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Proteomic changes in human lung epithelial cells (A549) in response to carbon black and titanium dioxide exposures

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ABSTRACT

This study combined cytotoxicity assays with proteomic analysis to characterize the unique biological responses of the A549 human lung epithelial cell line to two physicochemically distinct respirable particles titanium dioxide (TiO_2) and carbon black (CB). Cellular LDH, ATP, BrdU incorporation and resazurin reduction indicated that CB was more potent than TiO_2. Proteomic analysis was done using 2D-GE and MALDI-TOF-TOF-MS. Proteomic changes reflected common and particle-specific responses. Particle-specific proteomic responses were associated with cell death (necrosis and apoptosis), viability and proliferation pathways. Our results suggested that these pathways were consistent with the cytotoxicity data. For instance, increased expressions of anti-proliferative proteins LMNA and PA2G4 were in agreement with the decreased BrdU incorporation in A549 cells after exposure to CB. Similarly, increased expression of HSPA5 that is associated with ATPase activity was consistent with decreased cellular ATP levels in these cells. These findings reveal that proteomic changes can explain the cellular cytotoxicity characteristics of the particles. In essence, our results demonstrate that the *in vitro* toxicoproteomic approach is a promising tool to gain insight into molecular mechanisms underlying particle exposure-specific cytotoxicity.

Biological significance: In this study we have shown that toxicoproteomics is a sensitive and informative method to resolve the toxicity characteristics of particles with different physicochemical properties. This approach can be useful in the investigation of molecular mechanisms underpinning cellular cytotoxic responses elicited by particle exposures. Thus, the toxicoproteomic approach can be valuable in assessing the risk associated with particle exposures *in vitro*.

1. Introduction

Airborne particulate matter (PM) is a complex mixture of inorganic and organic compounds. Inhalation of airborne PM has been linked to the development or exacerbation of respiratory illnesses such as bronchitis [1–3], asthma [4–6], cystic fibrosis [7,8] and lung cancer [2,9–12].Toxicity of urban air particles and their associated adverse health outcomes can vary with particle composition. Insight into particle exposure-specific molecular mechanisms can provide a biological basis of particle toxicity. In this study, we focused on an *in vitro* toxicoproteomic approach that can distinguish the toxic effects of two particles that are chemically and physically different.

Carbon black (CB) is a manufactured product containing predominantly (95%) elemental carbon (EC) with negligible amounts of inorganic and organic materials [13,14] and should not be confused with black carbon (soot) that contains varying amounts of EC, organic materials and metals [14,15]. Recent reports have implied that exposure to elemental carbon (EC) can have a negative impact on the cardiovascular system [16–19]. Yet, toxicity mechanisms underpinning EC exposure-related adverse cardiovascular effects are not clear, and CB has been used as a surrogate for EC in *in vitro* and *in vivo* toxicity studies [20–25]. Meanwhile, titanium dioxide (TiO_2) is typically used as a "negative control" in many particle toxicology studies due to its relative low toxicity properties both *in vivo* and *in vitro*. Nevertheless, recent studies have reported that the toxicity of TiO_2 could depend on

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physicochemical parameters such as size, aggregation, crystal phase and surface modifications [26].

Cytotoxicity of particles has traditionally been assessed by endpoint assays such as resazurin reduction, cellular ATP, lactate dehydrogenase (LDH) release and 5-bromo-2′-deoxyuridine (BrdU) incorporation. These assays are attractive to researchers because they can be applied in a high-throughput manner to estimate the toxic potency of respirable particles. One of the drawbacks of these cytotoxic assays is that they do not reveal detailed information at the molecular level. In recent years, proteomic-based approaches have gained momentum in toxicology based on their ability to delineate the molecular mechanism underlying the toxicity of PM [27,28]. Protein separation by two-dimensional gel electrophoresis (2D-GE) followed by protein identification by mass spectrometry (MS) or tandem mass spectrometry (MS/MS) is a classical proteomic approach used to quantify and identify proteins in complex biological matrices. While shot-gun proteomic analyses (using MALDI-TOF-TOF/MS/MS or LC/MS/MS) are proposed alternatives to gel-based proteomic procedures, the advantage of 2D-GE is that it is relatively inexpensive and can provide high content data. Recent studies have used 2D-GE to identify proteomic changes in cells to help characterize the toxicity caused by exposure to particles [27,29–34].

In this study, in order to understand particle-specific cellular changes we exposed a human lung epithelial cell line (A549) to two chemically and physically different respirable materials, CB and TiO_2 particle. We then used a toxicoproteomic approach by conducting traditional cytotoxicity assays along with proteomics to identify the molecular signature of particle toxicity.

2. Materials and methods

2.1. Materials

Culture flasks (T-25 and T-75), 96-well plate and plastic cell scrapers were obtained from Corning Inc. (Corning, NY). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone (Logan, UT). Gentamicin, trifluoroacetic acid, α-cyano-4-hydroxy-cinnamic acid, Tris-HCl, NaCl, Tween-20 and Tween-80 were obtained from Sigma-Aldrich (Oakville, ON). Iodoacetamide, bis-acrylamide, ammonium persulfate, glycerol, immobilized pH gradient strips, Criterion Cassette (13.3 × 8.7 cm W × L), Tris/Glycine/SDS buffer, and BioSafe Coomassie Blue were purchased from Bio-Rad (Mississauga, ON). Trypsin, resazurin reduction (CellTiter-Blue®) and lactate dehydrogenase (LDH) cytotoxicity assay kits (CytoTox-96®) were from Promega Corporation (Madison, WI), ATP assay kit (ViaLight™ Plus) was purchased from Lonza Corporation (Rockland, ME), and 5-bromo-2′-deoxyuridine (BrdU) cell proliferation ELISA (chemiluminescent) assay kit was obtained from Roche Diagnostics (Laval, QC). All water used was deionized/demineralized (>16 MΩ resistivity).

2.2. Particles preparation

TiO_2 (SRM-154b) obtained from the National Institute of Standards and Technology (Gaithersburg, MD) was subjected to three successive washes with methanol and then phosphate buffered saline (PBS) to remove possible soluble metals and organic contaminants before use in the experiments [35]. Carbon black (Cas#1333-86-4) obtained from Cabot Corporation (Boston, MA) was used as received. Particles were resuspended at 10 mg/mL in particle buffer (0.19% NaCl and 25 μg/mL Tween-80) [36], vortexed (30 s), sonicated (20 min on ice), homogenized with a Dounce Homogenizer (25 strokes), and then heated (56 °C, 1 h). The particles were stored at −40 °C until use.

2.3. Cell culture and particle exposure

The A549 cell line (American Type Culture Collection - CCL-185; human, epithelial, lung carcinoma) was subcultured in DMEM supplemented with 50 μg/mL gentamycin and 10% FBS. The cells were maintained in T-75 flasks in a humidified atmosphere at 37 °C containing 5% CO_2 and 95% air. For experiments, the cells were seeded at 1.5×10^6 cells (T-25), 3.75×10^6 cells (T-75) or 2.0×10^4 cells/well (96-well plate for cytotoxicity assays) and incubated for 24 h, resulting in approximately 75% confluence prior to dosing with particles. The final volume of culture medium was 5 mL (T-25), 15 mL (T-75) or 200 μL/well (96-well plate). Solutions of particles were prepared by thawing the frozen stocks to aqueous solutions, sonicating on ice (20 min) then diluting in the culture medium to make up dosing concentrations of 0, 60, 140 and 200 μg/cm^2. The cells were exposed to the particles by replacing the existing culture medium with the particle solutions, and the flasks/plates were returned to the incubator and allowed to incubate for 24 h. To harvest the exposed cells, the medium in each flask was removed and the cells were detached from the flasks using a plastic scraper. The cell suspension was collected in cell culture medium and centrifuged at 350 ×g for 5 min, and the supernatant was removed. The cell pellet was then washed twice with PBS. The final cell pellet was aspirated dry and stored frozen at −80 °C until further use. The integrated cytotoxicity bioassay which combined endpoints of cell viability (resazurin reduction assay), cellular membrane integrity (intracellular LDH content) and energy metabolism (ATP assay) were conducted in 96-well plates as described in our previous study [37]. The cell proliferation (BrdU incorporation) assay was conducted in a separate 96-well plate.

2.4. Protein extraction

The cell pellets were solubilized in a protein extraction/rehydration buffer from Bio-Rad (8 M urea, 2% CHAPS, 50 mM dithiothreitol, 0.2% Biolyte 3/10), where the volume depends on the number of cells in the pellet to achieve 1–2 μg/μL, and 1×10^6 A549 cells was experimentally estimated to yield about 200 μg of protein. The samples were vortexed (30 s), sonicated (10 min), vortexed (30 s) and centrifuged (15,000 ×g, 10 min). The extracted protein in the supernatant was collected, and the concentration of protein in each extract was determined immediately using the Coomassie Plus Protein assay kit (Thermo Scientific). The extracted protein samples were stored at −80 °C until use.

2.5. Two-dimensional gel electrophoresis (2D-GE)

2D-GE was conducted as described in our previous study [38]. Briefly, an appropriate quantity of protein was suspended in a total volume of 200 μL of extraction buffer, and applied to an immobilized pH gradient (IPG) strips (11 cm, pH 3–10 or pH 5–8) in a clean disposable rehydration tray and allowed to incubate for 1 h at room temperature. The IPG strip was then overlaid with mineral oil and allowed to continue incubating overnight (16–20 h). The IPG strip was then moved to an isoelectric focusing tray, overlaid with mineral oil and subjected to isoelectric focusing using a PROTEAN IEF cell (BioRad). The focusing conditions were as follows: stage 1: linear ramp to 250 V for 20 min; stage 2: linear ramp to 8000 V for 2.5 h; stage 3: rapid ramp for 20,000 V h. The strip was then stored at −80 °C until use. The focused IPG strip was thawed and gently agitated for 10 min in equilibration buffer 1 (6 M urea, 2% SDS, 375 mM Tris-HCl, 20% glycerol, 130 mM dithiothreitol, 0.001% bromophenol blue). Then each strip was gently agitated for another 10 min in equilibration buffer 2 (6 M urea, 2% SDS, 375 mM Tris-HCl, 20% glycerol, 135 mM iodoacetamide, 0.001% bromophenol blue). The strip was then placed on a 12% SDS-PAGE gel casted in a 1.0 mm thick Criterion Cassette (13.3 × 8.7 cm W × L) and subjected to electrophoresis at 200 V for 65 min. Following electrophoresis, the gel was removed from the Criterion Cassette, washed for 30 min in water, stained in BioSafe Coomassie Blue (Bio-Rad) overnight (16–20 h), destained twice in water, and then imaged with a standard scanner.

To overcome the typical warping and distortion issues from gel to gel near the extremities of the pH and the molecular weight range, a

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