrays (Affymetrix). Differential gene expression comparing 3R4F smoke or THS2.2 aerosol–treated cells vs THS2.2 controls was computed with the limma R package employing a false-discovery rate (FDR) cut-off value of 0.05.

**Results**

**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**

**Introduction**

**Conclusions**

This study demonstrated that exposure of BEAS-2B cells treated with TPM from 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.