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Research Paper

Crucial role of chelatable iron in silver nanoparticles induced DNA damage and cytotoxicity

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ABSTRACT

Damage to mitochondria and subsequent ROS leakage is a commonly accepted mechanism of nanoparticle toxicity. However, malfunction of mitochondria results in generation of superoxide anion radical ($O_2^{\bullet-}$), which due to the relatively low chemical reactivity is rather unlikely to cause harmful effects triggered by nanoparticles. We show that treatment of HepG2 cells with silver nanoparticles (AgNPs) resulted in generation of H_2O_2 instead of $O_2^{\bullet-}$, as measured by ROS specific mitochondrial probes. Moreover, addition of a selective iron chelator diminished AgNPs toxicity. Altogether these results suggest that $O_2^{\bullet-}$ generated during NPs induced mitochondrial collapse is rapidly dismutated to H_2O_2, which in the presence of iron ions undergoes a Fenton reaction to produce an extremely reactive hydroxyl radical ($^{\bullet}OH$). Clarification of the mechanism of NPs-dependent generation of $^{\bullet}OH$ and demonstration of the crucial role of iron ions in NPs toxicity will facilitate our understanding of NPs toxicity and the design of safe nanomaterials.

1. Introduction

Iron is an essential component of many enzymes involved in a variety of biological processes, including electron transfer, oxygen transport, DNA synthesis and repair [1]. Despite its necessity for almost all living organisms, iron in excess is dangerous. In the presence of ferrous ions, hydrogen peroxide undergoes the Fenton reaction to produce an extremely reactive hydroxyl radical ($^{\bullet}OH$). Radical reactions initiated by $^{\bullet}OH$ may result in damage to the macromolecules, such as DNA, lipids and proteins [2,3]. Iron overload has been linked to increased risk of coronary heart disease, inflammation, neurodegenerative disease and cancer [4,5]. Iron content was also reported to correlate with the amount of oxidative damage to DNA [6,7] and with urinary excretion of 8-hydroxy-2-deoxyguanosine [8].

Iron uptake and storage are carried out by different proteins, thus there is a pool of chelatable iron ions (chelatable iron pool, CIP) that reflects a junction of metabolic pathways of iron-containing molecules. Although the CIP represents only a minor fraction of total cellular iron (3–5%), it is easily accessible and engaged in formation of reactive oxygen species (ROS) [9]. Increased ROS production leads to an imbalance between generation of free radicals and their neutralisation by cellular antioxidative defence mechanisms and causes disturbance of the redox equilibrium, known as oxidative stress. Being highly reactive, ROS are able to modify cellular components, causing cyto- and genotoxic effects.

An increase in ROS due to nanoparticles (NPs) treatment is well documented. It has been shown to be a key factor in the biological effects of NPs, both in vivo and in vitro [10–13]. Although low concentrations of ROS are generated during cell respiration under normal physiological conditions, the presence of NPs markedly increases ROS formation, likely due to the interference with mitochondrial or non-mitochondrial ROS producing enzymes. Indeed, an NPs-dependent increase in production of superoxide anion radical ($O_2^{\bullet-}$) by NADPH oxidase, accompanied by intracellular production of the other ROS was recently reported for AgNPs [14] and ultrafine particles [15]. It was also shown that different non-metal NPs co-localize with mitochondrial markers [16]. Also AgNPs of varying size and shape accumulate in the mitochondria [17]. Thus, it seems plausible to assume that AgNPs accumulation could be a direct cause of mitochondrial damage and malfunction of the respiratory chain resulting in ROS generation. For

Abbreviations: ROS, reactive oxygen species; TEM, transmission electron microscopy

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example, in BRL 3A rat liver cells exposed to AgNPs (15 and 100 nm) the cellular level of ROS increased in a AgNPs concentration-dependent manner and reached a maximum after 6 h [18]. Moreover, in three human cell lines treated with AgNPs, the extent of ROS production correlated with intracellular nanoparticle accumulation and genotoxicity, and negatively with long term cell survival [19]. The recent critical review on AgNPs toxicity leaves no doubts that ROS induction due to the malfunction of mitochondria might be a major cause of detrimental effects exerted by AgNPs on living cells [20].

Here we investigate the mechanism of AgNPs toxicity with special attention paid to generation of ROS and the role of iron in the formation of NPs-induced oxidative damage to DNA and toxicity.

2. Materials and methods

2.1. Chemicals and cell culture

All chemicals, cell culture media ingredients, etc. were purchased from Sigma-Aldrich (Poland) unless otherwise indicated. HepG2 cells were obtained from ATCC. Cells were cultured in cell culture flask with 75 cm^2 surface area (Nunc) in Williams' medium with 10% Fetal Bovine Serum at 37 °C in a 95% moist atmosphere with 5% carbon dioxide. Cells were repassaged when cultures reached 70–85% confluence. Cells were trypsynized, counted using a Countess Automated Cell Counter (Invitrogen) and plated at 4×10^4 per cm^2 new flask or used for experiments.

Depending on the exposure scenario cells were treated simultaneously with 25 μM deferoxamine (DFO) and AgNPs (50 or 100 μg/mL) for 2 h at 37 °C or pre-treated for 24 h with 25 μM DFO and then treated with AgNPs (50 and/or 100 μg/mL) for 2 h at 37 °C. After treatment AgNPs were washed out and cells were left for cytotoxicity assay. For comet assay experiments cells were pretreated for 2 h at 37 °C with DFO at concentration 100 μM to assure substantial removal of CIP and then treated with 10 μg/mL AgNPs for additional 2 h. After treatment cells were immediately processed for DNA damage estimation with the comet assay.

Since combination index approach described by Chuo et al. (for details see [21]) needs a different experimental design, to prevent cytotoxicity resulting from iron deprivation, DFO was used at low-toxic concentration and mixed with AgNPs at a fixed ratio of 1:10. Cells were treated with geometrically increasing concentrations of the mixture. Actual drugs concentrations are presented in CompuSync Report file AgNPs + DFO-d 2.pdf (Supplementary materials). After treatment nanoparticles were washed out and cells were left for cytotoxicity assay.

2.2. Nanoparticle preparation and characterization

AgNPs of nominal size 20 nm were purchased from Plasmachem GmbH, Germany. The stock solution (2 mg/mL) was prepared by suspending of 2 mg AgNPs in 800 μL of distilled water, followed by sonification (4.2 kJ/cm^3, Bronson, USA). Immediately after sonification 100 μL of 15% BSA and 100 μL of a 10× concentrated phosphate buffered saline [22]. Size and ξ-potential of AgNPs aggregates in suspension were determined by the dynamic light scattering (DLS) method (Zatasier S, Malvern Instruments, Malvern, United Kingdom).

2.3. Neutral Red assay

The Neutral Red (NR) assay was used to assess proliferation of HepG2 cells after treatment with AgNPs, DFO or their combination. The assay was performed as described in [22]. In brief, HepG2 cells were seeded in 96-well microplates (TPP, Switzerland) at a density of 1×10^4 cells/well in 100 μL of culture medium. Twenty four hours after cell seeding, cells were treated as described above. After treatment cell culture medium was removed, the cells were washed with 150 μL PBS and incubated for 3 h at 37 °C with 100 μL of neutral red solution at a final concentration of 50 μg/mL. Next the NR solution was aspirated, cells were washed with 150 μL of PBS and 200 μL of an acetic acid-ethanol solution (49% water, 50% ethanol and 1% acetic acid) was added to each well. After 15 min of gentle shaking, optical density was read at 540 nm in plate reader spectrophotometer Infinite M200 (Tecan, Austria). At least three independent experiments in six replicate wells were conducted per experimental point.

2.4. Alkaline comet assay

The comet assay (single cell gel electrophoresis) was performed as described in [23]. Briefly, an aliquot of cell suspension was mixed with an equal volume of 2% low melting point agarose (Type VII, Sigma), put on a microscope slide pre-coated with 0.5% normal agarose (Type I-A, Sigma) and left on ice. After agarose solidification, the slides were immersed in ice-cold-lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris and 1% Triton X-100, pH 10). After 1 h lysis, the slides were placed on a horizontal gel electrophoresis unit filled with a fresh electrophoretic buffer (1 mM Na_2EDTA (sodium ethylenediamine tetraacetate) and 300 mM NaOH) and allowed to stay in the buffer for 40 min for DNA unwinding. Next, electrophoresis was performed (1.2 V/cm, 30 min, 10 °C). After electrophoresis, the slides were washed with 0.4 M Tris, pH 7.5 (3 × 5 min) and stained with DAPI (4′,6-diamidino-2-phenylindole), 50 μL per slide (1 μg/mL).

Basically the same procedure was applied for the measurement of DNA base damage. The treated cells were incubated on slides with the formamido-pyrimidine DNA glycosylase (FPG, New England BioLabs, UK), as described in [24]. Briefly, after lysis, the slides were washed 3 × 5 min with the FPG buffer (40 mM Hepes (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid), 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL bovine serum albumin, pH 8) at 4 °C. Further, 50 μL of FPG solution (4.8×10^{-2} U) in the buffer was placed on each slide, covered with cover glass and incubated for 30 min in a light-protected box at 37 °C. The slides were stained with DAPI (1 μg/mL) and analysed as described above. Image analysis of the data was performed with the Comet Assay IV Image Analysis System (Perceptive Instruments, UK). Fifty randomly selected comets per slide were analysed, two slides per experimental point. The percentage of DNA in the comet tail was used in this study as a measure of DNA damage.

The induction of DNA damage by a combined treatment with AgNPs and DFO was compared to the "expected value". The "expected value" concept is based on the assumption that action of both factors (NPs and DFO) is independent, and their combined toxicity is a sum of toxicities of each factor alone (neutral effect). If the combined toxicity is lower than the"expected value", the sparring effect is observed. If the combined toxicity is higher than the"expected value", the synergistic (potentiating) effect is observed.

2.5. Detection of H_2O_2 in mitochondria

2.5.1. Cell transfection with Hyper Mito plasmid

pHyPer-dMito plasmid was purchased from Evrogen (Russia) To ensure that maximum transfection rate is achieved, the electroporation parameters have been optimized for HepG2 cells. Cells were harvested in the exponential growth phase, diluted in culture medium DMEM and the number of cells was determined using a Countess Automated Cell Counter (Invitrogen). One million HepG2 cells were span down and resuspended in 800 μL of Eppendorf Hypoosomal Electroporation Buffer. Ten micrograms of plasmid DNA were added and mixed well. Afterwards the cell suspension was transferred to 4 mm gap width electroporation cuvettes and electroporation was carried out using the Eppendorf Multiporator set to following parameters: 500 V, 3 pulses and 100 μs time constant at room temperature. After pulsing the cells were allowed to remain in the cuvettes for 5 min and then carefully transferred into 3 mL of fresh culture medium DMEM with 10% FBS and cultivated in 6-well culture plates at least 24 h.