TiO₂ particles induce ER stress and apoptosis in human hepatoma cells, HepG2, in a particle size-dependent manner

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Abstract The cytotoxicity of TiO₂ nanoparticles are well-known, but the particle size-dependent induction of ER stress and apoptosis by TiO₂ in hepatocytes has not been elucidated clearly. In the present study, we investigated whether a fine TiO₂ particle and two types of TiO₂ nanoparticles induce ER stress and apoptosis differently in HepG2 cells. A particle size-dependent decrease in cell viability was observed after exposure to the TiO₂ particles. The levels of ER stress-related proteins (BiP, CHOP, ATF6 α , and p-PERK) were increased with decreasing particle size. TiO₂ particles induced ER stress-mediated apoptosis in a particle size-dependent manner as seen by a decrease in the expression of Bcl-2, and increases in the expression of Bax, caspase-12, and cleaved caspase-3. These results indicated that the cytotoxicity produced by TiO₂ particles was related to particle size, with smaller TiO₂ nanoparticles producing greater toxic effects involving ER stress and apoptosis in the HepG2 cells.

Keywords Apoptosis · ER · HepG2 · Size-dependent · TiO₂

Introduction

Titanium dioxide (TiO₂) is used in diverse fields including the food, cosmetic, and medical industries in both bulk (fine) and nanoforms (Yoo et al., 2012). In particular, TiO₂ has been used as a color additive in various food products and as an engineered nanoparticle with new benefits in food, such as improved taste and texture, and prolonged shelf life (Chen et al., 2013). Therefore, the widespread application of TiO₂ nanoparticles creates possibilities of potential human exposure through gastrointestinal, dermal, and pulmonary absorption.

Toxicological studies have shown that TiO₂ nanoparticles could cause adverse effects mainly by inducing oxidative stress resulting in cell damage, inflammation, and immune responses (Skocaj et al., 2011). The damage induced by nanoparticles strongly depends on their characteristics. In particular, size is a critical parameter affecting both the cellular uptake and cytotoxicity of the nanoparticles (Sun et al., 2014). With increasing applications of TiO₂ nanoparticles, concerns regarding their toxicity to humans are also on the rise.

The use of TiO₂ nanoparticles as a food additive may allow these nanoparticles to reach the target or nontarget organs, including the liver, spleen, and lungs (Shukla et al., 2011). The biodistribution of TiO₂ nanoparticles leads to their accumulation in liver, which causes hepatic injury (Wang et al., 2014). Particle size-dependent toxicities by micro- and nanoscale TiO₂ particles have been demonstrated in Caco-2 cells (Tada-Oikawa et al., 2016) and other cell types (Sun et al., 2014; Tedja et al., 2011).

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However, to our knowledge, there has been little report on TiO₂ particle size-dependent cytotoxicity and its molecular mechanisms in hepatocytes.

The endoplasmic reticulum (ER) is an essential organelle responsible for protein folding and assembly, post-translational modification, vesicular trafficking, lipid biosynthesis, cellular calcium storage (Michalak, 2010). ER is prevalent in hepatocytes that perform a myriad of metabolic functions and are sensitive to changes in intracellular homeostasis (Malhi and Kaufman, 2011). Conditions that alter ER homeostasis and ER functioning generate ER stress (Bhandary et al., 2012), which activates a complex signaling network called the unfolded protein response (UPR) to reduce ER stress and restore homeostasis (Li et al., 2016). However, excessive and prolonged ER stress provokes apoptosis or cell death (Ron and Walter, 2007; Hitomi et al., 2004). In particular, various studies have shown that oral exposure to TiO₂ nanoparticles can induce liver damage (Chang et al., 2013). However, to our knowledge, there is no study of the effect of TiO₂ particle size on the induction of ER stress in hepatocytes or liver tissue.

Apoptosis is a type of programmed cell death in which molecules signal the initiation and execution of cell death in response to certain regulatory or stress-induced signals. Although there is a variety of conditions, ROS is an important factor in the apoptotic process. Excessive generation of ROS induces mitochondrial membrane permeability and damages the respiratory chain to eventually trigger apoptotic process (Bhandary et al., 2012). Sun et al. (2014) demonstrated the size-dependent induction of apoptosis in lung epithelial carcinoma cells following exposure to uniform polyethylene glycol-modified TiO₂ nanoparticles of various sizes (100, 200, and 300 nm). Prolonged ER stress also induces apoptosis through several mechanisms, including activation of ER-specific cysteine protease, caspase-12 (which is activated by calpains) and interaction with IRE-1 (Nakagawa et al., 2000). However, whether TiO₂ particles cause size-dependent ER stress and apoptosis, or whether ER stress is involved in particle-induced apoptosis in hepatocytes still remains unclear.

The main objective of this study was to examine relationship between the size-dependent cytotoxicity, ER stress and apoptosis of TiO₂ particles using one type of fine particle and two types of nanoparticles (100 nm particles, which are mainly used as food additive, and < 25 nm particles) on cultured HepG2 cells.

Materials and methods

TiO₂ particles

Three sizes of TiO₂ particles (nominally fine, 100 nm, and < 25 nm particles) were obtained from Hayashi Pure Chemical Industries (Osaka, Japan), MK Nano (Mississauga, Canada), and Sigma-Aldrich (St. Louis, MO, USA) respectively. Size and morphology of the TiO₂ particles were measured using a field emission scanning electron microscope (FE-SEM; Carl Zeiss, Oberkochen, Germany).

Cell culture and preparation of TiO₂ particles

The human hepatoma cell line, HepG2, was provided by Seoul National University (Seoul, South Korea). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (HyClone Laboratories, South Logan, UT) and 1% penicillin–streptomycin (Gibco). Cells were incubated at 37 °C with 5% CO₂. For experiments, the cells were seeded in 100 mm culture plates at 1.5×10^7 cells/mL and allowed to attach for 24 h. To suspend TiO₂ powder in cell culture medium, it was dispersed in sterile phosphate-buffered saline (PBS; Gibco) at pH 7.4 and the mixture was sonicated using a JAC-3010 instrument (KODO Technical Research, Hwaseong, Korea) for 20 min. After sonication, DMEM was transferred to a test tube, the TiO₂ stock solution was added to produce the end-point concentration, and the mixture stored in the dark at 4 °C until use.

Cell viability assay

HepG2 cells were seeded overnight at 1×10^4 cells per well in 96-well plates before the experiment. Particles were dispersed in each serum-containing cell culture medium at a final concentration ranging from 1 to 500 µg/mL. The effect of TiO₂ particles on cell viability was determined after incubation with the dispersed TiO₂ particles for 24 h, using an MTT assay, which quantitatively determined the mitochondrial conversion of the MTT salt into purple formazan crystals. The effect of particles on cell proliferation was expressed as the percentage of inhibition of cell growth relative to that in untreated controls. Data are expressed as the mean $\pm$ standard deviation (SD) from three independent experiments.

Determination of morphological integrity

HepG2 cells (8×10^3 cells) were added to each well of 96-well plates (PerkinElmer, Waltham, MA). After 24 h

from cell-seeding, each well was exposed to the various TiO₂ particle sizes at 100 µg/mL for 24 h, washed with PBS, and then fixed with 4% paraformaldehyde for 20 min. Following a further wash with PBS, cell nuclei were stained with DNA-specific Hoechst 33342 fluorescent dye, and mitochondria were stained with MitoTracker Deep Red (Thermo Fisher Scientific, Waltham, MA). Images of two stained cells were acquired using an Operetta high-content screening analysis system (PerkinElmer), which was designed by combining classical segmentation steps with morphological object measurements using Harmony 3.0 image analysis software (PerkinElmer). Briefly, the Hoechst dye channel was used to assess segmentation of nuclei and quantify nuclear size and staining pattern (texture) and the MitoTracker Deep Red was used to assess cell roundness, mitochondrial distribution and quantity, and mitochondrial texture. To quantify the morphological features of HepG2 cells exposed to the TiO₂ particles of three different average sizes, three types of features [including saddle, edge, and ridge (SER)], symmetry, threshold compactness, axial and radial (STAR) properties, and basic intensity and morphological measures were used. SER features such as spot, hole, edge, ridge, valley, saddle, bright, and dark features, and STAR properties such as compactness, radial, juxtamembrane, cytosolic, perinuclear, outer nuclear, and inner nuclear descriptors were used. Cell area and roundness, nuclear area, roundness, and intensity; and cytoplasmic area and intensity were used as the basic measures of intensity and morphology (Julia et al., 2012).

Measurement of intracellular ROS production

Conversion of 2',7'-dichlorofluorescein diacetate (DCFH-DA) from Sigma-Aldrich into 2',7'-dichlorofluorescein (DCF), a fluorescent dye, was used to assess oxidative stress and ROS production in HepG2 cells. The fluorescence intensity of DCF was positively correlated with the intracellular level of ROS. Cells were incubated with DCFH-DA (20 µM) for 30 min at 37 °C and washed twice with PBS. HepG2 cells were then cultured in the presence or absence of TiO₂ particles at 100 µg/mL for 24 h. H₂O₂ (200 µM) was used as a positive control to induce oxidative stress. After extensive washing, fluorescence was measured using a microplate reader (Molecular Devices, San Jose, CA) and inverted microscope (Nikon Eclipse Ti-U; Nikon, Melville, NY).

Quantitative real-time PCR analysis

HepG2 cells (8 × 10⁵ cells/well) were seeded in a dish of 60-mm diameter for 24 h. The cells were treated with TiO₂ (fine, 100 nm, and < 25 nm particles) particles at 100 µg/

mL for 24 h. Total messenger RNA from the cells was isolated using a RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was reverse-transcribed to synthesize cDNA using a QuantiTect Reverse Transcription kit (Qiagen). mRNA expression was determined using a SYBR Green kit (Toyobo, Osaka, Japan). The experiment was conducted using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). All measurements were performed in duplicate. Cycling (40 cycles) conditions were as follows: predenaturation at 95 °C for 3 min; denaturation at 95 °C for 15 s; annealing at 60 °C for 10 s; and extension at 72 °C for 30 s. The expression of ER stress marker genes was examined using Bio-Rad CFX Manager software and β-actin was used as the housekeeping gene against which the expression of the other genes was normalized. The following primers were used for qRT-PCR: (F) 5'-GGTGACCTGGTACTGCTTGATG-3' and (R) 5'-CCTTGGA TTCAGTTTGGTCATG-3' for binding immunoglobulin protein (BiP); (F) 5'-CTTGGCTGACTGAGGAGGAG-3' and (R) 5'-TCACCATTCGGTCAATCAGA-3' for CCAAT/enhancer-binding protein homologous protein (CHOP); (F) 5'-CCTTGGAATCAG TTTGGTCATG-3' and (R) 5'-GGGGCTTGGTATATATGTGG-3' (XBP1); (F) 5'-TCA TCACCATTTGGCAATGAG-3' and (R) 5'-CACTGTGTT GGCGTACAGGT-3' for β-actin.

Western blot assay

HepG2 cells were harvested, washed in ice-cold PBS once, gently lysed in radioimmunoprecipitation assay buffer containing 1% protease inhibitor cocktail (Quartett, Berlin, Germany) for 20 min, and centrifuged at 12,000 rpm at 4 °C. The protein concentration was measured using a BCA protein assay (Thermo Fisher Scientific). Protein (40 µg) was loaded into the wells of a 12% sodium dodecyl sulfate–polyacrylamide gel, separated by electrophoresis (150 V, 1 h), and transferred to poly(vinylidene) difluoride membranes (0.2 A, 3 h) using a TE22 transfer tank (Hoefer, Holliston, MA). After blocking the nonspecific binding sites on the membranes by 5% bovine serum albumin or skim milk in Tris-buffered saline containing Tween-20 (25 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, and 0.1% Tween-20) for 1 h, immunoblotting was conducted by incubating with the primary antibodies overnight at 4 °C. For the present study, BiP, CHOP, and cleaved-Caspase-3 antibodies were purchased from Cell Signaling (Beverly, MA). Activating transcription factor 6α (ATF6α) antibody was purchased from Novus Biological (Minneapolis, MN). Caspase-12 antibody was purchased from Abcam (Cambridge, MA). Bax, B cell lymphoma 2 (Bcl-2), phosphorylation of protein kinase-like endoplasmic reticulum kinase (p-PERK), β-actin antibodies, horseradish

peroxidase-conjugated secondary antibodies against rabbit, and mouse immunoglobulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The expression of protein was detected using enhanced chemiluminescence-detecting reagents (Bio-Rad) using Smart Chemi 500 apparatus (Beijing, China), and quantified using Lane ID software (version 4.0).

Statistical analysis

All experiments were performed in triplicate; the data are expressed as mean $\pm$ SD. The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 23 (Armonk, NY) and the individual comparisons were obtained by Duncan's multiple Range test. A value of $p < 0.05$ was considered to indicate a significant difference between groups.

Results and discussion

Dose- and size-dependent cytotoxicity of TiO₂ particles

To determine the cytotoxicity of TiO₂ particles, cell viability of HepG2 cells was measured after treatment with various sizes of TiO₂ particles (nominally fine, 100 nm, or < 25 nm particles). Figure 1 showed the size distribution and morphology of the purchased TiO₂ particles

measured by FE-SEM. TiO₂ particles had individual spherical shapes and uniform size. The average fine, 100 nm, or < 25 nm-sized distribution of TiO₂ particles were about 200 nm, 60 nm, and 37 nm, respectively. TiO₂ nanoparticles were more cytotoxic than fine particles, and while all three particle sizes inhibited cell viability, the latter decreased as the concentration increased (Fig. 2). An obvious particle size-dependent cytotoxicity of TiO₂ particles was shown at 100 μ g/mL. Compared to untreated controls, the survival of HepG2 cells decreased significantly ($p < 0.01$) as the nanoparticle size decreased.

The shape, size, monodispersity, and physiochemical properties of nanoparticles determine the different responses observed within biological systems (Dhawan et al., 2009). Coarse agglomerates of nanoparticles have been shown to exert different biological effects compared to well-dispersed nanoparticles (Buford et al., 2007). In the present study, stock suspensions of the particles were sonicated to disperse them in the culture medium, since sonication is more effective in dispersing nanoparticles than otherwise (Bihari et al., 2008). All three TiO₂ particles used in our study were spherical, consistent with the shape of the anatase structure used in other studies (Wei et al., 2014). In the present study, all three sizes (fine, 100-nm, and < 25 -nm) were cytotoxic at 24 h at different concentrations (1–500 μ g/mL) in a dose- and particle size-dependent manner. After 24 h, the liver cells exposed to TiO₂ nanoparticles showed 50% viability, whereas those exposed to fine TiO₂ particles showed 80% viability.

Fig. 1 FE-SEM image of TiO₂ particles. (A) nominally fine, (B) 100 nm, (C) < 25 nm particles

Table 1 Quantification of morphological changes in HepG2 cells after exposure to TiO₂ particles as evidenced by multiple parameters

Morphological property	Descriptor	CON	Fine size	100 nm size	< 25 nm size
Basic intensity and morphology	Cell area	0.99 ± 0.14 ^{b1}	1.00 ± 0.11 ^b	1.14 ± 0.14 ^a	1.18 ± 0.11 ^a
	Cell roundness	0.99 ± 0.04 ²	0.99 ± 0.01	1.00 ± 0.02	0.99 ± 0.02
	Nucleus area	0.99 ± 0.07	1.00 ± 0.05	1.00 ± 0.05	1.00 ± 0.05
	Nucleus roundness	0.99 ± 0.01 ^a	0.93 ± 0.03 ^b	0.99 ± 0.02 ^a	0.99 ± 0.01 ^a
	Nucleus intensity	0.99 ± 0.24 ^b	1.26 ± 0.31 ^a	1.17 ± 0.16 ^{ab}	1.14 ± 0.15 ^{ab}
	Cytoplasm area	0.99 ± 0.18 ^b	1.01 ± 0.14 ^b	1.20 ± 0.19 ^a	1.25 ± 0.15 ^a
	Cytoplasm intensity	0.99 ± 0.29 ^c	1.70 ± 0.71 ^a	1.37 ± 0.40 ^{ab}	1.17 ± 0.28 ^{bc}
STAR ³	Compactness 60%	1.00 ± 0.11	1.00 ± 0.02	0.93 ± 0.05	0.94 ± 0.04
	Radial	0.99 ± 0.08 ^{bc}	0.98 ± 0.04 ^c	1.03 ± 0.03 ^{ab}	1.06 ± 0.02 ^a
	Juxtamembrane	0.99 ± 0.02	0.99 ± 0.01	0.99 ± 0.01	0.99 ± 0.02
	Cytosolic	0.99 ± 0.02	1.00 ± 0.02	1.02 ± 0.03	1.00 ± 0.03
	Perinuclear	0.99 ± 0.04 ^{ab}	1.03 ± 0.05 ^a	0.98 ± 0.06 ^b	1.00 ± 0.07 ^{ab}
	Outer nuclear	0.99 ± 0.04 ^{bc}	1.16 ± 0.07 ^a	0.98 ± 0.09 ^c	1.03 ± 0.10 ^b
	Inner nuclear	0.99 ± 0.06 ^b	1.14 ± 0.08 ^a	0.94 ± 0.13 ^b	1.01 ± 0.14 ^b
SER ⁴	Spot	1.00 ± 0.08 ^{ab}	0.94 ± 0.11 ^b	1.03 ± 0.03 ^a	1.00 ± 0.06 ^{ab}
	Hole	0.99 ± 0.09 ^{ab}	0.96 ± 0.05 ^b	1.01 ± 0.02 ^{ab}	1.03 ± 0.04 ^a
	Edge	0.99 ± 0.19	1.15 ± 0.11	1.13 ± 0.15	1.08 ± 0.12
	Ridge	0.99 ± 0.06 ^b	0.94 ± 0.06 ^c	1.05 ± 0.06 ^a	1.02 ± 0.06 ^{ab}
	Valley	0.99 ± 0.08 ^b	1.13 ± 0.10 ^a	1.19 ± 0.17 ^a	1.12 ± 0.12 ^a
	Saddle	0.99 ± 0.09 ^b	1.06 ± 0.08 ^b	1.15 ± 0.14 ^a	1.09 ± 0.11 ^{ab}
	Bright	0.99 ± 0.07 ^a	0.93 ± 0.07 ^b	1.04 ± 0.05 ^a	1.01 ± 0.06 ^a
	Dark	0.99 ± 0.08 ^b	1.06 ± 0.06 ^{ab}	1.12 ± 0.12 ^a	1.09 ± 0.08 ^a

¹Values followed by different letters are significantly different to Duncan's multiple range test at $p < 0.05$. ²Not significant. ³Symmetry, threshold compactness, axial and radial property. ⁴Saddle, edge, and ridge property. Representative images of nuclei (blue, Hoechst 33342) and mitochondria (red, MitoTracker Deep Red). (A) Control, and (B) nominally fine, (C) 100 nm, (D) < 25 nm particle treated cells observed with fluorescence microscopy using the Operetta high content screening system. Original magnification: ×20. (Color figure online)

Particle size-dependent cytotoxicity was observed only when the particle concentration was 100 µg/mL. There was no significant difference in cell viabilities between 100 and < 25-nm nanoparticles at 500 µg/mL. Our results were consistent with those of Niska et al. (2015), who found that the viability of human osteoblasts was about 50% after treatment with 100 µg/mL of TiO₂ nanoparticles of size < 25 nm (15–20 nm).

Morphological changes of HepG2 cells induced by TiO₂ particles

The morphological and textural features of the TiO₂ particles-treated cells were analyzed (Table 1). Using the dye cocktail, notable changes in cellular morphology and

texture were detected, as indicated by changes in SER features, STAR properties, and basic intensity and morphological dimensions). Cellular and cytoplasmic areas were significantly increased in cells exposed to TiO₂ particles of reduced size. Although the nuclear roundness and intensity and cytoplasmic intensity were also significantly different between the untreated control and TiO₂ particle-treated cells, no particle size-dependent trend was observed. Among the STAR properties, radial property was significantly increased in a particle size-dependent manner. Peri-, outer, and inner nuclear descriptors were also significantly different between the control and TiO₂ particle-treated cells, but no particle size-dependent trends were observed. Among the SER features, valley and dark descriptors showed a significant size-dependent increase in

Fig. 2 The viability of HepG2 cells after 24 h exposure to TiO₂ particles of various sizes. Cell viability was measured after exposure of the cells to nominally fine, 100 nm-, and < 25 nm-sized particles with concentrations from 1 to 500 µg/mL. Data are expressed as mean ± SD from three independent experiments. ***p* < 0.01 compared with a control group using ANOVA

cells exposed to TiO₂ particles. Other SER descriptors such as spot, hole, ridge, saddle, and bright were also significantly different between the control and TiO₂ particle-treated groups, but no size-dependent trend was observed. Overall, the morphological and textural features such as cell and cytoplasmic area, and radial, valley, and dark properties showed a significant particle size-dependent increase in TiO₂ particles-exposed cells.

To our knowledge, no study of the effect of TiO₂ particles on morphological changes of various cell organelles has been conducted previously. Herein, a rapid and flexible image-based phenotypic characterization of HepG2 cells was described using their morphological and textural properties. Morphological and textural changes that reflect cell injuries were observed. With decrease in nanoparticle size, the cell size increased as determined by a significant increase in cellular and cytoplasmic area. TiO₂ particles damage the cell membrane structure, successively leading to an increase in cell and the cytoplasmic volume in a particle size-dependent manner. However, nuclear area and intensity, which is a simple and powerful indicator of cell toxicity; i.e., a characteristic morphological marker for nuclear shrinkage, swelling, or fragmentation (Martin et al., 2014) did not change in a particle size-dependent manner. STAR is used to generate a robust set of descriptors providing information about the subcellular localization and distribution of mitochondria within cells (Julia et al., 2012). Among the STAR descriptors, radial

property measures how closely the mitochondria are localized to the center of the cellular mass and how homogenous their distribution is near the cell border (Julia et al., 2012). Cells exposed to TiO₂ particles showed a significant size-dependent increase in radial property. SER features measure the local patterns of pixel intensity, which can indicate mitochondria structures (Julia et al., 2012). Two SER descriptors, i.e., valley and dark, indicated a significant particle size-dependent increase in TiO₂ particle-exposed cells. However, this study is likely to overestimate or underestimate the cytotoxic effects of TiO₂ particles since only one concentration was used to examine the morphological characteristics in the present study.

Intracellular ROS generation induced by TiO₂ particles

To further elucidate the mechanisms of cytotoxicity, the levels of intracellular ROS were determined. At a median concentration of 100 µg/mL, a size-dependent increase in ROS production was observed when HepG2 cells were exposed to TiO₂ particles (Fig. 3). Exposure to 100 µg/mL of < 25-nm particles resulted in higher levels of ROS than that in the same concentration of fine or 100 nm-particles. Fine, 100-nm, and < 25-nm TiO₂ particles elevated the levels of intracellular ROS significantly when compared to that in untreated control cells. These results indicated that the ROS generation by HepG2 cells, stimulated by TiO₂

Fig. 3 Intracellular generation of ROS by HepG2 cells after 24-h exposure to 100 $\mu\text{g/mL}$ of TiO₂ particles. Fluorescence micrographs of cells treated with (a) control, (b) positive control (200 μM H₂O₂), (c) nominally fine particles, (d) 100-nm particles, (e) < 25-nm particles are shown in (A). The corresponding flow cytometry results are shown in (B). Data are expressed as mean $\pm$ SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared to the control

particles, increased in a particle size-dependent manner; fluorescence micrographs showed that ROS levels increased with decreasing size of TiO₂ particles.

Generally, ROS generation and oxidative stress production by nanoparticles are considered to be the important factors associated with TiO₂ particle toxicity. Smaller the particle, larger is the surface area per unit mass (Dhawan et al., 2009). Therefore, a greater number of active sites is created on the particle surface, to capture oxygen molecules and produce superoxide radicals and other kinds of ROS through Fenton reaction (Nel et al., 2006). Previous studies using HepG2 cells (Shukla et al., 2013) found that exposure to TiO₂ nanoparticles at 40 and 80 $\mu\text{g/mL}$ led to a twofold increase in ROS level at 24 h, thereby causing DNA damage. We found an approximately threefold increase in the production of intracellular ROS after treatment with < 25-nm particles, and a particle size-dependent increase at 100 $\mu\text{g/mL}$. Additionally, our present findings are consistent with previous studies using various cell types where TiO₂ particles have been shown to produce ROS in a particle size-dependent manner (Autrup et al., 2009).

Effects of TiO₂ particle size on the ER stress-associated gene expression and protein levels

The expression of ER stress-related genes, including BiP, which serves as a gatekeeper for the activation of ER stress transducers, and CHOP, which is a transcription factor induced by ER stress, were analyzed by qRT-PCR. Gene expression levels in cells increased with decreasing size of TiO₂ particles (Fig. 4A, B). These results showed that exposure to TiO₂ particles increased ER stress markers,

such as BiP and CHOP in the cells in a particle size-dependent manner. However, only CHOP expression, a classical marker of ER stress response, was significantly increased in a particle size-dependent manner. Exposure to tunicamycin (TN), an ER stress inducer used as a positive control, significantly increased the expression of BiP and CHOP genes in the cells.

Diverse types of nanoparticle materials, including zinc oxide, silver, and TiO₂, have been shown to elevate ER stress (Yang et al., 2015; Yu et al., 2015). However, only a few studies have examined the effects of TiO₂ particle size, mainly on lung epithelial cells (Sun et al., 2014). BiP is an ER molecular chaperone that binds newly synthesized proteins during translation and is located in the ER lumen (Otero et al., 2010). CHOP, a protein induced by DNA damage, is expressed at a very low level under physiological conditions, but its expression significantly in the presence of severe or persistent ER stress (Otero et al., 2010). In the present study, the expression of BiP and CHOP genes in hepatocytes increased gradually after TiO₂ treatment in a particle size-dependent manner, but the increase was much smaller than that caused by TN, as a positive control. The reason for this difference may be that TN induces high ER stress in cells by inhibiting the first step in *N*-linked glycans biosynthesis in proteins, thereby resulting in several misfolded proteins (Banerjee et al., 2011).

To further assess whether ER stress is associated with the mechanism of apoptosis in HepG2 cells induced by TiO₂ particles, the expression of ER stress-related proteins was examined in TiO₂ particle-treated cells. TiO₂ particles increased BiP and CHOP levels, as seen by western blotting (Fig. 4C, D), in a TiO₂ particle-size dependent manner. Expression of BiP and CHOP were significantly

Fig. 4 Unfolded protein response and gene expression in HepG2 cells. HepG2 cells were treated with 100 μg/mL of TiO₂ particles for 24 h. mRNA was isolated for RT-PCR and protein was isolated for western blotting. Expression of ER stress marker genes (BiP (A) and CHOP (B)) in HepG2 cells was measured by qRT-PCR. The protein levels of BiP (C), CHOP (D), ATF6α (E) and p-PERK (F) were determined by western blotting. Quantification of western blot signals was normalized to β-actin expression and compared with controls. Results are presented as mean ± SD from three independent experiments. **p* < 0.05, ***p* < 0.01 compared to the control

increased in HepG2 cells exposed to < 25-nm TiO₂ particles. This suggested that TiO₂ nanoparticles upregulated BiP and CHOP, thereby leading to ER stress. The ATF6 α protein (90 kDa) is fragmented into a smaller protein (50 kDa) in the Golgi apparatus under ER stress. Expression of ATF6 α in HepG2 cells was increased in a TiO₂ particle size-dependent manner, and significant differences were observed except after treatment with 100-nm particles (Fig. 4E). Exposure of HepG2 cells to TiO₂ particles also resulted in a significant particle size-dependent increase in the phosphorylation of PERK (which is responsible for attenuating mRNA translation under ER stress) compared to that in untreated control cells (Fig. 4F).

Accumulation of un/mis-folded proteins in the ER lumen triggers an ER stress signal through ER stress markers. While < 25-nm particles produced significant increase in the levels of both BiP and CHOP protein, level of BiP was higher than that of CHOP. The gene expression and protein levels for these ER stress markers showed only minor differences, probably because mRNA levels do not always correlate with protein levels due to various reasons, such as mRNA half-life, translation efficiency, and protein stability (Koussounadis et al., 2015). The ER stress sensors, ATF6 α and p-PERK, were increased by TiO₂ particles in a size-dependent manner. The gene and protein expression levels suggested that stimulation with TiO₂ particles induces ER stress sensors (ATF6 α and p-PERK), BiP, and CHOP in a particle size-dependent manner.

TiO₂ particles induce size-dependent apoptosis in HepG2 cells

To determine whether apoptosis was induced in TiO₂ particle-treated cells, the ratio of Bax (proapoptotic)-to-Bcl-2 (antiapoptotic) expressions in HepG2 cells was assessed by western blotting. The Bax/Bcl-2 ratio serves as a marker to determine cell susceptibility to apoptosis. The smaller-sized TiO₂ particles induced expression of Bax, but inhibited that of Bcl-2 (Fig. 5A), which was consistent with the hypothesis that TiO₂ nanoparticles induce apoptosis in HepG2 cells. The Bax/Bcl-2 ratio was increased in a TiO₂ particle size-dependent manner (Fig. 5B). For cells exposed to < 25-nm TiO₂ particles, the Bax/Bcl-2 ratio was significantly greater than in untreated control cells. Our data suggested that TiO₂ particles induce apoptosis in a particle size-dependent manner by upregulating proapoptotic Bax and downregulating anti-apoptotic Bcl-2.

Prolonged ER stress is well known to cause cell apoptosis (Ron and Walter, 2007), which induces direct damage to not only cellular components, but also associated proteins that are localized on the mitochondrial membrane, particularly the Bcl-2 family members. The Bcl-2 family

consists of anti-apoptotic members such as Bcl-2, and proapoptotic proteins such as Bax (Bhandary et al., 2012). Our present results suggest that TiO₂ increased the level of Bax protein while decreasing the level of Bcl-2 protein, in a particle size-dependent manner. Only treatment with < 25-nm TiO₂ nanoparticles significantly increased the level of Bax protein, whereas the level of Bcl-2 was decreased significantly after treatment with either 100-nm or < 25-nm nanoparticles. The results observed in the present study are similar to those of Shukla et al. (2011), although they used slightly larger TiO₂ particles at different concentrations.

Treatment of HepG2 cells with TiO₂ particles for 24 h led to a particle size-dependent increase of caspase-12 activation (Fig. 5B). The expression levels of caspase-12 were significantly increased by 100-nm and < 25-nm TiO₂ nanoparticles. To further assess the relationship between nanoparticle size and TiO₂ particle-induced apoptosis, we compared the release of cleaved caspase-3 with that of the control cells. The cleaved caspase-3 activity increased significantly after exposure to TiO₂ particles and tended to increase with decreasing nanoparticle size (Fig. 5B). The increase of cleaved caspase-3 activity in cells exposed to < 25-nm particles was significantly greater than that in cells exposed to the fine and 100-nm particles. These results suggested that TiO₂ particles induce apoptotic cell death in HepG2 cells in a particle size-dependent manner via ER stress upregulation and subsequent caspase activation.

Caspase-12 is specifically activated by ER stress, but not by membrane- or mitochondria-targeted apoptotic signals (Nakagawa et al., 2000). In primary cultured Sertoli cells, TiO₂ nanoparticles significantly increased the expression of BiP, CHOP, and caspase-12, which were associated with ER stress-mediated apoptosis (Hong et al., 2016). The level of caspase-12 in our present study increased significantly in a TiO₂ particle size-dependent manner. Activated caspase-12 further cleaves and activates caspase-3, which subsequently results in apoptosis (Morishima et al., 2002). Consistent with previous studies, in the present study, TiO₂ particles produced a significant particle size-dependent effect on the level of cleaved caspase-3 (Shukla et al., 2011). The previous studies reported that TiO₂ nanoparticles induced apoptosis, and increased caspase-3 expression in cultured human lung epithelial cells (BEAS-2B), rat neuronal cells (PC-12), and HepG2. The apoptotic effect was larger with smaller particles than it was with larger particles.

Our present findings show that the size of TiO₂ particles from fine to < 25 nm significantly affected HepG2 cell viability, morphology, ER stress, and apoptosis. The present study also demonstrated that apoptosis induced by

Fig. 5 Expression of apoptotic marker proteins in TiO_2 particle-treated HepG2 cells. Representative images including Bax (proapoptotic) (A), Bcl-2 (antiapoptotic) (B), the ratio of Bax/Bcl-2 (C), caspase-12 (ER stress-initiated apoptosis) (D) and cleaved caspase-3 (E) were analyzed using western blotting. Quantification of western blot signals was normalized to β -actin expression and compared with control. Tunicamycin (1 $\mu\text{g/mL}$) was used as a positive control. Results are presented as mean $\pm$ SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared to the control group

TiO_2 particles could be attributed to ER stress, at least in part, as evidenced by particle size-dependent induction of caspase-12. Since only a single type of hepatocyte cell line,

HepG2, was used in the present study, the possibility of differences in sensitivity to TiO_2 particles across cell types cannot be excluded.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest.

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