Toxicology in Vitro 40 (2017) 223–233

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Silver nanoparticles induce hormesis in A549 human epithelial cells

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ARTICLE INFO

Article history:
Received 22 September 2016
Received in revised form 30 November 2016
Accepted 15 January 2017
Available online 18 January 2017

Keywords:
Silver nanoparticles
Acrolein
GSH
Hormesis
Nrf2

ABSTRACT

Despite the gaps in our knowledge on the toxicity of silver nanoparticles (AgNPs), the application of these materials is fast expanding, from medicine, to food as well as the use in consumer products. It has been reported that prolonged exposure might make cells more resistant to AgNPs. This prompted us to investigate if AgNPs may give rise to a hormetic response. Two types of AgNPs were used, i.e. colloidal AgNPs and an AgNP powder. For both types of nanosilver it was found that a low dose pretreatment of A549 human epithelial cells with AgNPs induced protection against a toxic dose of AgNPs and acrolein. This protection was more pronounced after pretreatment with the colloidal AgNPs. Interestingly, the mechanism of the hormetic response appeared to differ from that of acrolein. Adaptation to acrolein is related to Nrf2 translocation, increased mRNA expression of γGCS, HO-1 and increased GSH levels and the increased GSH levels can explain the hormetic effect. The adaptive response to AgNPs was not related to an increase in mRNA expression of γGCS and GSH levels. Yet, HO-1 mRNA expression and Nrf2 immunoreactivity were enhanced, indicating that these processes might be involved. So, AgNPs induce adaptation, but in contrast to acrolein GSH plays no role.

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1. Introduction

Silver nanoparticles (AgNPs) have found increasing applications in industry, medicine and consumer products and their use is fast expanding (Austin et al., 2014; Johnston et al., 2010). The main reason why silver is used in medical settings is because of its antibiotic properties (Batchelor-McAuley, et al., 2014). Nevertheless, there are fundamental gaps in the knowledge on their toxicity and mode of action (Beer et al., 2012; Braakhuis et al., 2014; Cronholm et al., 2013; De Matteis et al., 2015; Govender et al., 2013; Levard et al., 2013; McShan et al., 2014; Recordati et al., 2016). It is long known, that the adverse health effects of toxic fine particles like crystalline silica dust and asbestos are linked to reactive oxygen species (ROS) formation and induction of oxidative stress. Subsequently, redox dependent cellular processes and effects became the focus of investigations on the toxicity of ambient and engineered nanoparticles (Donaldson et al., 2003; Unfried et al., 2007). Indeed, also studies with AgNPs relate toxicity to their ability to generate ROS and to induce oxidative stress (Bohmert et al., 2015; Foldbjerg et al., 2012; Johnston et al., 2010; Liu et al., 2010; Xia et al., 2006). Paradoxically, AgNPs also activate the transcription factor nuclear factor erythroid-derived 2 related factor 2 (Nrf2) (Bohmert et al., 2015; Kang et al., 2012a; Kang et al., 2012b; Prasad et al., 2013; Sahu et al., 2015). This leads to upregulation of cellular antioxidant and detoxification enzymes and upgrades the cellular protection against oxidative stress (Xia et al., 2006; Zhang et al., 2012).

This prompted us to examine the potential relevance of both processes induced by AgNPs i.e. increased formation of ROS versus upregulation of the antioxidant defense. In recent years, peculiar findings on the toxicity of AgNPs have been reported. Aude-Garcia et al. (2015) compared the toxicity of AgNPs administered as a single high dose versus the same amount of AgNPs administered as repeated low doses on primary mouse macrophages. Intracellular accumulation of Ag was similar for both treatment regimes, while only the single high dose treatment caused a pro-inflammatory activation of these cells. Brzoska et al. (2015) reported a short term (2 h) increase in DNA damage returning to baseline after prolonged exposure (24 h) to AgNPs in HepG2 liver epithelial cells. This may point to a hormetic response to AgNPs. Hormesis is that "exposure to a low dose of a chemical that is damaging at higher doses, induces an adaptive upgrade of cellular protection" (Calabrese and Baldwin, 2002). In recent years, this adaptation concept has also gained more attention for nanoparticles (Bell et al., 2014; Iavicoli et al., 2010).

The process of hormesis can be illustrated with the ubiquitous environmental pollutant acrolein. Recently, we showed that in BEAS-2B lung epithelial cells a low dose of acrolein induces protection against a high

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http://dx.doi.org/10.1016/j.tiv.2017.01.010
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toxic dose of acrolein (Haenen et al., 1988; Sthijns et al., 2014). Acrolein can react with thiols of important cellular proteins, causing toxicity. The protagonist in the protective mechanism induced by this reactive unsaturated aldehyde is the thiol containing compound glutathione (GSH). GSH levels are upregulated by a low dose of acrolein via increasing the Nrf2-mediated gene expression of the rate limiting enzyme of GSH synthesis *gamma*-glutamylcysteine synthetase (γGCS) (Stevens and Maier, 2008). This protects against a second exposure to acrolein and thus gives the hormetic response.

The aim of our study was to investigate the hormetic adaptive response to AgNPs by investigating whether pretreatment with a low dose of AgNPs can protect against exposure to a high dose. Two contrasting types of AgNPs, i.e. colloidal versus powder, were selected and characterized for primary particle size, solubility and agglomeration behavior in this study. Additionally, the mechanism of adaptation was compared to acrolein. Experiments were performed in human A549 lung epithelial cells, which have been widely used to investigate the toxicity of various types of engineered nanoparticles including AgNPs (Beer et al., 2012; Brzoska et al., 2015; Foldbjerg et al., 2012; Han et al., 2014; Huk et al., 2014; Liu et al., 2010; Maurer et al., 2014; Singh et al., 2007). The use of this cell line also has practical relevance due to inhalation exposure to nanosilver caused by a growing number of consumer products such as disinfectants, deodorants, antimicrobial sprays and other applications where these particles also become airborne (Braakhuis et al., 2014; Christensen et al., 2010). Despite its potential limitations (i.e. transformation status), A549 cells were used in several recent and ongoing large-scale nanosafety projects to allow for the necessary bridging of data from different research labs. Their robustness for toxicity evaluation of nanomaterials was evaluated in a round robin approach within the EU-7th framework projects ENPRA (Kermanizadeh et al., 2016).

2. Materials and methods

2.1. Nanomaterials

Two types of silver nanoparticles were used, referred to in this study as AgNP1 and AgNP2. The first material, AgNP1, was purchased from Skyspring Nanomaterials, Inc. (US). This material is available as a powder. The AgNP2 represents a sample of the NM-300 reference nanomaterial and was received from the European Commission Joint Research Centre (Ispra, Italy) in the context of the EU FP7 project ENPRA. The nanomaterial NM-300 is a dispersion of silver nanoparticles in deionised water (85%) with 7% stabilising agent (ammonium nitrate) and 8% emulsifiers (4% each of polyoxyethylene glycerol Trioleate and Tween 20). This colloidal nanosilver samples were purchased and studied along with its dispersant control (i.e. NM-300dis) within the framework of the ENPRA project (Kermanizadeh et al., 2016). Because of absence of cytotoxicity for NM-300dis towards the A549 cells up to the highest test concentration equivalent to 80 μg/cm^2 for NM-300 (data not shown), it was decided not to include this dispersant control further in the present study. Representative scanning electron microscopy (SEM) images of both AgNPs are shown in Fig. 1. As can be seen in the figure, the AgNP1 consists of compact, near-spherical to elongated primary particles which tend to form compact agglomerates/aggregates. Detailed SEM analysis of the material revealed a primary particle size of 37.0 nm ± 13.0 nm. In contrast, the AgNP2 consists of homogenously distributed, merely spherical particles. For this sample a primary particle size of 16.6 nm ± 4.4 nm was determined. Both types of nanoparticles also revealed contrasting dissolution properties. We determined the solubility of the samples in deionised water (at room temperature) after continuous shaking, followed by syringe filtration and analysis by ICP-OES. Upon 72 h incubation, for the AgNP1 about 0.2% was found to be dissolved, whereas for the AgNP2 sample this was much larger, i.e. up to 5%.

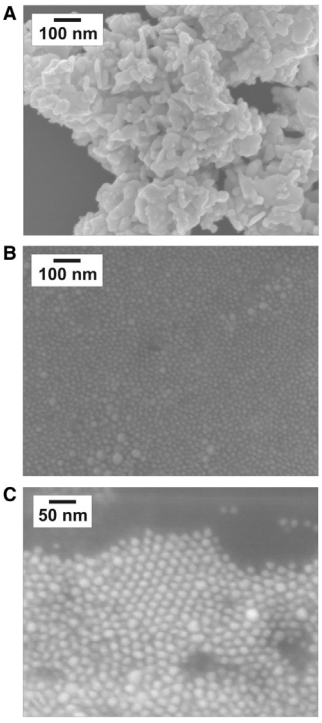

Fig. 1. Representative scanning electron micrographs of AgNP1 (A) and AgNP2 (B and C).

2.2. Culture and treatment of cells

Human lung adenocarcinoma cells (A549) were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Invitrogen, Bleiswijk, The Netherlands) supplemented with 10% Fetal Calf Serum (FCS, Sigma-Aldrich, St. Louis, MO, USA) and 1% Penicillin/Streptomycin (Life Technologies, Bleiswijk, The Netherlands) in an environment containing 5% CO_2 and 95% air at 37 °C. Experiments were performed with cells from passage 15–20.

All particle suspensions for the *in vitro* experiments were prepared on the basis of the nanoparticle dispersion protocols as developed within the EU-7th framework project ENPRA (http://www.enpra.eu) (Kermanizadeh et al., 2016; Kermanizadeh et al., 2013) and the SIINN-ERANET project NanOxiMet (http://www.nanoximet.eu) with following specific modifications: Stock solutions of 1 mg/ml were prepared in sterile RNase free water with 2% Adult Bovine Serum (ABS, Sigma-Aldrich, Darmstadt, Germany). The suspensions were then sonicated for 10 min with a Branson 450 Sonifier with Cuphorn at a power of 5.71 (200 W) and 20% duty cycle. The stock suspensions were quickly further diluted in exposure medium to a concentration of 256 μg/ml, equivalent to the treatment concentration of 80 μg nanoparticles per cm^2 of cell culture monolayer. The exposure medium for the AgNP samples consisted of phenol red free DMEM/F12 with 1% 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES, Sigma-Aldrich, Darmstadt, Germany) and 1% Penicillin/Streptomycin. The acrolein (Sigma-Aldrich, St. Louis, MO, USA) exposures were performed in Hank's Balanced Salt Solution (HBSS, Gibco, Bleiswijk, The Netherlands). For dose response relationship evaluations, the A549 cells were exposed to 0, 1, 3, 10, 30, 100 and 200 μM acrolein for 30 min and to 0, 1.25, 2.5, 5, 10, 20, 40, 80 μg/cm^2 AgNPs for 24 h, respectively. Toxicity and intracellular GSH levels were then determined as described in the subsequent sections.

To investigate adaptation processes, repeated exposures were performed at concentrations and treatment times based on the outcome of initially performed dose response analysis, and, for acrolein, in part

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