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Research Paper

TiO_2 nanoparticles cause mitochondrial dysfunction, activate inflammatory responses, and attenuate phagocytosis in macrophages: A proteomic and metabolomic insight

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ABSTRACT

Titanium dioxide nanoparticles (TiO_2 NPs) are widely used in food and cosmetics but the health impact of human exposure remains poorly defined. Emerging evidence suggests that TiO_2 NPs may elicit immune responses by acting on macrophages. Our proteomic study showed that treatment of macrophages with TiO_2 NPs led to significant re-organization of cell membrane and activation of inflammation. These observations were further corroborated with transmission electron microscopy (TEM) experiments, which demonstrated that TiO_2 NPs were trapped inside of multi-vesicular bodies (MVB) through endocytotic pathways. TiO_2 NP caused significant mitochondrial dysfunction by increasing levels of mitochondrial reactive oxygen species (ROS), decreasing ATP generation, and decreasing metabolic flux in tricarboxylic acid (TCA) cycle from ^{13}C-labelled glutamine using GC-MS-based metabolic flux analysis. Further lipidomic analysis showed that TiO_2 NPs significantly decreased levels of cardiolipins, an important class of mitochondrial phospholipids for maintaining proper function of electron transport chains. Furthermore, TiO_2 NP exposure activates inflammatory responses by increasing mRNA levels of TNF-α, iNOS, and COX-2. Consistently, our targeted metabolomic analysis showed significantly increased production of COX-2 metabolites including PGD_2, PGE_2, and 15d-PGJ_2. In addition, TiO_2 NP also caused significant attenuation of phagocytotic function of macrophages. In summary, our studies utilizing multiple powerful omic techniques suggest that human exposure of TiO_2 NPs may have profound impact on macrophage function through activating inflammatory responses and causing mitochondrial dysfunction without physical presence in mitochondria.

1. Introduction

Engineered Nanoparticles have been widely used in food, cosmetics, and medicine due to their unique physical and chemical properties [1]. In particular, titanium dioxide (TiO_2) nanoparticles (NPs) are added to cosmetics, chewing gum, beverage, sauce and many other products. There are increasing concerns regarding the health effects of human exposure of these TiO_2 NPs, which have not been well defined [2–4]. In 2006, the International Agency for Research on Cancer (IARC) classified pigment-grade TiO_2 as suspected carcinogen (class 2B) [5]. As the first line of defence, macrophages try to clear these TiO_2 NPs through phagocytosis and protect the body from potential harm. However, it remains poorly define how TiO_2 NPs affect the immune functions and metabolism of macrophages upon exposure [6]. Liu et al. reported decreased chemotactic ability, MHC-class II expression on the cell surface and increased secretion of nitric oxide (NO) and tumour necrosis factor-alpha (TNF-α) in rat pulmonary macrophages after intra-tracheal instillation of TiO_2 NPs [7]. Exposure of TiO_2 NPs also activated inflammatory responses through upregulation of inflammatory cytokines in mouse bronchial alveolar lavage (BAL) fluid [8]. Furthermore, gastric ingestion or oral exposure of TiO_2 NPs caused similar immune responses in other animal experiments. Sang et al. found that serious

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spleen injury, increased level of nucleic factor-kappa B, TNF-α and interleukin secretion and decreased immunoglobulin in mice for long time intra-gastric exposure to TiO_2 NPs [9]. It still poorly understood how TiO_2 NP exposure affects immune responses. Limited studies suggested that TiO_2 NP exposure led to activated immune responses and elevation of ROS production and upregulation of pro-inflammatory factors, such as IL-1β, TNF-α, IFN-γ and IL-10 [10]. The macrophages function appeared to be affected, such as decreased phagocytic ability and chemotactic ability in exposed primary PAMs [11,12]. Furthermore, it remains controversial for the cellular locations of the TiO_2 NPs once they enter the immune cells even though there are some data suggesting that mitochondrial functions are significantly affected [13–15]. Moreover, TiO_2 beads are routinely used in enrichment of phosphorylated peptides and proteins in proteomic studies due to its strong binding with phosphate groups [16,17]. Thus we hypothesized that exposure of TiO_2 NPs in macrophage might have profound impact on the membrane phospholipids and phosphorylated proteins, which represents a unique property of TiO_2 NPs different from other metal oxide NPs.

In this study, we found that treatment of macrophage cell line (RAW) or primary mouse bone marrow-derived macrophages (BMDM) with TiO_2 NPs had marginal effects on cell viability but caused mitochondrial dysfunction and upregulated inflammation. Proteomic analysis of the entire proteome and phosphorylated proteome revealed massive membrane reorganization and activation of inflammation. These results were further supported by targeted metabolomic analysis of inflammatory pathways including metabolites from cyclo-oxygenases (COX) pathway, lipidomic analysis of mitochondrial phospholipids cardiolipin and metabolic flux from ^{13}C-labelled glutamine. All these omic data strongly suggest that mitochondria are the primary cellular targets for TiO_2 NPs in macrophages. Surprisingly, however, Transmission Electron Microscopy (TEM) experiments demonstrated that TiO_2 NPs were not present in mitochondria but were trapped inside of multi-vesicular bodies (MVB), suggesting an indirect effect of TiO_2 on mitochondrial function of macrophages. Furthermore, we performed functional studies to investigate the consequences of TiO_2 NP exposure and found that TiO_2 NPs caused significant attenuation of phagocytic function of macrophages. In summary, our data shed new light on potential impact of long term human exposure of TiO_2 NPs.

2. Material and methods

2.1. TiO_2 NPs and characterization

TiO_2 NPs were purchased from Beijing DK Nano Technology Co., Ltd. (Beijing, China) and were used in a previous publication [18]. The average NPs size was 10 nm and 99.9% pure Anatase. There was no LPS contamination as determined by the same method previously used. Roswell Park Memorial Institute (RPMI, Hyclone) 1640 complete medium and Dulbecco's modified Eagle's medium (DMEM, Gibco) complete medium were used as suspending agents. TiO_2 NPs powder was dispersed into the suspending solutions and then were treated by ultrasonic agitation for 30 min for better diffusion. We used 1 mg/ml TiO_2 NPs as a stock solution, and diluted it for work solution. TiO_2 NPs size measurements were performed using dynamic light scattering (DLS) instrument (Malvern Zetasizer, Nano-ZS90). A stock solution of 1 mg/ml was immediately diluted to 10 μg/ml and 100 μg/ml and measure the size distribution.

2.2. Transmission electron microscopy (TEM)

Cells were harvested after 24 h exposure and fixed by 2.5% paraformaldehyde and 2.5% glutaraldehyde and stained by osmium tetroxide, then were scanned by transmission electron microscopy (TEM, FEI Tecnau G2 spirit).

2.3. Cell culture

RAW 264.7 cell line was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco), incubated in an atmosphere of 95% air and 5% CO_2 constant temperature of 37 °C. RAW cells were seeded 8×10^5 cells /well and BMDM were seeded 1×10^6 cells/well in 6-well plates. All experiments were performed at least triplicates.

2.4. Isolation and culture of mouse Bone marrow derived macrophages (BMDM)

BMDM was separated from bone and matured by L929 (cell line was purchased from ATCC® CCL-1TM) supernatant as protocol described in the literature [19]. We isolated bone from C57BL/6Jslac mice and cultured the matured BMDM with RPMI 1640 complete medium, incubated in an atmosphere of 95% air and 5% CO_2 constant temperature of 37 °C. The animal studies were approved by the review committee from the Institute for Nutritional Science (Shanghai Institutes for Biologic Sciences, Chinese Academy of Sciences) and performed in accordance with the institutional guidelines.

2.5. MTT assay

Cell viability was determined by the (3-(4, 5-dimethylthiazaol-2-yl)-2,5-diphenyltertrazoliun bromide) (MTT) assay. Cells were seeded in a 96-well plate, after 24 h the medium was replaced by medium containing 0–100 μg/ml for 24 h incubation and added 10 ul 5 mg/ml MTT for 4 h, then adding 200ul DMSO for dissolving crystals, after centrifugation the supernatant was placed in a new 96-well plate and measured by multilabel reader (PE Enspire, Beijing, China).

2.6. Proteomic analysis

BMDM cells were treated with 100 μg/ml TiO_2 NPs in triplicates. After 24 h the cells were washed with phosphate-buffered saline solution (PBS) three times. Proteins in cells were extracted with lysis buffer containing protease inhibitors. The phosphorylated proteins were enriched by TiO_2 beads. The proteomic analyses were performed in Shanghai Applied Protein Technology Co. Ltd. (Shanghai, China). Eight plex iTRAQ (isobaric tags for relative and absolute quantitation) was performed using high resolution Q Exactive mass spectormeter (Thermo Scientific, San Jose, CA) coupled with cation exchange (SCX) nano-liquid chromatography separation.

2.7. Gas Chromatography- Mass Spectrometry (GC-MS)

RAW264.7 cells were seeded at a density of approximately 2×10^6 cells per 10 cm dish. After adhering, the cell culture medium was changed for labelling medium with ^{13}C-glutamine containing different concentrations of TiO_2 NPs. The medium of ^{13}C-glutamine composed of low glucose DMEM (Gibco1, 1054-020) was supplemented with the unlabelled 1 g/L glucose (Sigma-Aldrich, G7528), 10% (v/v) FBS, the labelled 2 mM L-glutamine-$^{13}C5$ (Sigma-Aldrich, 605166), 1% (v/v) antibiotic/antimycotic solution (Omega). Following the 24 h labelling period, cells were rinsed with phosphate buffer sodium (PBS), detached with trypsin and subjected to centrifugation at 500 × *g* for 5 min. Cells were centrifuged at 500 × *g* for 3 min. Cell pellets were re-suspended in 0.6 ml cold (− 20 °C) 50% aqueous methanol containing 100 μM Norvaline as an internal standard, frozen on dry ice for 30 min, then thawed on ice for 10 min before centrifugation. The supernatant was then partitioned with 0.3 ml chloroform to reduce the fatty acid content. The methanol layer was dried by centrifugal evaporation and stored at − 80 °C before analysis. Metabolites were derivatized for GC/MS analysis as follows: First, 50 μL of 20 mg/ml O-Isobutyl