Toxicology in Vitro 28 (2014) 457–467

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Mechanism of uptake of ZnO nanoparticles and inflammatory responses in macrophages require PI3K mediated MAPKs signaling

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ARTICLE INFO

Article history:
Received 5 July 2013
Accepted 11 December 2013
Available online 22 December 2013

Keywords:
Zinc oxide nanoparticles
Proinflammatory cytokines
Macrophages
Phagocytosis
Endocytosis

ABSTRACT

The inflammatory responses after exposure to zinc oxide nanoparticles (ZNPs) are known, however, the molecular mechanisms and direct consequences of particle uptake are still unclear. Dose and time-dependent increase in the uptake of ZNPs by macrophages has been observed by flow cytometry. Macrophages treated with ZNPs showed a significantly enhanced phagocytic activity. Inhibition of different internalization receptors caused a reduction in uptake of ZNPs in macrophages. The strongest inhibition in internalization was observed by blocking clathrin, caveolae and scavenger receptor mediated endocytic pathways. However, FcR and complement receptor-mediated phagocytic pathways also contributed significantly to control. Further, exposure of primary macrophages to ZNPs (2.5 μg/ml) caused (i) significant enhancement of Ras, PI3K, (ii) enhanced phosphorylation and subsequent activation of its downstream signaling pathways via ERK1/2, p38 and JNK MAPKs (iii) overexpression of c-Jun, c-Fos and NF-κB. Our results demonstrate that ZNPs induce the generation of reactive nitrogen species and overexpression of Cox-2, iNOS, pro-inflammatory cytokines (IL-6, IFN-γ, TNF-α, IL-17 and regulatory cytokine IL-10) and MAPKs which were found to be inhibited after blocking internalization of ZNPs through caveolae receptor pathway. These results indicate that ZNPs are internalized through caveolae pathway and the inflammatory responses involve PI3K mediated MAPKs signaling cascade.

1. Introduction

Living beings have been exposed to nanoparticles (NPs) ever since the origin of life on earth. NPs are produced from various natural as well as anthropogenic sources such as, volcanic eruptions, physical and chemical weathering of rocks, diesel exhaust, electroplating, and welding (Rietmeijer and Mackinnon, 1997; Murr et al., 2004; Wigginton et al., 2007; Siegmann et al., 2008). These particles may aggregate to form large sized particles or may persist in air, water, soil and biological systems as such (Beduneau et al., 2009). Therefore, we may assume that living organisms have co-evolved with nanomaterials in the environment and may have developed efficient disposal or toxicity mitigating mechanisms for NPs. Nonetheless, it is also known that as particles approach the nano-scale, they often become more reactive, owing to increase in the surface area and predominance of surface related phenomenon (Monteiller et al., 2007). Importantly, materials that are inert in bulk form may become reactive and toxic in nano-sized form (Nel et al., 2006). For example, even an inert compound like gold tends to elicit a biological response when introduced in nano-form (Goodman et al., 2004). This clearly emphasizes the need for a careful evaluation of the toxicological manifestations rendered by NPs.

Zinc oxide nanoparticles (ZNPs) are among the most commonly used nanomaterials, with a wide range of applications in industrial and consumer products. Recently, ZNPs have been shown to specifically target and kill cancer cells and can possibly be developed as an alternative anticancer therapeutic agent (Hanley et al., 2008;

Abbreviations: AFI, absolute fluorescence intensity; CR, complement receptors; DMSO, dimethyl sulphoxide; DLS, dynamic light scattering; ELISA, enzyme linked immunosorbent assay; FBS, fetal bovine serum; MR, mannose receptors; MAPKs, mitogen-activated protein kinases; PBS, phosphate buffered saline; PI3K, phosphatidylinositol 3-kinase; SSC, side scatter angle; SR, scavenger receptors; TEM, transmission electron microscopy; ZNPs, zinc oxide nanoparticles.

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http://dx.doi.org/10.1016/j.tiv.2013.12.004

Moos et al., 2010). ZNPs have also been reported to have the potential to activate dendritic cells and stimulate the release of proinflammatory cytokines (Heng et al., 2011). In particular the workers in the manufacturing of ZNPs can be subjected to low level chronic exposure to ZNPs via different routes viz. inhalation, ingestion or dermal route. Ryman-Rasmussen et al. (2006), have shown that sub micrometer particles can penetrate even through the outer layers of skin.

In the present study, macrophages were chosen as an *in vitro* model to study the exposure to ZNPs because they are the primary scavenger cells of the body and form the first line of defense in the immune response to foreign materials (Germain and Margulies, 1993). When NPs enter the systemic circulation, they encounter a complex web of immune cells and plasma proteins. The recognition of NPs as non-self by the immune cells may result in a multilevel immune response against the NPs leading to generation of reactive oxygen species (ROS), reactive nitrogen species (RNS) and altered cytokine levels. The oxidative stress response and cytokine milieu in the body is delicately balanced and orchestrated according to the nature of physiological stress, infections and diseases. On exposure to xenobiotics, inflammatory cells produce hyper levels of ROS which may cause airway hyper-responsiveness, sepsis/multiple organ dysfunction, vascular dysfunction and reduced antioxidant levels (Busija et al., 2006; Dong et al., 2005; Hou et al., 2010). Similarly, abnormal levels of cytokines like TNF-α, IL-6, IL-17 and IFN-γ have been reported in rheumatoid arthritis, active psoriasis and allergic asthma (Wong et al., 2001; Arican et al., 2005; Gratacos et al., 1994).

Macrophages are professional phagocytes, which are capable of efficient uptake of foreign invaders by phagocytosis. Phagocytosis is a particle size-dependent phenomenon which is induced by the interaction of different specialized cell surface receptors with their specific ligands (Haberzettl et al., 2007; Kwiatkowska and Sobota, 1999). Scavenger receptors (SR), mannose receptors (MR), and complement receptors (CR) are some of the specialized receptors with respective specificities (Aderem and Underhill, 1999; Janeway, 1992; Underhill and Ozinsky, 2002). Phagocytosis could further be mediated by the family of Fc receptors (FcR), which recognize immunoglobulin coated particles and complexes (Aderem and Underhill, 1999). Though macrophages do not phagocytose NPs as efficiently as they do for larger particles, but the phagocytic process may get facilitated by the adsorption of opsonins (plasma proteins) onto the particle surface (Owens and Peppas, 2006).

Previously, we have reported that ZNPs have the immunomodulatory potential by altering the cytokines like TNF-α, IL-6, IL-1 and IL-17 in macrophages (Roy et al., 2011). Therefore, the aim of the present study was to identify the classic phagocytosis or endocytosis pathways involved in the uptake of ZNPs and then subsequent inflammatory consequences. Internalization of ZNPs was analyzed in ZNPs treated macrophages either in the presence or absence of inhibitors of different potentially involved receptors. Furthermore, we examined the morphological characteristics by TEM, mitogen activated protein kinases (MAPKs) signaling cascade, and the inflammatory responses, such as phagocytic activity, RNS generation and related inflammatory marker such as Cyclooxygenase-2 (Cox-2) and inducible form of Nitric oxide synthases (iNOS) expression in macrophages.

2. Materials and methods

2.1. Chemicals

ZnO nanopowder (<50 nm), RPMI 1640, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), Dimethyl sulphoxide (DMSO), Chlorpromazine, Filipin, Cytochalasin D and Amiloride were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Antibodies (CD16/32 and CD11b) were purchased from BD Biosciences. Anti-PI3K, anti-Ras, anti-p-JNK, anti-c-Fos and anti-α-Tubulin were purchased from BD Biosciences, USA. Anti-p-ERK1/2, anti-p-p38, anti-p-NF-κB, anti-c-Jun and anti-β-actin were purchased from Santa cruz, USA. Anti-Cox-2 and anti-iNOS were obtained from Calbiochem, USA. Phosphate buffered saline (PBS), fetal bovine serum (FBS) and antibiotic-antimycotic solution (10,000 U/ml penicillin, 10 mg/ml streptomycin sulfate) were purchased from Gibco, Invitrogen Cor. (Grand Island, N. Y., USA). Cell culture plastic-wares were obtained from Nunc (Roskilde, Denmark).

2.2. Nanoparticle suspension preparation and characterization

2.2.1. Preparation

100 μg/ml concentration of ZNPs suspension was prepared in RPMI 1640 containing 10% FBS by sonication (Sonics Vibra cell, Sonics and Material Inc., New Town, CT, USA) for 1.5 min at 32 W amplitude followed by the break for 1 min, and subsequently the same process was repeated twice.

2.2.2. Characterization

The nanoparticle suspensions were characterized by the following methods.

2.2.2.1. Transmission electron microscopy (TEM). The microstructure of ZNPs was studied using a TEM and selected-area electron diffraction was carried out on a Technai 30 G2 S-Twin electron microscope operated at 200 kV accelerating voltage. For TEM analysis, samples of ZNPs were prepared by solution-casting onto carbon coated copper grid.

2.2.2.2. Dynamic light scattering (DLS). The average hydrodynamic size, size distribution and zeta potential of ZNPs in suspension were determined by DLS and phase analysis light scattering, using a Zetasizer Nano-ZS equipped with 4.0 mW, 633 nm laser (Model ZEN3600, Malvern Instruments Ltd., Malvern, UK).

2.3. Isolation and culture of macrophages

Inbred strains of female Balb/c mice (8-10 weeks old) were sacrificed according to the guidelines for the care and use of laboratory animals of CSIR-Indian Institute of Toxicology Research, Lucknow, India. Peritoneal exudate cells were collected from the peritoneal cavity of mice by injecting chilled RPMI 1640 medium and added to 96-well cell culture flat bottom plate. After 3 h of incubation in a CO_2 incubator (5% CO_2) at 37 °C, the non-adherent cells were removed by vigorous washing (three times) with warm RPMI 1640 medium. Furthermore, adhered cells were incubated overnight in RPMI 1640 medium supplemented with heat-inactivated FBS (10%), penicillin (100 U/ml) and streptomycin (100 U/ml) at 37 °C in humid air containing 5% CO_2 to form macrophage monolayers. More than 95% of the adherent cell populations were macrophages as determined by morphology and non-specific esterase staining.

2.4. MTT cell viability assay

Stock suspension of 100 μg/ml particle concentration in RPMI1640 (supplemented with 10% FBS) was serially diluted to 1, 2.5, 5 and 10 μg/ml and vortexed before distribution in the culture wells. Macrophage monolayers (4×10^5 cells/well) were prepared as described above, and treated with the indicated concentrations of ZNPs for 24 h. The medium was removed and cells were washed with PBS. One tenth of total volume of MTT dye solution was added to each well. The 96-well plates were incubated in a humidified, 5% CO_2 incubator at 37 °C for 2 h. Next,