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Comparison of the Biological Activity between Ultrafine and Fine Titanium Dioxide Particles in RAW 264.7 Cells Associated with Oxidative Stress

Jihee Lee Kang[1], Changsuk Moon[1], Hui Su Lee[1], Hae Won Lee[1], Eun-Mi Park[2], Hee Sun Kim[3], and Vincent Castranova[4]

[1]*Departments of Physiology,* [2]*Pharmacology,* [3]*Neuroscience, Division of Cell Biology, Ewha Medical Research Center, School of Medicine, Ewha Womans University, Seoul, South Korea, and* [4]*Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA*

Ultrafine or fine titanium dioxide (TiO_2) particles are widely used in the production of white pigments, for sunscreens, and in cleanup techniques. However, currently knowledge is deficient concerning cellular responses to these particles. The study evaluated and compared the biological activity of ultrafine and fine TiO_2 particles in RAW 264.7 macrophages according to an oxidative stress paradigm. In vitro exposure of macrophages to ultrafine or fine TiO_2 in the range of 0.5–200 μg/ml did not significantly alter cell viability. However, ultrafine TiO_2 enhanced intracellular generation of reactive oxygen species (ROS) to a greater extent than fine TiO_2 at each exposure concentration. Ultrafine TiO_2 induced ERK1/2 activation in a concentration-dependent manner, while the fine TiO_2-induced changes were minimal. Phosphorylation of ERK1/2 occurred following 10 min exposure to higher concentrations of ultrafine TiO_2 (≥25 μg/ml). Similarly, ultrafine TiO_2 exposure significantly enhanced tumor necrosis factor (TNF)-α and macrophage inflammatory protein (MIP)-2 secretion in a concentration-dependent manner, and its potency was higher than fine TiO_2. These findings suggest that when exposure concentration is based upon equivalent mass, ultrafine TiO_2 exerts greater biological activity as measured by ROS generation, ERK 1/2 activation, and proinflammatory mediator secretion in RAW 264.7 macrophages than fine TiO_2.

As nanomaterials and nanodevices are developed rapidly, their novel physiochemical properties raise concerns about adverse effects on biological systems. Indeed, some studies suggest that nanomaterials affect biological behaviors at the cellular, subcellular, and protein levels (Oberdörster et al, 2005; Nel et al., 2006; Shvedova et al., 2003). Moreover, some nanoparticles may travel throughout the body, deposit in target organs, penetrate cell membranes, lodge in mitochondria, and trigger injurious responses.

Titanium dioxide (TiO_2) particles, in either fine or ultrafine sizes, are widely used in the cosmetic, photocatalyst, sensor, pharmaceutical, and paint industries. Fine (>100 nm in diameter)-sized TiO_2 was reported to be relatively biologically inert (Bernard et al., 1990; Hart & Hesterberg, 1998), such that it is often utilized as negative control particle in pulmonary toxicity studies (Tran et al., 2000; Hext et al., 2005). However, interleukin production from alveolar macrophages and goblet cell hyperplasia were induced in rats 24 h after exposure to a high concentration (4 mg/rat) of fine TiO_2 by intratracheal instillation (Ahn et al., 2005). Kim et al. (1999) showed TiO_2 induced cytotoxicity.

On the other hand, ultrafine (nano) particles (<100 nm in diameter) were found to possess a greater capacity to induce inflammation and cytotoxicity in the lungs than fine particles (Dick et al., 2003; Zhang et al., 1998). It was postulated that the high surface area or particle number of ultrafine particles per mass or the increased oxidative activity of the surface are important factors. In this regard, subchronic inhalation studies with ultrafine TiO_2 or fine TiO_2 particles demonstrated that approximately 20% of inhaled mass dose of the ultrafine TiO_2, compared with the fine TiO_2 produced equivalent pulmonary inflammation, fibrosis and lung tumors in rats (Bermudez et al., 2002). A single intratracheal instillation of ultrafine TiO_2 (500 μg/rat) induced more neutrophil recruitment, epithelial damage, and cytotoxicity than fine TiO_2 24 h after exposure (Renwick et al., 2005). The level of glutathione significantly

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Address correspondence to Dr. Jihee Lee Kang, Department of Physiology, School of Medicine, Ewha Womans University, 911-1 Mok-6-dong, Yangcheon-ku, Seoul 158-056, Korea. E-mail: jihee@ewha.ac.kr

decreased in in vivo ultrafine TiO_2-exposed rat alveolar macrophages (Afaq et al., 1998). The potent biological activity of ultrafine TiO_2 was associated with high surface reactivity leading to reactive oxygen species (ROS)-mediated cytotoxicity and genotoxicity (Wang et al., 2007; Gurr et al., 2005). However, there are limited reports on the use of in vitro models to evaluate and compare the effects of ultrafine and fine TiO_2 particles on the intracellular inflammatory signaling cascade induced via the oxidative stress paradigm. Moreover, recent studies suggest that delivering agglomerated nanoparticles in an in vitro or in vivo experimental situation might actually lead to the conclusion that the nanoparticle was less toxic than it actually is (Sager et al., 2007; Warheit et al., 2004; Shvedova et al., 2005). Thus, the particle agglomeration and poor dispersion may lead to misinterpretation of the biological activity of the particulate being assessed. Sager et al. (2007) demonstrated that suspension of nanoparticles in acellular bronchoalveolar lavage fluid (BALF) was effective in dispersing nanoparticles, such as carbon black and TiO_2, without masking the biological activity of the surface. A mixture of protein and dipalmitoyl phosphatidylcholine (DPPC) at levels found in BALF was reported to be an adequate substitution for BALF.

In the oxidative stress model, an intermediate amount of oxidative stress induces activation of mitogen-activated protein (MAP) kinases and nuclear factor (NF)-κB cascades, leading to proinflammatory responses (Nel et al., 2006). This oxidative stress model was adopted to the present comparative study of the biological activity of fine versus ultrafine TiO_2. Here, RAW 264.7 cells were treated with ultrafine or fine TiO_2 particles at various mass concentrations (0.5–200 μg/ml), using an improved method to disperse these particles. Parameters examined included cellular viability, intracellular ROS generation, MAP kinase activation, and proinflammatory mediator secretion.

METHODS

Particle Sieving

Ultrafine titanium dioxide (Aeroxide TiO_2 P-25, primary particle diameter of 21 nm) was obtained as a gift from the Degussa Corporation (Parsippany, NJ). Fine TiO_2 (titanium(IV) oxide, primary particle size of 1 μm) was purchased from Sigma-Aldrich (Atlanta, GA).

Each particulate sample was individually sieved in a Retsch AS 200 Sieve (Retsch GmbH, Haan, Germany) at a vibration amplitude of 50 for 15 min to break apart large clumps. Three different sieve sizes (1.18 mm, 250 μm, 45 μm openings) were used successively in the sieving process.

Suspension of Ultrafine and Fine TiO_2

Prior to suspension, the particle samples were sterilized by heating at 160°C for 90 min in a dry oven. Sager et al. (2007) reported Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) containing protein plus DPPC was a satisfactory medium to prepare nanoparticle suspensions. Thus, each particle was suspended in PBS containing 10 μg/ml DPPC and 0.6 mg/ml bovine serum albumin (BSA). DPPC was prepared as a 10-mg/ml stock solution in absolute ethanol. Thus, the final concentration of ethanol in the dispersion medium was 0.1% (v/v).

Suspensions were sonicated using a Cole-Palmer probe sonicator with a duty cycle setting of 10% and an output setting of 1. Samples were sonicated either with 5 individual pulses or continuously for 10 min. All TiO_2 particle suspensions were freshly prepared from sieved particles. This method greatly improved the dispersion of ultrafine or fine TiO_2 in suspension as demonstrated previously by light and electron microscopy (Sager et al., 2007). Microscopic evidence for particle dispersal of fine and ultrafine TiO2 in the present study is given in Figure 1 (A and B).

In Vitro Exposure of RAW 264.7 Cells

RAW 264.7 cells, a mouse peritoneal macrophage cell line, were obtained from American Type Culture Collection (Rockville, MD). The cells were maintained in DMEM (Dulbecco's modified Eagle's medium; Life Technologies) supplemented with 5% fetal bovine serum (FBS) (HyClone, Logan, UT), 2 m*M* glutamine, and 100 U/ml penicillin–streptomycin. Cells from passages 7 and 8 only were used in particle exposure experiments.

Measurement of Cell Viability

Lactate dehydrogenase (LDH) is an abundant intracellular enzyme, and its release into cell culture supernatants is a marker of lytic cell death. The activity of LDH was measured using an LDH determination kit (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, 100 μl of ultrafine or fine TiO_2 (0.2–100 μg/ml) was added to 100 μl of RAW 264.7 cells (10^6/ml) in given wells of a microplate. The cells were then incubated at 37°C in a

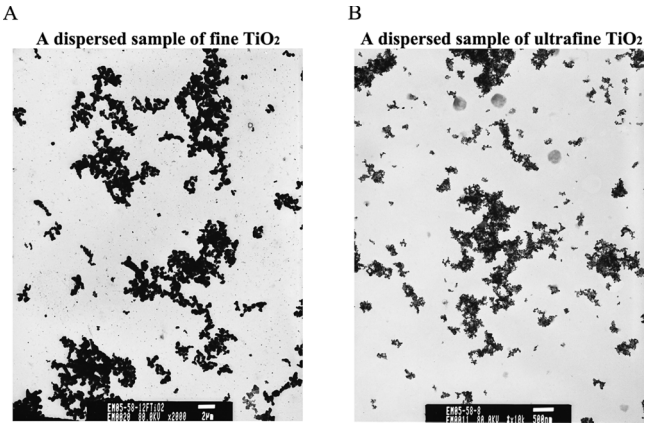

FIG. 1. Transmission electron micrographs of a dispersed sample of fine TiO_2 (A) and ultrafine TiO_2 (B). Scale bars are given in each micrograph.

humidified atmosphere of 5% CO_2 for 24 h. After incubation, 100 µl of supernatant was added to 100 µl of reaction mixture and incubated for 30 min at room temperature. Absorbance of the samples at 490 nm was measured using a microplate reader. Results were expressed as percent cell viability, referenced to the maximum LDH activity released when cells were lysed with detergent, using the formula: Percent viability = 100 × (1 – [experimental – untreated) / (detergent –untreated)]).

Measurement of Intracellular ROS Generation

Intracellular ROS generation was measured with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) using a modified method of Qin et al. (2005). The nonfluorescent compound H_2DCF-DA accumulates within cells upon deacetylation and then reacts with ROS to form fluorescent DCF. Briefly, 2×10^6cells/ml were stimulated with ultrafine or fine TiO_2 particles (0.5–100 µg/ml) or lipopolysaccharide (LPS) (1 µg/ml) for 4 or 24 h at 37°C and washed twice with warmed PBS. Then cells were stained with 10 µ*M* H_2DCF-DA in PBS for 30 min at 37°C. DCF fluorescence intensity was measured at 488 nm excitation and at 525 nm emission using a fluorescence microplate reader (Molecular Devices, CA). Increases in intracellular ROS after addition of TiO_2 or LPS were divided by the control value and expressed as fold induction.

For image analysis of intracellular ROS generation, dihydroethidium (DHE) assays were performed according to a modified protocol from Sanlioglu et al. (2001). Briefly, RAW 264.7 cells (1×10^5 cells) were plated in a 24-well tissue culture plate on glass coverslips and incubated in DMEM without serum containing 10 µ*M* DHE for 30 min. Ultrafine or fine TiO_2 particles (5 µg/ml) or LPS (1 µg/ml) was added for 30 min of incubation with DHE. The cells were fixed with 2% paraformaldehyde for 15 min. Nuclei were stained with Hoechst number 33258 (5 µg/ml, Calbiochem) for 10 min at room temperature, and slides were then mounted. Images were collected using an LSM image examiner on a confocal laser scanning microscope (LSM-5 Pascal Exciter, Carl Zeiss, Germany). In the presence of intracellular ROS, DHE is converted to ethidium and detected as bright red nuclear staining. The red DHE staining was quantified by creating masks and measuring fluorescent mean intensity of staining using LSM image examiner software (Carl Zeiss).

Measurement of TNF-α and MIP-2 Secretion

RAW 264.7 cells (10^5 cells) were incubated in 1 ml of RPMI media without serum containing ultrafine or fine TiO_2 (0.5 – 200 µg/ml) or LPS (1, 10 µg/ml) at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. After 24 h, the supernatant was collected and quantified for TNF-α and MIP-2 using a mouse enzyme-linked immunosorbent assay (ELISA) system (R&D Systems, Minneapolis, MN).

Western Blotting

Immunoblotting for phosphorylation of mitogen-activated protein (MAP) kinases (ERK1/2, JNK, and p38 kinase) was carried out as described in the protocol supplied by the manufacturer, using phospho-specific antibodies against phosphorylated sites of MAP kinases. Non-phospho-specific antibodies against MAP kinases provided in assay kit were used to normalize the phosphorylation assay using the same transferred membrane blot.

Densitometry Analysis

Westerns blots were scanned and analyzed using Scion Image. The scans were inverted and the background was subtracted before measuring the relative protein amounts. Densitometry numbers were calculated by measuring the ratio of phosphorylated kinase to nonphosphorylated kinase. All conditions were expressed as a density relative to the untreated control.

Statistics

Values are expressed as means ± standard errors. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Statistical significance was set at $p < .05$.

RESULTS

Cell Cytotoxicity Following Ultrafine and Fine TiO_2 Exposures

Cell cytotoxicity was measured to evaluate particle-induced cell death under the conditions used for the functional assays employed in this study. Viability of RAW 264.7 cells was not compromised after 24 h of exposure to ultrafine or fine TiO_2 in a concentration range of 0.5–200 µg/ml (Figure 2).

Intracellular ROS Generation

To clarify and compare the effects of ultrafine and fine TiO_2 on intracellular ROS generation, RAW 264.7 cells were

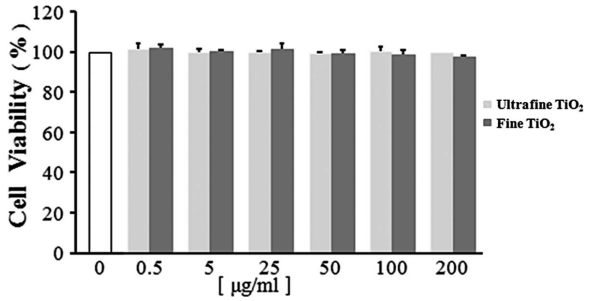

FIG. 2. Cell viability of RAW 264.7 cells after exposure to ultrafine and fine TiO_2 particles. RAW 264.7 cells were exposed to ultrafine or fine TiO_2 (0.5–200 µg/ml) for 24 h. The LDH activity in the supernate was detected, and results were expressed as percent cell viability, referenced to maximal LDH release. Data are the mean ± SE (n = 3 experiments).

exposed to the particles at various concentrations in the presence of the DCF-DA probe. The increase in DCF fluorescence of RAW 264.7 cells peaked at 4 h following exposure to 10 μg/ml ambient ultrafine particles (<100 nm) (Xia et al., 2006). Thus, ROS generation was first measured following 4 h of exposure to TiO_2 particles in the range of 0.5–100 μg/ml. Exposure to ultrafine TiO_2 at the lower concentrations (0.5, 1, and 5 μg/ml) increased intracellular ROS generation by approximate 2.4-fold compared with the untreated control, and its effect was comparable to that of LPS (1 μg/ml) stimulation (Figure 3A). At higher concentrations (>25 μg/ml), ultrafine TiO_2 -stimulated ROS generation declined slightly, but remained significantly greater than the untreated control. Compared to fine TiO_2 exposure at each exposure concentration, the levels of ROS induced by ultrafine TiO_2 were greater. Exposure to fine TiO_2 for 4 h resulted in small but significant increases in ROS generation. After 24 h of exposure to ultrafine or fine TiO_2, intracellular ROS levels in RAW 264.7 cells declined toward levels of untreated control cells (Figure 3B).

Intracellular ROS generation in RAW 264.7 cells exposed to ultrafine or fine TiO_2 was also qualitatively measured in the presence of the DHE probe, using confocal microscopy. DHE rapidly reacts with intracellular oxidants, resulting in the generation of ethidium, which intercalates with DNA and fluoresces in the Cy3 (red) channel. Treatment with ultrafine TiO_2 (5 μg/ml) or LPS (1 μg/ml) for 30 min markedly increased the level of ROS fluorescence in RAW 264.7 cells, while fine TiO_2 (5 μg/ml) induced a small increase (Figure 3C). Using LSM image examiner software, the amount of oxidant staining was quantified within the cells. Only ultrafine TiO_2 significantly increased fluorescence (300% increase vs. untreated cells) (Figure 3D). However, no significant change in the oxidant levels was observed when the cells were exposed to fine TiO_2 or LPS.

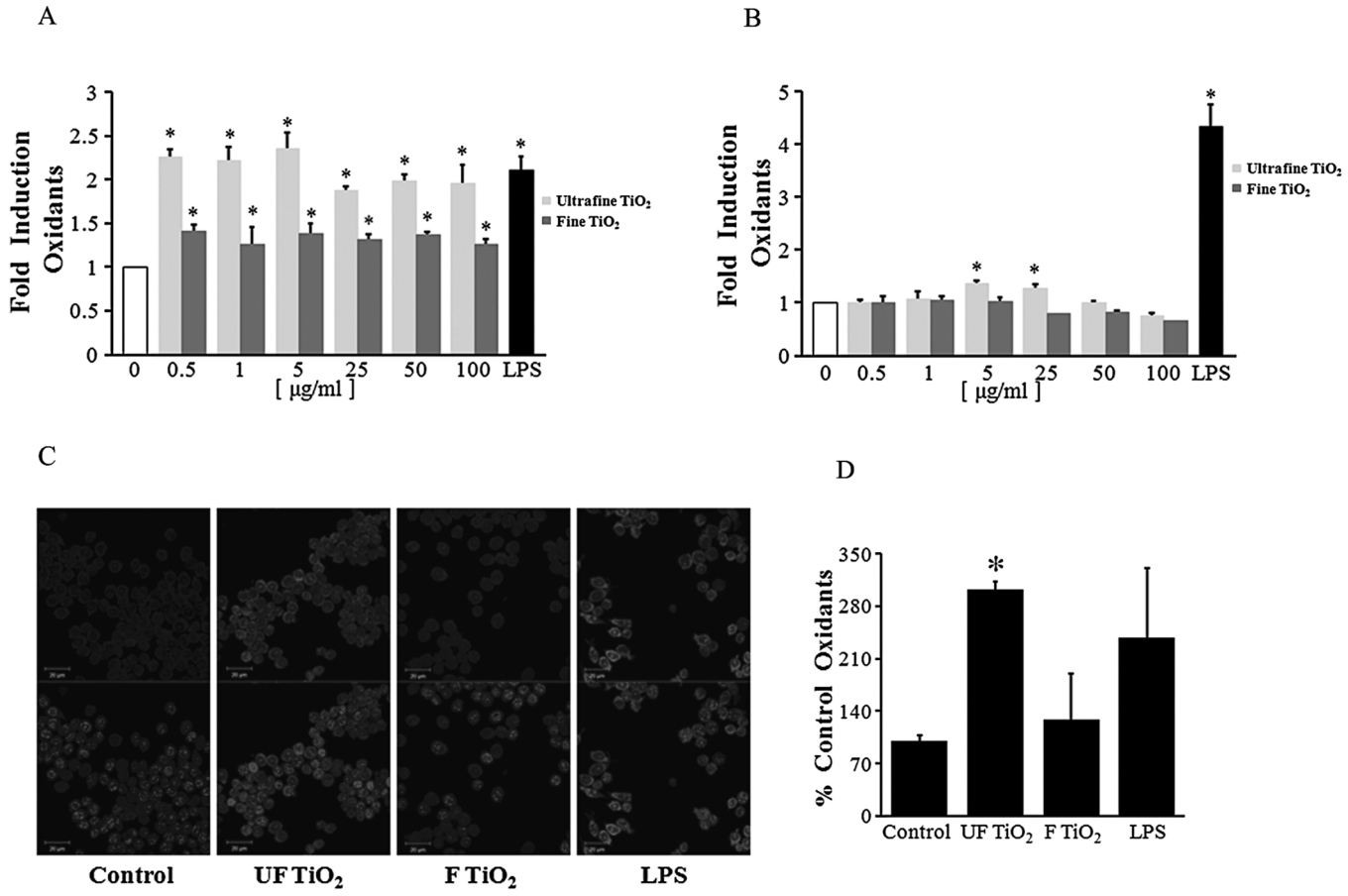

FIG. 3. Effects of ultrafine and fine TiO_2 on intracellular ROS generation. (A, B) RAW 264.7 macrophages exposed to ultrafine or fine TiO_2 (0.5–100 μg/ml) or LPS (1 μg/ml) for 4 and 24 h, respectively, and then incubated with 10 μ*M* H_2DCFH-DA for 30 min. DCF fluorescence intensity was measured using a fluorescence microplate reader. Increases in intracellular ROS were expressed as fold induction over the control value. (C) RAW 264.7 macrophages were incubated with DHE in the presence of ultrafine or fine TiO_2 (5 μg/ml) or LPS (1 μg/ml) for 30 min and analyzed with confocal fluorescence microscopy. In the presence of intracellular oxidants, DHE is converted to ethidium and detected as bright red nuclear staining in the Cy3 (red) fluorescence channel. (D) Quantification of DHE staining expressed as percent of control. Data represent the mean ± SE ($n \geq 3$ experiments). Asterisk indicates significant increase from the untreated control ($p < .05$).

MAP Kinase Activation

The MAP kinase cascades are redox-sensitive signaling cascades that are able to mediate cytokine and chemokine production. To determine the effects of the TiO_2 particles on MAP kinase activation, RAW 264.7 cells were exposed to ultrafine or fine TiO_2 particles at various concentrations for 10–60 min, and Western blot analysis with a phospho-specific MAP kinase antibody in cell lysates was conducted. Phosphorylation of ERK1/2 in RAW 264.7 cells was observed after 20 min of 0.5 μg/ml ultrafine TiO_2 stimulation and slightly declined after a 30-min exposure (Figure 4A). When the cells were stimulated with higher concentrations of ultrafine TiO_2 (25, 50, or 100 μg/ml), phosphorylation of ERK1/2 was observed after 10 min of stimulation and sustained through 30 min of exposure. The concentration response study with ultrafine TiO_2 confirmed that phosphorylated ERK1/2 levels following 30 min of exposure increased in a concentration-dependent manner (Figure 4B). The peak response was seen at 100 μg/ml, with an induction level comparable to the LPS (1 μg/ml) stimulation. However, the phosphorylated ERK1/2 levels in RAW 264.7 cells exposed to fine TiO_2 were lower at each exposure condition of both concentration and time compared with that of ultrafine TiO_2. Phosphorylation of p38 MAP kinase or JNK was not significantly changed in cells over this range of concentrations of ultrafine or fine TiO_2 particles (data not shown).

Proinflammatory Mediator Production

ROS mediate activation of MAP kinases and transcription factors, consequently resulting in proinflammatory mediator production. Therefore, the present study evaluated the effects of ultrafine or fine TiO_2 particles on TNF-α and MIP-2 secretion from RAW 264.7 macrophages. Ultrafine TiO_2 (0.5–200 μg/ml) significantly increased TNF-α and MIP-2 secretion in a concentration-dependent manner (vs. the untreated control) (Figure 5, A and C). The peak response was found at 200 μg/ml ultrafine TiO_2 with 6.6- and 5.8-fold

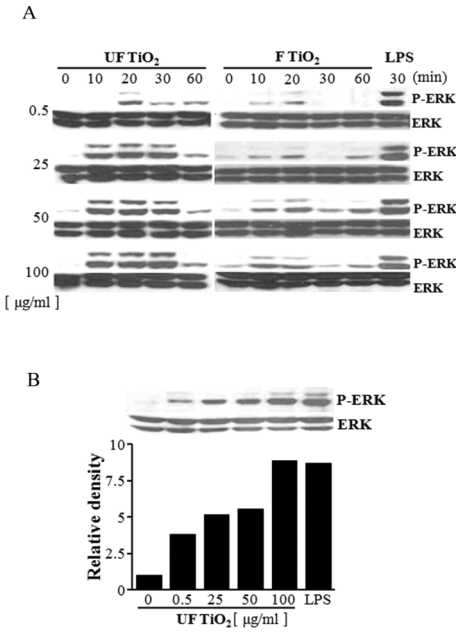

FIG. 4. Effects of ultrafine or fine TiO_2 on ERK 1/2 phosphorylation in RAW 264.7 macrophages. (A) Time courses of the phosphorylation of ERK1/2 in response to ultrafine or fine TiO_2. (B) Concentration response of the phosphorylation of ERK1/2 after 30-min exposure to ultrafine TiO_2. Lysates from RAW 264.7 cells stimulated with ultrafine or fine TiO_2 (0.5–100 μg/ml) for the indicated times or LPS (1 μg/ml) for 30 min were analyzed by anti-phospho-ERK1/2/ERK1/2 Western blotting. Relative values of the levels of phosphorylated ERK1/2 normalized versus non-phosphorylated ERK1/2 are presented below the gel. Data are representative of three separate experiments.

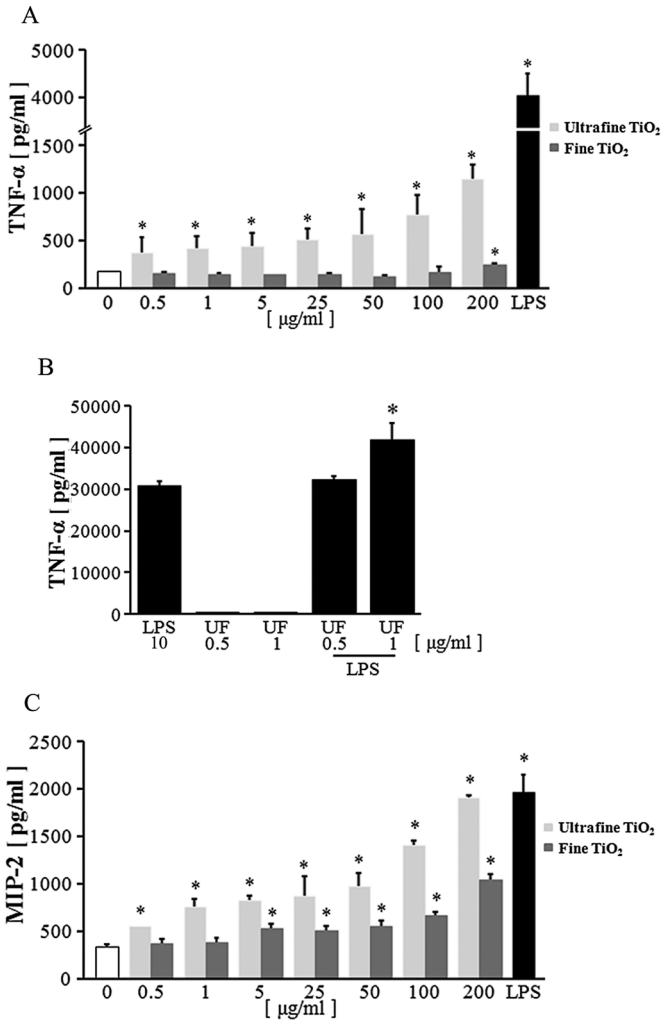

FIG. 5. Effects of ultrafine or fine TiO_2 on TNF-α (A, B) and MIP-2 (C) secretion from RAW 264.7 macrophages. RAW 264.7 macrophages (10^5/ml) were incubated with ultrafine or fine TiO_2 (0.5–200 μg/ml) or LPS (1, 10 μg/ml). TNF-α and MIP-2 levels were quantified by ELISA assay. Data represent the mean ± SE (n = 3 experiments). *(*A*, *C*), Asterisk in (A) and (C) indicates significant increase from the untreated control ($p < .05$), and in (B), significant increase above the sum of the separate effects of each stimulant ($p < .05$).

increases of TNF-α and MIP-2, respectively. These effects of ultrafine TiO_2 were greater than those of the fine particles at each exposure concentration. Fine TiO_2 (200 μg/ml) only induced a 1.4-fold significant increase in TNF-α and a 3.1-fold rise in MIP-2. Furthermore, addition of 1 μg/ml of ultrafine TiO_2 to the cell culture in the presence of LPS (10 μg/ml) further enhanced the LPS-induced response in RAW 264.7 cells by 36% (Figure 5B). This potentiation was not seen with 0.5, 5, 25, or 100 μg/ml ultrafine TiO_2. In addition, there was no further enhancement of MIP-2 secretion when ultrafine TiO_2 was added to the LPS-treated cells (data not shown).

DISCUSSION

The objective of the present investigation was to evaluate and compare the biological activity of ultrafine and fine TiO_2 particles in RAW 264.7 macrophages according to an oxidative stress paradigm. Recent investigations of nanoparticles indicated that nanoparticles agglomerate and clump in solution, making it difficult to accurately deliver them for in vivo or in vitro experiments (Donaldson et al., 2001; Warheit et al., 2004; Sager et al., 2007). Images taken using light microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM) showed that PBS is not a satisfactory medium to suspend nanoparticles, including TiO_2 particles, whereas PBS-containing protein plus DPPC was satisfactory (Sager et al., 2007). The level of phospholipid in the suspension solution (10 μg/ml) is much lower than in alveolar lining fluid and does not coat the particles in such a way as to mask their biological activity (Sager et al., 2007). Therefore, this new method was used to prepare dispersed suspensions of ultrafine or fine TiO_2 particles.

Data from our study indicate that in vitro exposure of RAW 264.7 cells to ultrafine or fine TiO_2 particles in the range of 0.5–200 μg/ml for 24 h did not significantly compromise cell viability. Thus, the biological effects of the TiO_2 particles may be evaluated under nontoxic conditions. Exposure of RAW 264.7 cells to ultrafine TiO_2 (0.5–100 μg/ml) for 4 h resulted in an increase in intracellular ROS generation that was greater than that observed for fine TiO_2 treatment. The peak increase in DCF fluorescence response occurred at 4 h after ultrafine TiO_2 exposure (data not shown). Concentration-response data indicated peak ROS levels following exposure to 5 μg/ml ultrafine TiO_2. At higher concentration of ultrafine TiO_2 (>25 μg/ml), the levels of intracellular ROS declined slightly. This decline in ROS levels is not due to membrane damage, because LDH activity leakage is not apparent in ultrafine TiO_2-exposed RAW 264.7 cells at these exposure levels. This decline might be due to the enhancement of antioxidant enzymes in cells exposed to higher concentrations of ultrafine TiO_2 for the protection of cells from damaging oxidants. Indeed, alveolar macrophages from rats exposed to ultrafine TiO_2 (2 mg per rat) showed an adaptive response because the activities of glutathione peroxidase, glutathuone reductase, glucose-6-phosphate dehydrogenase, and glutathione *S*-transferase were increased in these cells (Afaq et al., 1998). Data also suggested that the induction of antioxidant enzymes by these cells for self-protection is not sufficient to cope against the toxic action of ultrafine TiO_2, which may lead to oxidative stress. Assessing the antioxidant activities in cells in vitro exposed to ultrafine TiO_2 is needed in future study.

After 24 h of exposure to ultrafine TiO_2, intracellular ROS levels declined toward the control level. In addition, significant increases in ROS generation in ultrafine TiO_2-exposed cells, but not fine TiO_2-exposed cells, were also determined using confocal microscopic analysis (Figure 3, C and D) and in the luminol-dependent chemiluminescence assay (data not shown). Ultrafine TiO_2 was also associated with ROS production in vivo. There was a gradual increase in hydrogen peroxide generation by alveolar macrophages from rats exposed to ultrafine TiO_2 (2 mg per rat) following a single intratracheal instillation, reaching a maximum at d 16 (Afaq et al., 1998). Furthermore, exposure to ultrafine TiO_2 enhanced lipid peroxidation and produced an imbalance between oxidant generation and the antioxidant system in alveolar macrophages. In contrast to our data, Xia et al. (2006) reported that 10 μg/ml of ultrafine TiO_2 (the same P25 particles from Degussa used in the present study) did not increase DCF fluorescence in RAW 264.7 cells after a 4-h exposure. This disparity may be due to inadequate dispersion of the nanoparticles in that study. Indeed, they noted large agglomerations of ultrafine TiO_2 in their study. Sayes et al. (2006) proposed that phase composition of TiO_2 nanoparticles is more important for cytotoxicity and inflammatory response than nanoparticle size. For titania, anatase, rutile, and anatase/rutile particles differ substantially in their surface chemistry particularly as it relates to generating ROS under ultraviolet light, with ultrafine TiO_2 anatase exerting the greatest catalytic and biological activity. However, in the present study, cells were exposed to ultrafine or fine TiO_2 without direct ultraviolet (UV) irradiation of the particles. Therefore, differences in UV-dependent catalytic activity were avoided and direct particle size comparisons can be made.

Several previous studies showed that ultrafine particles are highly inflammogenic (Dick et al., 2003; Zhang et al., 1998). This has been linked to their small size and large surface area (Dick et al., 2003; Donaldson et al., 2002; Wilson et al., 2002). Renwick et al. (2005) reported that instillation of ultrafine TiO_2 and carbon black particles induced more neutrophil recruitment, epithelial damage, and cytotoxicity than their fine counterparts, exposed at equal mass. Epithelial injury and toxicity were associated with the development of inflammation after exposure to ultrafines. However, to date, inflammatory signaling cascades in cells exposed to ultrafine particles have not been evaluated. MAP kinases are redox sensitive (Ding et al., 1999; Nishida et al, 2000; Jimenez et al., 1997; Buder-Hoffmann et al., 2001). Their signaling cascades are linked to activation of nuclear transcription factors, leading to the induction of early response genes that are critical in inflammation

and carcinogenesis. In the present study, ultrafine TiO_2 significantly increased ERK1/2 phosphorylation in a concentration-dependent manner compared with the untreated control. The potency of ultrafine TiO_2 was substantially greater than an equivalent mass of fine TiO_2. Furthermore, time course study indicated that a higher concentration produced an earlier induction of ERK1/2 phosphorylation. ERK activation was demonstrated to be associated with integrin signaling bidirectionally: via activating an "inside-out" signal leading to integrin activation, and augmentation by "outside-in" signaling in cancer cell proliferation (Vellon et al., 2006) and cell migration (Sanders & Basson, 2003). Thus, further study is required to define the role of ultrafine TiO_2 in a functional link between ERK cascade and integrin signaling.

In addition to evidence that ultrafine TiO_2 stimulates intracellular ROS generation and ERK1/2 phoshorylation, this study clearly demonstrates that ultrafine TiO_2 stimulated TNF-α and MIP-2 secretion in RAW 264.7 cells in a concentration-dependent manner. These effects of ultrafine TiO_2 were greater than fine TiO_2 at each exposure concentration. Peters at al. (2004) showed that 50 μg/ml of TiO_2 nano-scaled particles (70 nm mean particle size) produced a slight but reproducible interleukin (IL)-8 secretion in endothelial cells, but a low particle concentration (5 μg/ml) did not induce this change. In the present study, at the low concentrations of ultrafine TiO_2 (≤5 μg/ml), there were significant increases in TNF-α and MIP-2 secretion from RAW 264.7 macrophages. This disparity may be due to differences in sizes of the particles, in methods for particle dispersion, and in cell types. Other nano-scaled particles, such as carbon black (CB, 200 μg/ml) and cobalt particles (50 μg/ml), also induced TNF-α and IL-8 secretion in alveolar macrophages (Brown et al., 2004) or endothelial cells (Peters et al., 2004), but fine CB had no significant effect on TNF-α secretion. The antioxidants nacystelin and the water-soluble vitamin E analog trolox both prevented the TNF-α secretion in response to ultrafine CB exposure, suggesting that ROS are involved in the regulation of TNF-α secretion (Brown et al., 2004). In addition to the direct effect of ultrafine TiO_2 on TNF-α secretion, a potentiative effect on LPS-induced TNF-α secretion when cells were exposed to 1 μg/ml ultrafine TiO_2 in the presence of LPS was observed. This finding suggests that ultrafine TiO_2 may be able to accelerate LPS-induced inflammatory responses.

Data from our in vitro study hypothesized that biological activity of ultrafine TiO_2 to generate ROS could be the central mechanism that regulates cellular inflammatory response. This might be also considered in a dose response of pulmonary inflammation to in vivo exposure to ultrafine TiO_2 (Renwick et al., 2005). Oxidant stress has been proposed as a major paradigm for the in vitro evaluation of the biological effects and potential health hazards due to inhalation of nanoparticles (Nel et al., 2006).

In conclusion, ultrafine TiO_2 at relatively low concentrations stimulated intracellular ROS generation and proinflammatory cellular cascades (ERK1/2 activation, TNF-α production, and MIP-2 secretion) in RAW 264.7 macrophages to a greater extent than fine TiO_2 on an equivalent mass basis. Evidence also supports that significant reduction of nanoparticle agglomeration increases the accuracy of the concentration being delivered to the testing system. At this time, the precise mechanism controlling the proinflammatory effects by ultrafine TiO_2 associated oxidative stress is unclear and thus warrants further investigation.

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