Toxicology in Vitro 23 (2009) 1365–1371

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Copper oxide nanoparticles induce oxidative stress and cytotoxicity in airway epithelial cells

Baher Fahmy [1], Stephania A. Cormier *

Department of Pharmacology and Experimental Therapeutics, Louisiana State University Health Sciences Center, New Orleans, LA, USA

ARTICLE INFO

Article history:
Received 5 May 2009
Accepted 13 August 2009
Available online 20 August 2009

Keywords:
Nanoparticles
HEp-2 cells
CuO
Oxidative stress
Resveratrol

ABSTRACT

Metal oxide nanoparticles are often used as industrial catalysts and elevated levels of these particles have been clearly demonstrated at sites surrounding factories. To date, limited toxicity data on metal oxide nanoparticles are available. To understand the impact of these airborne pollutants on the respiratory system, airway epithelial (HEp-2) cells were exposed to increasing doses of silicon oxide (SiO_2), ferric oxide (Fe_2O_3) and copper oxide (CuO) nanoparticles, the leading metal oxides found in ambient air surrounding factories. CuO induced the greatest amount of cytotoxicity in a dose-dependent manner; while even high doses (400 $\mu g/cm^2$) of SiO_2 and Fe_2O_3 were non-toxic to HEp-2 cells. Although all metal oxide nanoparticles were able to generate ROS in HEp-2 cells, CuO was better able to overwhelm antioxidant defenses (e.g. catalase and glutathione reductase). A significant increase in the level of 8-isoprostanes and in the ratio of GSSG to total glutathione in cells exposed to CuO suggested that ROS generated by CuO induced oxidative stress in HEp-2 cells. Co-treatment of cells with CuO and the antioxidant resveratrol increased cell viability suggesting that oxidative stress may be the cause of the cytotoxic effect of CuO. These studies demonstrated that there is a high degree of variability in the cytotoxic effects of metal oxides, that this variability is not due to the solubility of the transition metal, and that this variability appears to involve sustained oxidative stress possibly due to redox cycling.

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1. Introduction

The massive increase in manufacturing and utilization of metal oxide nanoparticles has lead to major concerns regarding the potential health impact of these particles on the pulmonary system. Since these particles have a small aerodynamic diameter (<0.1 μm), they can escape air filters, contaminate ambient air, penetrate deep into the lungs, reach the alveolar region and evoke adverse pulmonary effects (Oberdorster et al., 2005).

Many epidemiological studies have demonstrated a correlation between the level of nanoparticles ($PM_{0.1}$) in ambient air and a significant increase in pulmonary disease including exacerbation of bronchial asthma (Penttinen et al., 2001; Weichenthal et al., 2007). Since the chemical composition of airborne $PM_{0.1}$ varies significantly according to the location and time of sample collection, the exact particle responsible for adverse pulmonary effects has remained elusive. Experimental studies have supported the epidemiological findings and have provided evidence suggesting a role for oxidative stress in these events (Yang et al., 2009). Oxidative stress generated in cells exposed to nanoparticles may stimulate inflammatory responses, oxidize lipids or even lead to cell death. Chemical analysis of different populations of $PM_{0.1}$ has demonstrated the elevated presence of metal oxide nanoparticles at sites surrounding factories as compared to remote (i.e. "cleaner") areas (Rogaczewska and Matczak, 1985). Despite the increase in the levels of these particles in ambient air, epidemiological studies rarely focus on the health impact associated with the exposure to these specific particles.

At present, metal oxide nanoparticles are used in manufacturing of hundreds of commercial products, and their industrial applications are expected to expand during the next decade. Silica which is composed of SiO_2, is one of the most abundant oxides present in ambient air and comprises (up to 8%) of all total airborne nanoparticles (Balduzzi et al., 2004), typically in crystalline (quartz) or amorphous form. The amorphous form of silica is widely used in many industries and applications such as fillers in the rubber industry, anti-caking agents in powder materials such as paints and cosmetics (Merget et al., 2002). Copper oxide (CuO) nanoparticles are used in antimicrobial preparations, heat transfer fluids, semiconductors or intrauterine contraceptive devices (Aruoja et al., 2009). Ferric oxide (Fe_2O_3) nanoparticles are used as catalysts

* Corresponding author. Address: Louisiana State University Health Sciences Center, Department of Pharmacology and Experimental Therapeutics, 1901 Perdido Street, MEB, P7-1, New Orleans, LA 70112, USA. Tel.: +1 (504) 568 2810; fax: +1 (504) 568 2361.
E-mail address: scorm1@lsuhsc.edu (S.A. Cormier).
[1] Present address: Department of Pharmacology and Toxicology, Faculty of Pharmacy, Cairo University, Egypt.

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doi:10.1016/j.tiv.2009.08.005

and in the manufacture of pigments (Montes-Hernandez et al., 2006).

Oxidative stress is often used to explain toxicity associated with particle exposure. Although the ability of crystalline silica to generate oxidative stress in pulmonary cells has been demonstrated (Fanizza et al., 2007), little is known about the ability of amorphous silica nanoparticles to induce oxidative stress. Amorphous silica nanoparticles demonstrate less ability to induce pulmonary inflammation (Warheit et al., 1995) and fibrosis (Reuzel et al., 1991) as compared to quartz particles of the same size. Furthermore, silicosis is associated with the exposure to quartz but not amorphous silica particles suggesting that these particles may have different toxicity profiles (Reuzel et al., 1991).

Both copper and iron ions are able to generate oxidative stress (Moriwaki et al., 2008). Although, oral administration of copper oxide nanoparticles induces hepatotoxicity and nephrotoxicity in exposed rats (Lei et al., 2008), it is not known whether this toxicity is mediated by the generation of oxidative stress in the liver and the kidney tissues. Data demonstrating the toxic effect of ferric oxide nanoparticles remain controversial. While exposure to ferric oxide nanoparticles does not produce inflammation in vascular endothelial cells *in vitro* (Gojova et al., 2007), it significantly decreases cell viability in cancer cells (Choi et al., 2009). Although inhalation is the primary source of exposure to metal oxides in ambient air, data demonstrating the effect of metal oxide nanoparticles on the pulmonary system remain scarce. Therefore, comparative toxicological assessments need to be conducted to better understand the role of metal composition in the observed adverse pulmonary effects associated with the exposure to $PM_{0.1}$. Because of their presence in airborne particulate matter, we choose to investigate the biological effects of three nanoparticles: amorphous silicon oxide (SiO_2), ferric oxide (Fe_2O_3), and copper (II) oxide (CuO).

We hypothesized that different metal oxide particles will have different abilities to generate oxidative stress and alter cell viability based on the transition metal. The aim of this study was to compare the *in vitro* responses of respiratory epithelial cells following exposure to two types of commercially available metal oxide nanoparticles and amorphous SiO_2 nanoparticles. In particular, we investigated the intrinsic ability of silicon oxide, ferric oxide and copper (II) oxide nanoparticles to decrease cell viability and generate oxidative stress in respiratory epithelial cells. Human laryngeal epithelial cells (HEp-2) were chosen, since they are used in many pulmonary toxicological assays (Rudolf et al., 2001; Kvolik et al., 2005) and represent target cells which are usually subjected to significant amounts of airborne particles.

2. Materials and methods

2.1. Reagents

2′,7′-dichlorofluorescein-diacetate (H_2DCFDA), 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB), H_2O_2, oxidized glutathione (GSSG), reduced glutathione (GSH), β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH), sulfosalisilic acid (SSA), superoxide disumutas (SOD) determination kit and glutathione assay kit were all obtained from Sigma (St. Louis, MO). Copper (II) oxide (CuO) particles (30 nm, #45407) and ferric oxide (Fe_2O_3) particles (20–40 nm, #45007) were purchased from Alfa Aesar (Ward Hill, MA) and silicon oxide (SiO_2) (80 nm, #4830HT) was obtained from Nanostructured and Amorphous Materials, Inc. (Los Alamos, NM). Resveratrol was purchased from Axxora (San Diego, CA) and Alamar Blue was obtained from Invitrogen (Carlsbad, USA). All organic solvents were of Fisher optima grade (Fisher Scientific, Hampton, NH).

2.2. Methods

2.2.1. Cell culture and treatment

Human laryngeal epithelial cells (HEp-2 cells) were purchased from ATCC (Manassas, VA) and were cultured in 75 cm^2 flask at the density of 2×10^4 cell/cm^2 in Dulbecco's Modified Eagle's Medium-Reduced Serum (DMEM-RS), supplemented with 2% heat inactivated fetal bovine serum (FBS) at 37 °C in a humidified incubator containing 5% CO_2. At 85% confluence, cells were harvested using 0.25% trypsin and were sub-cultured into 75 cm^2 flasks, 6-well plates or 96 well plates. Cells were allowed to recover for 2 days prior to treatment. Particles were suspended in cell culture medium by pulse sonication (30 s on, 30 s off) using 50% amplitude (Sonics and Materials Inc., CT, USA) for 4 min to avoid particle agglomeration, followed by vigorous vortexing for 1 min prior to administration to the cells. A serial dilution was established by mixing equal volumes of particle suspension and cell culture medium followed by vigorous vortexing. All experiments were performed using HEp-2 cells at passage 10–20 and were replicated with at least two independent cell passages.

2.2.2. Cell viability assay

The cytotoxic effect of particles on HEp-2 cells was determined by the Alamar blue assay as previously described (Baudouin et al., 2007). Briefly, HEp-2 cells were cultured in 96 well plates at the density of 2×10^4 cell/cm^2 and then, incubated with particles (4–400 μg/cm^2), suspended in cell culture medium (200 μl/well) for 5 h to establish a dose response curve. Cell viability was estimated by measuring the emitted fluorescence of the reduced alamar blue using a plate reader (ex/em: 530/590) and was normalized to medium only treated cells (100% viability) and 0.1% saponin (0% viability). Unlike other cell viability assays, the presence of nanoparticles does not interfere with the Alamar blue assay (Simon-Deckers et al., 2008). A dose–response curve consisting of log doses of particles and percent cell viability associated with the exposure to each dose was plotted using the nonlinear fit (Third order polynomial; Graphpad Prism 5 software, La Jolla, CA). To investigate the influence of resveratrol, desferoxamine and D-penicillamine on the cytotoxic effect of copper oxide, cells were co-treated with 100 μM of resveratrol, 100 μM desferoxamine or 100 μM D-penicillamine prior to assessment of cell viability and the data were compared to medium containing 100 μM resveratrol, 100 μM desferoxamine or 100 μM D-penicillamine; respectively (100% viability) and 0.1% saponin (0% viability).

2.2.3. Measurement of cellular reactive oxygen species (ROS)

The production of reactive oxygen species in HEp-2 cells was measured by pre-loading the cells with 10 μM 2,7-dichlorofluorescin diacetate (H_2DCFDA) at 37 °C for 40 min in the dark in 6-well plate. The cells were then washed and incubated with particle suspension (80 μg/cm^2; 125 μg/ml) for 30 min at 37 °C. After the treatment, cells were washed, scraped, lysed, pulse sonicated for 15 s (1 s on, 1 s off) using 50% amplitude, and centrifuged at 12,000*g* for 15 min at 4 °C. The intensity of DCF fluorescence in the cell lysate was measured using a plate reader (ex/em: 485/530) and was normalized to protein content measured by BCA protein assay (Thermo Fisher Scientific Inc., Waltham, MA).

2.2.4. Antioxidant enzyme activity

After treating the cells for 4 h with particle suspension (80 μg/cm^2), cells were washed with PBS, scraped, lysed, sonicated for 15 s (1 s on, 1 s off) on ice and centrifuged at 12,000*g* for 15 min at 4 °C. The supernatant (cell lysate) was removed and the protein concentration was measured by the BCA method. The activities of different antioxidant enzymes were then measured in the cell lysates.

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