

Titanium Dioxide Nanoparticles-Mediated In Vitro Cytotoxicity Does Not Induce Hsp70 and Grp78 Expression in Human Bronchial Epithelial A549 Cells

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Abstract Titanium dioxide nanoparticles (TiO₂NPs) are increasingly being used in various industrial applications including the production of paper, plastics, cosmetics and paints. With the increasing number of nano-related products, the concern of governments and the general public about the health and environmental risks, especially with regard to occupational and other environmental exposure, are gradually increasing. However, there is insufficient knowledge about the actual affects upon human health and the environment, as well as a lack of suitable biomarkers for assessing TiO₂NP-induced cytotoxicity. Since the respiratory tract is likely to be the main exposure route of industrial workers to TiO₂NPs, we investigated the cytotoxicity of the anatase and rutile crystalline forms of TiO₂NPs in A549 cells, a human alveolar type II-like epithelial cell line. In addition, we evaluated the transcript and protein expression levels of two heat shock protein (HSP) members, Grp78 and Hsp70, to ascertain their suitability as biomarkers of TiO₂NP-induced toxicity in the respiratory system. Ultrastructural observations confirmed the presence of TiO₂NPs inside cells. In vitro exposure of A549 cells to the anatase or rutile forms of TiO₂NPs led to cell death and induced intracellular ROS generation in a dose-dependent manner, as determined by the MTS and dichlorofluorescein (DCF) assays, respectively. In contrast, the transcript and protein expression levels of

Hsp70 and Grp78 did not change within the same TiO₂NPs dose range (25–500 µg/ml). Thus, whilst TiO₂NPs can cause cytotoxicity in A549 cells, and thus potentially in respiratory cells, Hsp70 and Grp78 are not suitable biomarkers for evaluating the acute toxicological effects of TiO₂NPs in the respiratory system.

Keywords TiO₂ nanoparticles · Cytotoxicity · Heat shock protein · Hsp70 · Grp78 · A549 cells

Introduction

The industrial use of metallic oxide nanoparticles (NPs) in a wide variety of applications has rapidly expanded over the last decade. Because of its high stability, anticorrosiveness and photocatalytic properties, titanium dioxide nanoparticles (TiO₂NPs) are among the most frequently used metallic oxide NPs in various human products, such as cosmetics, sunscreen, toothpaste and paints [1]. Moreover, TiO₂NPs have been used in the manufacturing industry [2] and environmentally to decontaminate water, air and soil, including the decontamination of agrochemicals [3, 4]. With the rapid increase in the number of nano-related products comes an increasing concern of governments and the general public about their safety to humans and the environment. Indeed, the human health effects of TiO₂NP exposure, as well as their impact on the environment, are not under regulatory control [5], which then serves to raise concern further.

Because of their very small size, TiO₂NPs have specific physicochemical characteristics and can enter the human body through several potential routes, including inhalation, skin contact, ingestion and parenteral [6]. Among the different potential exposure routes, inhalation is likely to be the main entry route of manufactured TiO₂ into the body of

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industrial workers. Up to date, the safety aspects of TiO₂NPs in the respiratory system have been studied in various in vitro models that are derived from human bronchial epithelial cells or lung fibroblast cells, and in vivo models using rodent models. Most of the studies have focused on the cytotoxic effects, toxic mechanisms, accumulation and clearance of TiO₂NPs. For instance, it has been reported that TiO₂NPs are rapidly internalized in the A549 human alveolar derived cell line and become distributed in the cytoplasm and intracellular vesicles [7], whilst TiO₂NPs induce oxidative stress and cause genotoxicity in human lung fibroblast cells via DNA-adduct formation [8]. Furthermore, it has been reported that TiO₂NPs cause cell death via induction of mitochondrial apoptosis in the BEAS2B human bronchial epithelial cell derived cell line, and that this involves Bax, cytochrome C, p53 and Bcl-2 [9]. With respect to the particle uptake and clearance aspects of TiO₂NPs, lung surface macrophages do not efficiently phagocytose these ultrafines, but take them up in a rather sporadic and unspecific pathway in the rat model [10]. However, there are far fewer studies on the cellular responses and possible biomarker(s) for assessing the toxicity of TiO₂NPs in the respiratory system.

One of the key cellular responses to toxicant exposure which could potentially be used as an early marker of toxicity is the induction of heat shock protein (HSP) expression [11]. HSPs can function as molecular chaperones, facilitating protein folding, preventing protein aggregation and targeting improperly folded proteins to specific degradative pathways [12]. Among them, the 70-kDa HSP family (Hsp70) consist of at least 11 genes, including *Hsp70*, *Hsc70*, *Grp75* and *Grp78*, the protein products of which have housekeeping functions in the cell and are built-in components of protein folding, signal transduction pathways and quality control functions, where they proof read the structure of proteins and repair misfolded conformations [13–15]. The Hsp70 protein family differs from the other classes of HSPs in being one of the most highly conserved members and the first to be induced under stress conditions [14]. Among the members of the Hsp70 family, Hsp70 itself is an important cytosolic protein in the HSPA chaperone machine and the strongest stress induced protein [16]. Their main chaperone functions are to help the folding of proteins and the refolding of denatured proteins [15]. Another member of the HSP family, Grp78 (glucose-regulated protein of 78 kDa; BiP) is a major endoplasmic reticulum (ER) chaperone protein that facilitates protein folding and assembly, protein quality control, Ca²⁺-binding and regulating ER stress signaling [17]. Grp78 is induced by physiological stresses that perturb the ER function and homeostasis and protects against tissue or organ damage under these pathological conditions [18]. Grp78, therefore, is widely used as a marker for ER stress to toxicants. However, the involvement of Hsp70

and Grp78 in biological responses to TiO₂NPs in the respiratory system is currently unknown.

Therefore, in this study, we aimed to examine possible biomarker(s) in response to TiO₂NP-induced toxicity in A549 cells in vitro (representative of the respiratory system) by focusing on the transcriptional and post-translational expression levels of Hsp70 and Grp78.

Materials and Methods

Nanoparticles

Anatase and rutile crystalline forms of TiO₂NPs were purchased from Sigma-Aldrich (St. Louis, MO). The product insert information indicates particle sizes of less than 25 nm with a specific surface area of 200–220 m²/g.

Particles Characterization

The TiO₂NP morphology was analyzed by transmission electron microscopy (TEM) using a JEM-2010 microscope (Jeol, Japan). A drop of the TiO₂NP suspension (from a feedstock of 0.01 g TiO₂NP powder diluted to 20 ml by ethanol and sonicated for 5 min) was placed on a carbon film supported by a 200-mesh copper grid and the solvent was removed by drying overnight in a desiccator. TEM images of the TiO₂NPs were collected at 200 keV and 100,000 times magnification. An energy-dispersive X-ray analysis (EDX) was used to confirm the presence of titanium.

Particle size and polydispersity index (PDI) of TiO₂NPs were measured by dynamic light scattering (DLS) with a Zetasizer Nanoseries S4700 (Malvern, UK). TiO₂NPs were dispersed in ultrapure water or Ham's F12K at a concentration of 1,000 µg/ml. To this end, after sonication for 5 or 60 min, the TiO₂NP suspension was 10-fold diluted with distilled water or CM, and then analyzed by DLS.

Cell Culture and Preparation of Exposure Suspension

A549 cells, derived from the human alveolar type II-like epithelial cell line, were obtained from the American Type Culture Collection (CCL-185; ATCC, Manassas, VA). The cells were cultured in complete media (CM; Ham's F12K medium (Invitrogen, Carlsbad, CA) supplemented with 10 % (v/v) fetal bovine serum) at 37 °C in humidified atmosphere with 5 % (v/v) CO₂ and subcultured by trypsinization. Before the treatment, A549 cells at a passage number less than 40 were grown overnight to reach about 80 % confluence. TiO₂NPs were dispersed in the media without FBS at a concentration of 1,000 µg/ml by sonication for 5 min and diluted with CM prior to addition to the A549 cells.

Cytotoxicity Assay

A549 cells (5×10^3 cells/well) were seeded into each well of a 96-well plate in 100 μ l of CM and grown for 24 h before treatment. The medium was then removed and replaced with 100 μ l of the TiO₂NP suspension in CM at final concentrations of 5, 10, 25, 50, 100, 250 and 500 μ g/ml, which is equivalent to 1.563, 3.125, 7.813, 15.625, 31.25, 78.13 and 156.3 μ g/cm², respectively, and incubated for 24 h. The cytotoxicity of the added TiO₂NPs was subsequently assessed using the CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI), as per the manufacturer's instructions, which contains 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). The absorbance of the resulting solubilized formazan product was measured by monitoring the absorbance at 490 nm using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). Results are shown as the relative percentage cell viability (which assumes no differences between treatments in cell division or mitochondrial metabolism and hence is all due to the number of viable cells), calculated by comparing the observed absorbance to that of the control cells (no added TiO₂NPs).

Intracellular Reactive Oxygen Species (ROS) Generation

The induction of ROS generation by TiO₂NPs was measured by dichlorofluorescein (DCF) assay [20]. Briefly, A549 cells (1×10^4 cells/well) were seeded into each well of a 96-well culture plate and incubated at 37 °C for 24 h. The cells were subsequently incubated with 50 μ M 2',7'-dichlorodihydrofluorescein diacetate (H₂-DCF-DA; Molecular Probes, Grand Island, NY) dispersed in phosphate buffered saline pH 7.4 (PBS) for 40 min in the dark. The cells were washed with PBS and then exposed to various concentrations of the anatase or rutile crystalline forms of TiO₂NPs at final concentrations of 5, 10, 25, 50, 100, 250 and 500 μ g/ml for 2 h. Hydrogen peroxide (5 mM) was used as a positive control in the assay. The fluorescent intensity of 2',7'-dichlorofluorescein in the cells was measured at 485/528 nm by a microplate reader system (PowerWave XS2, BioTek, Winooski, VT).

Side Scatter Measurements

The degree of TiO₂NPs uptake or adsorption on cellular membranes was determined by measuring side scatter pulse area (SSC-A) parameter of FACS analysis as described previously [19]. Briefly, A549 cells were plated into a 12-well plate at 6×10^4 cells/well and cultured for 24 h. After TiO₂NP exposure, the cells were trypsinized, centrifuged and resuspended in PBS before subjected to FACS analysis using a flow cytometer (FACSaria II; Becton Dickinson, San Jose, CA). The

mean of SSC-A was measured by FACSDiva software (BD) based on 10,000 events.

TEM Analysis

A549 cells (8×10^5 cells) were plated into a 10-cm Petri dish and cultured for 24 h before treatment with 100 μ g/ml TiO₂NPs for 24 h. The cell pellets were collected, fixed with 2.5 % glutaraldehyde and post-fixed in 1 % osmium tetroxide. The specimens were subsequently dehydrated in a graded series of ethanol and embedded in a mixture of Spurr resin. After polymerized, ultra-thin sections were prepared with a Leica Ultracut microtome (Leica, Deerfield, IL), collected on copper grids, and contrasted with uranyl acetate and lead acetate. Samples were finally imaged using a TEM at 120 keV (FEI Tecnai T20, the Netherlands).

Quantification of the Expression Levels of *Grp78* and *Hsp70* mRNAs

A549 cells were seeded into each well of a 6-well culture plate at 1.5×10^5 cells/well and incubated at 37 °C for 24 h. The cells were then exposed to the anatase or rutile crystalline forms of TiO₂NPs at a final concentration of 25, 50, 100, 250 or 500 μ g/ml. Total RNA was isolated by using a FastPure RNA Kit (Takara, Japan) following the manufacturer's instructions. The isolated total RNA was subsequently treated with DNase I (Takara) to remove contaminating genomic DNA. The RNA concentration was determined at 260 nm by Nanodrop 2000C (Thermo Scientific, Wilmington, NC). The cDNA was then generated with a random hexamer by using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA). The expression level of *Grp78* and *Hsp70* mRNA transcripts in A549 cells were then measured by quantitative real-time PCR carried out on an ABI PRISM 7500 Fast (Applied Biosystems) using SYBR green (Applied Biosystems). Each PCR reaction mixture, at a final volume of 15 μ l, contained of 1 \times Power SYBR green Mastermix, 0.25 μ M of each of the forward and reverse primer (see Table 1) and 37.5 ng cDNA template. PCR reactions were performed at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C (62 °C for *Hsp70*) for 1 min. The expression levels of *Grp78* and *Hsp70* mRNAs were normalized to the expression level of GAPDH as an endogenous control gene. The primers were designed using the Oligo 4.0-s software (Molecular Biology Insights, Cascade, CO) and confirmed for likely gene specificity by Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) searching of the NCBI GenBank database.

Table 1 Sequences of the primers used in the real-time PCR

Gene name	GenBank accession No.	Location ^a (bp)	Expected size (bp) ^b	Sequence (5'–3') ^c
<i>Grp78</i>	NM005347.4	725–745 808–790	84	F: AAACCGCTGAGGCTTATTTGG R: CTTGGCGTTGGGCATCATT
<i>Hsp70</i>	NM005345.4	1,799–1,821 1,987–1,965	189	F: TGCAGGAGGCGGAGAAGTACAAA R: CCAGCCACGAGATGACCTCTTGA
<i>GAPDH</i>	NM002046.3	157–178 260–238	104	F: ACCAGGGCTGCTTTTAACTCTG R: TGGGTGGAATCATATTGGAACAT

^a The primer location is shown for the bp numbering of the sequence entry with the given NCBI GenBank accession number

^b Expected size of the PCR amplicon in bp

^c Primer sequences are shown 5' to 3' for: F, forward; R, reverse

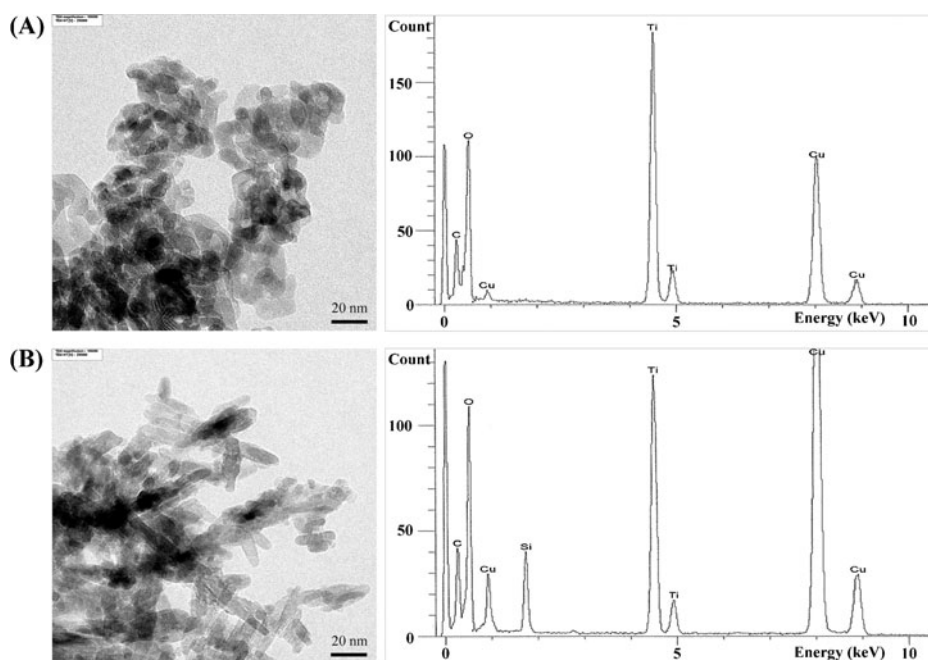
Cell Lysate Preparation

A549 cells (8×10^5 cells) were seeded in a 10-cm Petri dish in 10 ml of CM and incubated at 37 °C for 24 h. The cells were then exposed to various doses of anatase or rutile crystalline forms of TiO₂NPs at a final concentration of 25, 50, 100, 250 or 500 µg/ml for 6, 12 or 24 h. The cells were then washed and subsequently lysed in cell lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 % (v/v) Nonidet P-40, 1 mM EDTA, 100 mM NaF, 0.2 mM Na₃VO₄) supplemented with protease inhibitor cocktail (Sigma) for 30 min at 4 °C with gentle agitation. The cell debris was consequently removed by centrifugation at 10,000×g for 20 min at 4 °C. Cell lysates were kept at –80 °C until analysis. The protein concentration in each sample was measured by using a BCA protein assay kit (Thermo Scientific, Illinois, USA), as per the manufacturer's instructions.

Western Blot Analysis

The cell lysates (10 µg protein/lane) were separated on reducing SDS–polyacrylamide gel electrophoresis (7.5 % (w/v) acrylamide resolving gel) and transferred to a polyvinylidene fluoride membrane (Pall Life Sciences, Ann Arbor, MI) by Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, UK). The stress proteins, Grp78 and Hsp70, were specifically detected by incubating the membrane with anti-KDEL (SPA-827, Stressgen, San Diego, CA) and anti-HSP70 (SPA-820, Stressgen) antibodies (each diluted at 1:1,000) in PBS-S (5 % (w/v) skim milk in PBS) for 2 h at room temperature, respectively. Membranes were also incubated at this stage with anti-β-actin (sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:500 in PBS-S, for 2 h as a sample loading control. The membrane was subsequently washed with PBS and incubated with biotinylated-secondary antibodies (2,000 dilution) in PBS-S for 30 min. The membrane was then incubated with

Fig. 1 Characterization of the **a** anatase and **b** rutile crystalline forms of TiO₂NPs. The particle morphology was analyzed by TEM and imaged with a magnification of 100,000 times. The presence of titanium was confirmed by an energy-dispersive X-ray analysis (EDX). Micrographs and spectra shown are representative of those seen from five fields of view per sample



avidin–biotin complex (Santa Cruz Biotechnology) and finally incubated with 3,3'-diaminobenzidinetetrahydrochloride (DAB; a substrate for peroxidase). The protein bands were subsequently imaged by a gel imaging system (AlphaImager, San Jose, CA).

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. A value of $p < 0.05$ was considered statistically significant.

Results

TiO₂NP Characterization

According to the supplier's product information, the anatase and rutile TiO₂NPs were each 99.9 % pure with an average particle size of less than 25 nm and a specific surface area of 200–220 m²/g. The morphology and size distribution of the anatase and rutile TiO₂NPs were confirmed by dispersing TiO₂NPs in an aqueous solution, and then measured in the dry and hydrated states by TEM-EDX and DLS analyses, respectively. The size of the anhydrous anatase and rutile TiO₂NPs, as revealed by TEM analysis, revealed a median particle size of about 20 nm, and both forms showed a similar pattern of two peaks (high and small) of titanium in the EDX spectra (Fig. 1a and b). For the hydrated size, as evaluated by DLS, the TiO₂NPs dispersed in DI water tended to form agglomerates with a net size distribution at 542±8.21 and 321.53±16.18 nm for the anatase and rutile forms, respectively (Table 2). Although increasing the sonication time from 5 to 60 min reduced the diameter of the anatase

agglomerates ~1.1-fold, no significant change in agglomerate size was noted for the rutile NPs. Likewise, for the TiO₂NP size distribution, as measured by the DLS, the PDI was not decreased by increasing the sonication time from 5 to 60 min, but rather was increased. In the experimental condition of TiO₂NPs dispersed in CM, high degree of agglomeration was observed. The hydrodynamic diameter sizes of anatase TiO₂NPs in CM at 5 and 60 min sonication were ~5.3- and 5-fold larger than those in DI water, respectively. The increased sonication time appeared no effect on their hydrodynamic size. In contrast, the diameter sizes of rutile TiO₂NPs dispersed in CM were decreased up on increasing the sonication time from 5 to 60 min. Although diameter size of rutile TiO₂NPs at 5-min sonication was about twice larger than at 60-min sonication, the long period of sonication could generate heat and induce higher degree of sedimentation. At 5-min sonication, two populations of rutile TiO₂NP size distribution, in nano-sized range, were demonstrated (Suppl. 1). Therefore, a 5-min sonication of anatase and rutile TiO₂NPs was used in this study.

In Vitro Cytotoxicity of TiO₂NPs to the A549 Cell Line

To investigate the in vitro cytotoxicity of TiO₂NPs, A549 cells in tissue culture were used as an in vitro model system for the airway epithelium. The A549 cells were exposed to various concentrations of TiO₂NPs for 24 h and then analyzed by the MTS assay. The anatase form of TiO₂NPs caused a dose-dependent reduction in the amount of formazan produced, and thus an assumed decreased cell viability or increased cytotoxicity in A549 cells, but only the two highest concentrations tested (250 and 500 µg/ml) were statistically significant ($p < 0.05$) (Fig. 2a). Rutile TiO₂NPs also showed a dose-dependent decrease in the cell viability, but this numerical decrease was not statistically significant at the concentrations used in this study (Fig. 2b).

Intracellular ROS Generation

Since oxidative stress is one of the mechanisms of toxicity related to NP exposure in several cell lines, the intracellular ROS generation in A549 cells after exposure to TiO₂NPs was investigated using the DCF assay. As shown in Fig. 3, both the anatase and rutile forms of TiO₂NPs significantly increased the intracellular ROS levels, but this was only at the higher concentrations of 100 µg/ml numerically, and was statistically only significant at 250 and 500 µg/ml ($p < 0.05$). Nevertheless, consistent with the observed results for their ability to induce A549 cell cytotoxicity, the anatase and, to a lesser extent, the rutile forms of TiO₂NPs tended to induced ROS generation in a concentration-dependent manner.

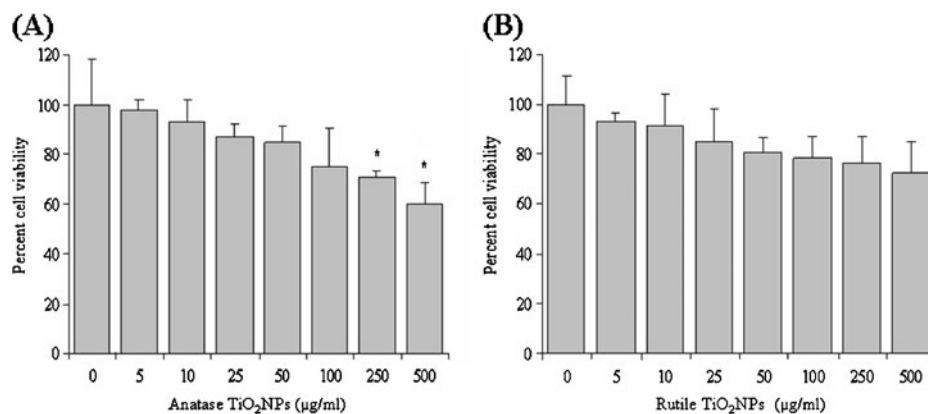
Table 2 Hydrated TiO₂NP (agglomerate) size and polydispersity index

Sample preparation ^a	Hydrodynamic diameter (nm) ^b	Polydispersity index (nm) ^b
Anatase TiO ₂ NPs		
DW, sonication for 5 min	542.5±8.2	0.258±0.014
DW, sonication for 60 min	499.0±3.2	0.304±0.026
CM, sonication for 5 min	2,865±381.7	0.593±0.163
CM, sonication for 60 min	2,503±328.2	0.504±0.189
Rutile TiO ₂ NPs		
DW, sonication for 5 min	321.5±16.2	0.370±0.021
DW, sonication for 60 min	338.5±17.1	0.469±0.029
CM, sonication for 5 min	4,235±662.7	1
CM, sonication for 60 min	2,623±326.6	0.842±0.075

^a Samples were suspended in distilled water (DW) or CM and sonicated for either 5 or 60 min

^b Data are shown as the mean ± SD and are derived from triplicate measurement

Fig. 2 In vitro cytotoxicity of the **a** anatase and **b** rutile crystalline forms of TiO₂NPs against A549 cells. A549 cells were incubated with various concentrations of the anatase or rutile crystalline forms of TiO₂NPs for 24 h and then the cell viability was determined by the MTS assay. Data are presented as mean ± SD of three independent experiments. **p*<0.05, using a one-way ANOVA for the comparison between untreated and TiO₂NP-exposed cells



Uptake of TiO₂NPs into the Cells

The uptake of TiO₂NPs into A549 cells was investigated by FACS and TEM analyses. Since, increase of SSC is proportional to cell granularity or complexity, endocytosis or adsorptive nanoparticle interactions to the cells were determined by SSC measurement following TiO₂NP exposure. As shown in Fig. 4, mean of SSC-A was significantly increased in cells treated with both anatase and rutile TiO₂NPs at even the low concentrations (25 and 50 µg/ml) used in this study. We further confirmed the internalization of TiO₂NPs to the cells by TEM analysis. Ultrastructure image demonstrated that TiO₂NPs could be internalized to the cells and appeared in the cytoplasm, but not in the nucleus (Fig. 5).

Effect of TiO₂NPs on Grp78 and Hsp70 Transcript and Protein Expression Levels in the A549 Cell Line

To examine the alteration of stress-related gene expression after exposure to TiO₂NPs, the mRNA transcript expression levels of *Grp78* and *Hsp70* were determined by real-time reverse transcriptase PCR. Exposure of A549 cells to

TiO₂NPs in the dose range of 25–500 µg/ml for 6 and 24 h did not significantly change the expression levels of either *Grp78* or *Hsp70* mRNA (Fig. 6). However, a slight dose-dependent numerical increase in the *Hsp70* transcript levels was noted after 24 h, but not 6 h, exposure to the TiO₂NPs, and especially for the anatase form, but as already noted this was not statistically significant.

The expression level of the Grp78 and Hsp70 proteins, relative to that of the housekeeping β-actin gene, in A549 cells after TiO₂NPs treatment in tissue culture were investigated by Western blot analyses. Similar to that observed for the mRNA transcript levels, both the anatase and rutile crystalline forms of TiO₂NPs did not significantly change the protein expression levels of Grp78 and Hsp70 after 6, 12 and 24 h of exposure, at least in the dose range examined in this study (Fig. 7).

Discussion

TiO₂NPs are increasingly being used in various commercial products, but there is still an inadequate knowledge concerning their risk to human health and the environment,

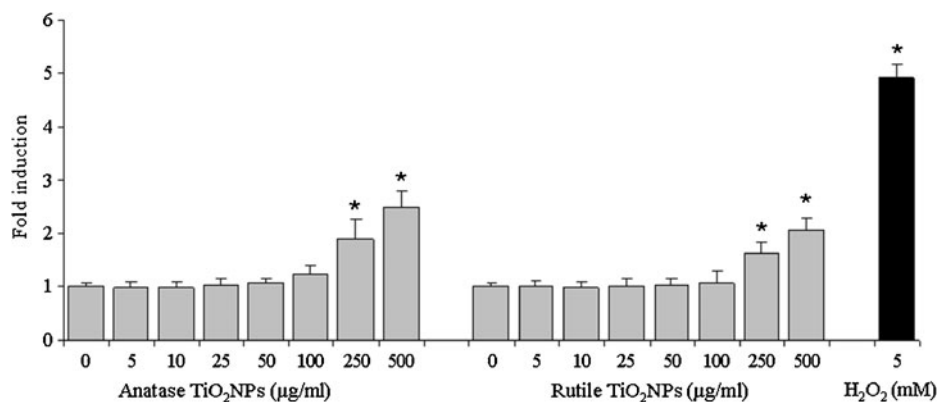


Fig. 3 Intracellular ROS generation in A549 cells after treatment with the **a** anatase and **b** rutile crystalline forms of TiO₂NPs. The cells were incubated with various concentrations of the anatase or rutile crystalline form of TiO₂NPs for 24 h and then the intracellular ROS levels were determined by the DCF assay. H₂O₂ at 5 mM was used as a

positive control. Data are presented as the mean ± SD of three independent experiments, and show the ROS levels relative to that of the no-TiO₂NPs control. **p*<0.05, using one-way ANOVA for the comparison between untreated and TiO₂NP-exposed cells

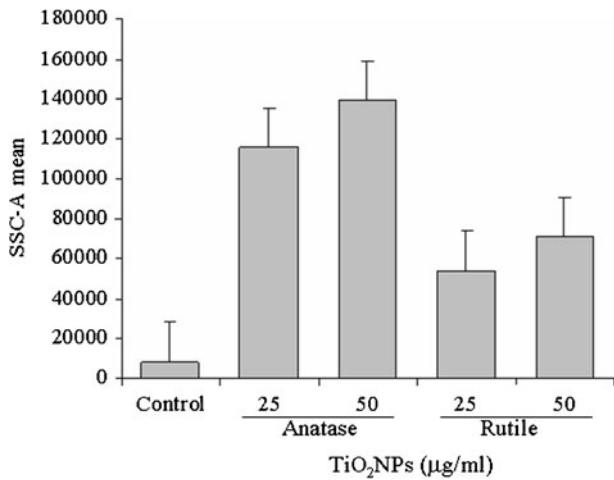
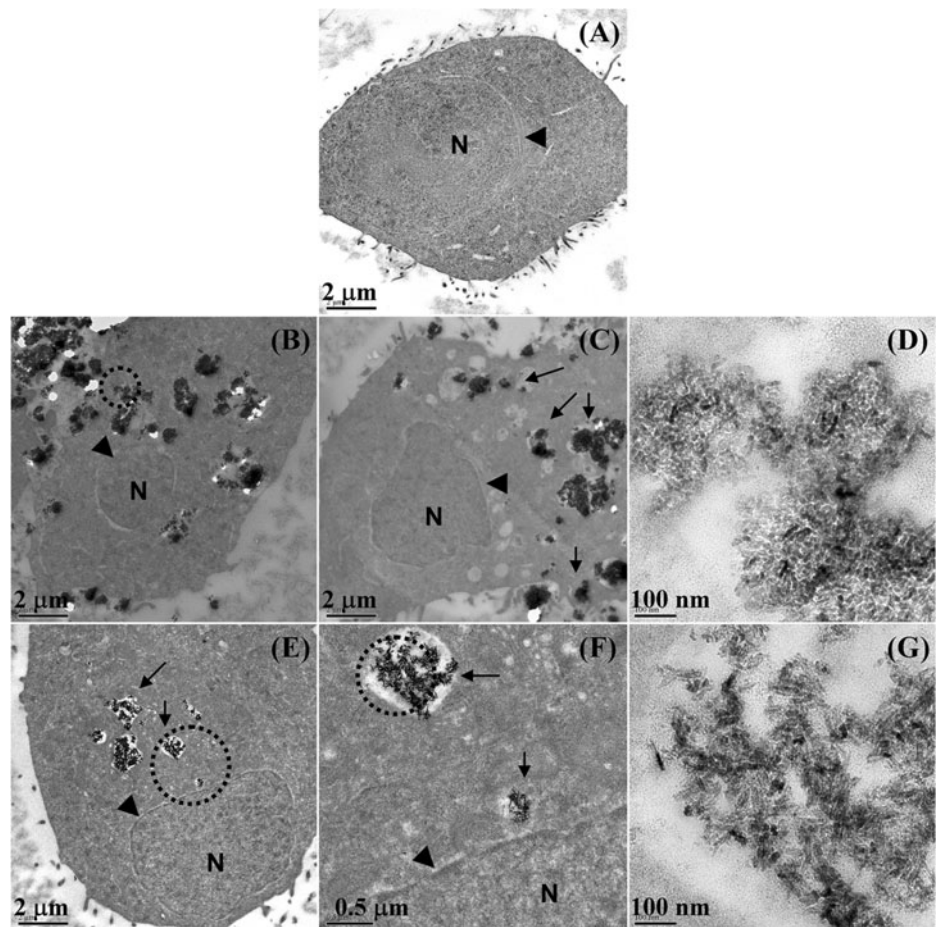


Fig. 4 Uptake of TiO₂NPs. A549 cells were treated with anatase and rutile TiO₂NPs at 25 and 50 µg/ml for 24 h and were subjected to FACS analysis. TiO₂NP uptake or cell surface adsorption is proportion to an increase in SSC-A of cell population

as well as on any suitable biomarker(s) for assessing the safety of TiO₂NPs in, for example, the human respiratory system. In this study, we, therefore, investigated the in vitro cytotoxicity of both the anatase and rutile crystalline forms of TiO₂NPs in A549 cells as an in vitro model of human

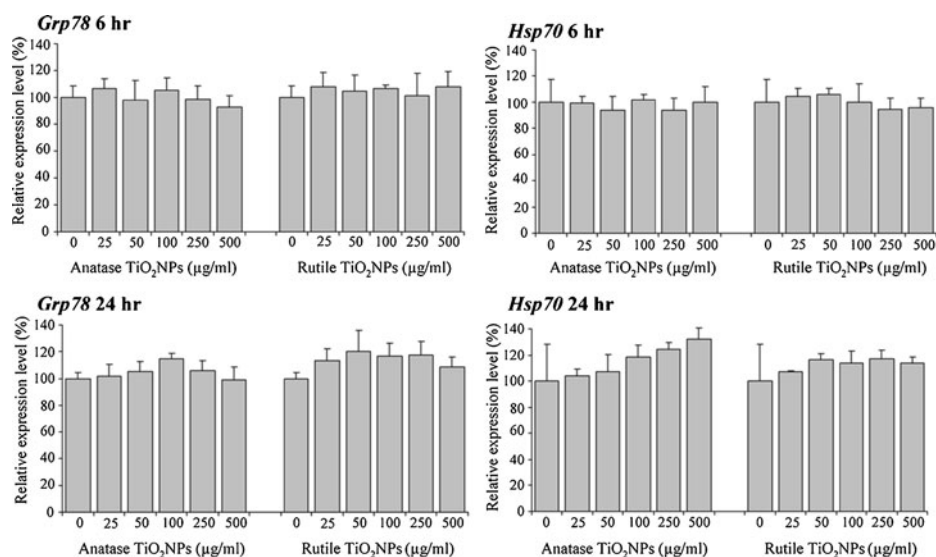
Fig. 5 TEM images of TiO₂NP inside the cells. A549 cells were treated with anatase and rutile TiO₂NPs at 100 µg/ml for 24 h. **a** Untreated control; **b–d** anatase TiO₂NPs, where **d** is an increased magnification image of the circle area in **b**; **e–g** rutile TiO₂NPs, where **g** is an increased magnification image of circle area in **e** and **f**. The arrows indicate clumps of TiO₂NPs in endosomes located in the cytoplasm. The arrowheads indicate nuclear membrane. *N* nucleus



bronchial epithelial cells and the possible biomarker(s) for evaluating the TiO₂NPs-induced cytotoxicity in respiratory system by focusing on HSPs family.

In toxicological studies, the drug or toxin-sensitive genes whose expression levels correlate with, or ideally are directly responsible for, the effect of the external stimuli (such as cytotoxicity here) can be useful as biomarkers for the effect. Among the biomarkers that have been studied, the expression levels of HSPs are widely used to monitor cellular hazards, because of their sensitivity to even minor assaults and their inducible nature against a wide range of chemicals [14]. Indeed, many toxicants alter HSP gene (transcript and protein) expression levels, in addition to various other stimuli including heat, pesticides, heavy metals, solvents, industrial and municipal effluents, some drugs, and mono- and polycyclic aromatic hydrocarbons [14, 21, 22]. Among the members of the Hsp70 family, the expression of Hsp70 and Grp78 are reported to be altered in response to various metal salts (including NPs) in several organs, including the lungs, both in the in vitro and in vivo models. For instance, the induction of Hsp70 protein expression was observed following silver NP treatment in *Drosophila melanogaster*, an often used in vivo model [23], in cadmium chloride treatment in LLC-PK1 renal epithelial cells in vitro [24], and in copper oxide NP and nickel exposure

Fig. 6 Effects of the anatase and rutile crystalline forms of TiO₂NPs on the *Hsp70* and *Grp78* transcript expression levels in A549 cells. A549 cells were treated with various concentrations of the anatase or rutile crystalline forms of TiO₂NPs for 6 or 24 h and then the mRNA expression levels of *Hsp70* and *Grp78* were determined by real time RT-PCR. Data are shown as relative to that of the transcript house-keeping gene (GADPH), and are presented as the mean \pm SD of three independent experiments



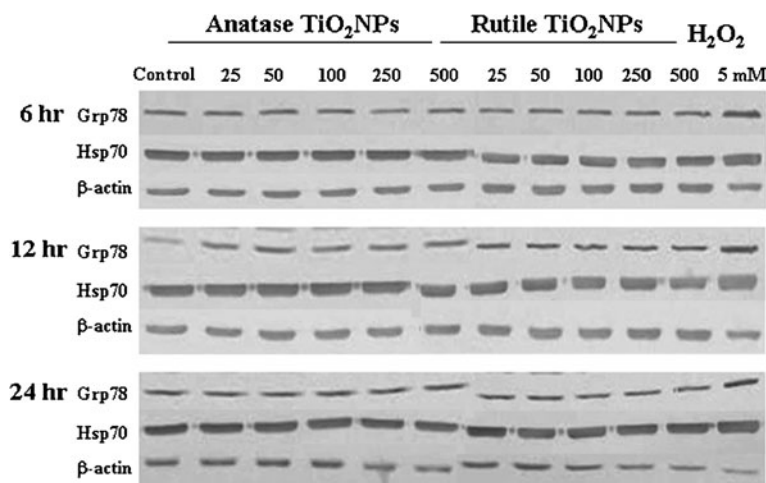
in A549 and Calu-3 human lung epithelial A549 cells [25, 26]. Likewise, an increased expression level of the Grp78 protein has been clearly observed after cadmium chloride exposure in several cell line types, including the LLC-PK1 renal epithelial cell line and rat lung epithelial cells [24, 27].

In the present study, we further investigated the possibility to apply the transcript and protein expression levels of Hsp70 and Grp78, two members of the HSPs family, as biomarkers of TiO₂NPs-induced cytotoxicity in alveolar epithelium using A549 cells as the exposure model [28]. For the TiO₂NPs used in this study, TEM-EDX confirmed that the primary size of the TiO₂NPs were in the “nano” range (Fig. 1). However, the TiO₂NPs tended to agglomerate in the hydrated forms, as determined by DLS (Table 2). TiO₂NPs dispersed in CM showed the larger agglomerated size than TiO₂NPs dispersed in DI water (Table 2). This might be because of the interaction between TiO₂NPs and serum components such as FBS and salts in the CM resulting in changing the agglomerated state [29, 30]. Nevertheless, these agglomerates can be taken up by

the cells and largely accumulated in the endosomes (Figs. 4 and 5), probably via endocytosis as reported previously shown the characteristic of lamellipodia engulfing TiO₂NP aggregates [31]. Our results showed that the anatase and, to a lesser extent, the rutile crystalline forms of TiO₂NPs caused cytotoxicity in a dose-dependent manner (Fig. 2), which correlates with the observed induction of intracellular ROS generation in the same cell line (Fig. 3). This is in agreement with a previous report that anatase TiO₂NPs (<25 nm) can cause cytotoxicity in A549 cells via oxidative stress and that pretreatment of the cells with dimethylthiourea, an OH• scavenger, improved the cell viability [32].

Alteration of the expression levels of HSPs after exposure to toxicants associated with the induction of ROS generation has been reported previously in several studies [11, 20]. This oxidative stress is also one of the toxic insults that can disrupt normal protein folding in cells and lead to activation of the unfolded protein response [33]. In this investigation, even though we found the induction of intracellular ROS by

Fig. 7 Effects of the anatase and rutile crystalline forms of TiO₂NPs on the *Hsp70* and *Grp78* protein expression levels in A549 cells. A549 cells were treated with various concentrations of the anatase or rutile crystalline forms of TiO₂NPs for 6, 12 or 24 h and then the expression levels of *Hsp70* and *Grp78* proteins were determined using Western blot analysis. Results represent blots from three independent experiments



TiO₂NPs, we could not, however, observe any significant alteration in the Hsp70 and Grp78 expression levels at either the level of mRNA or protein (Figs. 6 and 7). The sensitivity in any biological response to external stimuli via these two HSPs is potentially chemical and cell type specific [24]. Heme oxygenase (Hsp32), one of the HSP family members, is very sensitive to TiO₂NP exposure in the BEAS-2B human bronchial epithelial cell line, even at a dose that only causes 10 % or less cytotoxicity [34]. However, in our study, even at TiO₂NP doses that caused around 40 % cytotoxicity and induced more than a 2-fold elevated ROS level (40 % of the H₂O₂ control level), no significant change in the Hsp70 and Grp78 mRNA or protein expression levels were observed. This is despite the fact that it has been reported that Hsp70 and Hsp72 expression levels were increased in A549 cells after treatment with other metal NPs, such as copper oxide NPs, nickel and cadmium [25, 35, 36]. Therefore, the cellular responses of Hsp70 and Grp78 to TiO₂NPs in A549 cells might be metal specific, although we cannot exclude the possibility of a cell line-dependent effect, gene specific response, or due to experimental artifacts.

Up to date, the data on human exposure to TiO₂NPs via inhalation are limited. Based on chronic inhalation studies in rats, NIOSH recommends airborne exposure limits of 2.4 mg/m³ for fine TiO₂ and 0.3 mg/m³ for TiO₂NPs for up to 10 h/day, during 40 h/week, to reduce risk of lung cancer over working lifetime [37]. It should be noted that the concentrations (up to 500 µg/ml) used in our study were considerably high, in order to determine the mechanistic toxic effects. However, since there is less correlation between toxicity of nanoparticles in vitro and in vivo [38], these biological responses might not directly reflect in vivo results.

Taken together, our results suggest that TiO₂NPs can cause cytotoxicity and ROS generation, but independently of altered Hsp70 and Grp78 expression levels in A549 cells. The actual involvement of these two gene products, however, remains unknown and awaits further evaluation, such as by gene silencing. Regardless, Hsp70 and Grp78 expression levels would appear to not be useful biomarkers for the biological response to TiO₂NPs in the A549 cell line (the in vitro model of this study), and so potentially not in respiratory cells, although investigation in other models is still important.

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