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Research paper

Titanium dioxide nanoparticle ingestion alters nutrient absorption in an *in vitro* model of the small intestine

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ABSTRACT

Ingestion of titanium dioxide (TiO_2) nanoparticles from products such as agricultural chemicals, processed food, and nutritional supplements is nearly unavoidable. The gastrointestinal tract serves as a critical interface between the body and the external environment, and is the site of essential nutrient absorption. The goal of this study was to examine the effects of ingesting the 30 nm TiO_2 nanoparticles with an *in vitro* cell culture model of the small intestinal epithelium, and to determine how acute or chronic exposure to nano-TiO_2 influences intestinal barrier function, reactive oxygen species generation, proinflammatory signaling, nutrient absorption (iron, zinc, fatty acids), and brush border membrane enzyme function (intestinal alkaline phosphatase). A Caco-2/HT29-MTX cell culture model was exposed to physiologically relevant doses of TiO_2 nanoparticles for acute (4 h) or chronic (five days) time periods. Exposure to TiO_2 nanoparticles significantly decreased intestinal barrier function following chronic exposure. Reactive oxygen species (ROS) generation, proinflammatory signaling, and intestinal alkaline phosphatase activity all showed increases in response to nano-TiO_2. Iron, zinc, and fatty acid transport were significantly decreased following exposure to TiO_2 nanoparticles. This is because nanoparticle exposure induced a decrease in absorptive microvilli in the intestinal epithelial cells. Nutrient transporter protein gene expression was also altered, suggesting that cells are working to regulate the transport mechanisms disturbed by nanoparticle ingestion. Overall, these results show that intestinal epithelial cells are affected at a functional level by physiologically relevant exposure to nanoparticles commonly ingested from food.

1. Introduction

Engineered nanoparticles have become a commonly ingested material, and the effects of nanoparticles on gastrointestinal (GI) health and function are not well understood. Engineered nanoparticles (NP) exhibit specific physiochemical properties including unique optical effects, melting points, conductivity, ionization potential, electron affinity, magnetism, surface energy, reactivity, and potentially biological effects when compared to the bulk materials with the same mass dose (Auffan et al., 2009; Tiede et al., 2008; Jones and Grainger, 2009). The unique properties of NP stem from size, and the scale-dependent changes in the ratio of surface area to volume dramatically affect NP behavior. Volume decreases with size and the proportion of atoms at the particle surface increases, meaning that the number of atoms localized at the surface exponentially increases as the size decreases. This can increase the chemical reactivity and catalytic behavior per unit mass, and can alter absorption and excretion rates in biological systems such as DNA, proteins, and cell membranes (Auffan et al., 2009; Tiede et al., 2008; Jones and Grainger, 2009; Xia et al., 2009). Without a thorough understanding of the biological behavior of NP, it is impossible to predict the risks associated with NP exposure, and each new nanomaterial must be subject to health and safety assessment.

NP are increasingly used in food and food packaging applications, and companies are not required to seek regulatory approval before launching products containing nanosized ingredients made from approved bulk materials (Powell et al., 2010). There are currently 150–600 nano foods and 400–500 nanofood packaging applications containing nanotechnology-derived food ingredients, additives, supplements, and contact materials that are commercially available (Martirosyan and Schneider, 2014). The dietary consumption of NPs in developed countries is estimated at >1012 particles/day, consisting mainly of titanium dioxide (TiO_2) and mixed silicates (Martirosyan and Schneider, 2014). The European Food Safety Authority (EFSA), commissioning the Joint Research Centre (JRC), prepared an inventory of currently used and reasonably foreseen applications of nanomaterials in agriculture and food or feed production, and TiO_2 is the main type of engineered nanomaterial added to food (Picó, n.d.). TiO_2 NP exists in processed foods such as candies and chewing gums, and is primarily used as whitening agent due to its brightness, high refractive index, resistance to discoloration, and dispersion in water as a fairly stable colloid.

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Approximately 36% of food-grade TiO_2 (E171) are <100 nm in at least one dimension (Weir et al., 2012). Personal care products, like toothpastes and some sunscreens, contain 1% to over 10% titanium by weight (Weir et al., 2012; Chaudhry et al., 2008; Gitrowski et al., 2014). Human GI exposure to nanoparticles can occur in several ways. Nano-food ingredients, additives, and supplements from food packaging and contact materials that migrate into food can be ingested. Following consumption, nano-TiO_2 materials can enter the environment by treated effluent discharged to surface waters or biosolid application to agricultural land, for example, which can contribute to human exposure *via* drinking water or the food chain (Tiede et al., 2008; Weir et al., 2012). Due to the prevalence of nano-TiO_2 human ingestion is nearly unavoidable, which highlights the importance of studying the effects of TiO_2 NP ingestion.

In this study the two most common types of intestinal epithelial cells, absorptive and goblet, were represented by Caco-2 and HT29-MTX cells (Kararli, 1995; Forstner and Forstner, 1994). Caco-2 cells, which are derived from colonic epithelial adenocarcinoma cells, differentiate into a polarized, enterocyte-like epithelial barrier; express microvilli and tight junctions (TJ); and are capable of paracellular, transcellular, active, and transcytotic transport (Artursson and Karlsson, 1991; Artursson et al., 2001). Caco-2 cells also express all of the major amino acid, electrolyte, fatty acid, sugar/carbohydrate, iron, and zinc uptake, storage, transport, and carrier proteins (Kipp et al., 2003; Halleux and Schneider, 1991; Han and Wessling-Resnick, 2002; Shen et al., 2008; Levy et al., 1995; Hauri et al., 1985). The HT29-MTX cells are a subpopulation of HT29 human colonic adenocarcinoma cells selected for resistance to methotrexate (MTX), and mimic mucus secreting goblet-like cells (Lesuffleur et al., 1990). When seeded at a ratio of 75% Caco-2 to 25% HT29-MTX and cultured for two weeks, a mucus layer that completely covers the cell monolayer and is 2–10 μm thick is formed (Mahler et al., 2009). This *in vitro* mucus layer is ~2/3 of the thickness of the duodenal firmly adherent mucus layer in humans (15 μm) (Atuma et al., 2001).

Previous NP ingestion studies with this *in vitro* model (Mahler et al., 2012) showed that following exposure to 10^9 50 nm carboxylated polystyrene nanoparticles/cm^2, iron transport, which is representative of iron transfer into the bloodstream, was significantly lowered. Exposure to the same size and concentration of NP also affected iron absorption in an *in vivo* chicken model. Ferritin analysis, divalent metal transporter 1 (DMT1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB1) gene expression, and histological examination demonstrated that the changes in iron absorption were not due to changes in transport protein expression or an inflammatory response, but instead, that exposure to NP significantly increased the overall villi volume. These experiments showed that data from the *in vitro* system correlates well with *in vivo* models, and that nanoparticle consumption alters intestinal function.

The overall goal of the current study is to determine if acute or chronic exposure to TiO_2 NP, which are commonly added to food and food packaging, affects small intestinal function. The interaction of 30 nm TiO_2 NP with Caco-2/HT29-MTX monolayers was evaluated by examining molecular, functional, and structural characteristics of the cells including nutrient transporter gene expression, reactive oxygen species (ROS) generation, Fe, Zn, or fatty acid absorption, alkaline phosphatase enzyme activity, TJ functionality, and microvilli structure. Transepithelial nutrient permeability is regulated by amino acid, electrolyte, fatty acid, sugar/carbohydrate, and mineral protein transporters, and is a highly regulated process (Groschwitz and Hogan, 2009). Overall, these results show that exposure to physiologically relevant concentrations of TiO_2 NPs can have molecular, functional, and structural effects on intestinal epithelial cells.

2. Materials and methods

30 nm TiO_2 NP were purchased by US Research Nanomaterials, Inc. (Houston, TX). All culture flasks, plates, tubes, and pipette tips used for culturing cells were purchased from Corning (Corning, NY). All other chemicals, enzymes, and hormones were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated. Glassware used in sample preparation and analysis was washed, soaked in 10% hydrochloric acid and 10% nitric acid overnight, and rinsed with 18 MΩ water to avoid iron or zinc contamination. All reagents were prepared in 18 MΩ water.

2.1. Nanoparticle dose calculations

In vitro doses of NP were formulated to represent potential real-life exposure. The total intestinal surface area is approximately 2×10^6 cm^2 (DeSesso and Jacobson, 2001) and the daily intake of nano-TiO_2 has been estimated to be 10^{12}–10^{14} NP per day, which is approximately 10^{11}–10^{13} particles per meal (Lomer et al., 2002). Ingesting 10^{13} NP exposes the small intestine to 10^6 particles/cm^2. If 10^{11} or 10^{13} particles are ingested, the dose to the duodenum is approximately 10^8 or 10^{10} particles/cm^2, respectively. The duodenum is the first section of the small intestine, the site where most nutrient absorption occurs, and has approximately 900 cm^2 of absorbing surface area (Muir and Hopfer, 1985a; Kararli, 1995). Supplemental Table 1 describes the low, medium, and high concentrations of 30 nm TiO_2 nanoparticles used for this study. When 100 μL of these solutions were added to 0.33 cm^2 cell monolayers, the concentrations were 10^6 particles/cm^2 (low), 10^8 particles/cm^2 (medium), and 10^{10} particles/cm^2 (high) for acute exposures. Chronic doses were three times (3×) the acute doses, representing the NP consumed in one day instead of one meal.

TiO_2 NP powder was weighed in a polystyrene weighing dish, and dispersed in sterile 18 MΩ water. Solutions were mixed uniformly in a sterile tube using a Thermolyne Mixer (Maxi Mix II, Type 37600) for 1 min, and then serially diluted to the concentrations shown in Supplemental Table 1. Nanoparticles solutions were placed in a sonicator (VWR® symphony™ Ultrasonic Cleaners, RF-48 W) for 30 min to break down NP agglomerates. The aqueous dispersions were then measured and used for *in vitro* model exposures.

2.2. Nanoparticle characterizations

The distributions of TiO_2 NP sizes and average ζ- potentials were measured with a Zetasizer Nano ZS (Malvern Instruments Inc., Southborough, MA). Measurements were performed in Malvern disposable polycarbonate folded capillary cells with gold plated beryllium–copper electrodes (DTS1070), which were rinsed with ethanol, 18 MΩ water, and sample dispersions before readings. The Refractive Index (RI) value of TiO_2 is 2.42, and water RI is 1.33. Sample viscosities refer to the viscosity of water (0.8872 cP), and the dielectric constant of water is 78.5. The samples were equilibrated in the instrument chamber for 120 s, and measured at 25 °C.

The TiO_2 NPs moved randomly in dispersants *via* Brownian Motion, and the size (hydrodynamic diameter, $d_{h,z\text{-average}}$) of the NP determined the speed of movement. The translational diffusion coefficient of particles and the intensity fluctuations in the scattered light were expressed in hydrodynamic diameter by Dynamic Light Scattering (DLS). Polydispersity index (PdI), generated by the Malvern software, is dimensionless and refers to the range of hydrodynamic diameter distribution. If PdI > 0.5, NPs are polydisperse (polymodal) distributions (Camli et al., 2010); PdI < 0.1, represents monodisperse distributions (Bihari et al., 2008).

A potential exists between the surfaces of the TiO_2 NPs and the dispersants, and the charge measurement is expressed in terms of ζ-potential. The magnitude of the ζ-potential reflects the stability of the colloidal system. As the NPs in dispersants have a large negative or positive ζ-potential, they strongly repel each other to prevent particle aggregation. A solution is normally considered stable if the ζ-potential is more positive than +30 mV or more negative than −30 mV (Hanaor et al., 2012).