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Responses of human cells to ZnO nanoparticles: a gene transcription study[†]

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Abstract

The gene transcript profile responses to metal oxide nanoparticles was studied using human cell lines derived from the colon and skin tumors. Much of the research on nanoparticle toxicology has focused on models of inhalation and intact skin exposure, and effects of ingestion exposure and application to diseased skin are relatively unknown. Powders of nominally nanosized SiO₂, TiO₂, ZnO and Fe₂O₃ were chosen because these substances are widely used in consumer products. The four oxides were evaluated using colon-derived cell lines, RKO and CaCo-2, and ZnO and TiO₂ were evaluated further using skin-derived cell lines HaCaT and SK Mel-28. ZnO induced the most notable gene transcription changes, even though this material was applied at the lowest concentration. Nano-sized and conventional ZnO induced similar responses suggesting common mechanisms of action. The results showed neither a non-specific response pattern common to all substances nor synergy of the particles with TNF- α cotreatment. The response to ZnO was not consistent with a pronounced proinflammatory signature, but involved changes in metal metabolism, chaperonin proteins, and protein folding genes. This response was observed in all cell lines when ZnO was in contact with the human cells. When the cells were exposed to soluble Zn, the genes involved in metal metabolism were induced but the genes involved in protein refolding were unaffected. This provides some of the first data on the effects of commercial metal oxide nanoparticles on human colon-derived and skin-derived cells.

Introduction

The ongoing search for unique physical and chemical properties has motivated the development of numerous types of metal oxide nanomaterials, but concerns remain regarding biological effects of particles comparable in size to ultrafine air pollution ($d < 100$ nm). Much of the nanoparticle toxicological research has been on inhalation and intact-skin dermal exposures; however, there is also exposure and potential toxicity in the digestive tract and through damaged skin and other tissue epithelial barriers. Ingestion exposure can come from nano-sized materials used as food and drug ingredients, from hand-to-mouth contact, and through drinking water contamination. Dermal exposure can occur from nano-sized materials in sunscreens and cosmetics.

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[†]Electronic Supplementary Information (ESI) available: The significant results for each assessment of the gene expression experiments, including the annotation, statistical information, and the expression data, as well as the annotation analysis (EASE) for each assessment is provided in an Excel workbook. Additional data and methods are included in a supplemental document. See DOI: 10.1039/b000000x/

Numerous hypotheses have been proposed for potential toxicological responses induced by nanoparticle exposure including both chemical-specific effects of particle core composition or surface chemistry, and non-specific effects associated with the small size and high specific surface area of nano-sized particles.¹ Mechanistic hypotheses include particle-induced pro-inflammatory signaling responses, oxidative signaling responses, non-specific effects that are dependent primarily on available surface area and less on the chemical composition of the core substance, and size-dependent uptake of insoluble particles.²

There have been a number of genome-wide transcriptional studies of metal oxide nanoparticles. Nano-TiO₂ activates inflammatory response and cell adhesion, but not oxidative stress, genes in human keratinocyte cells.³ In mouse lungs nano-TiO₂ exacerbates LPS-induced inflammation, but does not alter the gene expression pattern.⁴ A comparison of 10- and 500-nm SiO₂ particles using RAW264.7 mouse macrophages showed high correlation in gene expression changes between the sizes, suggesting common genomic responses to nanosilica.⁵ Ultrafine NiO, but not C₆₀ fullerene particles, activated genes for chemokines, oxidative stress, and matrix metalloproteinase 12 in rat lungs.⁶ An inhalation comparison of multiwalled carbon nanotubes and α -quartz in rats revealed many interesting patterns such as expression of genes likely playing a role in fibrosis that were qualitatively similar for the two materials.⁷ The promotion of oxidative stress and inflammation are not restricted to engineered materials but have been associated with environmental nanoparticles. For example, environmental particulate like soot and diesel exhaust, are complex mixtures of organic and inorganic compounds, induce oxidative stress and inflammation pathways leading to disease.⁸

There have been a number of studies reporting preferential uptake of nanoparticles compared to larger particles of low-solubility substances. An ingestion study performed in rats showed that polystyrene particles smaller than 100 nm were taken up by the rat intestinal mucosa and enter systemic circulation.⁹ Indeed, inflamed colon cells internalize nanoparticles at a much greater rate than normal colon cells, and this has been exploited by studies using nanoparticles for drug delivery.¹⁰ Human epithelial cells take up and translocate nano-sized silica particles to the nucleus where the particles induced formation of aberrant clusters that inhibited replication, transcription, and cell proliferation.¹¹

The substances SiO₂, Fe₂O₃, and TiO₂ are all used in skin care and cosmetic formulations and are approved as food additives where they can be present at levels up to 1–2%, and current regulations do not restrict allowable particle size. ZnO is used in sunscreens, cosmetics, dietary supplements, and paints. Many sunscreens now contain nano-sized TiO₂ and ZnO, and there has been citizen concern about whether these nanomaterials pose different risks than the conventional forms of these oxides.¹² In addition to consumer uses, all these materials are widely used in industrial processes where occupational exposure and wastewater releases can occur. The U.S. Environmental Protection Agency has announced regulatory policies regarding certain nanomaterials, such as carbon nanotubes, as novel chemical entities, and is considering policies regarding nanoscale silver, TiO₂, and ZnO; however, most nanomaterials are still regulated only by their nominal chemical composition.

Given the limited data on the effects of nanoparticle ingestion we designed a screening study to evaluate gene regulation changes in colon cells treated with a suite of nanoparticles. The limitations of submerged cell culture using immortalized cell lines are acknowledged, but similar *in vitro* models are widely accepted for both preliminary screening and mechanistic studies. Based on the hypothesis that inflammation may increase the uptake and/or toxicity of ultrafine particles,¹³ we also evaluated the transcriptional responses to nanoparticulate with TNF- α pretreatment to mimic a pre-existing inflammatory state. Zinc oxide was found to be the most potent of tested nano-sized powders, so we extended the study to directly

compare gene transcription responses in colon cells exposed to ZnO sold as nano-sized and conventional powders. Next, we expanded these studies to evaluate ZnO and TiO₂-dependent alterations in gene expression on skin-derived cells and found that similar genes are altered in these cells and in the colon-derived cells. In our previous studies, we determined that commercial ZnO particulate required contact to promote cytotoxicity; therefore, we also evaluated gene expression changes dependent on soluble Zn. We determined that particulate ZnO promotes the induction of genes related to protein folding; these genes are not altered when exposed to soluble Zn, and therefore may represent early markers of ZnO-mediated toxicity.

Methods

Materials

TNF- α , Advanced DMEM, Glutamax, and Superscript III reagents were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Hyclone (Logan, UT). The RKO and SK Mel-28 cell lines were purchased from American Tissue Type Culture Collection (Manassas, VA). CaCo-2 cells were a gift from Dr. John G. Lamb (University of Utah). HaCaT cells were a gift of Dr. Doug Grossman (University of Utah). Antibodies directed against the gene products GADD45B (sc-8775), GAPDH (sc-25778), DNAJA4 (sc-100714), HMOX1 (sc1797), and secondary anti-goat, -mouse, and -rabbit antibodies were purchased from Santa Cruz Biotechnology; HSPA1A (C29F3A-5) was purchased from Stressgen; ZFP36 (ab36558) was purchased from Abcam. RNeasy Minikits were purchased from Qiagen (Valencia, CA). Cell Counting Kit-8 (CCK-8) was from Dojindo (Gaithersburg, MD). Lightcycler 480 SYBR Green I master mix was purchased from Roche Diagnostics (Indianapolis, IN). Reagent samples of anatase TiO₂, and amorphous SiO₂ were obtained from Nanostructured and Amorphous Materials, Inc. (Houston, TX), hematite Fe₂O₃, and nominally nanosized and conventional ZnO were obtained from Alpha Aesar (Ward Hill, MA).

Characterization of the particulate matter

The particle surface area per mass was measured by nitrogen adsorption (BET single point method) using a Quantachrome Monosorb analyzer. The surface-equivalent or surface mean diameter was calculated from the measured area assuming spherical particles and the appropriate mineral density. The geometric size of the particulate matter was verified by transmission electron microscopy. Briefly, samples of the powders were suspended at approximately 0.1 mg/ml in water containing 1% of FL-70 surfactant (Fisher Scientific), vortexed, and then dispersed using an ultrasonic tissue disruptor for 1 minute. A 1 μ l drop of the suspension was placed on a carbon grid, air dried, and examined on a Tecnai G2 transmission electron microscope at 120 kV.

Dynamic light scattering (DLS) was used to measure the size distribution of the agglomerates formed when the particles were added to either water or cell culture media. Suspensions were prepared at 0.1 mg/ml in water (for zeta potential and DLS measurement), or at 300 μ g/ml of particles in Advanced DMEM with Glutamax and 2% serum. The media-based DLS particle samples were prepared in the same manner as used for the cell treatments, below, and the tested suspension concentration corresponds to the 100 μ g/cm² cell exposures.

Soluble elements were measured by suspending 1 mg/ml of particles in Advanced DMEM culture media and incubating overnight at 37°C without cells. Composition was determined by ion coupled plasma spectrometry on a Perkin Elmer Optima 3100 XL.

Cell culture

All cells were maintained in Advanced DMEM supplemented with 1% Glutamax and 2% fetal bovine serum so that alterations in gene expression among the cell lines would not be due to distinct media. For experiments that used particulate matter, the nanomaterial was sterilized using 70% EtOH and vacuum drying, and then added to medium, vortexed, and sonicated for 15 min in a Cole-Parmer 8890 water bath sonicator to disperse the particulate matter. For experiments with TNF- α stimulation, cells were pretreated by incubation with 100 ng/ml TNF- α to create a drug-induced inflammatory state or as a control, respectively, for 1 hr, then the medium was exchanged with medium containing the particulate matter. For electron microscopy of the RKO cells with the ZnO and TiO₂ nanomaterials, cells were seeded on a plastic surface, treated with the nanomaterials, and then fixed, stained and sectioned for transmission electron microscopy using standard methods used at the University of Utah electron microscopy core.

Viability assay

Cellular viability was determined using a Cell Counting Kit-8 (CCK-8) which relies on tetrazolium salt reduction by NADH in viable cells.¹⁴ Briefly, cells were seeded into 48 well plates at 4×10^4 cells/well and allowed to recover overnight. Nanoparticles were evaluated at concentrations ranging from 1–100 $\mu\text{g}/\text{cm}^2$. Cells were treated as described above (*Cell culture*) and assessed for viability after 24 hrs. Following the treatment period, medium was aspirated and replaced with 4% CCK-8 in cell culture medium. Absorbance at 460 nm and 650 nm was measured after incubation at 37°C until the reagent developed sufficiently for maximal reading using a Perkin-Elmer VictorV³ Multimode Microplate Reader.

Microarray expression analysis

Cells were incubated as described above (*Cell culture*) and total RNA was collected after 4 hrs exposure to the particulate matter. For the expression analysis, we used 50 $\mu\text{g}/\text{cm}^2$ of SiO₂, TiO₂, 5 $\mu\text{g}/\text{cm}^2$ of ZnO (due to high toxicity) and 10 $\mu\text{g}/\text{cm}^2$ of Fe₂O₃ (due to interference with RNA collection at higher particle concentration) in the colon cancer cell lines; in the skin-derived cell lines, 1 or 5 $\mu\text{g}/\text{cm}^2$ of ZnO was used, and 5 or 10 $\mu\text{g}/\text{cm}^2$ of TiO₂. 100 μM ZnCl₂ was used for the soluble Zn treatment. RNA was collected and prepared for expression analysis from cells treated for 4 hrs. RNA concentration was determined with a Nanodrop spectrophotometer and sufficient total RNA was recovered using the Qiagen RNeasy minikit protocol for the analysis. The quality of the RNA was monitored using an Experion automated electrophoresis station (BioRad) with standard sensitivity RNA chips, all samples displayed RNA Quality Index (RQI) levels greater than 8. Agilent labeling kits were utilized to amplify and generate Cy-dye labeled cRNA for hybridization to Agilent oligonucleotide arrays. A minimum of four biological replicates were collected representing each condition, and samples were combined, in equal fractions, for the microarray analysis. For most sample types, each of the four replicates were mixed together as the input sample for the expression analysis. An exception was the TNF- α pretreatment samples where composed of two sets of three biological replicates were combined so that replicates of this condition would be represented by more than a single sample in the expression analysis.

Agilent 44K (human whole genome) oligonucleotide microarrays were processed on site in the Microarray Resource located within the Huntsman Cancer Institute. Transcript levels were assessed on each channel and quantified by Agilent Feature Extraction software. This software preprocesses the data as follows. Local background is subtracted, irregular spots flagged, and global linear regression (lowess) normalization is performed, and this ratio is log transformed. We import the data into TIGR MEV 3.1 software for further analysis. Generally, we use a supervised strategy to identify the genes with the greatest

significant differences between stimulated cells to unstimulated cells using multiclass SAM.¹⁵ In each SAM analysis, 500 iterations were used when evaluating the false discovery rate, and a conservative cutoff was used that gave a median false discovery rate of 0%. The gene expression profiles were hierarchically clustered for visualization. Gene Ontology analysis of the differentially expressed genes was accomplished with EASE.¹⁶

Immunoblot analysis

Cells were grown in 6-well plates and treated with ZnO, ZnCl₂ or were untreated as the control. Media was aspirated and cells were then washed with 1 ml of cold 1× PBS and the PBS aspirated. Cell lysates were collected in 8 M urea, 10 mM EDTA, 0.1 M Tris, pH 8, and the samples were reduced with the addition of dithiothreitol to 100 mM prior to protein estimation. Protein concentrations were determined using Coomassie Plus Protein Reagent (Pierce). Absorbance at 595 nm was measured using a Perkin-Elmer Victor³V plate reader. Fifteen micrograms of protein were separated on 4–12% NuPAGE Bis-Tris gels and transferred to PVDF membranes, transferred to PVDF membrane, blocked with 5% non-fat dry milk, incubated with primary antibody (1:200 for DNAJA4, GADD45B, HSPA1A, HMOX1, and ZFP36, and 1:400 for GAPDH) overnight at 4°C, washed 3×, incubated with secondary antibody (1:5000) for 45 min at 22°C, washed 3×, incubated with chemiluminescence reagents (SuperSignal West Dura, Thermo Scientific), and visualized on a Kodak ImageStation.

Results

Particle characterization

Documentation of particle characteristics is essential when reporting the toxicology of nanomaterials. Particle characteristics of the materials used in this study are summarized in Table 1. The TiO₂, SiO₂, and Fe₂O₃ nanomaterials were all over 60 m²/g by nitrogen absorption surface area measurement and had zeta potentials less than −40 mV. The ZnO nanoparticulate had specific surface of 24 m²/g and a zeta potential of −54 mV. Examination of the particles by TEM confirmed that all the nano-sized powders contained primary structures smaller than 100 nm (figure 1). The vendor's nominal size, the surface-equivalent diameter determined by gas adsorption, and the geometric size observed by TEM were reasonably consistent. Agglomerates much larger than the nominal particle size formed when the particles were suspended either in water or in Advanced DMEM cell culture media with 2% serum (as observed previously for ZnO¹⁷). The size distribution measured by dynamic light scattering was altered by water bath sonication and changed with time. The size of the major agglomerate mode in media after overnight incubation is listed in Table 1, and similar sized agglomerates were observed only minutes after sonication.

Soluble transition metals have been implicated as a cause of particle-induced oxidative stress, and trace metals can be a contaminant in insoluble materials. The concentration of trace elements, other than Zn, in cell culture media after overnight incubation with particles was near the blank media values. For example, based on an initial concentration of 1 mg/ml metal oxide concentration in media as a stock solution, soluble iron in the nano-Fe₂O₃ treated sample was 0.24 ppm compared to 0.17–0.19 ppm in blank media. Soluble zinc was 19 ppm in the nano-ZnO containing sample compared to 0.2 ppm in the blank media (~95× increase). Since there was no evidence that the particle suspensions, other than ZnO, resulted in soluble transition metals, this issue was not studied in greater detail herein.

Toxicity and comparisons with TNF-α in colon-derived cells

To determine if an induced inflammatory signaling condition altered the sensitivity of cells to the metal oxides, the effect of nanoparticulate matter on the viability of the RKO and

CaCo-2 cells in the presence or absence of TNF- α pretreatment were assessed. Cells were treated with the particles in a dose-dependent manner ranging from 1 to 100 $\mu\text{g}/\text{cm}^2$ using a formazan-style viability assay (Table 2). Initial experiments indicated that three of the metal oxide nanoparticles (TiO₂, SiO₂, and Fe₂O₃) had minimal toxicity below 100 $\mu\text{g}/\text{cm}^2$ and ZnO displayed the most toxicity. TNF- α pretreatment was used to mimic an inflamed state;¹⁸ however, we did not observe dramatic differences in cell viability whether they were pretreated with TNF- α or BSA.

Effects due solely to high number concentration or due to high surface area of insoluble particles have been hypothesized. To evaluate if there was a consistent or non-specific transcriptional effect of the nanoparticles at an early (4 hr) timepoint we first compared BSA pretreated RKO or CaCo-2 colon cancer cells to cells treated with TNF- α , nanoparticulate only, or TNF- α plus nanoparticulate by whole genome expression analysis. If TNF- α had an effect on the cellular responsiveness to nanoparticles in these cell lines, we expected to observe potentiation of the metal oxide response. However, the most prominent response resulted from the TNF- α treatment, independent of particle type (all significant genes of interest are listed in supplemental tables). The induced genes that were statistically enriched in the molecular function or biological process gene ontology categories following EASE analysis were primarily involved in the regulation of apoptosis and cytokine pathways (figure S1), consistent with TNF- α -stimulation. The genes were consistent with NF- κ B-mediated signaling pathways as these genes were primarily induced in the TNF- α -pretreatment groups but not in the BSA control group. No common set of genes modulated by the nano-sized TiO₂, SiO₂, Fe₂O₃, and ZnO was found, and this suggested that these materials did not induce a non-specific, nanoparticle-mediated response. Furthermore, co-treatment with TNF- α plus nanoparticles did not significantly alter the gene transcription response.

Particle-specific responses in colon-derived cells

Next we evaluated the same dataset but grouped the expression data by nanoparticle type to determine if the particulate matter gave a chemical species-specific nanoparticle response (figure 2 and supplemental tables). This analysis indicated that ZnO and TiO₂ displayed transcriptional effects with ZnO resulting in the largest number of gene expression changes. EASE annotation analysis indicated statistical enrichment of gene ontology categories related to unfolded protein, chaperon, and stress responses in ZnO-treated cells. Other enriched transcripts included genes whose products bind transition metals; many were Zn-finger containing proteins, so there was some overlap with the genes identified as transcriptional modulation as well as genes involved in metal metabolism. Genes involved in transcription regulation were also statistically enriched in the ZnO-treated cells, but many of these demonstrated decreased expression. In addition, decreased expression of genes involved in transcription were also identified in the TiO₂ treatment condition.

To evaluate whether ZnO and TiO₂ transit across the plasma membrane to promote these transcriptional responses, we examined cells treated with the nanomaterials by electron microscopy (figure 3). TiO₂ was clearly visible within RKO cells as large agglomerates while it was more challenging to identify the ZnO. The more pronounced ultrastructural features we found within the cells treated with ZnO were membrane whorls, a feature more consistent with late stage autophagosomes. Nevertheless, it did appear that the nanomaterials were entering the cells and ZnO-treatments demonstrated intracellular alterations consistent with autophagy (figures S3–S4).

Whether novel nano-sized particle formulations induce different toxicological responses than conventional powders of the same nominal substance is a question of great interest. Since ZnO was the most cytotoxic and also induced the most prominent alterations in early

transcriptional responses, experiments compared the nano-sized powder to a conventional ZnO powder that was nominally –325 mesh screen size (~44 μm) but actually contained a significant amount of submicron particles in agglomerates or aggregates (previously described¹⁷). While there does appear to be some differences, we found that the conventional- and nano-sized ZnO induced similar changes in expression (figure 4 and supplemental tables). Again, the most pronounced sets of genes affected by both ZnO particle sizes were those involved in unfolded protein, chaperon, cellular stress responses, and metal metabolism.

We wished to specifically evaluate the involvement of inflammation-associated genes since particulate matter has demonstrated proinflammatory characteristics in other *in vitro* systems. We evaluated each cell line and ZnO treatment using multiclass significance analysis so that we could determine if inflammation-related genes were part of an early response to ZnO under any condition other than TNF- α cotreatment; however, a broad pro-inflammatory response was not observed (figure S5).

We validated the microarray results by quantifying transcription of specific genes that are often used as biomarkers of specific pathways using quantitative PCR (QPCR). We measured the transcriptional levels of BIRC3, ZFP36, PTGS2, SLC30A1, MT1F, and HSPA1A as biomarkers for inflammation, metal response, and chaperonin genes, respectively. For all of these genes, the QPCR validated the microarray analysis (figure S6).

Comparison of responses in colon- and skin-derived cells

Since our results demonstrated the greatest gene transcription responses for ZnO and TiO₂, and these nanomaterials are frequently used for topical applications, we further evaluated these agents in HaCaT and SK Mel-28 cells as representative skin-derived keratinocytes and melanocytes. These cell lines displayed similar cytotoxic responsiveness to ZnO (table 3), and again, TiO₂ was minimally toxic (LD₅₀ >100 $\mu\text{g}/\text{cm}^2$) in both cell lines. Next we evaluated the transcriptional response of these cell lines to ZnO and TiO₂. With the additional gene expression experiments, we found a broader range of gene expression differences (figure 5). Still, the strongest changes in gene expression were related to metal metabolism, stress, and protein folding. There were also more modest changes in gene expression related to nucleic acid metabolism, and transcription (see gene expression supplemental tables). The genes that increased expression following exposure to ZnO were primarily those related to protein folding responses and stress-related transcriptional programs. The genes that were attenuated were related to nucleic acid metabolism.

Our previous studies with these same nanomaterials have demonstrated that ZnO liberates Zn²⁺;¹⁷ therefore, we identified the gene expression changes among the cell lines with direct contact to the ZnO and TiO₂ compared soluble Zn (liberated Zn from the nano-ZnO at 5 $\mu\text{g}/\text{cm}^2$ was ~ 50 μM). To evaluate responses to soluble Zn, we measured the gene expression changes in the HaCaT cells treated with 100 μM ZnCl₂, or ZnO (5 $\mu\text{g}/\text{cm}^2$) that was separated from the cells with a Transwell insert, and SK Mel-28 cells treated with 100 μM ZnCl₂. We then determined which genes are differentially expressed among these samples with soluble Zn compared to the ZnO direct contact samples. The genes associated with metal ion binding (copper and cadmium binding gene ontology categories; GO:0005507 and GO:0046870) are still induced by soluble Zn ions present in the media. However, the genes associated with protein turnover, cellular stress, nucleic acid metabolism, and transcription related pathways are the genes that no longer demonstrate changes in expression (figure 6 and S7).

Finally, to validate that chaperon and stress response genes displayed differential expression between cells exposed to soluble Zn compared to ZnO, the gene products were confirmed

and validated by immunoblot analysis (figure 7). The gene products of HSPA1A, DNAJA4, and GADD45B are representative of chaperon and stress pathways. Additional gene products that could represent oxidative stress (HMOX1) and inflammation (ZFP36) were also evaluated. However, since there is a paucity of other antioxidant and inflammatory genes, it is more likely that ZFP36 expression is responding to Zn levels, and that HMOX1 responds as a stress response gene under ZnO stimulation.

Discussion

The SiO₂, Fe₂O₃, and TiO₂ nanoparticulate displayed little toxicity and had limited effects on gene transcription in RKO and CaCo-2 colon cells, even at concentrations above likely physiological exposure. This adds to the growing evidence that low solubility inorganic particles do not have unique or unusual mechanisms of toxicity solely because of size by providing genome-wide expression profiling data for lesser-studied cell types. A TNF- α induced pro-inflammatory environment did not alter the responses of RKO and CaCo-2 cells to these metal oxide nanoparticles, refuting our initial hypothesis that inflammation would enhance response to nanoparticles. ZnO was the most toxic of the tested metal oxides and microarray evaluation of the whole human genome suggests that the cells produced a generalized stress response that affected the expression of Zn response proteins, heat shock proteins, early response proteins, and metal metabolism. Genomic analysis supplement studies that measure only a few protein expression biomarkers, as indicated by the observation that there was a limited pro-inflammatory response to the ZnO that involved a few genes, but not the entire pathway. Significantly, contact with nano-ZnO particles produced a different response than that resulting from an equivalent concentration of ZnCl₂.

ZnO and TiO₂ are common ingredients in topical consumer products. Therefore, we expanded our study to evaluate the cytotoxicity and transcriptional responses in skin-derived cells. The cytotoxicity was similar in these cells to the colon-derived cells. We also observed significant overlap in the gene expression from the ZnO treated cells indicating that ZnO likely has a common mechanism of action.

ZnO has displayed potential for numerous beneficial applications including food additives, sunscreens, and pigments as well as a broad range of engineering applications.¹⁹ ZnO has traditionally demonstrated utility by its antimicrobial activity.²⁰ In addition, ZnO has anti-cancer activity, that may be associated with rapid cell division compared to non-malignant cells.²¹ Our studies utilized a variety of transformed cell lines and ZnO was cytotoxic in all. In addition, the cellular transcriptional response was similar in all cases. Therefore, ZnO may have a common mechanism of cytotoxicity among all the cells. Our previous observations, as well as those of other groups,^{17, 22} suggests that cytosolic Zn²⁺ liberated over time from ZnO taken up by cells promotes mitochondrial dysfunction, stress at the endoplasmic reticulum, and superoxide production that promote cell death and loss of membrane integrity.

Gene expression analysis provides insight into particular cellular response pathways in an unbiased manner. For example, the gene expression data strongly suggest that Zn ions are available to the cells exposed to ZnO. The upregulation of genes like the metallothioneins (MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1L, MT1M, MT1X, and MT2A) and the Zn efflux transporter (SLC30A1) are genes that are under the control of the metal transcription factor that requires Zn metal ions for transactivation.²³ ZnO toxicity for some organisms is related to Zn solubility,²⁴ and the development of ZnO tolerance appears to enlist genes that sequester Zn²⁺ like metallothionein.²⁵ This early transcriptional response we observed is consistent with cells managing Zn but also showed additional transcription responses not related to the metal transcription factor.²⁶ Since the metallothionein response

was also observed when the cells were exposed to soluble Zn, this response appears to be an adaptive response to the increased Zn^{2+} in the cellular environment. Nevertheless, the induction of metallothioneins as well as additional stress response genes have been characterized as a gene expression response for toxic metals like cadmium.²⁷

Besides the induction of metal responsive agents, the most characteristic cellular pathways influenced by ZnO were transcriptional regulation and protein folding responses. The repression of transcription and nucleic acid metabolism is consistent with Zn toxicity.²⁸ At physiological doses, the transition metal, Zn, is critical for many proteins containing Zn-finger motifs; however, these protein motifs are subject to disruption by other transition metals, and even high concentrations of Zn.²⁹ While it is unlikely that this is cell type specific response to nano-ZnO since others have shown Zn-dependent heat shock protein responses,³⁰ this response was absent in experiments where the cells were exposed to soluble Zn instead of ZnO particles. Indeed, these stress responses appear to be common responses to metal toxicity,³¹ and suggests that the cytotoxicity of metals like Zn may be due to a broad disruption of protein function, analogous to thiol oxidation. The induction of a common set of genes involved in stress responses and protein folding (ATF3, BAG3, DNAJA1, DNAJA4, DNAJB1, DNAJB4, DNAJB6, ERFF1, FOS, GADD454B, HSPA1A, HSPA1L, HSPA4L, HSPA6, and HSPH1) provides an opportunity to investigate the relationship of these gene products with Zn-mediated cytotoxicity since many heat shock proteins have roles in cell survival and apoptosis.³² Therefore, further studies are warranted to determine if the induction of these genes is consistent with disruption of the mitochondrion as previous studies have observed,¹⁷ as well as determining if impairment of protein translocation to the mitochondria³³ are components of Zn-mediated cytotoxicity

The size-dependent variation in potency of nanomaterials is often explained in terms of surface area effects, as in a recent gene array comparison of 10 and 500 nm SiO_2 .⁵ In this study, we also observed similar genes responding to ZnO regardless of the primary particle size. However, the lack of stability of the nanomaterial dispersions, as defined by the agglomeration observed in aqueous solutions, limits our ability to explicitly assess the concentration-dependent interactions of the ZnO with the cells. It is possible that the agglomerates promote interactions with the cells due to settling. This hypothesis is supported by the conventional ZnO generating more pronounced responses by QPCR than the nano-sized ZnO. However, the conventional ZnO has been shown to be very polydisperse and contains a significant amount of submicron particles.¹⁷ Therefore, we cannot rule out that the smaller size particles from the conventional ZnO may contribute disproportionately to the cellular responses.

ZnO has demonstrated induction of oxidative stress responses and DNA damage in A431 cells,³⁴ as does environmental particulate contaminated with Zn.³⁵ The possibility of inhalation of Zn-containing compounds in environmental particulate has spurred an investigation of responsive genes in mice instilled with a Zn salt,³⁶ and these investigators identified genes similar to those reported herein. Our gene expression data, evaluating early responses to ZnO, did not demonstrate widespread pro-inflammatory or oxidative stress responses gene expression induction. We did observe select gene modulation like ZFP36 and PTGS2 representing inflammation associated genes, and HMOX1 may be the most responsive gene, and could represent an oxidative response. However, there are few additional genes modulated by ZnO that were consistent with these functional categories. Indeed, the transcriptional regulation of HMOX1 by the transcriptional repressor, Bach1, has demonstrated cadmium responsiveness³⁷ and HMOX1 may be the most responsive gene repressed by Bach1 in keratinocytes.³⁸ Therefore, these genes are most likely responding to the ZnO explicitly and are not components of broader transcriptional responses.

The exposure to different nanomaterials via oral human consumption is an outstanding question as it is unknown whether dietary metal oxides nanomaterials have chronic effects on the colon. SiO₂, and TiO₂, are commonly used as food ingredients. They are used in cosmetics, drugs, and consumer products where exposure can result from hand-to-mouth contact and drinking water contamination. We have shown that colon cell responses to ZnO are mediated by cell-particle contact, and not by soluble Zn.¹⁷ Nanomaterial exposure to gastric acid may alter their properties, particularly since ZnO is more soluble in acidic solutions compared to other metal oxides. However, the ingestion of large amounts of ZnO has been reported to cause gastroduodenal corrosive injury in humans without systemic toxicity.³⁹

Conclusions

This study has provided some of the first data on the effects of commercial metal oxide nanopartilces on human colon-derived as well as skin-derived cells. These gene transcription data are consistent with other recent work suggesting that low solubility metal oxide nanoparticles do not have unique mechanisms of action or orders-of-magnitude greater potency when compared to conventional powders of the same nominal substance.⁵ In this study we observed similar patterns of gene transcription in colon cells treated with materials marketed as nano-sized and conventional ZnO powders. Continued research on the environmental and health effects of new industrial chemical formulations, including smaller primary particle sizes and novel surface treatments, is consistent with the precautionary principle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

BIRC3	baculoviral IAP repeat-containing 3
B2M	beta-2 microglobulin
DLS	dynamic light scattering
DNAJA4	DnaJ (Hsp40) homolog, subfamily A, member 4
EASE	expression analysis systematic explorer
Fe₂O₃	iron oxide
GADD45B	growth arrest and DNA-damage-inducible, beta
HSPA1A	inducible heat shock protein 70
HMOX1	heme oxygenase 1
MT1F	metallothionein 1F

PTGS2	prostaglandin-endoperoxide synthase 2 (also known as COX-2, cyclooxygenase-2)
SLC30A1	solute carrier family 30 (zinc transporter) member 1 (also known as ZnT1, zinc transporter 1)
TIGR MEV	the institute for genomic research multiple experiment viewer
TNF-α	tumor necrosis factor alpha
SAM	significance analysis of microarrays
SiO₂	silicon dioxide
TEM	transmission electron microscopy
TiO₂	titanium dioxide
ZFP36	zinc finger protein 36 (also known as TTP, tristetraprolin)
ZnO	zinc oxide

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Fig. 1. TEM images of nanoparticles suspended in water and dried on a grid; A) Fe₂O₃, B) TiO₂, C) SiO₂, and D) ZnO.

Fig. 2.

Gene expression changes of RKO and CaCo-2 cells following treatment with metal oxide nanomaterials. A) Multiclass SAM comparison indicated schematically. of nanoparticle-specific categories regardless of TNF- α pre-treatment was performed on the following categories (as represented schematically at the top of the figure): nanoparticle B) Hierarchical cluster visualization and heatmap of gene expression changes. nanoparticle-induced gene expression changes. Treatments are denoted: 1) Zn for ZnO; 2) Ti for TiO₂; 3) Fe for Fe₂O₃; and 4) Si for SiO₂, and 5) pre-treatments with T, T1 or T2 for TNF- α ; samples are denoted with a B for BSA. 435 elements, representing 392 distinct probes, or 332 unique genes were identified as significant. ZnO treatment altered the expression of most of these genes, TiO₂ inhibited the expression of a smaller set of genes, but Fe₂O₃ and SiO₂ treatment did not generate coherent transcriptional responses. C) The top ten biological processes or molecular functions gene ontology (GO) categories as determined by EASE with the

Bonferroni corrected probability (cyan bars), and the number of genes that identified the gene ontology category (■) displayed. The dashed line represents category significance at the $p=0.05$ level. The complete geneset and annotation analysis is provided in the microarray supplementary tables.

Fig. 3.

TEM evaluation of ZnO and TiO₂ nanoparticulate in RKO cells. A) ZnO; electron dense material was observed at the surface (*light arrow right panel*) and within membrane bound structures (*heavy arrow lower panel*). B) The major ultrastructural change following ZnO treatment was membrane-bound structures and whorls. C) TiO₂; agglomerates were visible (*light arrow*) but did not always elicit membrane encapsulation. The solid bar on the images represents ~600 nm.

Fig. 4.

Similar gene expression changes occur in RKO and CaCo-2 cells following treatment with nano-sized and micron-sized ZnO commercial powders. A) Schematic representation of the multiclass SAM performed on the following categories 1) TNF- α pre-treatment, 2) nanoZnO treatment, and 3) conventional ZnO treatment (designated by the μ ZnO). B) Hierarchical cluster visualization of ZnO particulate transcript profiles. 217 elements, representing 181 distinct probes, or 162 unique genes were identified as significantly different among these groups. C) The top ten gene ontology (GO) categories determined by EASE with the Bonferroni corrected probability (bars), and the number of genes that identified the gene ontology category (■) as described in figure 2. D) The Venn diagram represents the significant GO categories from figure 2C (dark grey) and this analysis (light

grey) and shows that five of the top ten gene ontology categories were in common among the significantly enriched groups included the response to unfolded protein and response to protein stimulus categories.

Fig. 5.

Gene expression in colon- and skin-derived cells. A) Schematic representation of the multiclass SAM performed on the following categories: 1) control samples (C1, C2) and TNF- α samples (T1, T2), 2) 5 $\mu\text{g}/\text{cm}^2$ ZnO treatments, and 3) TiO₂ treatments regardless of dose. B) Hierarchical cluster visualization of nanoparticle treatment transcript profiles. 917 elements, representing 818 distinct probes, or 690 unique genes were identified. C) The top ten gene ontology (GO) categories determined by EASE with the Bonferroni corrected probability (bars), and the number of genes that identified the gene ontology category (■) as described in figure 2. D) The Venn diagram represents the significant GO categories from figure 2C (dark grey) and this analysis (light grey) and depicts that the only GO categories

that were consistent in the top ten categories were the protein stimulus and response to unfolding protein.

Fig. 6.

ZnO contact dependence for gene expression modulation. A) Schematic of the multiclass SAM of skin-derived and colon-derived cells performed on the following categories: 1) control samples (C1, C2) and TNF- α samples (T1, T2), 2) all ZnO treatments, 3) all TiO₂ treatments, and 4) skin-derived cells exposed to soluble Zn (ZnCl₂) or ZnO separated from cells using a Transwell insert (TW). B) Hierarchical cluster visualization of nanoparticle treatment transcript profiles. 274 elements, representing 234 distinct probes, or 186 unique genes were identified. C) The top ten gene ontology (GO) categories determined by EASE with the Bonferroni corrected probability (bars), and the number of genes that identified the gene ontology category (■) as described in figure 2. D) The Venn diagram represents the significant GO categories from figure 5C (dark grey), this analysis (light grey), and the

smaller circle represents the significant GO categories observed from analysis of soluble Zn samples. Two GO categories were in common among all these analyses (cadmium ion binding and zinc ion binding); categories that loose representation in the soluble Zn analysis are those relating to protein folding and transcriptional regulation.

Fig. 7.

Immunoblot analysis of select gene products demonstrating ZnO-dependence of expression in RKO, CaCo-2, HaCaT, and SK Mel-28 cells. Protein levels in untreated cells or following 8 hrs of stimulation with 100 µM ZnCl₂ or 5 µg/cm² ZnO. Gene products representing protein folding and stress responses include; HSPA1A, DNAJA4, GADD45B, ZFP36, and HMOX1; while GAPDH represents a control protein. Contact with ZnO demonstrates gene product modulation across all 4 cell lines derived from different tissues while soluble Zn did not elicit similar responses.

Table 1

Particulate Matter Characterization

Material and Abbreviation	Supplier's Nominal Size	Surface Area (BET) ^a (m ² /g)	Surface equivalent sphere ^b (nm)	Agglomerate mode: water ^c (nm)	Agglomerate mode: media ^d (nm)	Zeta Potential ^e (mV)	Vendor ^f	Comment
Iron Oxide Fe ₂ O ₃	3 nm	222	5	557, >1000 (bimodal)	1300	-62	AA	
Titanium Dioxide TiO ₂	5 nm	240	6	749	1000	-45	NS	anatase
Silicon Dioxide SiO ₂	10 nm	124	20	181	600	-44	NS	amorphous
Zinc Oxide, Nano ZnO	8–10 nm	24	40	161, 615 (bimodal)	650	-54	AA	
Zinc Oxide, Conventional ZnO	–325 mesh (<44 µm)	3.2	300	ND	>5000	ND	AA	

^a Surface area measured by nitrogen adsorption using a Quantachrome Monosorb analyzer.

^b Surface equivalent sphere is defined as the size of uniform spheres that have the same ratio of mass to surface area as the powder. Data were rounded to one figure due to uncertainties introduced by calculation assumptions.

^c Volume-weighted agglomerate mode in water at 0.1 mg/ml, vortexed, and sonicated immediately prior to DLS.

^d Volume-weighted agglomerate mode in cell culture media at 300 µg/ml as determined by DLS after 24 hr incubation.

^e Zeta potential were measured using a Malvern Zetasizer Nano ZS in water at 0.1 mg/ml, vortexed, and sonicated immediately prior analysis.

^f Vendor codes: NS, Nanostructured and Amorphous Materials; AA, Alpha Aesar.

ND = not done.

Table 2NanoParticulate LC₅₀ (μg/cm²) in Colon-derived Cell Lines

Material and Abbreviation	RKO		CaCo-2	
	–TNF	+TNF	–TNF	+TNF
Iron Oxide Fe ₂ O ₃	>100	>100	>100	>100
Titanium Dioxide TiO ₂	>100	>100	>100	>100
Silicon Dioxide SiO ₂	>100	>100	>100	>100
Zinc Oxide, Nano ZnO	27 ± 3.6	16 ± 2.6	28 ± 4.6	27 ± 1.8

Formazan-style viability assays were performed with each nanomaterial between 0–100 μg/cm². In all cases, 100 μg/cm² completely coated the bottom of the well. If a 50% loss of viability was not observed within this range the LD₅₀ was designated >100 μg/cm².

Table 3NanoParticulate LC₅₀ in Skin-derived Cell Lines

Material and Abbreviation	HaCaT	SK Mel-28
Titanium Dioxide TiO ₂	>100	>100
Zinc Oxide, Nano ZnO	11.8 ± 0.54	11.5 ± 2.6

Formazan-style viability assays were performed with each nanomaterial between 0–100 µg/cm². In all cases, 100 µg/cm² completely coated the bottom of the well. If a 50% loss of viability was not observed within this range the LD₅₀ was designated >100 µg/cm².