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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 (TiO_2NPs) 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 TiO_2NP-induced cytotoxicity. Since the respiratory tract is likely to be the main exposure route of industrial workers to TiO_2NPs, we investigated the cytotoxicity of the anatase and rutile crystalline forms of TiO_2NPs 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 TiO_2NP-induced toxicity in the respiratory system. Ultrastructural observations confirmed the presence of TiO_2NPs inside cells. In vitro exposure of A549 cells to the anatase or rutile forms of TiO_2NPs 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 TiO_2NPs dose range (25–500 μg/ml). Thus, whilst TiO_2NPs 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 TiO_2NPs in the respiratory system.

Keywords TiO_2 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 (TiO_2NPs) are among the most frequently used metallic oxide NPs in various human products, such as cosmetics, sunscreen, toothpaste and paints [1]. Moreover, TiO_2NPs 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 TiO_2NP 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, TiO_2NPs 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 TiO_2 into the body of

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industrial workers. Up to date, the safety aspects of TiO_2NPs 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 TiO_2NPs. For instance, it has been reported that TiO_2NPs are rapidly internalized in the A549 human alveolar derived cell line and become distributed in the cytoplasm and intracellular vesicles [7], whilst TiO_2NPs induce oxidative stress and cause genotoxicity in human lung fibroblast cells via DNA-adduct formation [8]. Furthermore, it has been reported that TiO_2NPs 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 TiO_2NPs, 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 TiO_2NPs 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 *Hsp*70, *Hsc*70, *Grp*75 and *Grp*78, 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, Ca^{2+}-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 TiO_2NPs in the respiratory system is currently unknown.

Therefore, in this study, we aimed to examine possible biomarker(s) in response to TiO_2NP-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 TiO_2NPs 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 m^2/g.

Particles Characterization

The TiO_2NP morphology was analyzed by transmission electron microscopy (TEM) using a JEM-2010 microscope (Jeol, Japan). A drop of the TiO_2NP suspension (from a feedstock of 0.01 g TiO_2NP 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 TiO_2NPs 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 TiO_2NPs were measured by dynamic light scattering (DLS) with a Zetasizer Nanoseries S4700 (Malvern, UK). TiO_2NPs 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 TiO_2NP 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) CO_2 and subcultured by trypsinization. Before the treatment, A549 cells at a passage number less than 40 were grown overnight to reach about 80 % confluence. TiO_2NPs 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 μl of CM and grown for 24 h before treatment. The medium was then removed and replaced with 100 μl of the TiO_2NP suspension in CM at final concentrations of 5, 10, 25, 50, 100, 250 and 500 μg/ml, which is equivalent to 1.563, 3.125, 7.813, 15.625, 31.25, 78.13 and 156.3 μg/cm^2, respectively, and incubated for 24 h. The cytotoxicity of the added TiO_2NPs 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)-2*H*-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 TiO_2NPs).

Intracellular Reactive Oxygen Species (ROS) Generation

The induction of ROS generation by TiO_2NPs 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 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2-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 TiO_2NPs at final concentrations of 5, 10, 25, 50, 100, 250 and 500 μg/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 TiO_2NPs 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 TiO_2NP 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 μg/ml TiO_2NPs for 24 h. The cell pellets were collected, fixed with 2.5 % glutaraldehyde and posted-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 TiO_2NPs at a final concentration of 25, 50, 100, 250 or 500 μg/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 *Hsp*70 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 μl, contained of 1× Power SYBR green Mastermix, 0.25 μM 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 *Grp*78 and *Hsp*70 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]
*Grp*78	NM005347.4	725–745	84	F: AAACCGCTGAGGCTTATTTGG
		808–790		R: CTTGGCGTTGGGCATCATT
*Hsp*70	NM005345.4	1,799–1,821	189	F: TGCAGGAGGCGGAGAAGTACAAA
		1,987–1,965		R: CCAGCCACGAGATGACCTCTTGA
GAPDH	NM002046.3	157–178	104	F: ACCAGGGCTGCTTTTAACTCTG
		260–238		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 TiO_2NPs 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 Na_3VO_4) 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 polyvinylidine 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

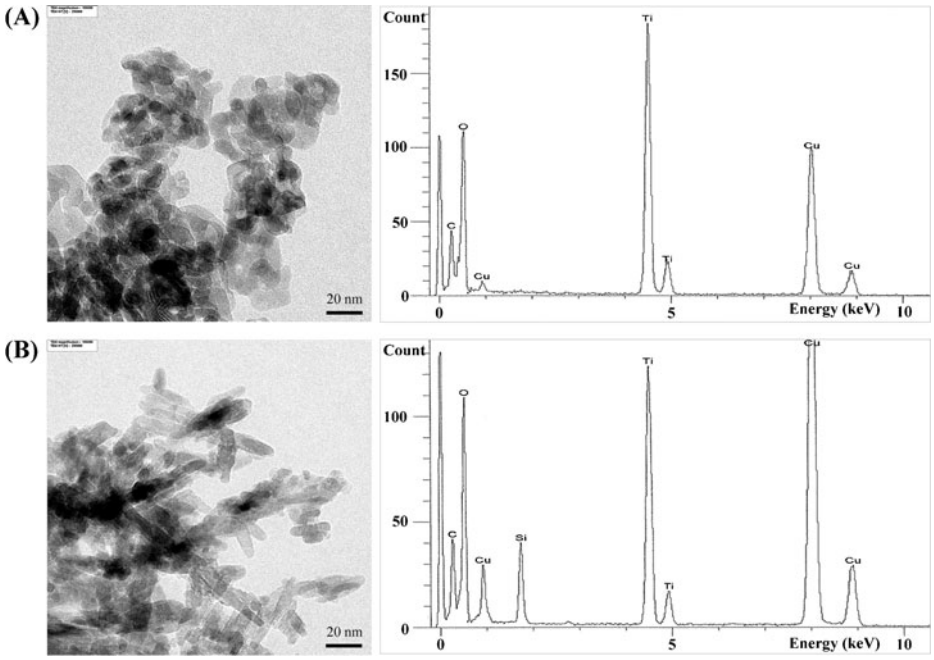

Fig. 1 Characterization of the **a** anatase and **b** rutile crystalline forms of TiO_2NPs. The particle morphology was analyzed by TEM and imaged with a magnification of 100,000 times. The presence of titanium was confirmed by an energy-dispersive X-ray analysis (*EDX*). Micrographs and spectra shown 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

TiO_2NP Characterization

According to the supplier's product information, the anatase and rutile TiO_2NPs were each 99.9 % pure with an average particle size of less than 25 nm and a specific surface area of 200–220 m^2/g. The morphology and size distribution of the anatase and rutile TiO_2NPs were confirmed by dispersing TiO_2NPs 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 TiO_2NPs, 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 TiO_2NPs 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 TiO_2NP 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 TiO_2NPs dispersed in CM, high degree of agglomeration was observed. The hydrodynamic diameter sizes of anatase TiO_2NPs 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 TiO_2NPs dispersed in CM were decreased up on increasing the sonication time from 5 to 60 min. Although diameter size of rutile TiO_2NPs 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 TiO_2NP size distribution, in nano-sized range, were demonstrated (Suppl. 1). Therefore, a 5-min sonication of anatase and rutile TiO_2NPs was used in this study.

Table 2 Hydrated TiO_2NP (agglomerate) size and polydispersity index

Sample preparation[a]	Hydrodynamic diameter (nm)[b]	Polydispersity index (nm)[b]
Anatase TiO_2NPs		
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_2NPs		
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

In Vitro Cytotoxicity of TiO_2NPs to the A549 Cell Line

To investigate the in vitro cytotoxicity of TiO_2NPs, 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 TiO_2NPs for 24 h and then analyzed by the MTS assay. The anatase form of TiO_2NPs 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 TiO_2NPs 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 TiO_2NPs was investigated using the DCF assay. As shown in Fig. 3, both the anatase and rutile forms of TiO_2NPs 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 TiO_2NPs tended to induced ROS generation in a concentration-dependent manner.

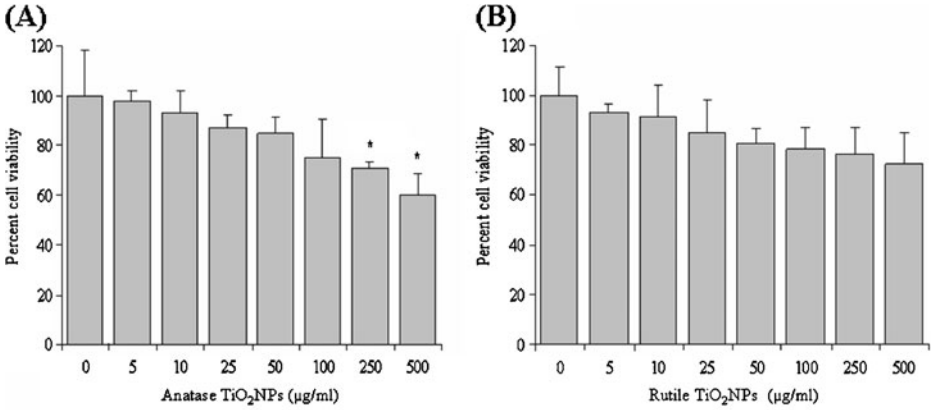

Fig. 2 In vitro cytotoxicity of the **a** anatase and **b** rutile crystalline forms of TiO_2NPs against A549 cells. A549 cells were incubated with various concentrations of the anatase or rutile crystalline forms of TiO_2NPs for 24 h and then the cell viability was determined by the MTS assay. Data are presented as mean ± SD of three independent experiments. *$p<0.05$, using a one-way ANOVA for the comparison between untreated and TiO_2NP-exposed cells

Uptake of TiO_2NPs into the Cells

The uptake of TiO_2NPs 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 TiO_2NP exposure. As shown in Fig. 4, mean of SSC-A was significantly increased in cells treated with both anatase and rutile TiO_2NPs at even the low concentrations (25 and 50 μg/ml) used in this study. We further confirmed the internalization of TiO_2NPs to the cells by TEM analysis. Ultrastructure image demonstrated that TiO_2NPs could be internalized to the cells and appeared in the cytoplasm, but not in the nucleus (Fig. 5).

Effect of TiO_2NPs 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 TiO_2NPs, the mRNA transcript expression levels of *Grp*78 and *Hsp*70 were determined by real-time reverse transcriptase PCR. Exposure of A549 cells to TiO_2NPs in the dose range of 25–500 μg/ml for 6 and 24 h did not significantly change the expression levels of either *Grp*78 or *Hsp*70 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 TiO_2NPs, 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 TiO_2NPs 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 TiO_2NPs 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

TiO_2NPs are increasingly being used in various commercial products, but there is still an inadequate knowledge concerning their risk to human health and the environment,

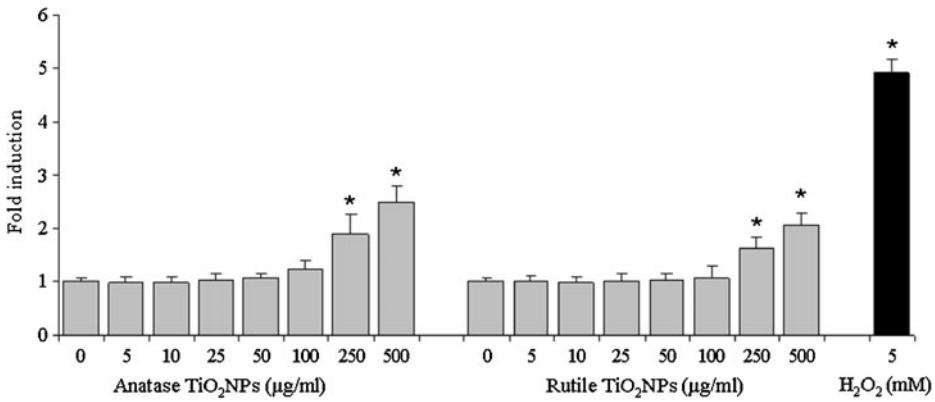

Fig. 3 Intracellular ROS generation in A549 cells after treatment with the **a** anatase and **b** rutile crystalline forms of TiO_2NPs. The cells were incubated with various concentrations of the anatase or rutile crystalline form of TiO_2NPs for 24 h and then the intracellular ROS levels were determined by the DCF assay. H_2O_2 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-TiO_2NPs control. *$p<0.05$, using one-way ANOVA for the comparison between untreated and TiO_2NP-exposed cells

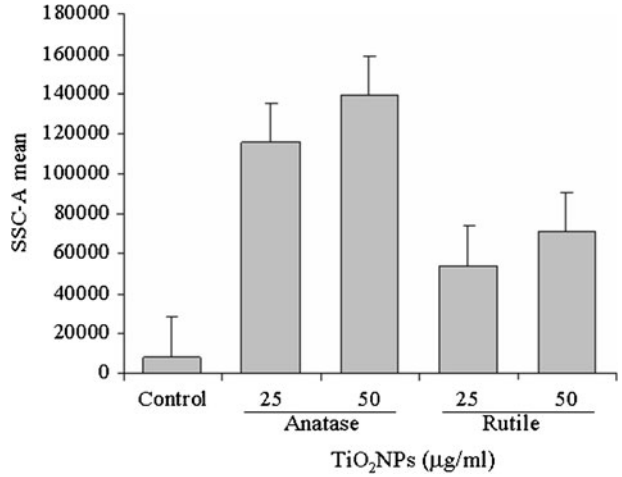

Fig. 4 Uptake of TiO_2NPs. A549 cells were treated with anatase and rutile TiO_2NPs at 25 and 50 μg/ml for 24 h and were subjected to FACS analysis. TiO_2NP uptake or cell surface adsorption is proportion to an increase in SSC-A of cell population

as well as on any suitable biomarker(s) for assessing the safety of TiO_2NPs 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 TiO_2NPs in A549 cells as an in vitro model of human bronchial epithelial cells and the possible biomarker(s) for evaluating the TiO_2NPs-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

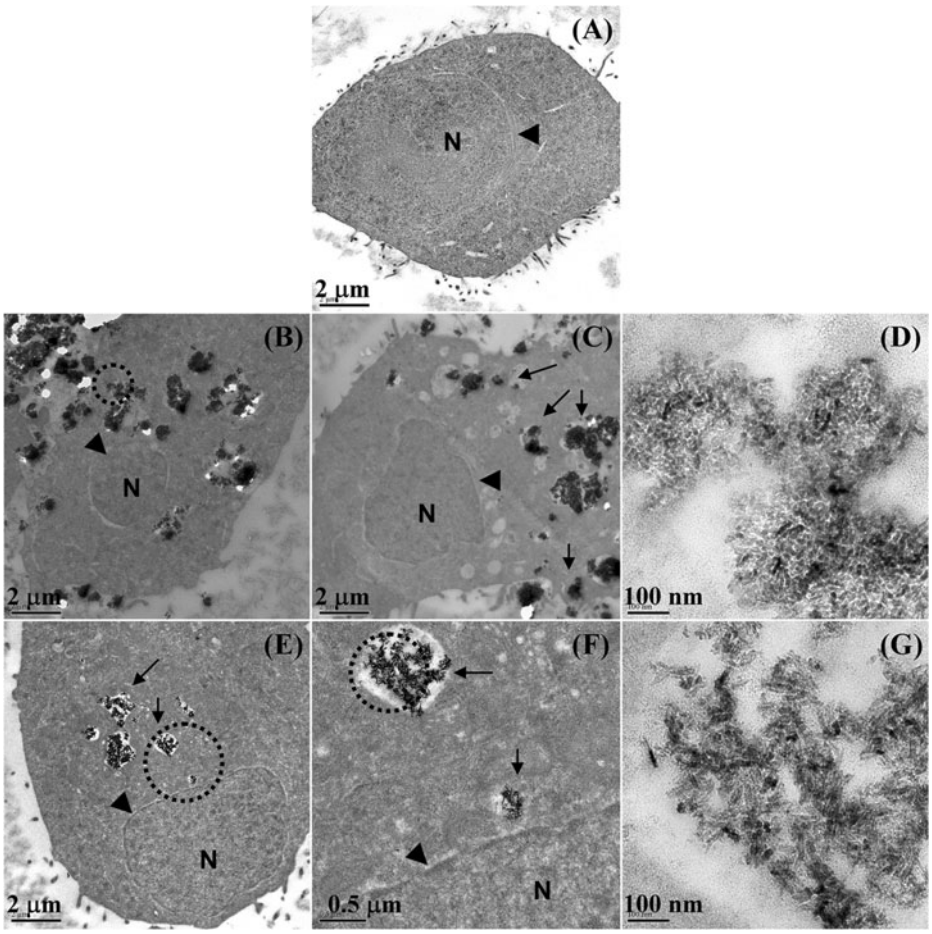

Fig. 5 TEM images of TiO_2NP inside the cells. A549 cells were treated with anatase and rutile TiO_2NPs at 100 μg/ml for 24 h. **a** Untreated control; **b**–**d** anatase TiO_2NPs, where **d** is an increased magnification image of the circle area in **b**; **e**–**g** rutile TiO_2NPs, where **g** is an increased magnification image of circle area in **e** and **f**. The *arrows* indicate clumps of TiO_2NPs in endosomes located in the cytoplasm. The *arrowheads* indicate nuclear membrane. *N* nucleus

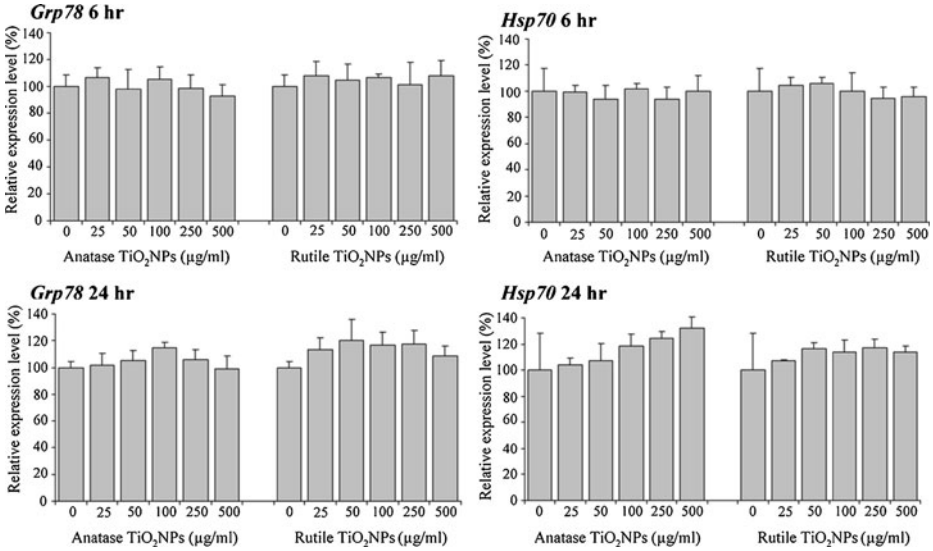

Fig. 6 Effects of the anatase and rutile crystalline forms of TiO_2NPs on the *Hsp*70 and *Grp*78 transcript expression levels in A549 cells. A549 cells were treated with various concentrations of the anatase or rutile crystalline forms of TiO_2NPs for 6 or 24 h and then the mRNA expression levels of *Hsp*70 and *Grp*78 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 ± 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 TiO_2NPs-induced cytotoxicity in alveolar epithelium using A549 cells as the exposure model [28]. For the TiO_2NPs used in this study, TEM-EDX confirmed that the primary size of the TiO_2NPs were in the "nano" range (Fig. 1). However, the TiO_2NPs tended to agglomerate in the hydrated forms, as determined by DLS (Table 2). TiO_2NPs dispersed in CM showed the larger agglomerated size than TiO_2NPs dispersed in DI water (Table 2). This might because of the interaction between TiO_2NPs 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 TiO_2NP aggregates [31]. Our results showed that the anatase and, to a lesser extent, the rutile crystalline forms of TiO_2NPs 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 TiO_2NPs (<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

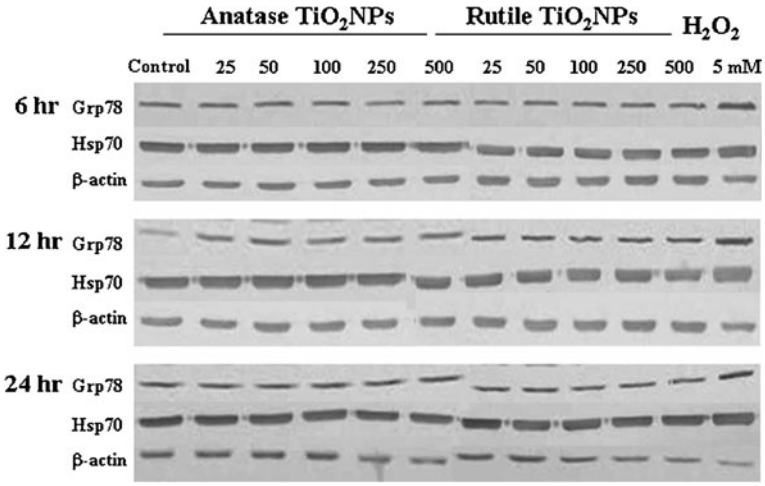

Fig. 7 Effects of the anatase and rutile crystalline forms of TiO_2NPs on the Hsp70 and Grp78 protein expression levels in A549 cells. A549 cells were treated with various concentrations of the anatase or rutile crystalline forms of TiO_2NPs for 6, 12 or 24 h and then the expression levels of Hsp70 and Grp78 proteins were determined using Western blot analysis. Results represent blots from three independent experiments

TiO_2NPs, 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 TiO_2NP 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 TiO_2NP doses that caused around 40 % cytotoxicity and induced more than a 2-fold elevated ROS level (40 % of the H_2O_2 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 TiO_2NPs in A459 cells might be metal specific, although we cannot exclude the possibility of a cell line-dependent affect, gene specific response, or due to experimental artifacts.

Up to date, the data on human exposure to TiO_2NPs via inhalation are limited. Based on chronic inhalation studies in rats, NIOSH recommends airborne exposure limits of 2.4 mg/m^3 for fine TiO_2 and 0.3 mg/m^3 for TiO_2NPs 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 TiO_2NPs 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 TiO_2NPs 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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