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Oxidative and pro-inflammatory effects of cobalt and titanium oxide nanoparticles on aortic and venous endothelial cells

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ABSTRACT

Ultra-fine particles have recently been included among the risk factors for the development of endothelium inflammation and atherosclerosis, and cobalt (CoNPs) and titanium oxide nanoparticles (TiNPs) have attracted attention because of their wide range of applications. We investigated their toxicity profiles in two primary endothelial cell lines derived from human aorta (HAECs) and human umbilical vein (HUVECs) by comparing cell viability, oxidative stress, the expression of adhesion molecules and the release of chemokines during NP exposure. Both NPs were very rapidly internalised, and significantly increased adhesion molecule (ICAM-1, VCAM-1, E-selectin) mRNA and protein levels and the release of monocyte chemoattractant protein-1 (MCP-1) and interleukin 8 (IL-8). However, unlike the TiNPs, the CoNPs also induced time- and concentration-dependent metabolic impairment and oxidative stress without any evident signs of cell death or the induction of apoptosis. There were differences between the HAECs and HUVECs in terms of the extent of oxidative stress-related enzyme and vascular adhesion molecule expression, ROS production, and pro-inflammatory cytokine release despite the similar rate of NP internalisation, thus indicating endothelium heterogeneity in response to exogenous stimuli. Our data indicate that NPs can induce endothelial inflammatory responses via various pathways not involving only oxidative stress.

1. Introduction

Nanotechnologies based on the chemical, mechanical, optical, magnetic and biological properties of nanomaterials are being increasingly used in a wide range of industries, and there are now more than 1500 commercial products available on the world market (EPA, 2007). However, their use (which is still largely unregulated) has become a recognised social health problem because the inhalation, dermal absorption or ingestion of particles of various sizes and compositions leads to increased rates of chronic respiratory and cardiovascular diseases (Borm et al., 2004; Byrne and Baugh, 2008; Donaldson et al., 2013; Donaldson and Seaton, 2012; Oberdorster et al., 2005; Xia et al., 2009). It is currently difficult to quantify the risk because the available information is contradictory, and there is a lack of definite toxicological data or shared guidelines (Borm et al., 2006; Iavicoli et al., 2009; Schulte and Salamanca-Buentello, 2007), but there is clearly an urgent need to develop a rapid, accurate and efficient means of assessing the effects of nanoparticles (NPs) on human health.

Despite their limitations, *in vitro* studies are still fundamental when assessing dosing ranges and probable mechanisms of toxicity. In the case of metal or metal oxide NPs, besides modifications

Abbreviations: BCA, bicinchoninic acid; CoNPs, nanoparticles of cobalt oxide; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate; EC, endothelial cells; FSC, forward scatter; GSH, glutathione; GSSG, glutathione disulfide; HAECs, human aortic endothelial cells; HO-1, heme oxygenase-1; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; IL-8 (CXCL-8), interleukin 8; LDH, lactate dehydrogenase; MCP-1, monocyte chemoattractant protein-1; MDA, malondialdehyde; NPs, nanoparticles; SELE, E-selectin; ROS, reactive oxygen species; SSC, side scatter; SOD-1, superoxide dismutase 1; SOD-2, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TEM, transmission electron microscopy; TiNPs, nanoparticles of titanium dioxide; VCAM-1, vascular cell adhesion molecule 1..

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of cellular functions, such as repression/activation of genes and mitochondrial dysfunction (Comfort et al., 2014; Jeng and Swanson, 2006; Jugan et al., 2012; Karlsson et al., 2008; Soto et al., 2007), oxidative stress is one of the most studied mechanism of cytotoxicity. It occurs at early stages of interaction and is relevant to potential negative effects on cell functions and DNA damage (Carlson et al., 2008; Choi et al., 2009; Jugan et al., 2012; Karlsson et al., 2008; Liu et al., 2010; Moller et al., 2010; Papis et al., 2009).

Cobalt [Co (II,III)] oxide is one of the most interesting NP chemicals because it can be used in pigments, catalysts, electrochemical sensors, and magnetism and energy storage. However, it has been reported that cobalt oxide NPs are associated with genotoxicity, an increased production of reactive oxygen species (ROS), and the induction of DNA fragmentation in humans (Alarifi et al., 2013; Colognato et al., 2008; De Boeck et al., 2003; Horev-Azaria et al., 2011; Papis et al., 2009).

The most widely synthesised and distributed of the metal oxide NPs are titanium dioxide (TiO_2) NPs. Micro- or submicro-particles have been commercially used as white pigments in paints, plastics, paper, pharmaceuticals, cosmetics and toothpastes. Furthermore, in addition to its industrial and medical applications, TiO_2 is also a common additive in many foods (Weir et al., 2012), and its use is exponentially increasing because of its stability, anti-corrosiveness and photocatalytic properties. It has been estimated that the global production of Ti nano-scaled particles was 5000 t in 2010, and this is expected to increase further because of the greater use of personal care products such as topical sunscreens and cosmetics (EPA, 2009; Hendren et al., 2011; Robichaud et al., 2009). Consequently, there are many potential environmental and occupational sources of exposure to nanoscale TiO_2, but no definite toxicological profile has yet been published. The National Institute for Occupational Safety and Health (NIOSH) considers that occupational exposure (mainly via inhalation and dermal contact) to low concentrations of TiO_2 lead to a negligible risk of lung cancer in workers. Therefore, time-weighted average (TWA) airborne concentration limits of 2.4 mg/m^3 for fine and 0.3 mg/m^3 for ultra-fine TiO_2 for up to 10 h/day during a 40-h working week is recommended (NIOSH, 2011).

The inhaled, dermal or gastrointestinal intake of NPs can reach the bloodstream and be distributed to target organs distant from the site of adsorption (Christensen et al., 2011; Kreyling et al., 2002; Landsiedel et al., 2012; Nemmar et al., 2002). The endothelium lining the inner surface of blood vessels therefore comes into direct contact with NPs in a potentially pathogenic manner. Endothelial cells play a very important role in inflammation, and pro-inflammatory stimulation enhances the expression of adhesion molecules on cell membranes and thus mediates leukocyte attachment. Furthermore, these cells release potent cytokines, thus leading to the migration of leukocytes from blood into the perivascular space. The ability of metal NPs such as Co and Ti oxide to activate endothelial cells and induce pro-inflammatory events and the expression of early and late adhesion molecules has been demonstrated by *in vitro* studies (Duffin et al., 2007; Gojova et al., 2007; Han et al., 2013; Iavicoli et al., 2012; Montiel-Davalos et al., 2012; Moschini et al., 2013; Peters et al., 2004; Strobel et al., 2014).

The aim of this *in vitro* study was to compare the anti-proliferative activity and cytotoxic effects of commercially available Co_3O_4 and TiO_2 nanopowders on human aortic endothelial cells (HAECs) and human umbilical vein endothelial cells (HUVECs) in an attempt to cast some light on their role and possible mechanisms of action in determining cell behaviour and fate. Previous studies of NPs have examined only one endothelial cell line, but studies of endothelial cell diversity have shown that ECs from different vascular beds have distinct sensitivities to oxidative stress and phenotypes that may contribute to the site specificity of vascular pathogenesis (Cai, 2005; Chi et al., 2003; Deng et al., 2006).

2. Experimental procedures

2.1. Reagents

The sterile plastic material for the tissue cultures was purchased from Costar, Corning (Amsterdam, The Netherlands), and phosphate buffered saline (PBS) from Euroclone (Milan, Italy). The ApoTox-Glo™ Triplex assay, the CytoTox-One™ homogeneous membrane integrity assay, and the CellTiter-Glo® luminescent cell viability assay were obtained from Promega (Madison, WI, USA), and the Annexin V/FITC kit from Bender MedSystems GmbH (Vienna, Austria). DCFH-DA was provided by Molecular Probes (Eugene, OR, USA), the JC-1 mitochondrial membrane potential assay kit by Biotium Inc. (Hayward, CA, USA), the GSH colorimetric kit and GRP78 enzyme-linked immunosorbent assay (ELISA) by Enzo Life Sciences International Inc. (Plymouth Meeting, PA, USA), and the BCA protein assay by Thermo Scientific (Rockford, IL, USA). The MCP-1 ELISA kit was purchased from R&D Systems (Minneapolis, MN, USA) and the IL-8 US ELISA kit from Invitrogen (Camarillo, CA, USA). The FITC mouse anti-human CD106 (VCAM-1), PE mouse anti-human CD62E (E-selectin), and APC mouse anti-human CD54 (ICAM-1) antibodies and their respective isotype controls were purchased from Becton Dickinson (Lincoln Park, NJ, USA). The commercially available cobalt (II,III) oxide (<50 nm) and TiO_2 (<100 nm) nanopowders were provided with physico-chemical characterisation by Sigma (St. Louis, MO, USA), which also supplied all of the other reagents unless otherwise specified.

2.2. Particle preparation

The nano-sized Co (II,III) oxide and TiO_2 powders were suspended in ultra-pure water (2 mg/ml), sonicated on ice at 50 W using a probe sonicator (Heat Systems Ultrasonics Inc., Farmingdale, NY, USA) in order to minimise particle aggregates, stabilised by adding PBS 10× and bovine serum albumin (BSA) (final concentration 0.15%), and finally diluted in culture medium to the final working concentrations.

In order to distribute the particles in the working solution as evenly as possible before each cell culture experiment, the samples were processed three times by means of 20-s sonications immediately before use.

2.3. Characterisation of nanoparticles

The CoNPs and TiNPs were structurally and morphologically characterised by means of transmission electron microscopy (TEM) using a 200 kV analytical JEM 2200-FS (JEOL Inc., Peabody, MA, USA). As the behaviour and aggregation of the NPs in different media greatly depends on the surface charge of the NPs and the ionic strength of the suspension, the samples were further characterised using dynamic light scattering (DLS) and *Z*-potential techniques, and measurements made using a 90Plus PALS instrument (Brookhaven Instruments Corporation, Holtsville, NY, USA). In order to estimate the Stokes–Einstein or hydrodynamic radius (R_H) of the suspended NP agglomerates, we measured the autocorrelation function, which was fitted using the minimisation by non-negative least-squares (NNLS) technique, assuming the log-normal distribution of relaxation times in order to take into account the poly-dispersion of the not-ideal colloidal systems.

The specimens for TEM analysis were prepared by depositing one drop of a colloidal suspension of the nanoparticles in water (concentration: 0.1 mg/mL) on a TEM grid after 10 min of ultra-