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## Nanoparticles-Induced Apoptosis of Human Airway Epithelium is mediated by ProNGF/p75<sup>NTR</sup> Signaling

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### Abstract

Environmental and occupational exposures to respirable ultrafine fractions of particulate matter (PM) have been implicated in the initiation and exacerbation of lung diseases. However, the precise mechanisms underlying production of cell damage and death attributed to nanoparticles (NP) on human airway epithelium are not fully understood. This study examined the role of neurotrophic pathways in NP-induced airway toxicity. Size and agglomeration of TiO<sub>2</sub> nano (TiO<sub>2</sub>-NP) and fine (TiO<sub>2</sub>-FP) particles were measured by dynamic light scattering. Expression and signaling of key neurotrophic factors and receptors were assessed by real time polymerase chain reaction, flow cytometry, immunostaining, and Western blot in various respiratory epithelial cells after exposure to TiO<sub>2</sub>-NP or TiO<sub>2</sub>-FP. Particle-induced cell death was measured by flow cytometry after annexin V/propidium iodide staining. The role of neurotrophin-dependent apoptotic pathways was analyzed with specific blocking antibodies or siRNAs. Exposure of human epithelial cells to TiO<sub>2</sub>-NP enhanced interleukin (IL)-1 $\alpha$  synthesis, as well as nerve growth factor (NGF) gene expression and protein levels, specifically the precursor form (proNGF). TiO<sub>2</sub>-NP exposure also increased expression of p75<sup>NRF</sup> receptor genes. These neurotrophic factor and receptor responses were stimulated by IL-1 $\alpha$  and abolished by its specific receptor antagonist (IL-1-ra). TiO<sub>2</sub>-NP also increased JNK phosphorylation and apoptosis, which was prevented by anti-p75<sup>NRF</sup> or NGFsiRNA. Data demonstrated that TiO<sub>2</sub>-NP exerted adverse effects in the respiratory tract by inducing unbalanced overexpression of immature neurotrophins, which led to apoptotic death of epithelial cells signaled through the death receptor p75<sup>NTR</sup>. This may result in airway inflammation and hyperreactivity after exposure to TiO<sub>2</sub>-NP.

### Keywords

Air pollution; Asthma; Lung injury; Neurotrophins; Particulate matter

## INTRODUCTION

Because of their size ( 100 nm diameter), engineered nanoparticles (NP) and the ultrafine fraction of ambient air pollution display greater ability to be inhaled and deposited in the fragile epithelial structures of the distal airways compared to larger particles (Tsuda et al. 2009; Kermanizadeh et al. 2016; Oberdorster et al. 2015). Indeed, a number of epidemiologic studies reported that exposure to ambient particulate matter, which includes a NP component, exerts deleterious effects on human lungs leading to obstructive and fibrotic disease, exacerbation of pre-existing airway disease, and increased risk of respiratory infections (Chen et al. 2015; Costa et al. 2014; de Oliveira et al. 2014; Nel 2005; Pietropaoli et al. 2004; Tsai et al. 2015). In particular, the toxicology of inhalable titanium dioxide (TiO<sub>2</sub>) NP has drawn significant attention because of its extensive commercial use (Kermanizadeh et al. 2016; Xia et al. 2006), with most studies focusing on TiO<sub>2</sub>-induced oxidative stress mediated by reactive oxygen species (ROS) (Gurr et al. 2005; Olmedo et al. 2005).

Previously, Scuri et al (2010a) reported that exposure to NP upregulates the expression of lung neurotrophins which subsequently leads to airway hyperresponsiveness and inflammation. The neurotrophin family includes the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins (NT) 3 and 4 (Robinson et al. 1999). By binding to high-affinity tropomyosin receptor kinases (TrkA, TrkB, TrkC) or to the pan-neurotrophin receptor p75<sup>NTR</sup>, neurotrophins not only play a key role in neuronal survival, development, and function (Scuri et al 2010b), but also exert direct and indirect (i.e., nerve-mediated) effects on innate and adaptive immune pathways involved in allergic inflammation (Braun et al. 1999). In addition, mature NGF non-specifically modulate cell death in a variety of tissues by increasing expression of the anti-apoptotic *Bcl-2* family members (Othumpangat et al. 2009).

The aim of this study was to determine the role played by neurotrophic pathways in the interactions between TiO<sub>2</sub>-NP and human airways. To this end, the effects of nano-size (21 nm primary particle diameter) and fine-size (<5 μm) TiO<sub>2</sub> particles were first compared *in vitro* on viability of human epithelial cells sampled from different anatomical levels of the respiratory tract (nasal, tracheal, bronchial, and alveolar). Therefore, the influence of these particles on gene and protein expression of key neurotrophic factors and receptors in the airway epithelium were systematically analyzed. These initial experiments directed attention to the immature dimeric precursor of the NGF protein (proNGF), which binds with high affinity to p75<sup>NTR</sup> death receptor and initiates apoptosis in target cells by signaling through downstream stress-activated protein kinase/c-Jun-amino-terminal kinase (SAPK/JNK). Finally, specific inhibitors were used to determine the contribution of this pathway in the apoptotic or necrotic death of primary human epithelial cells exposed to titanium dioxide nanoparticles (TiO<sub>2</sub>-NP).

## MATERIALS and METHODS

### Materials

Commercial grade titanium dioxide nanoparticles (TiO<sub>2</sub>-NP) and titanium(IV) oxide fine particles (TiO<sub>2</sub>-FP) were kindly provided by the National Institute for Occupational Safety and Health (NIOSH). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell proliferation assay kits were purchased from Cayman Chemical (Ann Arbor, MI); FITC Annexin-V Apoptosis Detection kits from BD Biosciences (San Jose, CA); and RNeasy kits for total RNA isolation from Qiagen (Valencia, CA). Primers for human NGF, BDNF, TrkA, TrkB and p75<sup>NTR</sup> were purchased from SABiosciences (Valencia, CA) and primers for the β<sub>2</sub> microglobulin (B2M) housekeeping gene from RealTimePrimers (Elkins Park, PA).

The following primary antibodies, secondary antibodies, and isotype controls were used for immunostaining: NGF, BDNF, p75<sup>NTR</sup>, GAPDH and JNK (Santa Cruz Biotechnology, Santa Cruz, CA); phospho-SAPK/JNK (Cell Signaling, Danvers, MA); pro-NGF (Millipore, Billerica, MA); TrkA and TrkB (R&D Systems, Minneapolis, MN); AlexaFluor 488-conjugated secondary antibodies (Invitrogen, Carlsbad, CA); and horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology). Western blotting reagents were purchased from Amersham Life Science (Buckinghamshire, United Kingdom). Interleukin 1 alpha (IL-1α) and IL-1 receptor antagonist (IL-1-ra) were purchased from Santa Cruz Biotechnology.

### Preparation of particles

Stock solutions (10 mg/ml) of TiO<sub>2</sub>-NP or TiO<sub>2</sub>-FP were prepared in aseptic phosphate-buffered saline (PBS) by sonicating for 30 sec (Misonix Sonicator XL-2000; Qsonica, Newtown, CT) and cooling on ice for 15 sec for a total of 3 min. Before use, particles were diluted under aseptic conditions to 0–100 μg/ml in PBS, and then sonicated for another 1 min. Particle size distribution was measured with a Nanotrak Particle Size Analyzer (Microtrac, Montgomeryville, PA) performing dynamic light scattering (DLS) analysis of 100 μg/ml of particles suspended in PBS and sonicated for 2 min (TiO<sub>2</sub>-FP) or 1 min (TiO<sub>2</sub>-NP) in a Vibra-Cell ultrasonic processor (Sonics & Materials, Newtown, CT). Sonication of TiO<sub>2</sub>-NP for a longer than 1 min resulted in increased agglomeration. To monitor structure size and agglomeration pattern of the particles as delivered in cell culture medium, 10 μg/ml of sonicated particle suspensions were added to the medium and then reanalyzed by DLS.

### Cell culture

Human nasal epithelial cells from PromoCell GmbH (Heidelberg, Germany); human tracheal and bronchial epithelial cells from Cell Applications (San Diego, CA); and Type I alveolar cells from ScienCell Research Laboratories (Carlsbad, CA) were used. All these primary human epithelial cells were grown with their respective medium at 37°C in 5% CO<sub>2</sub> atmosphere. All experiments were performed when the cells reached 70–80% confluence and within 5–6 passages, except alveolar cells that were used within 3–4 passages.

### Cytotoxicity assay

Epithelial cells were plated at a density of  $5 \times 10^3$  per well in flat bottom 96-well plates (CellStar; Greiner Bio-One, Frickenhausen, Germany). Once cells reached approximately 70% confluence, medium was replaced with 200  $\mu$ l fresh medium containing different concentrations of TiO<sub>2</sub>-FP or TiO<sub>2</sub>-NP (0–100  $\mu$ g/ml) and incubated for 24 hr. Following exposure, cell viability was measured by adding 10  $\mu$ l MTT reagent and incubating for 3 hr at 37°C. Crystallized MTT was dissolved, and its optical density was measured at 575 nm with a reference wavelength of 690 nm (PowerWave XS ELISA plate reader; BioTek, Winooski, VT). Viability was calculated as the ratio of the average OD obtained for each treatment to that of untreated cells using the Gen5™ data analysis software (BioTek). Data shown are means of 3 separate experiments.

### Reverse transcription-polymerase chain reaction (RT-PCR)

Gene expression of neurotrophic factors and receptors was analyzed by RT-PCR. Total RNA (100 ng) isolated with the RNeasy kit was amplified with QuantiTect One-step SYBR Green and specific primers using an Applied Biosystems 7500 real-time thermocycler (Foster City, CA). Fold changes in neurotrophins expression were normalized to B2M and calculated with the Ct method (Livak and Schmittgen 2001).

### Fluorescence-activated cell sorting (FACS)

Neurotrophins protein expression after 24 hr exposure to TiO<sub>2</sub> particles was measured using FACS. NGF, BDNF and their TrkA, TrkB and p75<sup>NTR</sup> receptors were stained with appropriate primary antibodies. Approximately  $1 \times 10^5$  airway epithelial cells were isolated by trypsinization, fixed in 4% paraformaldehyde, and permeabilized with 0.5% Triton X-100 in PBS. Nonspecific binding was removed by adding human IgG on ice for 20 min. Cells were incubated separately with primary antibodies for NGF, BDNF, TrkA, TrkB, or p75<sup>NTR</sup> followed by the respective fluorescent conjugated secondary antibodies, and analyzed using FACSCalibur with CellPro software (BD Biosciences). Data were further analyzed with the Windows Multiple Document Interface (WinMDI) software version 2.9 (Scripps Research Institute, La Jolla, CA).

### Immunostaining

Imaging studies were performed on  $1 \times 10^3$  human airway epithelial cells grown on poly-L-lysine coated glass coverslips in 6-well plates and exposed to 10  $\mu$ g/ml of TiO<sub>2</sub>-FP or TiO<sub>2</sub>-NP for 24 hr. Cells were then rinsed in PBS, fixed with 4% paraformaldehyde, and immunostained with the anti-NGF antibody followed by AlexaFluor 488-labeled secondary antibody. Coverslips were mounted with Prolong Gold anti-fade reagent containing 4,6-diamidino-2-phenylindole (DAPI). Cells were visualized using EC Plan-Neofluar 40x1.30 oil DIC M27 objective and photographed using a confocal microscope (LSM510; Carl Zeiss, Jena, Germany) with 405-diode laser to excite DAPI and Argon laser to excite the AlexaFluor 488-labeled secondary antibody.

### Immunoprecipitation/Immunoblot

Protein was extracted from control and TiO<sub>2</sub>-treated cell pellets using RIPA lysis buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing a cocktail of protease inhibitors (Calbiochem, San Diego, CA). Protein concentration was determined using bicinchoninic acid (Pierce, Rockford, IL). Equal amounts of protein (50 µg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to a nitrocellulose membrane at 4°C. Membranes containing the protein samples were blocked with 5% milk in TBS-T buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 0.05% Tween 20) followed by incubation with anti-NGF antibody or with phospho-JNK. Protein bands were visualized by chemiluminescence detection (ECL, Amersham Life Sciences, United Kingdom). Immunoblots were subsequently stripped, blocked and re-probed with antibodies specific for β-actin, GAPDH, or total JNK and quantified using Image J1.43u chemiluminescent densitometry software (National Institutes of Health, Bethesda, MD).

To study the role of p75<sup>NTR</sup>-mediated activation of the JNK cell death pathway, 200 µg of protein from cells exposed to TiO<sub>2</sub> particles or untreated controls was incubated overnight at 4°C with anti-p75<sup>NTR</sup> mouse monoclonal antibody (sc-56448). Antigen-antibody complexes were precipitated by binding to A/G plus-agarose beads (sc-2003) for 2 hr at 4°C, and once eluted from the beads were subjected to immunoblotting as described earlier. Membranes were then incubated overnight at 4°C with rabbit polyclonal anti-NGF antibody (sc-33602) in blocking buffer, and chemiluminescent signals were developed as detailed above.

### Role of IL-1α

To study the role of IL-1α in modulating NGF expression in bronchial epithelial cells exposed to TiO<sub>2</sub>-NP, confluent (80%) plates of bronchial epithelial cells were exposed to varying concentrations of recombinant IL-1α (0–5 ng/ml) or were pre-treated with IL-1-RA (0–100 µM) for 3 hr followed by TiO<sub>2</sub>-NP (10 µg/ml) for an additional 24 hr. Following incubation, cells were either processed for RNA extraction and stored at –80°C, or processed immediately for Western blot and immunofluorescence assays.

To confirm the role of p75<sup>NTR</sup> in mediating neurotrophin-induced death, a specific blocking antibody (anti-human NGFR p75<sup>NTR</sup>, SPM299) was employed. Bronchial and alveolar epithelial cells were pre-incubated for 24 hr with 1 µg/ml antibody before exposure to TiO<sub>2</sub>-NP for another 24 hr. To assess specificity of the blocking antibody, an isotype IgG antibody was used as control. Cell death was measured by FACS as described previously.

### Apoptosis/necrosis assay

To understand the mode of TiO<sub>2</sub>-induced cell death, the annexin V/propidium iodide (PI) assay was utilized. Airway epithelial cells were grown in 6-well plates and exposed to TiO<sub>2</sub>-NP or TiO<sub>2</sub>-FP for 24 hr. All floating cells were collected by centrifugation and adherent cells were detached by trypsinization. These cells (10<sup>6</sup> cells/ml) were incubated with fluorescein isothiocyanate (FITC)-conjugated annexin V and PI for 15 min at room temperature in the dark and analyzed with a FACSCalibur flow cytometer and CellQuest Pro software. Cells in the right lower quadrant represented early apoptosis, while cells in the

right upper quadrant represented late apoptosis or post-apoptotic necrosis. Necrosis is represented by the left upper quadrant. A total of 20,000 events were acquired for each sample, and data expressed as means of 3 separate experiments.

### NGF silencing

To silence NGF gene expression, a specific siRNA (Dharmacon, Chicago, IL) with Lipofectamine™2000 as transfection reagent (Invitrogen, Carlsbad, CA) was used. Silencing efficiency was measured by RT-PCR using scrambled siRNA to control for both transfection and gene silencing. NGF-deficient bronchial epithelial cells were then exposed to TiO<sub>2</sub> particles (10 µg/ml) for another 24 hr. Cells were collected by trypsinization. Apoptosis and necrosis were measured by FACS. To further confirm the role of p75<sup>NTR</sup> in proNGF-induced apoptosis, confluent bronchial epithelial cells were pre-treated with 1 µg/ml anti-human p75<sup>NTR</sup> antibody for 24 hr and then exposed to TiO<sub>2</sub>-NP for an additional 24 hr. Anti-human IgG antibody was used as the isotype control. Cells were harvested after incubation and apoptosis was measured by FACS.

### Statistical analysis

All data are expressed as means ± standard deviations of the mean (SD). Data were analyzed by ANOVA for multiple comparisons and t-test for comparisons within a group using SigmaStat 3.5 (Systat Software, San Jose, CA). Post-hoc comparisons between groups were analyzed with the Holm-Sidak method or the Tukey-Kramer test. P-value <0.05 was considered significant.

## RESULTS

### TiO<sub>2</sub>-induced cytotoxicity

Size and distribution pattern of TiO<sub>2</sub> particles in PBS and cell culture medium were measured by DLS. According to the manufacturer, TiO<sub>2</sub>-NP (80/20% anatase/rutile) consisted of small aggregates with primary particles having a mean diameter of approximately 21 nm and specific surface of 50 m<sup>2</sup>/g, while TiO<sub>2</sub>-FP (100% rutile) had an average diameter <5 µm with specific surface area of 2–3 m<sup>2</sup>/g. However, TiO<sub>2</sub>-NP agglomerated in suspension with mean structure diameters of 2.6 µm in PBS and 0.7 µm in cell culture medium (Table 1), whereas TiO<sub>2</sub>-FP showed less agglomeration with a mean structure size of 1 µm in PBS and 1.2 µm in medium.

The viability of nasal, tracheal, bronchial, and alveolar human type I epithelial cells was measured after exposure to increasing concentrations of TiO<sub>2</sub>-NP at 37°C for 24 hr (Figure 1). Alveolar and bronchial cells tended to be more susceptible to TiO<sub>2</sub> toxicity than tracheal and nasal cells. The MTT assay also showed numerically higher fall in cell viability after exposure to TiO<sub>2</sub>-NP than to TiO<sub>2</sub>-FP using equal mass concentrations. Specifically, 69.9% of bronchial cells remained viable after exposure to 10-µg/ml TiO<sub>2</sub>-NP in comparison to 79.4% of cells exposed to TiO<sub>2</sub>-FP.

### TiO<sub>2</sub>-induced neurotrophins expression

Exposure to TiO<sub>2</sub>-NP resulted in elevated neurotrophins synthesis and surface receptors expression in airway epithelial cells, with important differences dependent on airway type (Figure 2). TiO<sub>2</sub>-NP exposure significantly enhanced expression of NGF and p75<sup>NTR</sup> receptor genes in nasal, bronchial, and alveolar epithelial cells, whereas TrkA receptor expression was only increased in alveolar epithelial cells. In contrast, BDNF and TrkB gene expression remained near basal levels in all cell types except for TrkB expression in nasal epithelial cells. Further, tracheal epithelial cells were the least responsive to TiO<sub>2</sub>-NP with no significant changes in neurotrophin gene expressions.

TiO<sub>2</sub>-FP at the same mass concentrations increased NGF gene expressions in tracheal, bronchial, and alveolar epithelial cells, together with a marked rise in p75<sup>NTR</sup> in tracheal epithelial cells and elevated TrkA in bronchial epithelial cells. In general, the lower respiratory tract epithelium, particularly alveolar and bronchial epithelial cells, exhibited the greatest rise in transcripts encoding NGF and its cognate receptors, without associated significant changes in the BDNF-TrkB axis.

Whether increased levels of NGF transcripts in epithelial cells exposed to TiO<sub>2</sub>-NP translated into higher protein concentrations was subsequently examined at the single cell level by FACS (Figure 3). Alveolar epithelial cells exhibited significant upregulation of NGF protein synthesis after exposure to 10 µg/ml TiO<sub>2</sub>-NP or TiO<sub>2</sub>-FP compared to non-exposed controls. Nasal epithelial cells also expressed markedly higher NGF protein concentration when exposed to TiO<sub>2</sub>-NP, whereas tracheal epithelial cells did not display any significant change in NGF compared to controls independent from particle size.

RT-PCR and FACS data were further confirmed by confocal immunostaining analysis. Nasal and alveolar cells treated with TiO<sub>2</sub>-NP demonstrated significant increase in intracellular staining for NGF as opposed to near-baseline levels following TiO<sub>2</sub>-FP exposure (Figure 4). However, bronchial epithelial cells significantly upregulated endogenous NGF when exposed to either TiO<sub>2</sub>-NP or TiO<sub>2</sub>-FP.

As in preliminary studies, IL-1 $\alpha$  synthesis was elevated in cells exposed to TiO<sub>2</sub>-NP. Therefore, experiments were conducted to determine whether this pro-inflammatory cytokine played a role in modulation of NGF gene expression in bronchial epithelial cells exposed to TiO<sub>2</sub>-NP. IL-1 $\alpha$  protein concentration rose significantly in cell supernatants after exposure to either TiO<sub>2</sub>-NP or TiO<sub>2</sub>-FP (Figure 5A). Exogenous IL-1 $\alpha$  increased NGF gene expression to levels similar to TiO<sub>2</sub>-NP, and pretreatment with a specific receptor antagonist (IL1-ra) abolished the effect of TiO<sub>2</sub>-NP exposure on NGF gene expression. IL1-ra exerted a similar inhibitory effect on TiO<sub>2</sub>-NP induced NGF protein synthesis measured by either Western blot (Figure 5B) or FACS (Figure 5C) analysis of bronchial epithelial cells.

### ProNGF/p75<sup>NTR</sup> mediated cell death

Immunoblot studies revealed a dimeric form of NGF (proNGF, 27 kDa) with highest concentrations in bronchial epithelial cells following TiO<sub>2</sub>-NP exposure (Figures 6A–B-C). Immunoprecipitation showed that proNGF binds with high affinity to its p75<sup>NTR</sup> receptor (Figure 6D). Western blot analysis demonstrated significant increase in JNK

phosphorylation following TiO<sub>2</sub>-NP exposure in bronchial epithelial cells (Figure 6E), suggesting that this pathway may play a critical role in cell death initiated by signaling from the proNGF-p75<sup>NTR</sup> axis.

TiO<sub>2</sub>-NP induced marked airway epithelial cell death primarily by apoptosis, particularly among bronchial epithelial cells ( $22 \pm 5.6\%$  vs.  $10.4 \pm 0.7\%$ ; Figure 7). Similarly, an increased proportion of early apoptotic cells (lower right quadrant) was noted following treatment with TiO<sub>2</sub>-NP in alveolar>bronchial>nasal epithelial cells. In contrast, exposure to TiO<sub>2</sub>-FP induced cell death primarily via necrosis (upper left quadrant) in nasal>alveolar>tracheal>bronchial epithelial cells.

To further understand the role of p75<sup>NTR</sup> in proNGF-mediated cell death, bronchial and alveolar epithelial cells were exposed to TiO<sub>2</sub>-NP after pretreatment with anti-p75<sup>NTR</sup> blocking antibody (Figure 8). This inhibitor prevented TiO<sub>2</sub>-NP induced cell death and significantly enhanced survival of both bronchial and alveolar epithelial cells, confirming that signaling through the proNGF-p75<sup>NTR</sup> axis is essential for NP-induced apoptosis. Further, bronchial cells transfected with NGF-specific siRNA (NGFsiRNA) before exposure to TiO<sub>2</sub>-NP for 24 hr displayed significant reduction in early apoptosis along with increased necrosis compared to controls transfected with scrambled siRNA (scRNA), although apoptosis remained significantly higher than in non-exposed cells (Figure 9). Viability of non-exposed scrambled siRNA transfectants was similar to untreated cells (89.3 vs. 90.9%).

## DISCUSSION

In humans, it can generally be considered that inhaled particles with diameter 5–10 μm are deposited in the large conducting airways and those <5 μm in the small airways and alveoli; thus, particles with diameter <5 μm constitute the breathable fraction of an aerosol (Oberdorster et al, 2015). As the epithelial lining of the respiratory tract is the primary target of inhaled particles, our study sought to clarify the cytotoxic mechanisms activated by TiO<sub>2</sub> in the form of NP or FP after deposition onto human airway epithelium.

*In vitro* exposure to both particle sizes induced concentration-dependent cytotoxicity, and cells derived from more distal segments of the respiratory tract tended in general to be more susceptible than those from more proximal segments. Some previous studies reported that NP are more toxic than FP on a mass dose basis (Oberdorster 1996; Okuda-Shimazaki et al. 2010). Oberdorster (1996) proposed that surface area increases as particle size becomes smaller, and thus NP tend to be more toxic when compared with the same mass of FP. However, in the present study, the difference in potency of TiO<sub>2</sub>-NP vs. TiO<sub>2</sub>-FP was slight. Shvedova et al. (2007) noted that degree of agglomeration markedly impacts the bioactivity of NP. In the present study, TiO<sub>2</sub>-NP tended to agglomerate in serum-free culture media generating submicronic structures (0.7 μm) that were only slightly smaller than agglomerates derived from FP (1.2 μm). Several studies suggested that singlet NP are more active than agglomerates in their ability to evoke a response (Oberdorster 1996; Oberdorster et al 2005; Shvedova et al. 2007; Hartmann et al. 2015). Therefore, agglomeration of TiO<sub>2</sub>-NP in the present study likely decreased their bioactivity.

Although NP pose an emergent challenge to toxicologists, no apparent limit for their average ambient air concentration has been set by any regulatory agency (OSHA or EPA). The American Conference of Governmental Industrial Hygienists (ACGIH, Cincinnati, OH, 1992) assigned to FP a threshold limit value of 10 mg/m<sup>3</sup> (total dust) as a time-weighted average for a normal 8-hr workday and a 40-hr workweek, although data suggest that high-risk individuals (e.g., patients with asthma or COPD) may be exposed to ambient levels of atmospheric particles close to 20 µg/cm<sup>2</sup> (Phalen et al 2006). More recently the National Institute for Occupational Safety and Health (NIOSH) proposed size-dependent exposure limits, i.e. 0.3 mg/m<sup>3</sup> for TiO<sub>2</sub>-NP vs. 2.4 mg/m<sup>3</sup> for TiO<sub>2</sub>-FP.

Another source of concern is that novel therapeutics in the form of nanosize preparations of molecules, proteins, DNA or small inhibitory RNAs are advancing at a rapid rate toward the clinic. Indeed, NP derived from a variety of different molecular approaches provide a means to deliver drugs to target organs and cells, lowering dose requirements and potentially overcoming problems associated with systemic drug delivery. Because of their accessibility, the respiratory and digestive systems are natural targets for NP therapies. On the other hand, data such as those reported in the present study raise awareness of potential and previously unknown side effects that may limit the use of otherwise promising strategies.

### Neurotrophins

Exposure to TiO<sub>2</sub>-NP or TiO<sub>2</sub>-FP resulted in complex changes in the pattern of neurotrophins expression in airway epithelial cells. Specifically, exposure to 10 µg/ml TiO<sub>2</sub>-NP for 24 hr upregulated expression of genes encoding NGF and its low-affinity p75<sup>NTR</sup> receptor in all respiratory tract epithelial cell types, except for tracheal cells. Further, TiO<sub>2</sub>-NP selectively co-upregulated TrkA and p75<sup>NTR</sup> surface expressions in alveolar epithelial cells. In contrast, TiO<sub>2</sub>-FP upregulated the NGF-p75<sup>NTR</sup> axis only in tracheal epithelial cells, and no cell type expressed a coordinated BDNF-TrkB upregulation after exposure to either particle size. Data indicate responses of airway epithelium to fine and ultrafine particles are consistent with the upregulated expression of lung neurotrophins previously observed in murine airways exposed to TiO<sub>2</sub>-NP *in vivo* (Scuri et al. 2010a).

Previous *in vivo* studies showed IL-1α-dependent pro-inflammatory activity of TiO<sub>2</sub>-NP in lung and peritoneum of murine inflammation models (Yazdi et al. 2010), and the same cytokine is known to potently induce NGF expression (Pons et al. 2001). IL-1α precursor is constitutively expressed in airway epithelial cells and immediately released if these constituents become damaged, thereby initiating a cascade of cytokines and chemokines that play a key role in innate immunity and inflammation. In the present study, bronchial epithelial cells incubated with low concentrations of recombinant human IL-1α showed NGF upregulation similar to that observed when treated with TiO<sub>2</sub>-NP, and pretreatment with IL-1 receptor antagonist (IL-1ra) virtually abolished the NGF upregulation in response to TiO<sub>2</sub>-NP.

### ProNGF/p75<sup>NTR</sup>

After defining the early events triggering neurotrophins synthesis, the present study focused attention on the signaling pathways triggered by TiO<sub>2</sub>. Results demonstrated that TiO<sub>2</sub>-NP

modulate airway epithelial cell death predominantly through proNGF binding to its p75<sup>NTR</sup> receptor. Blocking p75<sup>NTR</sup> with a selective antibody increased significantly the viability of bronchial epithelial cells and - to a lesser extent - alveolar epithelial cells. Similarly, inhibiting endogenous NGF production by siRNA interference also significantly lowered the rate of apoptosis in TiO<sub>2</sub>-NP treated bronchial epithelial cells, but with a concomitant rise in the proportion of necrotic cells.

Data are consistent with the notion that neurotrophins elicit cell survival or death depending on which receptor and signaling pathway is activated. In many neuronal and non-neuronal cell types, binding and activation of the Trk family of tyrosine kinase receptors regulates survival and differentiation (Barde 1994; Snider and Silos-Santiago 1996). In contrast, when expressed in the relative absence of Trk receptors, NGF binds p75<sup>NTR</sup> and initiates apoptotic cell death (Frade and Barde 1998; Majdan et al. 2001) via downstream activation of the mitochondrial stress kinase JNK (Verheij et al. 1996). Similarly, TiO<sub>2</sub>-NP exposed bronchial epithelial cells exhibited enhanced expression of p75<sup>NTR</sup> without any change in TrkA receptors, which resulted in upregulated p-JNK activity and significantly higher proportion of apoptotic cells. A lower apoptotic rate in TiO<sub>2</sub>-NP exposed alveolar epithelial cells may be due to co-expression of p75<sup>NTR</sup> with TrkA receptors, as co-expression may modify neurotrophin binding (Barker and Shooter 1994; Hempstead et al. 1991), ligand discrimination (Chao 1994), retrograde transport (Curtis et al. 1995), and signal transduction (Verdi et al. 1994).

All neurotrophins are initially synthesized as precursors consisting of an N-terminal pro-domain and a C-terminal mature domain. Pro-neurotrophins are normally cleaved by furin in the trans-Golgi, and the mature domain is then trafficked by secretory vesicles to the extracellular space. However, immature isoforms might also reach the extracellular space, where they might be degraded by matrix metalloproteinase-7 (MMP-7) or converted by plasmin into mature neurotrophins (Bruno and Cuello 2006). Binding of mature neurotrophins to p75<sup>NTR</sup> expressing cells induces apoptosis but only at high concentrations. Whereas, proNGF binds with high affinity to a complex consisting of p75<sup>NTR</sup> and the transmembrane protein sortilin to induce apoptosis at subnanomolar concentrations (Lee et al. 2001). Thus, proNGF is a distinct, biologically active ligand that might induce actions opposing the anti-apoptotic effects of mature NGF binding to its TrkA receptor.

ProNGF is constitutionally expressed at low levels in the central and peripheral nervous systems of children and young adults, but it is markedly upregulated in adults of advanced age or with Alzheimer's disease, suggesting that imbalance of the proNGF:mature NGF ratio in the CNS is responsible for the cholinergic neuron loss seen in aging and dementia (Lee et al. 2001). This imbalance implies that proteolysis of extracellular proNGF is impaired in pathological states and may result from the coordinate induction of inhibitors of matrix metalloproteinases (MMPs) and plasmin, such as tissue inhibitors of metalloproteinase (TIMPs), neuroserpin, and alpha-2 macroglobulin (Bruno and Cuello 2006). Similarly, dysregulation of NGF expression or impaired conversion of proNGF to mature NGF might contribute to pathology in other organs. Data from the present study suggest that chronic inhalation of NP in highly polluted areas may generate "premature

senescence” of the lower airways by shifting balance between pro-apoptotic and pro-survival neurotrophic pathways.

## Conclusions

Data demonstrated that exposure to TiO<sub>2</sub>-NP produce complex changes in expression pattern of key neurotrophic proteins and their cognate receptors in the respiratory tract epithelium with differences dependent on airway depth in the respiratory tract. In particular, NGF precursors are overexpressed in bronchial epithelial cells exposed to TiO<sub>2</sub>-NP, but fail to convert into mature neurotrophins and therefore bind predominantly the p75<sup>NTR</sup> death receptor. In the same cells, p75<sup>NTR</sup> is significantly overexpressed in parallel to its protein ligand and vis-a-vis of unchanged expression of the anti-apoptotic TrkA receptor. The resulting increase in proNGF/p75<sup>NTR</sup> signaling activates downstream the SAPK-JNK pathway, resulting in apoptotic death and premature aging of the bronchial epithelium. Thus, imbalance between immature NGF precursors and its mature form, paired to preferential expression of receptors favoring cell death, contributes to the adverse effects of airborne NP on the respiratory tract.

## Acknowledgments

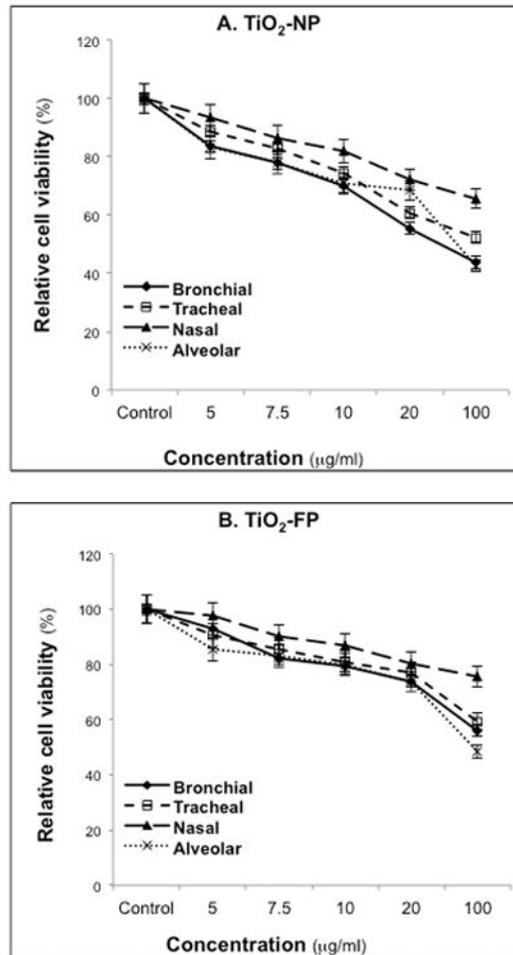
This work was supported in part by the U.S. National Institutes of Health grants RO1-HL61007 to Dr. Giovanni Piedimonte. Image acquisition and data analysis were performed at the West Virginia University Imaging Core Facility, which was supported in part by the NIH grant P20RR016440. FACS experiments were performed in the WVU Flow Cytometry Core Facility, which was supported in part by NIH grants RR106440 and RR020866. We also would like to thank Drs. Min Ding and Sreekumar Othumpangat (NIOSH, Morgantown, WV), as well as Lennie Samsell and Cheryl Walton (WVU Pediatric Research Institute, Morgantown, WV) for providing the titanium dioxide particles and technical help.

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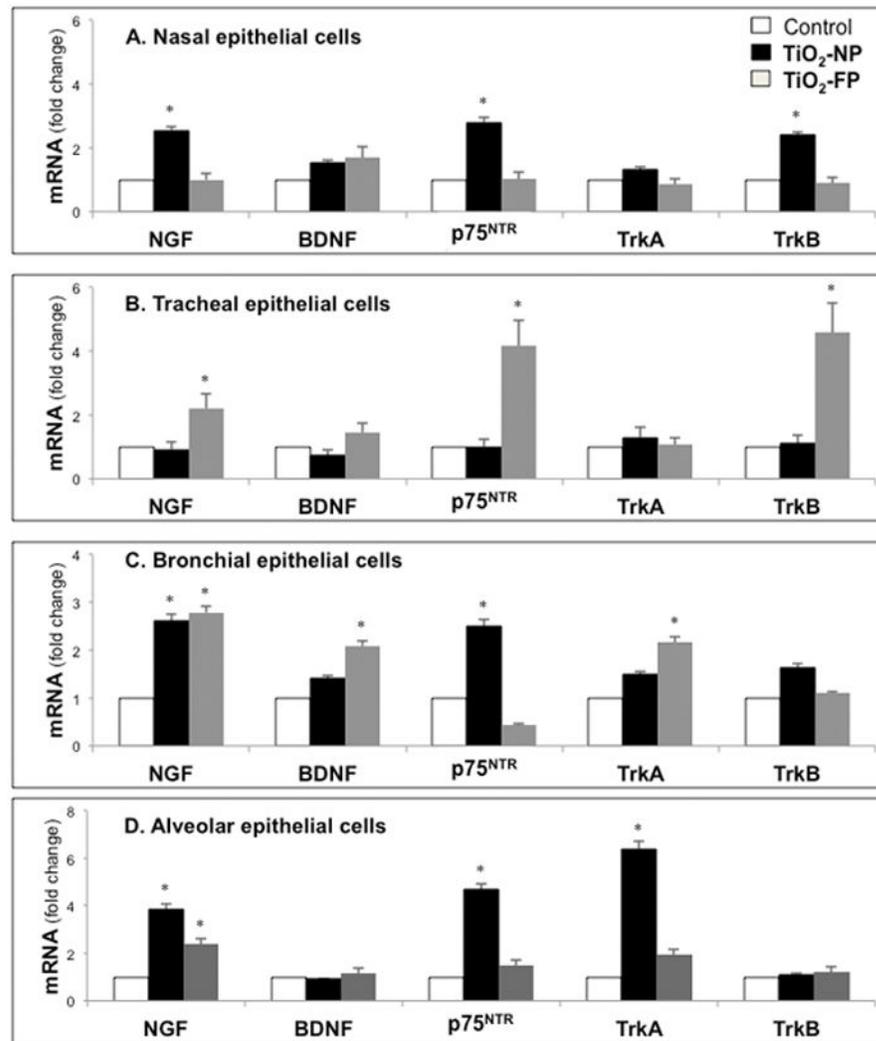
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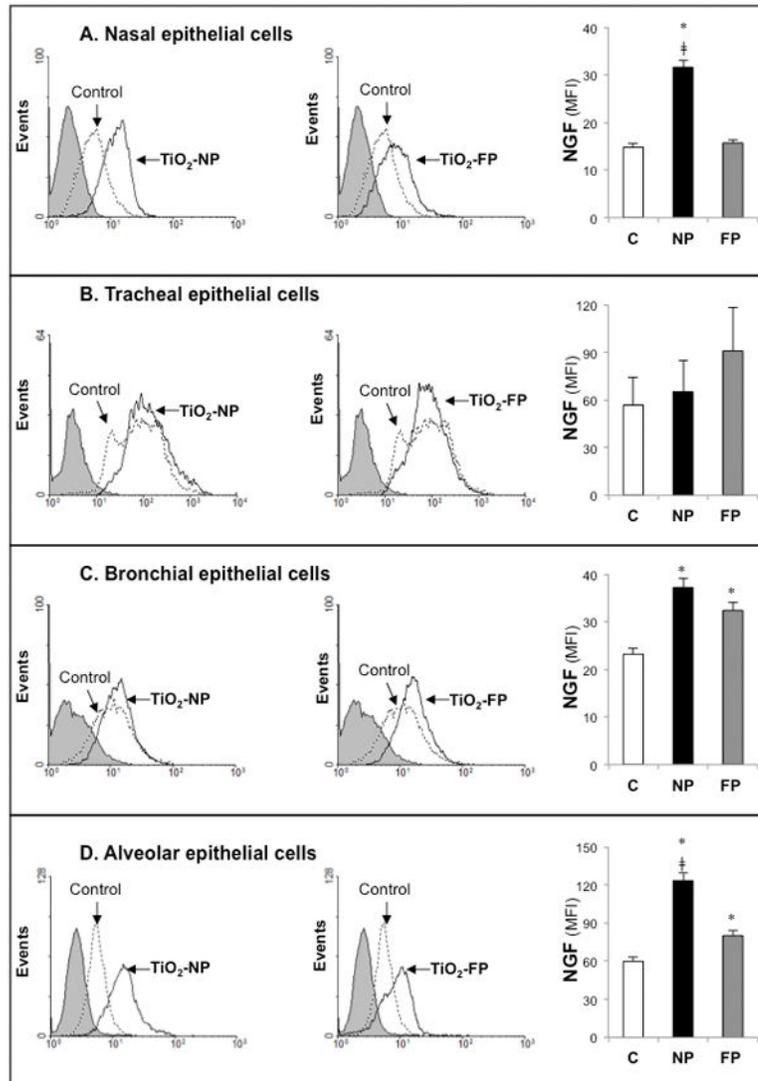


### Figure 1. Viability of TiO<sub>2</sub>-exposed airway epithelia

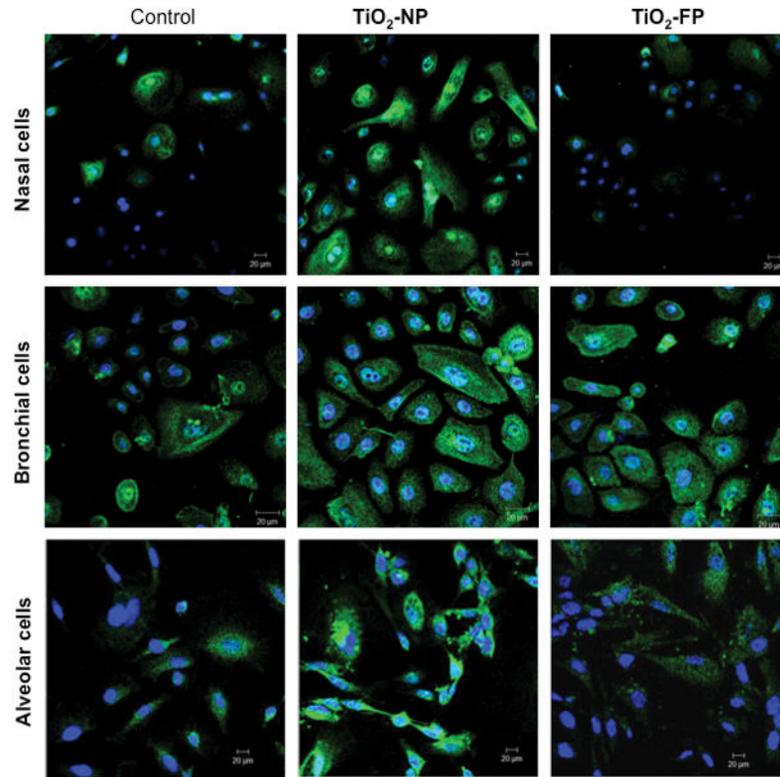
Human nasal, tracheal, bronchial and alveolar epithelial cells were exposed to various concentrations of TiO<sub>2</sub>-NP (A) or TiO<sub>2</sub>-FP (B) for 24 hr. Relative cell viability was measured by the MTT assay. Data are expressed as means ± SDs of 3 independent experiments.



**Figure 2. Neurotrophins gene expression in TiO<sub>2</sub>-exposed airway epithelia**  
 Total cellular RNA was isolated from human nasal (A), tracheal (B), bronchial (C), and alveolar (D) epithelial cells after exposure to 10 µg/ml of TiO<sub>2</sub>-NP or TiO<sub>2</sub>-FP for 24 hr. Gene expression was analyzed by real-time PCR and cycle threshold values were normalized to the housekeeping gene B2M. Data are expressed as means ± SDs of 3 independent experiments. \*p<0.05 compared to control cells not exposed to TiO<sub>2</sub>.

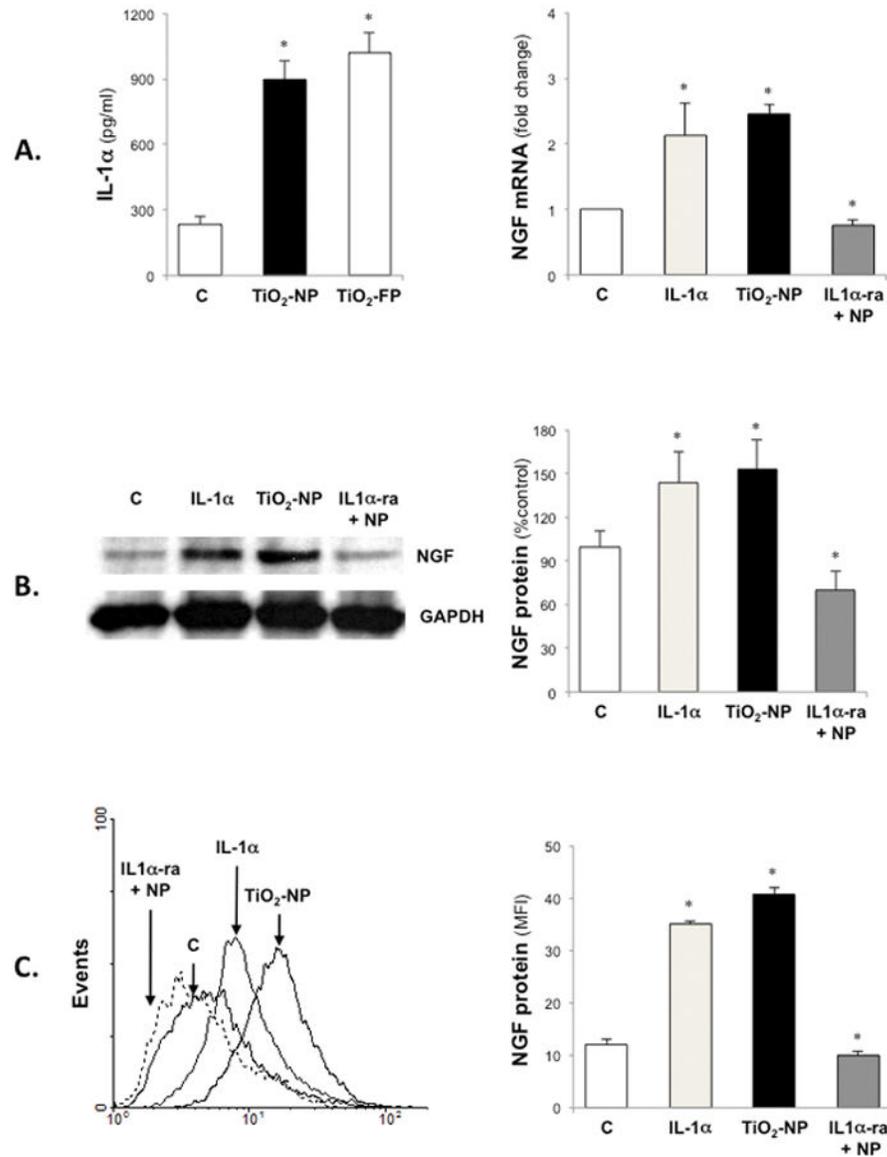


**Figure 3. NGF protein expression in TiO<sub>2</sub>-exposed airway epithelia**  
 Human nasal (A), tracheal (B), bronchial (C), and alveolar (D) epithelial cells exposed to TiO<sub>2</sub>-NP or TiO<sub>2</sub>-FP were stained with anti-human rabbit polyclonal NGF primary antibody and anti-rabbit secondary antibody, and changes in intracellular protein expression were measured by FACS. Mean fluorescent activity (MFI) data are expressed as means ± SDs of 3 independent experiments. \*p<0.05; compared to control cells not exposed to TiO<sub>2</sub>. †p<0.05 compared to cells exposed to TiO<sub>2</sub>-NP. ‡p<0.05 compared to cells exposed to TiO<sub>2</sub>-FP.



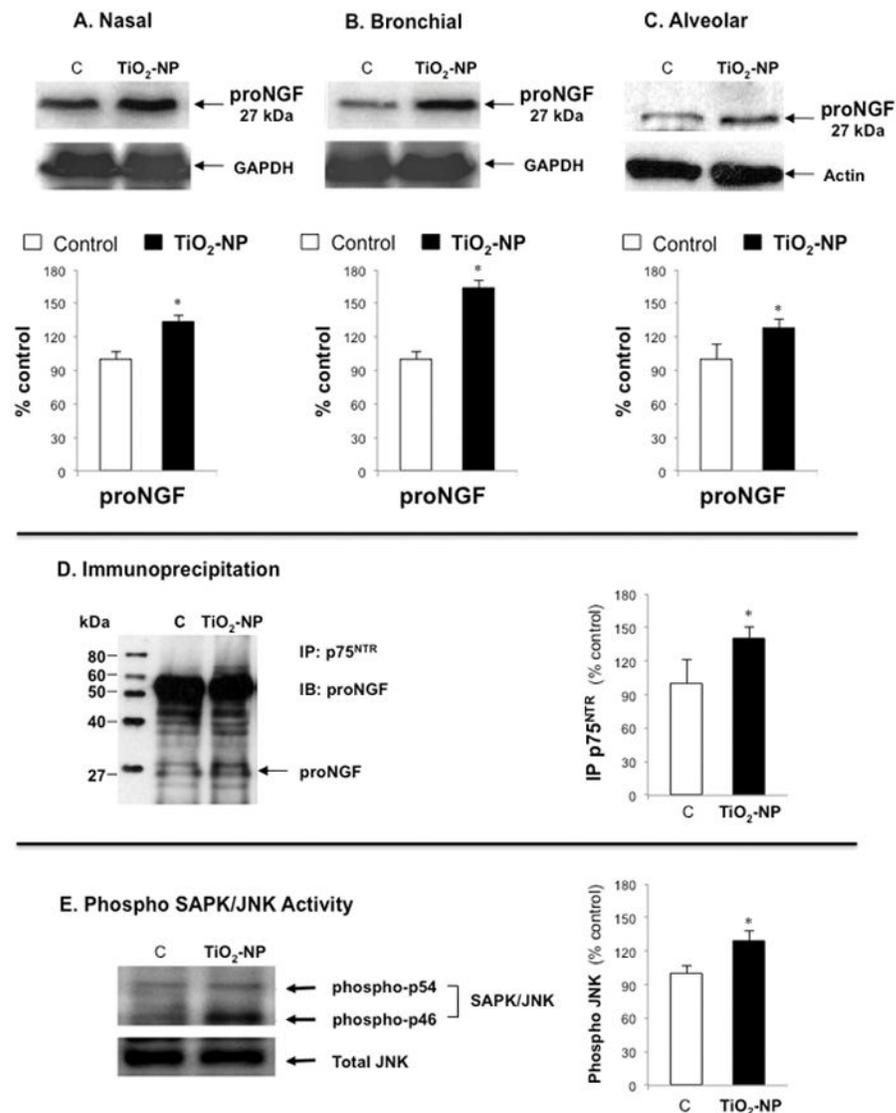
**Figure 4. NGF immunoreactivity in TiO<sub>2</sub>-exposed airway epithelia**

NGF immunoreactivity increased in human nasal, bronchial, and alveolar epithelial cells exposed to 10 μg/ml of TiO<sub>2</sub>-NP for 24 hr. Immunostaining was done with rabbit polyclonal NGF antibody and Alexa-Fluor 488 conjugated secondary anti-rabbit antibody. Nuclei were stained with DAPI. Cells were visualized using an EC Plan-Neofluar 1.30 oil DIC M27 objective. Magnification = 40X.



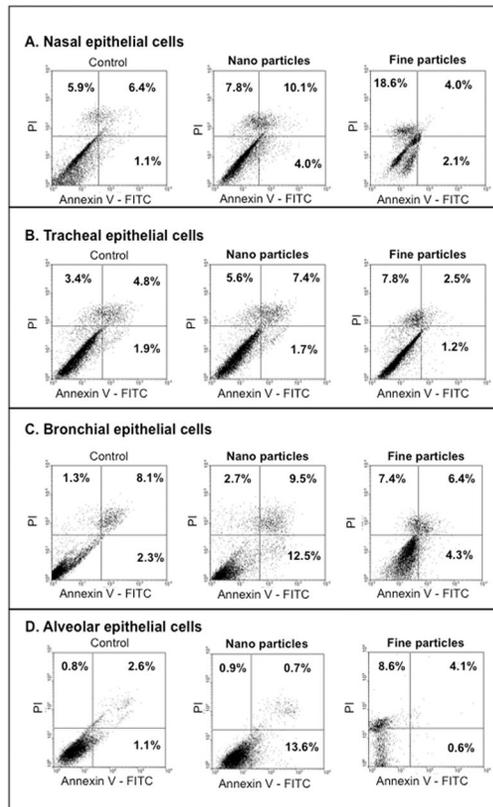
### Figure 5. IL-1 $\alpha$ modulation of NGF expression

TiO<sub>2</sub>-FP exposure increased IL-1 $\alpha$  synthesis in bronchial epithelial cells and exogenous IL-1 $\alpha$  increased NGF gene expression to levels similar to TiO<sub>2</sub>-NP, while pretreatment with a specific receptor antagonist (IL1 $\alpha$ -ra) abolished the effect of TiO<sub>2</sub>-NP exposure on NGF gene expression (A). Similar results were obtained by analyzing the expression of proNGF protein by Western blot (B) or FACS (C). Data are expressed as means  $\pm$  SDs of 3 independent experiments. \* $p$ <0.05 compared to control cells not exposed to TiO<sub>2</sub>.



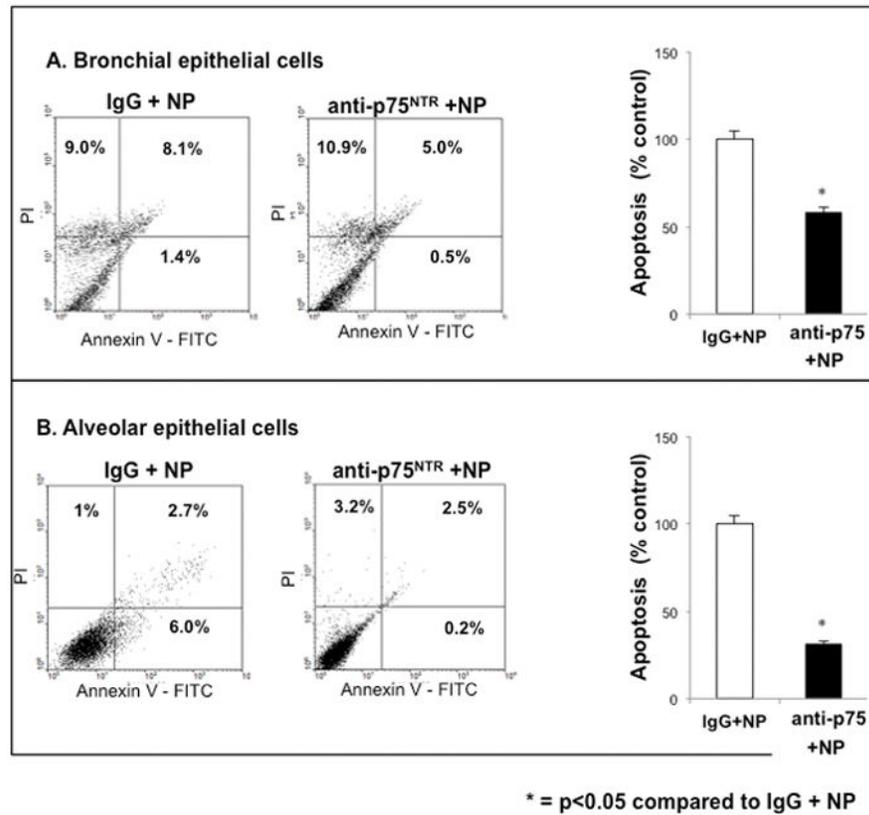
### Figure 6. ProNGF/p75<sup>NTR</sup> signaling

Top panel shows Western blot and densitometry measurements of dimeric forms of NGF (proNGF, 27kDa) in nasal (A), bronchial (B), and alveolar (C) epithelial cells exposed to 10  $\mu\text{g/ml}$  of TiO<sub>2</sub>-NP for 24 hr. Middle panel (D) shows the interaction between proNGF and its p75<sup>NTR</sup> receptor in bronchial epithelial cells exposed to TiO<sub>2</sub>-NP with the corresponding densitometry analysis. Lower panel (E) shows the Western blot analysis of downstream stress-associated protein kinase/Jun-amino-terminal kinase (SAPK/JNK) in bronchial epithelial cells. Blots were probed with phospho SAPK/JNK [Thr183/Tyr185] and re-probed with JNK antibody as an internal control. Densitometry data are expressed as percentage of non-exposed controls after normalizing with GAPDH for NGF or with total JNK for p-JNK in corresponding graphs. Data shown are the means  $\pm$  SDs of 3 independent experiments. \* $p < 0.05$  compared to non-exposed control cells (C).



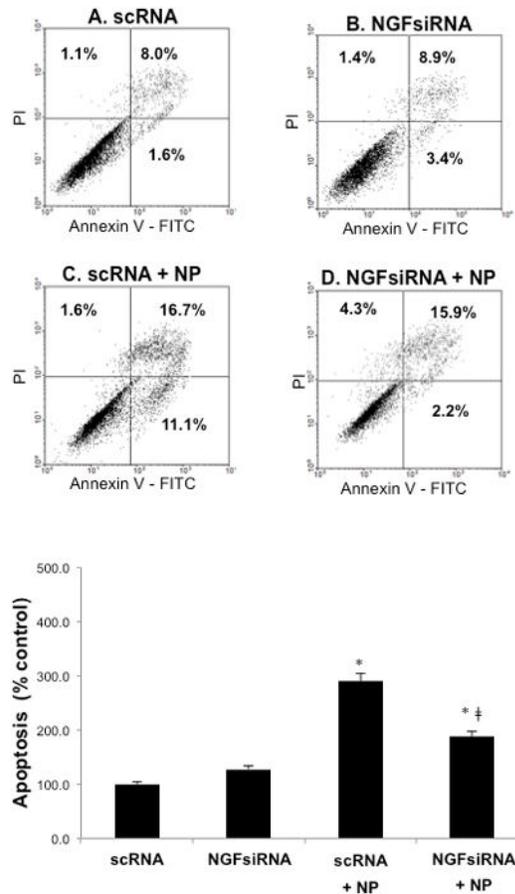
**Figure 7. Nano vs. fine particles-induced cell death**

FACS data for nasal (A), tracheal (B), bronchial (C) and alveolar (D) epithelial cells are shown. Airway epithelial cells were exposed to 10  $\mu\text{g}/\text{ml}$  of  $\text{TiO}_2\text{-NP}$  or  $\text{TiO}_2\text{-FP}$ , then stained with annexin V-FITC and propidium iodide (PI), and cytotoxicity was measured by FACS. Bronchial and alveolar epithelial cells showed increased fractions of early apoptotic cells after  $\text{TiO}_2\text{-NP}$  exposure, while  $\text{TiO}_2\text{-FP}$  diverted cells to necrosis. Data shown are representative of 3 independent experiments.



**Figure 8. Anti-apoptotic effect of anti-p75<sup>NTR</sup>**

Selective immunologic blockade of the cell death receptor p75<sup>NTR</sup> inhibited TiO<sub>2</sub>-NP induced apoptosis. Lower respiratory tract bronchial (A) and alveolar (B) epithelial cells were pretreated anti-human p75<sup>NTR</sup> antibody for 24 h before exposure to 10 μg/ml of TiO<sub>2</sub>-NP. Control group was treated with human IgG antibody. Cells were harvested, stained with annexin V-FITC and propidium iodide (PI), and viability was analyzed by FACS. Data shown are the means ± SDs of 3 independent experiments. \*p<0.05 compared to control cells.



### Figure 9. Anti-apoptotic effect of NGF gene silencing

NGF gene knockdown by siRNA reduced TiO<sub>2</sub>-NP mediated apoptosis. After transfection with either scrambled siRNA (scRNA) or NGF-specific siRNA (NGFsiRNA) for 48 hr, bronchial epithelial cells were exposed to 10 µg/ml TiO<sub>2</sub>-NP, stained with annexin V-FITC and propidium iodide (PI), and analyzed by FACS. Each experiment included: unexposed cells treated with scRNA (A); unexposed cells treated with NGFsiRNA (B); cells transfected with scRNA before exposure to TiO<sub>2</sub>-NP (C); and cells transfected with NGFsiRNA before exposure to TiO<sub>2</sub>-NP (D). Data shown are the means ± SDs of 3 independent experiments.

\*p<0.05 compared to scRNA; ‡p<0.05 compared to scRNA + TiO<sub>2</sub>-NP.

**Table 1**Physical properties of nano and fine TiO<sub>2</sub> particles

| Description                               | Nano particles Titanium Oxide P <sub>25</sub> | Fine particles Titanium[IV] oxide |
|---|---|-----------------------------------|
| Purity                                    | 99%   | 99.9%                             |
| Primary particle diameter                 | 21 nm   | <5 μm                             |
| Specific surface area (m <sup>2</sup> /g) | 50 ± 15                                       | 2–3                               |
| Particle form (crystal phase)             | 80/20 anatase/rutile                          | rutile                            |
| Average size in PBS (μm)                  | 2.6 ± 0.3 (2.3–3.16)                          | 1.0 ± 2.4(0.8–6.2)                |
| Average size in medium (μm)               | 0.7 ± 0.1 (0.4–0.9)                           | 1.2 ± 0.1 (0.9–1.5)               |
| Suspension in medium                      | 100%  | 100%                              |

Physical properties provided by nanomaterial manufacturers.

Specific surface area measured with the Brunauer-Emmett-Teller (BET) method.

Particles suspended in PBS or cell culture medium by sonication.

Agglomerate structure diameter measured by dynamic light scattering (DLS).

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