Nanosize Titanium Dioxide Stimulates Reactive Oxygen Species in Brain Microglia and Damages Neurons in Vitro

Thomas C. Long,1 Julianne Tajuba,1 Preethi Sama,1 Navid Saleh,2 Carol Swartz,1 Joel Parker,3 Susan Hester,4 Gregory V. Lowry,2 and Bellina Veronesi4

1Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, North Carolina, USA; 2Department of Civil and Environmental Engineering, Carnegie Mellon University, Pittsburgh, Pennsylvania, USA; 3Constella Inc., Research Triangle Park, North Carolina, USA; 4National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina, USA

The increased use of engineered nanoparticles in medical, agricultural, industrial, manufacturing, and military sectors Nanosize titanium dioxide is used in a variety of consumer products (e.g., toothpastes, sunscreens, cosmetics, food products) (Kaida et al. 2004), paints and surface coatings (Fisher and Egerton 2001) and in the environmental decontamination of air, soil, and water (Choi et al. 2006; Esterkin et al. 2005). Such widespread use and its potential entry though dermal, ingestion, and inhalation routes suggest that nanosize TiO2 could pose an exposure risk to humans, livestock, and eco-relevant species. Recent studies indicate that TiO2 is toxic to eco-relevant species (i.e., Escherichia coli, daphnia) (Adams et al. 2006) and mammals (Warheit et al. 2006, 2007). Numerous in vitro studies have reported OS-mediated toxicity in various cell types (Afaq et al. 1998; Beck-Speier et al. 2001; Gurr et al. 2005; Sayes et al. 2006; Wang et al. 2007b; Zhang and Sun 2004). However, the response of nerve cells to nanosize TiO2 has not been investigated in vitro or in vivo, except for a companion study (Long et al. 2006).

Because of their size and unusual properties, nanoparticles can enter the body and cross biological barriers relatively unimpeded. Several studies have reported that inhaled or injected nanosize particles enter systemic circulation and migrate to various organs and tissues (Kreyling et al. 2002; Takenaka et al. 2001) where they could accumulate and damage organ systems that are especially sensitive to oxidative stress (OS). The brain is one such organ, being highly vulnerable to OS because of its energy demands, low levels of endogenous scavengers (e.g., vitamin C, catalase, superoxide dismutase) and high cellular concentration of OS targets (i.e., lipids, nucleic acids, and proteins). Recent experimental studies indicate that nanoparticles can cross the blood–brain barrier (Lockman et al. 2004) and enter (in low numbers) the central nervous system (CNS) of exposed animals (Kreyling et al. 2002; Oberdörster et al. 2004).

In the brain, OS damage is mediated by the microglia, a macrophage-like, phagocytic cell that is normally inactive unless confronted by potentially damaging xenobiotics. Their immediate and characteristic response (i.e., oxidative bursts) to foreign stimuli involves cytoplasmic engulfment (i.e., phagocytosis), an increase in metabolic activity, and a change in cell shape, size and proliferation (Block et al. 2007). The NADPH-oxidase driven oxidative burst can be monitored by the immediate production and release of superoxide anions (O2•−) that convert to multiple ROS such as hydrogen peroxide (H2O2), hydroxyl radicals, and peroxyoxinitrites. The excess O2•− arising from the oxidative burst can diffuse from the microglial plasma membrane and damage the proteins, lipids, and DNA of neighboring cells, especially neurons. Current thinking indicates that microglial-generated ROS underlie neurodegeneration (Block et al. 2007). Although the oxidative burst is the major source of OS in the activated microglia, O2•− is also generated as a by-product of normal mitochondrial energy production (i.e., bioenergetics). This results from the inefficient transfer of electrons along the electron transport chain (ETC) (Fariss et al. 2005). The levels of O2•− generated from the ETC are relatively low and efficiently neutralized by matrix-located antioxidant enzyme systems (i.e., endogenous scavengers). However, the levels of ETC-generated O2•− can increase significantly if one or more of the enzymatic complexes in the ETC is inhibited.

To examine the possible neurotoxicity of TiO2, nerve cells critical to the pathophysiology of neurodegeneration (i.e., microglia, neurons) were exposed to a commercially available

Address correspondence to B. Veronesi, U.S. EPA, NHEERL, NTD B105-06, 109 T.W. Alexander Dr., Research Triangle Park, NC 27711 USA. Telephone: (919) 541-5780. Fax: (919) 541-4849. E-mail: veronesi.bellina@epa.gov

The authors acknowledge the kind gift of P25 from Degussa Corp. (Frankfurt/Main, Germany) and also thank J. Hong of the National Institute of Environmental Health Sciences, Research Triangle Park, NC, for his gift of the BV2 and N27 cell lines. The authors also acknowledge the expert preparation of electron microscopy samples by the Microscopy Services Laboratory, University of North Carolina, Chapel Hill, North Carolina.

The technical support of T.C. Long was supported by U.S. EPA Professional Services Contract X3C247. T.L. was supported under U.S. EPA service contract X4247.

This document has been reviewed by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute the endorsement of recommendation for use. The authors declare they have no competing financial interests.

Received 2 March 2007; accepted 3 August 2007.

1631
nanomaterial, Degussa P25. This material is an uncoated photo-active, largely anatase form of nanosize TiO₂, not to be confused with the nonphotoactive nanomaterial currently used in sun blocks and cosmetics. P25 is a widely distributed material used for water treatment, self-cleaning windows, and antimicrobial coatings and paints. The BV2 microglia is an immortalized mouse cell line that responds to pharmacological agents, particulates, and environmental chemicals with characteristic signs of OS (Block et al. 2004; Wu et al. 2005). Its biochemical, morphological and genomic response to P25 exposure was examined in the present study. Because certain neuronal populations [such as dopaminergic (DA) neurons found in the brain striatum] are especially vulnerable to OS (Mattson 2001), the neurotoxicity of P25 was studied in the N27, an immortalized rat DA neuronal cell line (Zhou et al. 2000) and complex CNS cultures of embryonic rat striatum, which contains high numbers of DA neurons (Maier et al. 1994). Throughout the study, the physicochemical properties of P25 were described under exposure conditions that paralleled the biological response of these cells.

**Methods**

**Physicochemical characterization.** Commercial grade, uncoated nanosize Degussa P25 is a mixture of the anatase (70%) and rutile (30%) forms of TiO₂. Anatase is the preferred form for use in catalysis because of its enhanced redox activity (Shi et al. 2007). Several physicochemical properties of nanosize particles such as zeta potential (i.e., surface charge) and particle aggregate size (Wiesner et al. 2006; Xia et al. 2006) have been associated with toxicity (Wiesner et al. 2006; Xia et al. 2006). A companion study measured the effect of P25 concentration on aggregate size in physiological buffer and culture media (Long et al. 2006). In the current study, the aggregate size and zeta potential of P25 at a median concentration (20 ppm) is studied under conditions (vehicle, time point, temperature) that parallel the biological response. Physicochemical properties of P25 (20 ppm) were measured in Hanks balanced salt solution (HBSS) at 25°C over a 120-min period to parallel the exposure parameters of ROS release in microglia. Measures were also taken in low serum (1%) and culture media (RPMI 1640) at 37°C over 48 hr to parallel the neurotoxic response of N27 neurons. A Zeta Sizer Nano ZS (Malvern, Inc., Southborough, MA) was used to measure the hydrodynamic diameter (size) of P25 using the intensity-averaged distribution and the electrophoretic mobility of P25 was used to calculate its zeta potential using the Helmholtz-Smoluchowski equation.

**Cell culture.** Immortalized mouse BV2 microglia and rat N27 mesencephalic neurons were grown, respectively, in Dulbecco’s modified Eagle’s medium (DMEM) or RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (ATCC, Manassas, VA). Neurotoxicity studies used low (1%) serum RPMI 1640 exposure media. Tissue plugs of embryonic rat (Sprague-Dawley) brain striatum were purchased (BrainBits LLC, Springfield, IL; http://www.brainbitsllc.com) and upon receipt were triturated and plated on polystyrene-lyso-separated 96-well plates (Nalge Nunc International, Rochester, NY) in Neurobasal/B27 media (Invitrogen, Carlsbad, CA).

**Assays.** Fluorescent and chemiluminescent probes were chosen to measure the changes resulting from the oxidative burst and interference with mitochondrial ETC (Invitrogen Corp. 2005). The immediate production of intracellular H₂O₂ generated from the oxidative burst was measured in BV2 microglia with Image-it LIVE Green a dichlorohydrofluorescein diacetate-based compound that reacts with intracellular esterases and fluorresces in the presence of ROS. The production of O₂⁻ resulting from interference with the mitochondria’s ETC was measured with MitoSOX Red. The viability and cytotoxicity of neuronal (N27) cultures were monitored using a luciferase-based, chemiluminescence assay that measures intracellular levels of ATP (CellTiter-Glo; Promega, Inc., Madison, WI). Increases of caspase activity, an index of apoptotic entry was measured in both BV2 microglia and N27 neurons with Caspase-Glo 3/7 (Promega, Inc.). Loss of nuclear material (i.e., cytotoxicity) was measured with Hoechst 33342 (Invitrogen), a fluorescent probe that binds to adenine-thymine-rich regions of double-stranded nuclear DNA and indicates apoptotic loss of nuclear material (Oancea et al. 2006). All fluorescent probes were purchased from Molecular Probes (Eugene, OR) except for the chemiluminescent assays, CellTiter-Glo and Caspase-Glo 3/7.

For exposures, P25 (2.5–120 ppm) was ultrasonicated (~ 1 min) in 10× stock concentrations in either HBSS or low serum exposure media. For ROS measurements, cells were exposed to the fluorescent probe (i.e., “loaded”) and washed with HBSS to remove any extracellular probe from the cell’s external environment.

**Genomics and bioinformatics.** BV2 microglia were exposed (n = 5 wells/treatment) in 6-well plates to P25 (20 ppm) for 3 hr. Total RNA was extracted using TRIzol reagent (Invitrogen), purified, and its concentration determined using a NanoDrop ND-1000 Spectro-photometer (NanoDrop Technologies, Wilmington, DE). Large-scale gene analysis was performed by Expression Analysis (Durham, NC) using the Affymetrix Mouse Genome 430 2.0 GeneChips oligonucleotide array (Affymetrix, Santa Clara, CA) that measures approximately 39,000 transcripts. Target was prepared according to protocols outlined in the Affymetrix Technical Manual (Affymetrix Inc. 2004).

**Data analysis.** Affymetrix CEL files were analyzed using GC-robust multivariate (Wu et al. 2004) for array normalization and estimation of probe set intensities. Significance analysis of microarrays (SAM) (Tusher et al. 2001) was used to identify genes differentially expressed between P25-treated samples and the media control. Significantly different up- and down-regulated genes were analyzed by Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA; http://ingenuity.com/index.html) to determine p-values associated with Core canonical (metabolic and signaling) pathways and Tox Solution, which identifies relevant toxicity phenotypes and clinical pathology end points. Probesets that related to OS genes were analyzed separately using an IPA master list. In the graphic depiction of these analyses. The ratio of list genes to pathway genes is presented along with the Fisher exact test p-value. Pathways above a p-value threshold of 0.1 were discarded.

**Immunohistochemistry (IHC) and morphometry.** Cultures were fixed for 30 min in 3.7% paraformaldehyde, blocked with a mixture of 1% BSA, 0.4% Triton X-100, and 4% normal horse serum (20 min at room temperature, RT), and incubated in a 1:200 dilution of monoclonal mouse anti-human, neuron-specific enolase (NSE) for 30 min at RT (Dako Inc., Ft. Collins, CO). Visualization with streptavidin followed protocol of the LSAB 2 System-HP kit from Dako. IHC stained striatal cultures were analyzed morphometrically for neuronal loss. Six (10×) photographs of each well (n = 3/treatment) were taken using a Nikon TE300 inverted microscope and a cooled-frame CCD camera (Orca 1; Hamamatsu Photonics, Hamamatsu City, Japan). Each digitized image was analyzed using MetaMorph 7.0 software (Molecular Devices, Sunnyvale, CA). Populations of control, NSE-stained neurons were “binned” according to size and shape parameters using the integrated morphometric analysis mode. The total area of NSE-stained figures (cell bodies with attached axons) that fell within these parameters was calculated and compared with cultures treated with P25 (5 ppm; 6–48 hr).

Data were collected in Excel 2003 (Microsoft Corp., Redmond, WA) and transferred to GraphPad Prism 5 for graphing of the histogram (Graphpad Software, Inc., San Diego, CA; www.graphpad.com).

**Light (LM) and transmission electron microscopy (TEM).** For TEM examination, cells were exposed in 6-well plates to P25 particles (20 ppm) for 3 hr. After exposure, cells
were washed in warm HBSS to remove all noninternalized particles and fixed overnight in cold 2.5% cacodylate-buffered glutaraldehyde (Poly Scientific, Bayside NY). Cells were processed for TEM using standard procedures (Phillips 1998) and examined with a Zeiss LEO electron microscope (Carl Zeiss SMT Inc., Peabody, MA). LM preparations were examined as toluidine blue stained 1-µm epoxy sections or in unstained glutaraldehyde-fixed samples. Both types of LM samples were photographed with a Nikon TE300 inverted microscope.

Statistics. Spectrophotometric data were collected using SoftMax Pro 4.8 software (Molecular Devices). Graphing and statistics were done using Excel 2003 or GraphPad Prism 5. The mean response value (n = 6) of each concentration treatment was calculated. Data from several time intervals were normalized to show a time-course response. Data were analyzed using a one-way analysis of variance (ANOVA) with Dunnett’s test to determine significance (*p < 0.05) relative to its unexposed control.

Results

BV2 (ROS). Measures of H2O2, released from both the oxidative burst and inhibition of the ETC were collected. BV2 microglia responded to P25 at ≥ 60 ppm with a rapid (1–5 min) release of H2O2 as measured with Image-iT LIVE Green (Figure 1A). Significant release of O2•− as measured by MitoSOX Red first occurred at 30 min in response to ≥ 100 ppm P25 and only responded to concentrations ≥ 60 ppm after 70-min exposure (Figure 1B). Significant increases in caspase 3/7 activity, which signal the cell’s entry into apoptosis (Fariss et al. 2005), were first measured at 6 hr which signal the cell’s entry into apoptosis. Significant increases in caspase 3/7 activity were first seen by 6 hr in response to ≥ 40 ppm P25 and remained at this level for 24 hr. The production of O2•− resulting from interference with the mitochondria’s ETC was measured with MitoSOX Red. BV2 microglia, incubated in 2 µM (10 min, 37°C) showed a delayed but significant increase in fluorescence after 30-min exposure to ≥ 100 ppm P25. (C) Significant increases of caspase 3/7 activity were first seen by 6 hr in response to ≥ 40 ppm P25 and remained at this level for 24 hr. (D) Apoptotic loss of nuclear material, as measured with Hoechst stain, was first noted after 24 hr in response to P25 (≥ 100 ppm) and involved all concentrations by 48 hr.

Figure 1. (A) The immediate production of intracellular H2O2 generated from the oxidative burst was measured in BV2 microglia with Image-iT LIVE Green. Cells were incubated (30 min, 37°C) in 25 µM Image-iT LIVE Green and exposed to P25. Significant increases of fluorescence first occurred in response to P25 (≥ 60 ppm; ≥ 1 min). (B) The production of O2•− resulting from interference with the mitochondria’s ETC was measured with MitoSOX RED. BV2 microglia, incubated in 2 µM (10 min, 37°C) showed a delayed but significant increase in fluorescence after 30-min exposure to ≥ 100 ppm P25. (C) Significant increases of caspase 3/7 activity were first seen by 6 hr in response to ≥ 40 ppm P25 and remained at this level for 24 hr. (D) Apoptotic loss of nuclear material, as measured with Hoechst stain, was first noted after 24 hr in response to P25 (≥ 100 ppm) and involved all concentrations by 48 hr.

Figure 2. BV2 microglia exposed to P25 (20 ppm) were examined with both LM and TEM. (A) The toluidine blue stained cytoplasm of BV2 microglia housed numerous, light-refractive P25 aggregates after 3-hr exposure. (B) LM examination of unstained, fixed cells exposed to P25 for 48 hr indicated that the cellular membranes were fragmented and showed granular cytoplasm and centralized nuclei. Magnification × 1,200. (C) TEM examination of the P25 exposed BV2 microglia indicate phagocytic internalization of the P25 aggregates after 3 hr. (D) Higher magnification of the BV2 microglial cytoplasm indicated swollen and disrupted mitochondria (circles) in proximity to the P25 aggregates.
gene function or pathway. The wavy line on the
lower-axis represents the ratio or percent of
P25-affected genes relative to a particular
IPA pathway.

Core canonical analysis (Figure 3A) indicated
that P25 up-regulated genes were clus-
tered around signaling pathways involved
with B-cell receptor (gene transcription in the
immune response), the Death receptor
tumor necrosis factor receptor family; apop-
totic initiating pathways; caspase activation),
apoptosis, calcium, and inflammation
[death receptor families (i.e., caspase activa-
tion)]. Toxicity analy-
factors for cell proliferation, differentia-
tion, migration, survival, and fate). Toxicity
alysis (Figure 3B) indicated a strong pathway
association with pathways associated with
inflammation (NF-κB), cell cycling, oxidative
stress (peroxisomes) and pro-apoptotic activi-
ties. P25’s down-regulated genes (Figure 4A)
were associated with adaptive change (e.g.,
B-cell receptor, ERK/MAPK) and energy pro-
duction (glycolysis, gluconeogenesis, oxidative
phosphorylation). Toxicity analysis indicated
that down-regulated genes were associated
with pathways triggered by response to low
oxygen availability (i.e., hypoxia-inducible
factor), peroxisomes, and Nrf2-mediated OS
(Figure 4B). Canonical analysis of the P25
affected genes associated with OS indicated
that the majority clustered around key energy
pathways involving oxidative phosphoryla-
tion, biosynthesis of ubiquinone (involved in
shuttling electrons in the ETC) and the citric
acid cycle (Figure 5A). Toxicity Pathway
analysis indicated that these pathways were
associated with mitochondrial dysfunction
(Figure 5B).

**Neurotoxicity.** The direct (in the absence
of microglia) neurotoxicity of P25 and that
mediated by microglia-generated ROS was
addressed, respectively, in isolated rat DA
neurons (N27) and in primary cultures of rat
striatum. P25 increased intracellular levels of
ATP in N27 beginning at 1 hr (≥ 80 ppm)
and continued over 48 hr (≥ 40 ppm)
(Figure 6A). Caspase 3/7 activity, an indicator
of apoptosis, significantly increased at both
24- and 48-hr exposure (≥ 40 ppm)
(Figure 6B). Apoptotic damage to isolated
N27 neurons, using Hoechst 33342 nuclear
stain, was not seen even after a 72-hr exposure
to P25 (2.5–120 ppm) (Figure 6C).

Ultrastructurally, both nanosized and
large aggregates of P25 were seen in the N27
cytosplasm after 3-hr exposure to P25
(20 ppm). P25 aggregates were randomly
located throughout the neuronal cytoplasm
(Figure 6D) and appeared to be encased in

---

**Figure 3.** BV2 microglia were exposed to P25 (20 ppm) for 3 hr and prepared for
microarray analysis. IL-4, interleukin 4; PPARα, peroxisome proliferator-activated
receptor α; TR/RXR, thyroid hormone receptor/retinoid X receptor. (A) IPA’s Core analysis
(metabolic/signaling pathways) indicated that up-regulated genes were clustered around
pathways involving with apoptosis, Death receptor families (i.e., caspase activation),
calcium signaling, inflammation (NF-κB), and cell cycling and maintenance. (B) Toxicity
Pathway analysis indicated that P25 up-regulated pathways were primarily associated
with inflammatory (NF-κB), cell cycling and pro-apoptotic activities.

**Figure 4.** BV2 microglia were exposed to P25 (20 ppm) for 3 hr. IL-6, interleukin 6,
PPARα, peroxisome proliferator-activated receptor α; TR/RXR, thyroid hormone
receptor/retinoid X receptor. (A) Core analysis of the down-regulated genes showed clustering around pathways associated with adaptive change and key energy production pathways. (B) Toxicity Pathway analysis indicated that P25 down-regulated genes in pathways associated with hypoxia, peroxi-
somes, and Nrf2-mediated oxidative stress.
membrane-bound vacuoles (Figure 6D, inset). No evidence of phagocytosis or pinocytosis (i.e., elaboration of pseudopodia) was observed, suggesting that the particles impacted the cell body by sedimentation. In contrast to the disrupted organelles noted above in BV2 microglia, mitochondria appeared ultrastructurally normal in the N27 neurons, in spite of their close proximity to P25 aggregates.

IHC stained cultures of rat brain striatum, exposed to P25 (5 ppm) were photographed (Figure 7A–D) and analyzed morphometrically. Results indicated that the total area of NSE-stained neurons were reduced by 14% after 6-hr exposure and 19% after 24-hr exposure (Figure 8A,B).

Physicochemistry. The aggregate size and zeta potential of P25 (20 ppm) were measured in relevant exposure vehicles (HBSS, RPMI) at time points that paralleled the biological response (Figure 9A,B). In HBSS, the hydrodynamic diameter of P25 aggregates ranged from 800 to 1,900 nm (30 min) and decreased to 770 nm (2 hr) as the larger aggregates settled from solution. The zeta potential of P25 (20 ppm) in HBSS (pH 7.6) ranged from –9.78 to –13.8 mV after 2 hr (25°C) (Figure 9A). In low-serum RPMI exposure medium, P25 quickly aggregated but remained relatively stable in suspension (300–350 nm) over the 48-hr exposure period. The zeta potential ranged from –8.54 to –10.1 mV over 0–48 hr (37°C) in low-serum RPMI media (Figure 9B).

Discussion

The present data indicate that Degussa P25 stimulates BV2 microglia to release ROS and affects genomic pathways associated with cell cycling, inflammation, apoptosis and mitochondrial bioenergetics. Although, several adaptive pathways (neuregulin ERK/MAP kinase) (Pagès et al. 1999) were differentially affected, the ubiquinone, biosynthetic pathway which functions as an electron carrier in the mitochondrial ETC and also acts as antioxidant (Artuch et al. 1999), and mitochondrial bioenergetics pathways involving oxidative phosphorylation, glycolysis, etc. were severely depressed which would create levels of ROS and ultimately OS in the cell (Fariss et al. 2005). P25 appeared to be nontoxic to isolated DA neurons (N27) even after 72 hr. However, when examined in primary cultures of brain striatum which contain microglia, neuronal loss occurred by 6 hr in response to only 5 ppm. This shift in dose-response, coupled with cellular and genomic evidence of P25’s effect on inflammatory and apoptotic pathways and disruption of energy pathways in BV2 microglia, suggest that the potent neurotoxicity of P25 seen in complex cultures was mediated though microglia-generated ROS. The microglia’s release of H₂O₂ from the oxidative burst and ETC, if excessive, can activate caspase 8 and its downstream effectors caspase 3/7, inducing apoptosis though extrinsic cell death pathways (De Giorgi et al. 2002). Stimulation of mitochondrial apoptotic pathways (e.g., caspase 3/7) was noted biochemically and genomically in BV2 microglia, and apoptotic morphology was shown in both isolated BV2 microglia and in cultures of striatum. These data indicate that OS-mediated apoptosis played a signature role in P25 neurotoxicity.

Ultrastructurally, the phagocytosis of P25 aggregates by BV2 microglia and the strong association of mitochondrial disruption with these aggregates have been previously

Figure 5. BV2 microglia were exposed to P25 (20 ppm) for 3 hr. (A) Canonical analysis of all P25 affected genes associated with OS indicated that they largely clustered around key energy pathways involving oxidative phosphorylation, biosynthesis of ubiquinone (involved in shuttling electrons in the ETC) and the citric acid cycle. (B) Toxicity Pathway analysis localized these pathways further to mitochondrial dysfunction.

Figure 6. (A) N27 neurons were exposed (1–72 hr) to P25 (2.5–120 ppm) and intracellular ATP levels measured with CellTiter-Glo. Significant increases were seen as early as 1 hr postexposure to ≥ 80 ppm and continued until 48 hr in response to ≥ 40 ppm. (B) Significant increases (p < 0.05) in caspase 3/7 activity were first seen in N27 neurons after 24 hr in response to ≥ 40 ppm P25. (C) Significant reductions of Hoechst stain did not occur in response to P25 (2.5–120 ppm) at any time point. (D) TEM of P25 (20 ppm, 3 hr) treated N27 neurons showed numerous membrane-bound aggregates. An amorphous substance was seen within the vacuoles (insert). In addition, individual nanosize P25 particles (circle) were noted throughout the cytoplasm. Mitochondria in nearby proximity showed no evidence of disruption or swelling.
reported (Long et al. 2006). Fractal aggregates can maintain the large surface area, sharp crystallite edges, and other characteristics of individual nanoparticles (Phenrat et al. 2007). Membrane-bound P25 aggregates were also seen within the N27 cytoplasm. However, no morphological evidence of phagocytosis, pinocytosis or endocytosis was noted. Because of this, the possibility that P25 aggregates sedimented from the exposure medium onto the cells and became incorporated into cytoplasmic lysosomes cannot be excluded. Nanosize particles were also documented laying free in the neuronal cytoplasm. The manner by which such nanoparticles particles enter the cell cytoplasm is still a matter of discussion and is thought to involve mechanisms distinct from phagocytosis and endocytosis (Rothen-Rutishauser et al. 2006).

The biological interactions of nanoparticles are associated with physical properties such as surface area, particle shape, zeta potential, and aggregate size (Wiesner et al. 2006). For valid interpretation of nanotoxicity data, these properties must be determined under conditions that parallel the biological exposures. Our data indicated that the exposure conditions (i.e., vehicle, temperature) significantly modified P25’s particle size and zeta potential which could affect its interaction with biological systems and its ultimate toxicity. Particle (or aggregate) size determines if a particle enters the cellular environment through ROS-producing phagocytosis, through endocytosis, or some undefined mechanisms (Champion and Mitragotri 2006; Sabokbar et al. 2003). The surface charge or zeta potential of a particle affects its aggregation in solution and its behavior in an electric or ionic field. The surface charge of a particle also determines its interactions with specific biological receptors. Polymodal receptors located in the cellular membrane of microglia and macrophages (e.g., TRPV1, Mac-1) are sensitive to protons (i.e., charge) or repeating patterns of charge (Block et al. 2007; Husemann et al. 2002; Reilly et al. 2006) like those found on crystalline metal oxide nanoparticles. The activation of these receptors triggers various signal transduction pathways that determine the cell’s ultimate fate. Scavenger receptors have been implicated in mediating the cytotoxicity of alveolar macrophages exposed to TiO2 (Kim et al. 1999). Studies have also shown that TRPV1 receptors located on rat primary microglia stimulate OS-sensitive neurons in cultures of brain striatum.

In summary, this study describes the in vitro neurotoxicity of a widely used nano-material, P25. This material appears to be non-toxic to isolated N27 neurons but stimulates BV2 microglia to produce ROS and damages OS-sensitive neurons in cultures of brain striatum.

Figure 7. LM histology of IHC rat embryonic striatum. Confluent cultures of embryonic were exposed to 5 ppm P25 for 6–48 hr. IHC stained with NSE and morphometrically analyzed. (A) Untreated cultures consisted of a dense plexus of neurons and glia. (B) Axonal beading and cellular granularity were seen as early as 6 hr postexposure. (C) Evidence of apoptosis (circles) was documented by 24 hr. (D) Complete disruption and loss of cellular integrity was noted by 48 hr postexposure to 5 ppm P25.

Figure 8. Morphometric analysis was conducted on NSE-stained cultures of mouse striatum. These data indicated that the total area of NSE-stained neurons was reduced by 14% after 6-hr exposure (A) and 19% after 24 hr (B) to P25 (5 ppm).

Figure 9. The aggregate size and zeta potential of P25 (20 ppm) were measured in both HBSS and RPMI at conditions that paralleled the biological responses. (A) P25 aggregates reached 1,900 nm in size over a 30-min measurement in HBSS (25°C) and maintained > 1,000 nm size for the 2-hr exposure period. The zeta potential of P25 (blue triangles) initially measured –9.8 mV and decreased slightly over the 2-hr period. (B) Both the aggregate size (black squares; 300–350 nm) and zeta potential (blue triangles) (~8 mV to ~10 mV) of P25 remained stable when measured in RPMI over the 48-hr period.
Afaq F, Abidi P, Matin R, Rahman Q. 1998. Cytotoxicity, pro-oxidant effects and antioxidant depletion in rat lung alveolar macrophages exposed to ultrafine titanium dioxide. J Appl Toxicol 18:307–312.

Affymetrix Inc. 2004. Affymetrix Technical Manual. Available: www.expressionanalysis.com (accessed 2 October 2007).

Artuch R, Colome C, Vlaseca MA, Pineda M, Campistol J. 1999. Ubiquinone: metabolism and functions. Ubiquinone deficiency and its implication in mitochondrial encephalopathies. Treatment with ubiquinone. Rev Neurol 293:69-75.

Beck-Speier I, Dayal N, Karg E, Maier KL, Roth C, Ziesenis A, et al. 2001. Agglomerates of ultrafine particles of elemental carbon and TiO2 induce generation of lipid mediators in alveolar macrophages. Environ Health Perspect 109(suppl 4):613-618.

Block ML, Wu X, Li G, Wang T, Qin L, et al. 2004. Nanometer size diesel exhaust particles are selectively toxic to dopaminergic neurons: the role of microglia, phagocytosis, and NADPH oxidase. FASEB J 18:1618–1620.

Block ML, Zecca L, Hong JS. 2007. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci 8:57-69.

Champion JA, Mitragotri S. 2006. Role of target geometry in phagocytosis. Proc Natl Acad Sci USA 103:4930–4934.

Choi H, Stathatos E, Dionysiou DD. 2006. Sol-gel preparation of mesoporous photocatalytic TiO2 films and TiO2/Al2O3 composite membranes for environmental applications. Appl Catal B:Environ 63:90–97.

De Giorgi F, Lartigue L, Bauer MK, Schubert A, Grimm S, Wiesner MR, Lowry GV, Alvarez P, Dionysiou D, Biswas P. 2004. Nano-particle surface charges after blood-brain barrier integrity and permeability. J Drug Target 12:625-641.

Long TC, Saleh N, Tilson RD, Lowry GV, Veronesi B. 2006. Titanium dioxide (TiO2) produces reactive oxygen species in immortalized brain microglia (BV2): implications for nanoparticle neurotoxicity. Environ Sci Technol 40:4346–4352.

Maier WE, Kodavanti PR, Harry GJ, Tilson HA. 1994. Sensitivity of adenosine triphosphatases in different brain regions to polychlorinated biphenyl congener. J Appl Toxicol 14:225–229.

Mattson MP. 2001. Mechanisms of neuronal apoptosis and excitotoxicity. In: Pathogenesis of Neurodegenerative Disorders (Mattson M, ed). Baltimore:Humana Press, 1–20.

Oancea M, Mazumder S, Crosby ME, Almasan A. 2006. Apoptosis assays. Methods Mol Med 129:279–290.

Oberdörster G, Sharp Z, Atudorei V, Elder A, Gelein R, Kreyling W, et al. 2004. Translocation of ultrafine insoluble iridium particles from lung epithelium to extrapulmonary organs is size dependent but very low. J Toxicol Environ Health A 58:437–450.

Oancea M, Mazumder S, Crosby ME, Almasan A. 2006. A model-based background adjustment for oligonucleotide expression arrays. J Am Stat Assoc 99:909–917.

Sabokbar A, Pandey R, Athanassou NA. 2003. The effect of particle size and electrical charge on macrophage-osteoclast differentiation and bone resorption. J Mater Sci Mater Med 14:731–738.

Sayes CM, Wahi R, Kupan PA, Liu Y, West JL, Auman KD, et al. 2006. Correlating nanoscale titanium structure with toxicity: a cytotoxicity and inflammatory response study with human dermal fibroblasts and human lung epithelial cells. Toxicol Sci 92:174–185.

Shi JY, Chen J, Feng ZG, Chen T, Lian YX, Wang XL, et al. 2007. Photoluminescence characteristics of TiO2 and their relationship to the photoassisted reaction of water/methanol mixture. J Phys Chem C 111:693–699.

Takenasa S, Karg E, Roth C, Schulz H, Ziesenis A, Heinzmann U, et al. 2001. Pulmonary and systemic distribution of inhaled ultrafine silver particles in rats. Environ Health Perspect 109:547–551.

Tusher VG, Tibshirani R, Chu G. 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 98:5116–5121.

Wang J, Zhou G, Chen C, Yu H, Wang T, Ma Y, et al. 2007a. Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. Toxicol Lett 168:176–185.

Wang J, Sanderson SJ, Wang H. 2007b. Cyto- and genotoxicity of ultrafine TiO2 particles in cultured human lymphoblastoid cells. Mutat Res 629:99–106.

Warheit DB, Webb TR, Reed KL, Frerichs S, Sayes CM. 2007. Pulmonary toxicity study in rats with three forms of ultrafine TiO2 particles: differential responses related to surfactant properties. Toxicology 230:90–104.

Warheit DB, Webb TR, Sayes CM, Colvin VL, Reed KL. 2006. Pulmonary instillation studies with nanoscale TiO2 rods and dots in rats: toxicity is not dependent upon particle size and surface area. Toxicol Sci 91:227–236.

Wixens MR, Lowry GV, Alvarez P, Dionysiou D, Biswas P. 2006. Assessing the risks of manufactured nanomaterials. Environ Sci Technol 40:4336–4345.

Wu X, Block ML, Zhang W, Qin L, Wilson B, Zhang WQ, et al. 2005. The role of microglia in paraglutam-induced dopaminergic neurotoxicity. Antioxid Redox Signal 7:654–661.

Wu X, Irizarry RA, Gentleman R, Martinez-Murillo F, Spencer F. 2004. A model-based background adjustment for oligonucleotide expression arrays. J Am Stat Assoc 99:909–917.

Xia T, Kovochich M, Brant J, Hotze M, Sempf J, Oberley T, et al. 2006. Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm. Nano Lett 6:1794–1807.

Zhang AP, Sun YP. 2004. Photocatalytic killing effect of TiO2 nanoparticles on L929-T4 human colon carcinoma cells. World J Gastroenterol 10:3191–3193.

Zhou W, Hurlburt MS, Schack J, Prasad KN, Freed CR. 2000. Overexpression of human alpha-synuclein causes dopamine neuron death in rat primary culture and immobilized mesencephalon-derived cells. Brain Res 866:33–43.

Husemann J, Loike JD, Anakov R, Febbraio M, Silverstein SC. 2002. Scavenger receptors in neurobiology and neuropathology: their role on microglia and other cells of the nervous system. Glia 40:195–205.