Chemico-Biological Interactions 228 (2015) 57–68

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

Recovery of redox homeostasis altered by CuNPs in H4IIE liver cells does not reduce the cytotoxic effects of these NPs: An investigation using aryl hydrocarbon receptor (AhR) dependent antioxidant activity

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ARTICLE INFO

Article history:
Received 5 September 2014
Received in revised form 26 November 2014
Accepted 7 January 2015
Available online 21 January 2015

Keywords:
Copper nanoparticles (CuNPs)
Oxidative stress
H4IIE
Aryl hydrocarbon receptor (AhR)
ß-Naphthoflavone (ßNF)
Co-incubation

ABSTRACT

The generation of reactive oxygen species (ROS) and consequent oxidative stress is regarded as a relevant mechanism for nanoparticle toxicity. In cells, the activation of the aryl hydrocarbon receptor (AhR) triggers a cascade of defence responses against oxidative stress. By increasing AhR dependent cellular anti-oxidant activity, we tested the extent to which the cytotoxic effect of copper nanoparticles (CuNPs) is governed by oxidative stress. H4IIE rat hepatoma cells were challenged with high ROS levels after exposure to CuNPs, while the AhR-induced cellular anti-oxidant defence was simultaneously activated by the AhR ligand beta-Naphthoflavone (ßNF). Activation of phase II detoxification enzymes (as glutathione-S-transferases, GSTs) and anti-oxidants (glutathione, GSH) led to a complete abrogation of CuNP-induced ROS production. However, a concurrent reduction in cytotoxicity was not detected, thereby indicating that CuNPs exert non-oxidative stress mediated cytotoxic effects. Transmission electron microscopy analysis pointed to a direct physical perturbation of cellular structures by CuNPs, thus contributing to their cytotoxicity. Our observations highlight that distinct mechanisms underlie the toxicity of ions and NPs and indicate that while ROS elicitation is CuNP-specific, the cytotoxic action of these particles may not be directly related to their pro-oxidative activity. These findings have important implications with respect to the oxidative stress paradigm used to explain NP toxicity.

1. Introduction

The increasing use of nano-sized materials in a variety of products has raised serious concerns about the possible release of this new class of anthropogenic contaminants into the environment [1]. Consequently, the assessment of the risks associated with these substances has become of great importance. Toxicity assessments using CuNPs currently lags behind research efforts made for other nanomaterials. However research that has been performed shows striking evidence of grave toxicological effects of CuNPs on the kidney, liver and spleen in mouse models [2]. This, along with the fact that heightened toxic efficacy of Cu at nanoscale compared to bulk counterpart has been highlighted, points to possible increased hazards associated with nanoforms [3].

The mechanisms underlying CuNPs toxic insults (and many other nanomaterials) are currently under debate. Together with the physical damage of cell membranes caused by direct contact with NPs [4,5], one of the most accepted theories to explain the toxicity of these particles is the oxidative stress paradigm [6]. Countless studies point to and highlight the association between increased reactive oxygen species (ROS) levels and cytotoxicity after exposure to NPs [7–9].

Oxidative stress is the result of a redox imbalance between the generation of ROS and the compensatory metabolic response of the endogenous anti-oxidant networks of the cell. Cells respond to ROS

Abbreviations: CuNP, copper nanoparticle; ROS, reactive oxygen species; AhR, aryl hydrocarbon receptor; ßNF, ß-Naphthoflavone; αNF, α-Naphthoflavone; GST, glutathione-S-transferase; GSH, glutathione; GSSG, oxidised glutathione; Nrf2, nuclear factor erythroid-2-related factor; HO-1, heme oxygenase-1; SnPP, tin protoporphyrin IX; TEM, transmission electron microscopy; AREs, anti-oxidant response elements; XREs, xenobiotic response elements; LOEC, lowest observed effect concentration; CYP1A1, cytochrome P450, family 1, subfamily A, polypeptide 1; RR, resazurin reduction; EROD, 7-ethoxyresorufin-*O*-deethylase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; P/S, penicillin and streptomycin; EMEM, Eagle Minimum Essential Medium; NEAA, non-essential amino acids; BSA, bovine serum albumin; DLS, dynamic light scattering; PBS, phosphate-buffered saline; FBS, foetal bovine serum; NADPH, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate.

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http://dx.doi.org/10.1016/j.cbi.2015.01.012
0009-2797/

insult by activating signalling pathways that trigger the expression of genes that participate in the anti-oxidative response. One such pathway involves the activation of the nuclear factor erythroid-2-related factor (Nrf2), which is responsible for the induction of genes that hold anti-oxidant response elements (AREs) in their promoters. These genes encode for a range of cytoprotective enzymes, including phase II detoxifying enzymes such as glutathione-S-transferase (GST), the stress-protein heme oxygenase 1 (HO-1), and also key players in the synthesis of glutathione (GSH) [10–12]. GSTs catalyse the nucleophilic addition of GSH to electrophiles, making them less reactive and more soluble.

The induction of phase II GST and other detoxification activities directed at reducing oxidative stress is also triggered by other transcription factors, as for instance the aryl hydrocarbon receptor (AhR) [13]. This receptor can be activated by a variety of xenobiotics sharing some structural features (polycyclic aromatic compounds that can easily take a planar conformation) [14,15]. This receptor induces genes that hold xenobiotic response elements (XREs) in their promoter regions. A prototypical AhR activator used in a variety of studies is ß-Naphthoflavone (ßNF). Activation of AhR results in an increase in phase I cytochrome P4501A (CYP1A) mono-oxygenase enzyme induction involved in oxidation reactions but also of phase II GST detoxification activity [13]. CYP1A can be evidenced at the enzymatic level by measuring the associated ethoxyresorufin-*O*-deethylase (EROD) activity. Recent data provide evidence for a cross-talk between the Nrf2 pathway and the pathway leading to the induction of XRE-driven genes by the AhR [14,16–18]. Unlike the AhR, the Nrf2 transcription factor is not ligand activated, rather it responds to xenobiotics and endogenous compounds that are thiol reactive, such as reactive oxygen species or electrophilic insults.

A variety of modes of toxicity have been suggested for metal ions and NP fractions [19,20]. The relative contribution of free ions to NP toxicity and ROS generation is debatable and the role of dissolution in nanoparticle toxicity further increases the complexity of this process. Kim and colleagues [21] provide evidence that NP cytotoxicity is caused by NP-specific oxidative stress responses, independently of the toxicity of ions. Other studies support the hypothesis that the toxic effects of these particles are simply due to the release of ions into the surrounding environment, with a limited material effect [22], while others argue joint action [23,24]. Recent papers also suggest that ROS-independent mechanisms involving cell cycle arrest are associated with NP toxicity [25] and that oxidative stress is merely a secondary effect of direct physical damage by NPs [26,27]. Also, metal NPs show anti-oxidative properties that promote cell survival under conditions of oxidative stress. For example, cerium oxide (CeO_2) and yttrium oxide (Y_2O_3) have been reported to exhibit ROS scavenging capacity and neuroprotective activity in rodent nervous system HT22 cells [28,29].

Copper is a redox active metal. It directly catalyses the formation of ROS through redox cycling between different valence states either through Haber-Weiss reactions or Fenton chemistry [30]. Copper capacity to stimulate Nrf2-dependent transcriptional activity and induce HO-1 activity has been reported [30,31]. In a previous study, we showed that copper nanoparticles (CuNPs) induce high levels of ROS in a variety of mammalian and piscine cell lines [32]. Following this line of work, the main objective of the present work was to determine if CuNP cytotoxicity is associated with the observed increase in ROS levels. We have exposed H4IIE cells (a rat hepatoma cell line) to two of the previously used CuNPs (CuNP 25 and CuNP 78, with 25 and 78 nm in size, respectively), or to $Cu(NO_3)_2$, used as a source of ions that served as a control, and characterised the induction of ROS. Then to test to what extent the cytotoxic effect of CuNPs is governed by ROS generation and oxidative stress we increased endogenous cellular antioxidant activity through activation of the AhR signalling pathway. We have chosen ß-Naphthoflavone (ßNF), as a prototypical AhR inducer for the co-incubation experiments. Co-incubations allowed us to simultaneously study the responses of H4IIE cells to activation of the ßNF detoxification pathway and CuNP-induced ROS generation. We characterised the redox status in cells exposed to CuNPs and to $Cu(NO_3)_2$ alone and compared the responses with those obtained after simultaneous co-incubation with the AhR ligand ßNF.

2. Materials and methods

2.1. Chemicals and reagents

Uncoated CuNPs of 25 and 78 nm (CuNP 25 and CuNP 78), in size according to the manufacturer's information, were purchased from IoLiTec, Inc. (Heilbronn, Germany) and NanoAmor® USA (Houston, TX, USA), respectively. L-Glutamine (200 mM), foetal bovine serum (FBS), penicillin, and streptomycin (10,000 U/mL, 10 mg/mL, respectively) (P/S), 100X non-essential amino acids (NEAA), and Eagle's Minimum Essential Medium (EMEM) were purchased from Lonza (Barcelona, Spain). Phenol-red free, serum-free Minimum Essential Medium (MEM) was supplied by PAN Biotech GmbH, (Aidenbach, Germany). Acetonitrile (HPLC grade) was provided by Panreac (Barcelona, Spain). Bovine serum albumin (BSA) was purchased from Merck group KGaA (Darmstadt, Germany). Glutaraldehyde (Electron Microscopy grade, 25%), osmium tetroxide, and Spurr's resin were from TAAB Laboratories Equipment Ltd. (Aldermaston, UK). Paraformaldehyde (16%) was sourced from Electron Microscopy Sciences (Hatfield, UK). All other chemicals and reagents used, including ßNF and α-Naphthoflavone (αNF), were supplied by Sigma Aldrich (Madrid, Spain).

2.2. Cell culture conditions

The rat hepatoma cell line H4IIE was cultured in EMEM supplemented with 10% FBS, 1% P/S, 1% L-glutamine, and 1% NEAA at 37 °C in a humidified atmosphere of 5% CO_2. Cells were seeded at a density of 2.5×10^4 cells/well in 96-well plates (Greiner Bio-One GmbH, Germany). For TEM analysis, they were seeded on poly-L-lysine-coated glass coverslips (BD Biosciences, Erembodegem, Belgium) in 24-well plates (Greiner Bio-One GmbH, Germany).

2.3. Nanoparticle preparation and physico-chemical characterisation

We prepared 200 μg/mL suspensions of CuNP 25 and CuNP 78 in EMEM (10% FBS) directly prior to exposure experiments. In order to improve dispersion, the suspensions were sonicated for 10 min in a water bath sonicator (S 40 H Elmasonic, Elma, Germany). Dynamic light scattering (DLS) using a Zetasizer Nano-ZS apparatus (Malvern Instruments Ltd., Malvern, UK) was then used to measure the hydrodynamic size of the particles upon dispersion (time 0) and after 24 h of incubation under culture conditions (37 °C/5% CO_2). Supernatants were prepared from these NP suspensions after a 24 h incubation under cell-free culture conditions and 20 min centrifugation at 13,362×*g* (5415 R series centrifuge, Eppendorf, Hamburg, Germany), as described by Song and colleagues [32]. These supernatants contained only copper ion fractions, as shown by DLS analysis. The ion concentration in each CuNP suspension was determined in a previously published study [32], which established the dissolution rate of a 200 μg/mL suspension of CuNPs. In the present study, DLS was also performed with suspensions of CuNPs co-incubated with ßNF (2.5 μM) for 24 h in order to characterise the size distribution of NPs in co-incubation experiments. Size distributions are reported on the basis of the mean of four