Free Radical Biology and Medicine ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

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

Original Contribution

Titanium dioxide nanoparticles induce strong oxidative stress and mitochondrial damage in glial cells

Elizabeth Huerta-García [a,b,1], José Antonio Pérez-Arizti [a,1], Sandra Gissela Márquez-Ramírez [a,b], Norma Laura Delgado-Buenrostro [c], Yolanda Irasema Chirino [c], Gisela Gutierrez Iglesias [b], Rebeca López-Marure [a,*]

[a] Departamento de Fisiología (Biología Celular), Instituto Nacional de Cardiología "Ignacio Chávez," Tlalpan, CP 14080 México DF, Mexico
[b] Departamento de Posgrado, Escuela Superior de Medicina, Instituto Politécnico Nacional, México DF, Mexico
[c] Unidad de Biomedicina, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, México DF, Mexico

ARTICLE INFO

Article history:
Received 10 January 2014
Received in revised form
25 April 2014
Accepted 28 April 2014

Keywords:
Nanoparticles
Titanium dioxide
Glial cells
Oxidative stress
Lipoperoxidation
ROS
Mitochondrial damage
Free radicals

ABSTRACT

Titanium dioxide nanoparticles (TiO_2 NPs) are widely used in the chemical, electrical, and electronic industries. TiO_2 NPs can enter directly into the brain through the olfactory bulb and can be deposited in the hippocampus region; therefore, we determined the toxic effect of TiO_2 NPs on rat and human glial cells, C6 and U373, respectively. We evaluated some events related to oxidative stress: (1) redox-signaling mechanisms by oxidation of 2′,7′-dichlorodihydrofluorescein diacetate; (2) peroxidation of lipids by *cis*-parinaric acid; (3) antioxidant enzyme expression by PCR in real time; and (4) mitochondrial damage by MitoTracker Green FM staining and Rh123. TiO_2 NPs induced a strong oxidative stress in both glial cell lines by mediating changes in the cellular redox state and lipid peroxidation associated with a rise in the expression of glutathione peroxidase, catalase, and superoxide dismutase 2. TiO_2 NPs also produced morphological changes, damage of mitochondria, and an increase in mitochondrial membrane potential, indicating toxicity. TiO_2 NPs had a cytotoxic effect on glial cells; however, more in vitro and in vivo studies are required to ascertain that exposure to TiO_2 NPs can cause brain injury and be hazardous to health.

Nanoparticles (NPs)[2] are a subset of nanomaterials defined as single particles with a diameter < 100 nm that can form aggregates larger than 100 nm in diameter [1]. Titanium dioxide nanoparticles (TiO_2 NPs) are used extensively in many commercial products, including paints, cosmetics, plastics, paper, and food, as an anticaking or whitening agent [2]. It is likely that during their production, distribution, and use, TiO_2 NPs may become released into the environment and become suspended in air, representing a consumer and occupational risk in the long term, and potentially causing harm [3]. Despite this, it has been described that TiO_2 NPs are not dangerous to human health; these particles are in the breathable size range and several toxic effects have been described after their inhalation [4,5].

Tissue deposition of NPs and their toxicity are closely related to the route of exposure and, with this point of view, keratinocytes have been studied as the primary target of dermal exposure, lung tissue as the target for inhalation, and intestines, kidney, and liver for oral exposure. The lung is the best characterized organ regarding the toxic effects induced by TiO_2 NPs. For example, cellular uptake and distribution of TiO_2 NPs have been shown in A549 lung epithelial cells [6]. In addition, in rat pulmonary alveolar macrophages, TiO_2 NPs induce membrane and ultrastructure damage and dysfunction mediated by an increase in the phagocytic ability of macrophages and a decrease of their chemotactic ability [7]. All the reported effects induced by TiO_2 NPs are associated with adverse effects on mammalian cells such as increase in reactive oxygen species (ROS) production and cytokine levels, reduction in cell viability and proliferation, induction of apoptosis, and genotoxicity [8].

TiO_2 NPs can be translocated to all regions of the respiratory tract. Their small size facilitates uptake into cells and transcytosis across epithelial and endothelial cells into the blood and lymph circulation to reach potentially sensitive target sites, such as bone marrow, lymph nodes, spleen, and heart [9]. However, there is increasing evidence that NPs can cross the blood–brain barrier independent of the route of exposure. In this regard, it has been demonstrated that even after 4 months of oral exposure, NPs can

Abbreviations: NP, nanoparticle; C6, rat brain glial tumor cell line; U373, human astrocytoma cell line grade III; GPx, glutathione peroxidase; SOD2, superoxide dismutase 2

* Corresponding author. Fax: +52 55 55 73 09 26.
E-mail address: rlmarure@yahoo.com.mx (R. López-Marure).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.freeradbiomed.2014.04.026
0891-5849/

persist in the brain [10]. Specifically, nasal exposure to TiO_2 NPs can not only allow for their deposition in the brain [11] and induce release of proinflammatory cytokines, such as IL-1β and TNF-α [12], but can also induce alterations in genes related to memory formation and learning [13]. Recently, it was reported that instilled TiO_2 NPs enter the brain directly through the olfactory bulb during the whole exposure period and are deposited in the hippocampus region, causing significant pathologic changes [14], as well as the release of monoaminergic neurotransmitters in the brain [15]. One study showed that TiO_2 NPs injected into the abdominal cavity can be translocated into the brain and, in turn, cause brain injury [16]. In addition, maternal exposure of mice to TiO_2 NPs affected the expression of genes related to the development and function of the central nervous system (CNS) [17]. In brain microglia (BV2) exposed to noncytotoxic (2.5–120 ppm) concentrations of TiO_2 NPs a rapid and sustained release of ROS occurs [18].

Oxidative stress, which is an imbalance between ROS production and the antioxidant systems favoring a ROS excess, has been identified as an important contributor to neurodegeneration associated with acute CNS injuries, such as DNA damage, which can affect viability of cerebral cells [19]. During oxidative stress, ROS are produced mainly from the mitochondrial electron transport chain. Several molecules/factors have been suggested as regulators of mitochondrial ROS production, including an elevated inner membrane potential, as well as of Ca^{2+} and nitric oxide (NO). The exogenous stimulators of mitochondrial ROS have not been elucidated yet, but there is evidence that lipid oxidation products play a major role [20]. At the molecular level, TiO_2 NPs induce oxidative stress by activating the Nrf2 pathway [21] but gene expression of DNA repair, immune response, and apoptosis can also be altered after intranasal exposure [13].

To minimize the damage induced by ROS, free radicals can be transformed to other less toxic molecules, for example, the superoxide anion is enzymatically converted into hydrogen peroxide by superoxide dismutase (SOD), and hydrogen peroxide may be enzymatically converted into water by catalase or glutathione peroxidase enzymes [22].

Considering that NPs can translocate into the brain, it is important to investigate the potential health effects of these particles on brain cells. We hypothesized that TiO_2 NPs could cause oxidative stress and mitochondrial damage to glial cells; therefore, we determined the effects of TiO_2 NPs on cellular redox state, lipid peroxidation, mitochondrial membrane potential, integrity of mitochondria, and several antioxidant enzymes in human (U373) and rat (C6) glial cells.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), antibiotic–antimycotic 100× solution, minimal essential medium (MEM) nonessential amino acids and pyruvate solutions (100×), 0.25% trypsin–EDTA solution, and newborn calf serum (NBCS) were purchased from Gibco/BRL (Grand Island, NY, USA). Sterile plastic material for tissue culture was from Corning (Corning, NY, USA). Flow cytometry reagents were purchased from Becton–Dickinson Immunocytometry Systems (San Jose, CA, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) and *cis*-parinaric acid were purchased from Molecular Probes, Invitrogen (Carlsbad, CA, USA). Perfect RNA Eukaryotic, Mini solution was from Eppendorf (Hamburg, Germany). DNase was from Invitrogen. Transcriptor First Strand cDNA Synthesis kit, the human Universal ProbeLibrary, LightCycler TaqMan master reaction mixture, and borosilicate glass capillaries were from Roche Diagnostics (Mannheim, Germany). MitoTracker Green FM was from Invitrogen (Cat. No. M-7514). TiO_2 NPs were purchased from Paris Drugstore (Mexico City, Mexico). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Titanium dioxide nanoparticles

TiO_2 NPs were previously characterized by our work group [23]. TiO_2 NPs were sterilized by autoclave (1.5 atm, 20 min). A stock solution was prepared by suspending 1 mg of sterile particles in 1 ml of Hepes–phosphate buffer solution (HPBS: 150 mM NaCl, 4.4 mM KCl, 10.9 mM Hepes, 12.2 mM glucose, pH 7.4), mixing in a vortex at high speed for 2 min, and sonicating at 33 W for 1 min before being used to treat cell cultures. TiO_2 NPs were characterized as used for the biological assays in supplemented medium as described above, and they were observed by means of scanning electron microscopy in a field emission scanning electron microscope (JEOL Model JSM-7401-F; see supplementary material). The size of the nanoparticles and the ζ potential were analyzed in a Zetasizer Nano Series Model ZS. For this measurement, nanoparticles were suspended in culture medium plus 10% NBCS and sonicated at 33 W for 15 min to attain a better dispersion. Additionally, nanoparticles were analyzed by X-ray diffraction to determine composition (Bruker D8 Advance with Cu Kα radiation and a Lynxeye Bruker detector; Bruker, Karlsruhe, Germany; see supplementary material). Transmission electron microscopy was used to determine the uptake and internalization of TiO_2 NPs by cells. BET surface area of nanoparticles was determined in an ASAP 2050 Xtended Pressure Sorption analyzer (Micromeritics Instrument Corp., Norcross, GA, USA).

Culture of cerebral cells

U373 cells (ATCC HTB-17) were kindly provided by Dr. Ignacio Camacho Arroyo from the Facultad de Química, Departamento de Biología, Universidad Nacional Autónoma de México, Mexico. C6 (ATCC CCL-107) and U373 cells were cultured in DMEM supplemented with 4500 mg/L glucose, 10% NBCS, 2 mM glutamine, 1 mM pyruvate, antibiotic–antimycotic, MEM nonessential amino acids 1×, as previously described [24]. Cell cultures were exposed to 20 μg/cm^2 of TiO_2 NPs (concentration that was shown to have a strong biological effect on endothelial and glial cells in our previous studies) [23,25] for various times depending on the biological assay.

Determination of oxidative stress

Cellular redox state

Oxidation of H_2DCFDA into 2′,7′-dichlorodihydrofluorescein (DCF) was used as a redox indicator probe [26]. Redox-sensitive cellular targets provide sensitive and rapid responses to changes in metabolism and fluxes of ROS. DCFH-DA is a nonfluorescent and nonpolar dye that is converted into the polar derivative, DCFH, by cellular esterases, but it is switched to the highly fluorescent DCF when oxidized by intracellular ROS, including hydrogen peroxide, organic hydroperoxides, NO, and other peroxides, mainly peroxynitrite [27,28]. H_2DCFDA can also be oxidized directly by factors that generate oxidative stress per se, such as metal ions in high oxidation states [29]; to perform this assay, cells were cultured without or with TiO_2 NPs (20 μg/cm^2) for 2, 4, 6, and 24 h. H_2O_2 (500 μM) was used as positive control to induce oxidative stress. After treatment, the cells were incubated with H_2DCFDA (10 μM) for 30 min at 37 °C and washed twice with HPBS. After an extensive wash, fluorescence was evaluated by flow cytometry (FACSCalibur, Becton–Dickinson). The mean fluorescence intensity was calculated by multiplying the number of events (fluorescent

Please cite this article as: Huerta-García, E; et al. Titanium dioxide nanoparticles induce strong oxidative stress and mitochondrial damage in glial cells. *Free Radic. Biol. Med.* (2014), http://dx.doi.org/10.1016/j.freeradbiomed.2014.04.026