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Silver nanoparticles activate endoplasmic reticulum stress signaling pathway in cell and mouse models: The role in toxicity evaluation

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ABSTRACT

Silver nanoparticles (AgNPs) attract considerable public attention both for their antimicrobial properties and their potential adverse effects. In the present study, endoplasmic reticulum (ER) stress was used as a sensitive and early biomarker to evaluate the toxic potential of AgNPs in three different human cell lines *in vitro* and *in vivo* in mice. In 16HBE cells, the activation of ER stress signaling pathway was observed by upregulated expression including *xbp-1s*, *chop/DDIT3*, *TRIB3*, *ADM2*, BIP, Caspase-12, ASNS and HERP at either the mRNA and/or protein levels. However, these changes were not observed in HUVECs or HepG2 cells. Furthermore, mice experiments showed that different tissues had various sensitivities to AgNPs following intratracheal instillation exposure. The lung, liver and kidney showed significant ER stress responses, however, only the lung and kidney exhibited apoptosis by TUNEL assay. The artery and tracheal tissues had lower ER stress and apoptosis after exposure. The lowest observable effect concentrations (LOEC) were proposed based on evaluation of AgNP induced ER stress response in cell and mouse models. In summary, preliminary evaluation of AgNP toxicity by monitoring the ER stress signaling pathway provides new insights toward the understanding the biological impacts of AgNPs. The adverse effects of exposure to AgNPs may be avoided by rational use within the safe dose.

1. Introduction

Silver nanoparticles (AgNPs) are widely used in consumer and medical products based on their unique antimicrobial properties. If AgNPs released from these products, they could create potential human exposure [1,2]. Studies have shown that exposure to AgNPs induces cytotoxicity, immunotoxicity and genotoxicity to vertebrates *in vitro* and *in vivo* [3—6]. With the advances of molecular biology, toxicity testing on the cellular response pathways responsible for adverse health effects had been recommended by the U.S. Environmental Protection Agency (EPA) and the National Research Council (NRC) as the preferred toxicity testing strategy in the 21st century (NRC 2007, U.S. EPA 2009). For the toxicity of AgNPs, clear dosage guidelines are important for achieving the goal of safe applications. Failure to consider dosimetry may lead to the wrong conclusions when the toxicity of nanomaterials is being compared. In the face of such a reality, it might be best for scientists to specify the possible target organs and the safe application concentration of AgNPs before seeking an alternative to this nanomaterial.

AgNPs usually exhibit an antimicrobial function at a relatively low concentration. The minimal inhibitory concentration (MIC) of AgNPs against yeast was from 6.6 nM to 13.2 nM, and only 3.3 nM—6.6 nM for *Escherichia coli* under the test conditions used [7]. However, cellular toxicity to cell lines of human origin usually has been reported at higher concentrations, ranging from 1 to 50 μg/mL (about 0.01—0.5 mM) [3,8,9]. This wide toxic dosage rage of human origin cell lines usually comes from different synthesis

methods, various sizes, and the presence or absence of capping agents of AgNPs [10]. AgNPs are usually less toxic than silver ions although there has been no conclusive evaluation of their toxicity until now [11,12]. The ultimate question is that what concentration of AgNP is high enough for its antimicrobial function, but low enough to avoid toxicity *in vivo*. In answering this question objectively, we should avoid exaggeration of NP toxicity by drawing conclusions from obvious adverse outcomes of varying experimental conditions. We propose that a simple testing based on cell signaling pathways is a good option to predict AgNP toxicity.

In our previous report [13], we have found that endoplasmic reticulum (ER) stress, also known as unfolded protein response (UPR), is an important cellular self-protection mechanism that could be used as an early biomarker for evaluating the toxicity of an exogenous stimulator. Toxicosis only occurs when the concentration exceeds the physiological threshold of cellular self-protection capacity. The ER stress system contains three dominant stress sensors that include inositol-requiring protein 1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor-6 (ATF-6). In its normal state, the ER-chaperone protein 78 kDa glucose-regulated protein (GRP78/BIP) binds these three stress sensors, and in its active state releases them when stress occurs. IRE1, which functions as an endoribonuclease, initiates the splicing of X-box binding protein 1 (*xbp-1*) mRNA after activation. Then, the newly produced spliced form of *xbp-1* (*xbp-1s*) induces the transcription of chaperone protein-encoding genes for self-protection functions. The *xbp-1* splicing and induction of BIP and CHOP (also known as DNA-damage-inducible transcript 3, DDIT3) have been identified as the specific markers of ER stress responses [13,14]. Several papers have shown that AgNPs could induce apoptosis *via* modulation of the ER stress reaction in mammalian cells and in zebrafish [15–17]. However, the detailed mechanism of AgNP-induced ER stress has not been clearly determined. Further, ER stress as a result of AgNP exposure has not been reported in animal models. Our study focuses on the AgNPs induced ER stress response in human cells and in a mouse model, with the intention of outlining the use of the adverse outcome pathway for predicting nanotoxicity.

2. Materials and methods

2.1. Nanoparticles and characterization

AgNPs (NM-300K) and the corresponding stabilizing agents (NM-300K DIS) were provided by the European Commission Joint Research Center (Ispra, Italia). The NPs were stabilized in the same solutions to NM-300K-DIS (4 wt% polyoxyethylene glycerol trioleate and 4 wt% Tween 20). They were sub-sampled under Good Laboratory Practice conditions and preserved under argon in the dark until use. Same volume NM-300K-DIS to the NPs (NM-300K) was used as the control sample in the experiments of this study. In characterization tests, NM-300K was dispersed in sterile Milli-Q water to prepare stock solutions (1 mg/mL). Stock dispersions were sonicated for two times 10 min on ice in an ultrasound bath (100 W) to break up aggregates and diluted in Milli-Q water or cell culture medium for transmission electron microscopy (TEM) assays, dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) (LM10-Base, Nanosight, UK).

2.2. Cell culture and AgNP treatment

Human bronchial epithelial cells (16HBE), human hepatocellular liver carcinoma cells (HepG2) and human umbilical vein endothelial cells (HUVECs) were cultured at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA)

supplemented with 10% fetal bovine serum (FBS, Gibco, USA). Cells were seeded and incubated for 24 h before exposure to AgNPs or Ag^+ in 96-well plates, 6-well plates and 6 cm cell dishes for the cell viability assay, RNA extraction and protein collection, respectively.

2.3. Cellular toxicity test, real-time PCR, ICP-MS, Western blot, PCR array and immunofluorescence assay

Procedures were the same as previously described [13]. The expression of 84 key genes recognizing and responding to misfolded protein accumulation in the endoplasmic reticulum was determined using the Human Unfolded Protein Response RT^2 Profiler™ PCR Array (Qiagen, Germany).

2.4. Intratracheal instillation of mice and preparation of tissue samples

Male ICR mice of 19–21 g were obtained from Vital River Laboratory Animal Technology Co., Ltd. Animals were housed in standard cages in an isolated and air-conditioned animal room. Experiments were approved by the Ethics Committee of Animal Care and Experimentation of the National Institute for Environmental Studies, China. Mice were randomly allocated into six subgroups (n = 6), including two doses each with two time points (8 and 24 h), a normal control, and a vehicle control. AgNP (NM-300K) and NM-300K-DIS were suspended in physiological saline solution and ultrasonicated for 15 min before intratracheal instillation. A final volume of 0.1 ml was intratracheally instilled for all exposure groups except the blank control, which was performed non-surgically using a ball-tipped needle after animals were anesthetized by ether. Detailed instillation procedures can be found in our previous paper [18]. Mice were sacrificed at 8 and 24 h after AgNP exposure. Then, tissue samples were excised and parts allocated to being quickly frozen in liquid nitrogen before storage at −80 °C or being immersed in formalin solution. The quick-freeze samples were used for RNA extraction and Western blot sample preparations. The fixed samples were embedded in paraffin blocks, cut into 4-μm-thick sections, and mounted onto glass for HE staining and TUNEL assays.

2.5. Terminal deoxynucleotidyl TUNEL assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick endlabelling (TUNEL) assay could specifically detect the fragmented genomic DNA usually caused from sequential activation of Caspases and endonucleases in apoptosis [19]. Dewaxing, tissue rehydration and staining were carried out according to the recommended procedures from the fluorescence-conjugated TUNEL kit manufacturer (Roche, Mannheim, Germany). For counting the total number of cells in tissue samples, DAPI was added before mounting the coverslip to stain the nuclei. Images were captured under a fluorescence microscope (Olympus BX61W1with Fluoview FV1000 software, Japan), and then analyzed by ImagePro software. Three different image areas of at least 500 cells were counted to obtain the apoptosis rate.

3. Results

3.1. Characterization of silver nanoparticles

The diameter of AgNPs employed in this study was d = 20 nm by TEM and 38 nm by DLS (Fig. 1A and Table S1). According to the published report of manufacturer [20], the primary crystal size is about 15 nm with 99% of particles below d = 20 nm and with a narrow size distribution. NTA test showed that AgNPs were