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Investigating mitochondrial dysfunction in human lung cells exposed to redox-active PM components

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ABSTRACT

Exposure to ambient particulate matter (PM) causes cardiopulmonary morbidity and mortality through mechanisms that involve oxidative stress. 1,2-naphthoquinone (1,2-NQ) is a ubiquitous component of PM and a potent redox-active electrophile. We previously reported that 1,2-NQ increases mitochondrial H_2O_2 production through an unidentified mechanism. We sought to characterize the effects of 1,2-NQ exposure on mitochondrial respiration as a source of H_2O_2 in human airway epithelial cells. We measured the effects of acute exposure to 1,2-NQ on oxygen consumption rate (OCR) in the human bronchial epithelial cell line BEAS-2B and mitochondrial preparations using extracellular flux analysis. Complex-specific assays and NADPH depletion by glucose deprivation distinguished between mitochondrial and non-mitochondrial oxygen utilization. 1,2-NQ exposure of BEAS cells caused a rapid, marked dose-dependent increase in OCR that was independent of mitochondrial respiration, exceeded the OCR observed after mitochondrial uncoupling, and remained sensitive to NADPH depletion, implicating extra-mitochondrial redox cycling processes. Similar effects were observed with the environmentally relevant redox-cycling quinones 1,4-naphthoquinone and 9,10-phenanthrenequinone, but not with quinones that do not redox cycle, such as 1,4-benzoquinone. In mitochondrial preparations, 1,2-NQ caused a decrease in Complex I-linked substrate oxidation, suggesting impairment of pyruvate utilization or transport, a novel mechanism of mitochondrial inhibition by an environmental exposure. This study also highlights the methodological utility and challenges in the use of extracellular flux analysis to elucidate the mechanisms of action of redox-active electrophiles present in ambient air.

1. Introduction

Ambient particulate matter (PM) is a global public health concern. Exposure to ambient PM is estimated to cause 3.3 million premature deaths worldwide annually and is linked to increased cardiovascular and respiratory morbidity and mortality (Pope and Dockery 2006; Lelieveld et al. 2015). Despite intensive investigation, the mechanism (s) that initiate the adverse health effects of PM exposure are not known. However, oxidative stress has been specifically implicated in the responses to PM inhalation (Kelly 2003; Li et al. 2008).

Mitochondrial dysfunction has been identified as a key event in PM-induced cytotoxicity (Hiura et al. 2000; Li et al. 2003; Xia et al. 2004). In addition to their crucial role in cellular bioenergetics, mitochondria are important regulators of oxidative stress. In resting cells, mitochondria generate reactive oxygen species (ROS), the levels of which are highly controlled by multiple antioxidant systems (Murphy 2009; Brand 2010). A variety of xenobiotics disrupt mitochondrial function by inhibiting the electron transport chain or uncoupling mitochondrial membrane potential, processes which increase ROS generation (Kovacic et al. 2005).

Typically found on particles surfaces, quinones are ubiquitous organic components of PM that have been implicated in PM-induced

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mitochondrial dysfunction (Flowers-Geary et al. 1996). Quinones commonly found in ambient air include 1,2-naphthoquinone (1,2-NQ), 1,4-naphthoquinone (1,4-NQ), 9,10-phenanthrenequinone (9,10-PQ), and 1,4-benzoquinone (BQ) (Cho et al. 2004; Delgado-Saborit et al. 2013). Polyaromatic hydrocarbons (PAHs) can also be oxidized by cytochrome *p*450 to form quinones *in vivo* (Penning et al. 1999). Quinones have been proposed to contribute to the health effects of PM inhalation (Valavanidis et al. 2005).

Previous work on environmentally relevant quinones has largely focused on their electrophilic properties, specifically their adduction of cellular macromolecules through Michael addition (Iwamoto et al. 2007; Kumagai et al. 2012). Less studied is the role played by ROS generated through redox cycling of specific quinone species (Watanabe and Forman 2003). Redox-cycling quinones can undergo one-electron reduction to form unstable semiquinones (Fig. S1). This reduction may be catalyzed by several flavin-containing enzymes, such as NADH:ubiquinone oxidoreductase (Complex I) of the mitochondrial electron transport chain and the cytochrome P450 system of the endoplasmic reticulum (Henry and Wallace 1996). Semiquinones can donate an electron to oxygen (O_2) to form superoxide ($O_2^{\cdot}$), which has an extremely short half-life due to its rapid dismutation to the more stable species, hydrogen peroxide (H_2O_2), by abundant superoxide dismutases. Individual quinone species vary in their propensity to adduct and redox cycle, dependent on their electrophilicity and redox potential, respectively (Song and Buettner 2010). Thus, 1,2-NQ and 1,4-NQ are capable of both mechanisms, while BQ is exclusively an electrophile and 9,10-PQ only redox cycles in the cell (Kumagai et al. 2012). Prior studies have demonstrated that both redox-cycling and electrophilic quinones can depolarize mitochondrial membrane potential and deplete ATP production (reviewed in Henry and Wallace 1996).

Previous work implicated mitochondria as a source of H_2O_2 production in human airway epithelial cells exposed to environmentally relevant concentrations of 1,2-NQ (Cheng et al. 2012), but the mechanism remains to be elucidated. We hypothesized that 1,2-NQ impairs mitochondrial function, leading to increased mitochondrial H_2O_2. In the present study, we investigated whether 1,2-NQ disrupts mitochondrial function, as measured through extracellular flux analyses. We show here that 1,2-NQ exposure of the human bronchial epithelial cell line BEAS-2B impairs complex I-linked mitochondrial substrate oxidation, a novel mechanism of mitochondrial impairment by 1,2-NQ, and markedly increases oxygen consumption through a redox cycling mechanism, both potential sources of H_2O_2.

2. Methods

2.1. Materials

Tissue culture media and supplements were purchased from Lonza (Walkersville, MD, USA). The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO): 1,2-naphthoquinone (1,2-NQ), 1,4-naphthoquinone (1,4-NQ), 9,10-phenanthrenequinone (9,10-PQ), 1,4-benzoquinone (BQ), DMSO, oligomycin, Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), rotenone, Antimycin A, glucose, sodium pyruvate, fatty acid-free bovine serum albumin (BSA), mannitol, sucrose, potassium phosphate monobasic (KH_2PO_4), magnesium chloride ($MgCl_2$), EGTA, H_2O_2, and HEPES. XF Base Medium, XF Flux Paks, and XF PMP were purchased from Seahorse Bioscience (Billerica, MA, now Agilent Technologies). L-glutamine (Gibco) was purchased from ThermoFisher Scientific (Waltham, MA). Basic laboratory supplies were purchased from Fisher Scientific (Raleigh, NC, USA).

2.2. Cell culture

SV40 large T antigen-transformed human airway epithelial cells [BEAS-2B cells, subclone S6 (Reddel et al. 1988)] were cultured in serum-free keratinocyte growth medium (KGM). BEAS-2B have been used extensively for *in vitro* testing of inhaled toxicants as a model of airway epithelium (Veljkovic et al. 2011; Persoz et al. 2012). BEAS-2B cell cultures were continually renewed from frozen stocks every 2–3 months for the duration of the study. Cells were deprived of growth factors overnight prior to experiment by changing the medium to keratinocyte basal medium (KBM), to induce cellular quiescence.

2.3. Quinone preparation

100 mM stocks of 1,2-NQ and 1,4-NQ and 15 mM stocks of 9,10-PQ were prepared in DMSO. Working solutions were diluted in cell media with final DMSO concentrations < 0.1%. 50 mM stocks of BQ were prepared directly in cell media or mitochondrial assay solution. All stocks and working solutions were made fresh on the same day as exposure.

2.4. Protein assay

Where indicated, protein concentration was determined by a modified Bradford Assay (Bio-Rad Laboratories, Inc.).

2.5. Extracellular flux analysis

Oxygen consumption rate (OCR) was measured at 37 °C using the Seahorse XFe96 Analyzer (Agilent Technologies). BEAS-2B were seeded at 16,000 cells per well two days prior to assay in XF96 cell plates with KGM. Four background wells without cells were included in all assays. XFe96 sensor cartridges were hydrated overnight with XF Calibrant at 37 °C. Media was replaced the night before assay to KBM without growth factors. For intact cell experiments, XF Cell Mito Assay Media (XF Base Media with 10 mM glucose, 1 mM sodium pyruvate, 2 mM glutamine) was prepared fresh on the day of assay and adjusted to pH 7.4 with 100 mM NaOH. Cell media was changed 1 h prior to assay and cells were placed in a 37 °C incubator without CO_2. Stock concentrations of oligomycin, FCCP, rotenone, and antimycin A were prepared in DMSO and stored at −20 °C. All compounds used for in-assay injections were prepared in XF Cell Mito Assay Media with final DMSO concentrations < 0.1%. The concentration of FCCP that effects maximal OCR was optimized in preliminary experiments. For Cell Mito Stress Tests, the injections were ordered as follows unless otherwise noted: A) quinone; B) 1 μM oligomycin; C) 0.25 μM FCCP; D) 1 μM rotenone, 1 μM antimycin A. Mix-wait-measure times were 3 min-0 min-3 min.

Bioenergetic parameters were calculated through the standard Cell Mito Stress Test Assay (Agilent Technologies). Briefly, change in basal OCR after quinone addition was calculated as the measurement immediately after quinone addition minus the basal measurement directly before addition. ATP production was measured as the decrease in OCR after oligomycin addition. Non-mitochondrial respiration was measured as the minimum OCR after rotenone/antimycin A injection. Reserve capacity was measured as the increase in OCR after FCCP was added. Spare respiratory capacity was calculated as the maximal respiration after quinone treatment minus the basal respiration.

For glucose starvation experiments, cell media was changed to either XF Cell Mito Assay Media or XF Glycolysis media (XF Base Media supplemented with 2 mM glutamine) two hours prior to start of assay.

2.6. Cell viability

Cell viability was measured by monitoring retention of pre-loaded calcein-AM (Molecular Probes, Eugene, OR, USA). BEAS-2B were plated in black-walled, clear-bottom 96-well plates (Costar, Corning Inc., Corning, NY) and grown until confluency. Cells were deprived of growth factors overnight before the experiment. Two hours before the assay, cell media was changed to XF Cell Mito Assay Media or XF Glycolysis media, and cells were placed in a non-CO_2 incubator at 37 °C.