Toxicology 306 (2013) 1–8

Contents lists available at SciVerse ScienceDirect

Toxicology

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

Titanium dioxide nanoparticles increase inflammatory responses in vascular endothelial cells

Sung Gu Han[a,b,d], Bradley Newsome[a,b,c], Bernhard Hennig[a,b,*]

[a] Superfund Research Program, University of Kentucky, Lexington, KY 40536, USA
[b] Molecular and Cell Nutrition Laboratory, College of Agriculture, University of Kentucky, Lexington, KY 40536, USA
[c] Department of Chemistry, College of Arts and Sciences, University of Kentucky, Lexington, KY 40506, USA
[d] Department of Food Science of Animal Resources, College of Animal Bioscience and Technology, Konkuk University, Seoul 143-701, Republic of Korea

ARTICLE INFO

Article history:
Received 29 October 2012
Received in revised form 10 January 2013
Accepted 22 January 2013
Available online xxx

Keywords:
Nanoparticle toxicity
Titanium dioxide nanoparticles
Endothelial cells
Adhesion molecules

ABSTRACT

Atherosclerosis is a chronic inflammatory disease that remains the leading cause of death in the United States. Numerous risk factors for endothelial cell inflammation and the development of atherosclerosis have been identified, including inhalation of ultrafine particles. Recently, engineered nanoparticles (NPs) such as titanium (TiO_2) NPs have attracted much attention due to their wide range of applications. However, there are also great concerns surrounding potential adverse health effects in vascular systems. Although TiO_2 NPs are known to induce oxidative stress and inflammation, the associated signaling pathways have not been well studied. The focus of this work, therefore, deals with examination of the cellular signaling pathways responsible for TiO_2 NP-induced endothelial oxidative stress and inflammation. In this study, primary vascular endothelial cells were treated with TiO_2 NPs for 2–16 h at concentrations of 0–50 μg/mL. TiO_2 NP exposure increased cellular oxidative stress and DNA binding of NF-κB. Further, phosphorylation of Akt, ERK, JNK and p38 was increased in cells exposed to TiO_2 NPs. TiO_2 NPs also significantly increased induction of mRNA and protein levels of vascular cell adhesion molecule-1 (VCAM-1) and mRNA levels of monocyte chemoattractant protein-1 (MCP-1). Pretreatment with inhibitors for NF-κB (pyrrolidine dithiocarbamate), oxidative stress (epigallocatechin gallate and apocynin), Akt (LY294002), ERK (PD98059), JNK (SP600125) and p38 (SB203580) significantly attenuated TiO_2 NP-induced MCP-1 and VCAM-1 gene expression. These data indicate that TiO_2 NPs can induce endothelial inflammatory responses via redox-sensitive cellular signaling pathways.

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1. Introduction

Nanotechnology and the production of nano-sized particles have emerged as promising areas of study due to their many applications in industry and medicine (Lam et al., 2006; McIntyre, 2012). Particularly, TiO_2 NPs are produced on a large scale and are being employed in a variety of consumer products, such as sunscreens, cosmetics, pharmaceutical additives and food colorants (Nohynek et al., 2007; Skocaj et al., 2011). Although TiO_2 is considered to be a safe material, concerns have been raised about the potential adverse health effects in occupational and environmental settings (Elsaesser and Howard, 2012; Linkov et al., 2008). Because of its toxic potential, TiO_2 has been classified by the International Agency for Research on Cancer as "possibly carcinogenic to humans" by inhalation (IARC, 2006). Uptake of TiO_2 NPs can occur through multiple routes, including inhalation, ingestion and transdermal. Transdermal exposure of TiO_2 NPs is linked to the use of sunscreen and cosmetics, although there is no evidence demonstrating that TiO_2 can penetrate into normal skin (Schilling et al., 2010). Actually, the major route of human exposure to TiO_2 NPs is through its use as a pharmaceutical additive and through food intake, where TiO_2 has been widely used as a coloring agent for the food industry. Additionally, studies dealing with oral exposure of TiO_2 NPs in mice have demonstrated the presence of particles in distant organs such as the liver, spleen, kidneys and lungs (Wang et al., 2007). These data suggest that TiO_2 particles can travel to other tissues and organs following uptake by the gastrointestinal tract, with blood circulation primarily implicated in its biodistribution. A number of studies for TiO_2 toxicity in animals have focused on inhalation although potential inhalation exposure to TiO_2 NPs occurs mostly in the workplace (Hext et al., 2005; Skocaj et al., 2011). Because of the physicochemical similarities between ambient, nanoscale particles and NPs, there is a strong rationale linking exposure to TiO_2 NPs with adverse cardiovascular effects. Past studies have demonstrated that inhaled ambient ultrafine particles can reach deep into the lungs where

* Corresponding author at: Molecular and Cell Nutrition Laboratory, Room 599, Wethington Building, 900 South Limestone Street, Department of Animal and Food Sciences, University of Kentucky, Lexington, KY 40536, USA. Tel.: +1 859 218 1387; fax: +1 859 257 1811.
E-mail address: bhennig@uky.edu (B. Hennig).

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http://dx.doi.org/10.1016/j.tox.2013.01.014

Table 1
TiO_2 nanoparticle physicochemical properties.

Vendor	Vendor crystal structure	Vendor size (nm)	Vendor purity	Vendor surface area (m^2/g)	Analyzed SEM Avg. size (size range) (nm)	Analyzed DLS Avg. aggregate size (size range) (nm)	Analyzed purity (EDS)	Analyzed surface area (BET) (m^2/g)
Alfa Aesar Stock # 44689 Lot # C22S013	Anatase	5	99%	200–220	79 (7–232)	349 (250–396)	99.7%	93.9

they can enter the circulatory system, resulting in cardiovascular diseases (Kreyling et al., 2002). Also, multiple studies reporting epidemiological animal data have established a link between ambient particles and the etiology of cardiovascular disease (Floyd et al., 2009; Terzano et al., 2010). Other exposure routes include surgical implant-derived wear debris and intravenously administered contrast agents (Chandran et al., 2011; Umbreit et al., 2012). Although intravenous exposure cases are rare, there is potential concern because a direct injection of TiO_2-containing contrast agents into the circulatory system may confer a greater impact on the vascular endothelium due to near-100% bioavailability.

Vascular endothelial cells are potential targets for TiO_2 NP toxicity in human exposure. There are several cellular events responsible for the initiation of atherosclerosis in the vascular endothelium, including oxidative stress, inflammation and activation of endothelial cells (Businaro et al., 2012). Previous studies have evaluated endothelial activation and dysfunction in endothelial cells but the intracellular signaling pathways are not fully identified (Montiel-Davalos et al., 2012). The purpose of this study is to determine the intracellular signaling pathways by which TiO_2 NPs induce inflammatory responses in vascular endothelial cells. Our data demonstrate that TiO_2 NPs increase vascular adhesion molecules such as monocyte chemoattractant protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1), and that this is mediated by multiple intracellular signaling pathways including mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-3 kinase (PI3K)/Akt.

2. Materials and methods

2.1. Materials

Commercial grade uncoated TiO_2 NPs (anatase, metal basis, stock# 44689, 99.9% purity, 5 nm) were purchased from Alfa Aesar (Ward Hill, MA). DMSO, apocynin, PD98059, SB203580, SP600125 and LY294002 were purchased from Sigma–Aldrich (St. Louis, MO). The NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) was obtained from Calbiochem (Darmstadt, Germany). Epigallocatechin gallate (EGCG) was purchased from Cayman Chemical (Ann Arbor, MI). VCAM-1, IκBα and p-IκBα antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for extracellular signal-regulated kinase-1/2 (ERK1/2), p42/44, p38 MAPK, c-Jun-N-terminal kinase (JNK) and Akt were obtained from Cell Signaling Technology (Danvers, MA). LC3 antibody was purchased from Novus Biologicals (Littleton, CO).

2.2. Nanoparticle characterization

TiO_2 nanoparticle size, surface area and purity were evaluated in comparison to vendor specifications. Elemental analysis of dry TiO_2 samples was performed using a Hitachi S-4300 scanning electron microscope (Dallas, TX) and Princeton Gamma-Tech energy dispersive X-ray spectroscopy (EDS) Microanalysis System (Princeton, NJ), primary and aggregate particle sizes were determined using scanning electron microscopy (SEM), and surface area was determined through the Brunauer–Emmett–Teller (BET) method using a Micromeritics TriStar 3000 surface area and pore size analyzer (Norcross, GA), following overnight nitrogen degassing at 120 °C. Characterization results can be found in Table 1 as compared to vendor-supplied values. Suspensions of TiO_2 NPs in cell culture media were prepared at a concentration of 5 mg/mL. Nanoparticle dispersion and size characteristics were analyzed by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 particle size analyzer (Malvern Instruments, Westborough, MA), which measures hydrodynamic diameters of nanoparticles (Narband et al., 2009). TiO_2 NP suspensions ($n = 3$) were suspended in deionized H_2O, sonicated briefly using a probe sonicator (Heat Systems, Inc., Farmingdale, NY) for 15 min to minimize particle aggregates, added to cell culture medium and analyzed via DLS with five measurements per sample. The same dispersion procedure was also used prior to in vitro studies. Sizing data, including mean nanoparticle size (nm) and particle size ranges, was determined using Malvern DTS Software, v. 6.32 (Table 1).

2.3. Primary cell culture and endothelial cell treatments

Primary vascular endothelial cells were isolated from porcine pulmonary arteries and characterized as described previously (Han et al., 2010; Hennig et al., 1984). Cells were cultured in M199 media (Gibco, Grant Island, NY) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad). Cells were grown to confluence and serum starved overnight in medium containing 1% FBS prior to initiation of cell treatments. A stock suspension of 5 mg/mL TiO_2 NPs was prepared and dispersed by probe sonication for 15 min. Based on our preliminary studies, we chose to treat cells with TiO_2 NPs at 10 and 50 μg/mL, which corresponded to 2 and 10 μg nanoparticles/cm^2, respectively. Of particular relevance to the present study, TiO_2 NPs have been suggested for use in intravenous applications as contrast agents (Chandran et al., 2011; Umbreit et al., 2012). Due to near-100% bioavailability, potential intravenous applications could allow nanoparticles to achieve significantly higher concentrations in the blood circulation than that from translocation of nanoparticles following occupational and environmental exposure. These nanoparticle concentrations (10 and 50 μg/mL) were selected not only to address potential intravenous and environmental exposure levels but also to correspond with previous studies showing increased expression of inflammatory genes without cell death (Montiel-Davalos et al., 2012; Sanders et al., 2012). Equal volumes of water (up to 1% of media; no hypotonic conditions were produced as shown by autophagy analysis in Fig. 6) were used in place of NP-suspension volumes to serve as controls in cell culture. The TiO_2 NP concentrations and treatment intervals employed in these studies did not lead to significant cytotoxicity, as seen by trypan blue exclusion staining (data not shown).

2.4. Assessment of superoxide ($O_2^{\bullet -}$) levels

Endothelial cells were grown to confluence in 8-chamber culture slides (BD Biosciences, Bedford, MA). Following treatment, cells were incubated with a final concentration of 5 μM dihydroethidium (DHE), MitoSOX™ Red mitochondrial superoxide indicator (MitoSOX) or DMSO (blank) in a 5% CO_2 incubator for 15 min. Cells were washed 3× with PBS, fixed with 4% formaldehyde, and washed again 3× with PBS. Slides were mounted with ProLong Gold Antifade reagent containing 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA) to visualize the nuclei. Slides were evaluated under a Nikon ECLIPSE TE2000-U fluorescence microscope and the images were captured digitally using a Nikon LH-M100CB-1 camera and NIS-Elements BR 4.00.08 software (Nikon Instruments Inc., Melville, NY).

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts of endothelial cells were prepared using NE-PER nuclear extraction reagents (Thermo, Rockford, IL). The concentration of nuclear extract was determined using Bradford reagent (Bio-Rad, Richmond, CA). DNA binding activities of NF-κB were determined using a LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL) according to the manufacturer's protocol. DNA-binding reactions were performed with a final volume of 20 μL buffer containing 5 μg of nuclear extract, 50 ng/μL poly(dI·dC) and biotin end-labeled oligonucleotides. Synthetic 5′-biotinylated complementary oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). The oligonucleotides containing the NF-κB consensus sequence (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were described previously (Lim et al., 2007). EMSA gels were quantified by Image J software (NIH, Bethesda, MD).

2.6. Quantitative real-time PCR

Cells were grown in 6-well plates, and total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Reverse transcription was performed using the AMV reverse transcription system (Promega, Madison, WI). The levels of mRNA expression were then assessed by quantitative real-time PCR using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) and SYBR Green master mix (Applied Biosystems). Data

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