Toxicology in Vitro 29 (2015) 1053–1059

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Toxic mechanisms of copper oxide nanoparticles in epithelial kidney cells

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ARTICLE INFO

Article history:
Received 25 September 2014
Accepted 31 March 2015
Available online 7 April 2015

Keywords:
Cell line
DNA damage
Mechanistic toxicology
Reactive oxygen species
Xenopus laevis

ABSTRACT

CuO NPs have previously been reported as toxic to a range of cell cultures including kidney epithelial cells from the frog, *Xenopus laevis* (A6). Here we examine the molecular mechanisms affecting toxicity of Cu in different forms and particle sizes. A6 cells were exposed to ionic Cu (Cu^{2+}) or CuO particles of three different sizes: CuO NPs of 6 nm (NP6), larger Poly-dispersed CuO NPs of <100 nm (Poly) and CuO Micro particles of <5 μm (Micro), at 200 μM, equal to 12.7 mg Cu/L. Poly was significantly more toxic than NP6, Micro and Cu^{2+} to A6 cells, causing DNA damage, decreased cell viability and levels of reduced glutathione (GSH) and eventually cell death. We show that ROS (Reactive Oxygen Species) generation plays a key role and occurs early in Poly toxicity as Poly-induced DNA damage and cell death could be mitigated by the antioxidant NAC (N-acetyl-cysteine).

Here we propose a model of the sequence of events explaining Poly toxicity. Briefly, the events include: cellular uptake, most likely via endocytosis, production of ROS, which cause DNA damage that activates a signaling pathway which eventually leads to cell death, mainly via apoptosis.

1. Introduction

Copper oxide nanoparticles (CuO NPs) are being increasingly used in consumer products such as conductive inks, antimicrobial coatings, textiles for biocidal activity, lubricants, plastics, cosmetics, environmental remediation and electronics (Cioffi et al., 2005; Park et al., 2007; Wang et al., 2008; Perelshtein et al., 2009; Ren et al., 2009; Unrine et al., 2010; Nanotechproject, 2014; Rubilar et al., 2013). We have previously shown that poly-dispersed CuO NPs are more toxic to A6 cells from the kidney of the frog *Xenopus laevis* than 6 nm CuO NPs and Copper (Cu) ions administered as $CuCl_2{\cdot}2H_2O$, causing cell death, at least partly in the form of apoptosis (Thit et al., 2013). Here we examine the molecular mechanisms controlling CuO NP toxicity.

Cu is an essential metal serving as a co-factor for a variety of enzymes, including cytochrome c oxidase in respiration and superoxide dismutase (SOD) in free radical eradication (Zhou and Gitschier, 1997). However, Cu is highly toxic in excess amounts because it can interfere with homeostasis of other metals, cause DNA damage, and has the ability to generate Reactive Oxygen Species (ROS, H_2O_2, $O_2^{\cdot-}$ and $OH^{\cdot}$) that can adversely modify proteins, lipids and DNA (Aruoma et al., 1991; Banci et al., 2010; Halliwell and Gutteridge, 1984; Xie et al., 2006). As Cu can cycle between two redox states (oxidized, Cu^{2+} and reduced, Cu^{+}) it has the ability to catalyze the transfer of electrons from a donor molecule to an acceptor such as O_2 to yield the superoxide radical, $O_2^{\cdot-}$. $O_2^{\cdot-}$ can also be produced for example by an incomplete electron transfer in the electron transport chain in the mitochondria, if the electron leaks to O_2 instead of cytochrome c oxidase (Halliwell, 1987). In living cells the $O_2^{\cdot-}$ can be reduced to hydrogen peroxide, H_2O_2, by Cu^{+} itself or by the oxidative defense enzyme superoxide dismutase. H_2O_2 can be scavenged by the enzyme glutathione peroxidase, with GSH (reduced glutathione) as electron donor. Alternatively, H_2O_2 and $O_2^{\cdot-}$ can be converted into the hydroxyl radical, $OH^{\cdot}$, in the presence of Cu^{+} (Halliwell, 1987), which is very reactive and can cause DNA-strand breaks, attack proteins and membrane lipids. Oxidation of a membrane lipid can cause a radical chain reaction, leading to the formation of peroxides (lipid peroxidation), which can severely damage the cell membrane (Halliwell, 1987).

CuO NPs have previously been shown to cause greater effects to human epithelial cells compared with bulk CuO (Karlsson et al., 2009; Wang et al., 2012) and compared to other metal NPs (Sun et al., 2012). Metal and metal oxide nanoparticles have been hypothesized to promote cytotoxicity and apoptosis via generation

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http://dx.doi.org/10.1016/j.tiv.2015.03.020
0887-2333/

of ROS, activation of intracellular signaling pathways, DNA damage and autophagic cell death (Fahmy and Cormier, 2009; Misra et al., 2014; Sun et al., 2012; Xu et al., 2012). In addition, CuO NPs have been observed in the cell nucleus and have been hypothesized to disrupt the nuclear membrane, allowing the particles to enter the cell nucleus and interact with DNA directly (Wang et al., 2012).

Here we investigate the ability of Cu in different forms (Cu ions versus CuO NPs) and varying particle sizes (mono-dispersed 6 nm NPs, NP6; larger poly-dispersed <100 nm CuO NPs, Poly; and Bulk micro-sized CuO <5 μm NPs, Micro) to induce ROS generation, DNA damage and cytotoxicity in a cultured epithelial kidney cell line from *X. laevis* (A6). We aim at determining the role of ROS and establishing the sequence of events in CuO NP toxicity by using the antioxidant N-acetyl-Cysteine (NAC) to examine if DNA damage and cytotoxicity can be mitigated by increasing the cells oxidative defense.

2. Materials and methods

2.1. Cell culture

The A6 cell line used was purchased from American Type Culture Collection (Rockville, MD, USA) at serial passage 67. The cell line was originally isolated from the distal kidney tubule of the aquatic frog *X. laevis* (Rafferty, 1969). Cells were maintained as previously described (Thit et al., 2013). Briefly, cells were grown to confluence in falcon T25 culture flasks (BD bioscience, CA, USA) with 10 ml growth medium at 26 °C in humidified atmosphere of 5% CO_2 in a CO_2 incubator (Flow laboratories incubator 1500, CA, USA). The growth medium was made from Dulbeccós modified Eaglés medium (Gibco, InVitrogen Corporation, CA, USA) diluted 25% with autoclaved MilliQ water to adjust to amphibian osmolarity. In addition, the growth medium contained 2% penicillin/streptomycin (5000 units/ml Penicillin + 5000 μg/ml Streptomycin, Gibco, InVitrogen Corporation, CA, USA) and 10% Fetal Bovine Serum (Biochrom AG, Berlin, Germany).

2.2. Exposure chemicals

CuO NPs (NP6) were provided and characterized by Natural History Museum (NHM, London, UK) in an aqueous suspension as mono-dispersed spherical particles (6 ± 1 nm). Particles had a hydrodynamic diameter ranging roughly from 9 to 40 nm with the majority around 19 nm and a zeta potential of 44.0 ± 0.5 mV in deionized water. Further details on the characterization are found within Pang et al. (2013). Larger CuO NPs (Poly) were produced by Intrinsiq Materials Ltd. (UK) as nano-powder and were characterized by NHM. Particles were Poly-dispersed (size and shape), with a primary particle size below 100 nm. The average hydrodynamic diameter of Poly in deionized water was 204 ± 1 nm, with sizes ranging from 40 to 500 nm. Hence, the average hydrodynamic diameter of Poly was about 10 times higher than for NP6. The zeta potential of Poly was 42 ± 1 mV. Further details on the characterization of Poly are found within Pang et al. (2013). In order to make comparisons between the toxicity of CuO NPs with that of larger particles, micro-sized CuO particles (Micro, <5 μm) were tested. Micro particles were purchased from Sigma–Aldrich, Denmark and characterized by NHM, London, UK. Particles were highly polydispersed with presence of particles both in the NP range (10–50 nm) and micro size-range. The average hydrodynamic diameter of Micro in deionized water was 813 ± 141 nm (the average hydrodynamic diameter about 4 times larger than Poly and 40 times larger than NP6) and the zeta potential was 16.9 ± 2 mV. Further details on the characterization are found within Pang et al. (2013). Copper chloride dihydrate ($Cu(II)Cl_2{\cdot}2H_2O$, Merck, Dk) was used as a reference for the ionic form. Stock suspensions of the three Cu forms (0.01 M) were made by transferring the needed amount of the respective compound into MilliQ water.

2.3. Experimental setup

Cells were seeded in sterile tissue culture treated polystyrene 24-well microtiter plates (Costar, USA) except for cells used in qualitative assessments of ROS generation and amount of GSH using Fluorescence Activated Cell Sorting (FACS) and NucleoCounter (NC). Here 6-well plates were used in order to obtain sufficient amount of cells for analysis (10,000 cells/replicate). In both scenarios cells were seeded in concentrations of $5–10 \times 10^3$ cells/cm^2. After 48 h cultivation the experiment was initiated by exchanging the growth medium with experimental medium containing copper (Cu^{2+}, NP6, Poly or Micro) at a concentration of 200 μM, equal to 12.7 mg Cu/L, or clean growth medium for controls. The exposure concentration was selected based on previous findings (Thit et al., 2013). The total volume of growth medium added was 1 ml per well, for 24-well plates (4 wells per treatment) and 3 ml for 6-well plates (3 wells per treatment), respectively. To assess the effects of increasing the cells oxidative defence on cell death and DNA damage, selected cells (4 wells per treatment) were pre-incubated for 3 h with the antioxidant NAC (Sigma–Aldrich, 1 mM) before exposure initiation and continuously during the exposures. The experiments conducted to assess DNA damage and cell death were repeated two more times.

2.4. Toxicity: Qualitative and quantitative determination of cell death

Toxicity of the different Cu treatments was assessed following the procedure described in Thit et al. (2013). Briefly, exposed cells were observed with an inverted microscope (DMIRB/E Leica), and digital image acquisition was performed with a Leica DC 300 F camera and software from Leica Microsystems Ltd. (Leica Microsystems A/S, Dk). Pictures were taken of each well of the microtiter plate, just before cell treatment was initiated (0 h) and several times during the subsequent exposures (i.e., after 24 h for quantitative analysis and after 48 h for qualitative assessments of cell death). When unhealthy (dead or dying) cells are excluded from the cell monolayer they become spherical, and appear white on the microscope images. Therefore dead or dying cells were quantified based on this distinct morphology, and images were analyzed with ImageJ software as previously described (Thit et al., 2013). Confirmation of cell death was conducted by staining with the viability DNA stain Propidium Iodide (PI), as cells with intact membranes are impermeable to PI, whereas the membranes of dead or damaged cells are permeable to PI. Cells were incubated in the dark with 4 mg/ml PI for 15 min, washed with PBS (phosphate buffered saline) and observed with epi-fluorescence microscopy. During the washing procedure a small loss of undetached dead cells may have occurred. Therefore the observations of PI fluorescence were purely conducted to confirm cell death and not for quantification. Four wells were used per treatment, equal to 24 images in total. Selected representative images are presented in Fig. 1 in supplementary information (SI). Effects of Cu treatments on cells that had been incubated with and without the antioxidant NAC were compared to assess whether toxicity of 24 h Cu treatments was a result of intracellular ROS generation. A total of 82 images of individual exposure wells were obtained in four different experiments and used for quantitative analysis of cell death.