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Intracellular accumulation dynamics and fate of zinc ions in alveolar epithelial cells exposed to airborne ZnO nanoparticles at the air–liquid interface

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Abstract

Airborne nanoparticles (NPs) that enter the respiratory tract are likely to reach the alveolar region. Accumulating observations support a role for zinc oxide (ZnO) NP dissolution in toxicity, but the majority of *in-vitro* studies were conducted in cells exposed to NPs in growth media, where large doses of dissolved ions are shed into the exposure solution. To determine the precise intracellular accumulation dynamics and fate of zinc ions (Zn²⁺) shed by airborne NPs in the cellular environment, we exposed alveolar epithelial cells to aerosolized NPs at the air–liquid interface (ALI). Using a fluorescent indicator for Zn²⁺, together with organelle-specific fluorescent proteins, we quantified Zn²⁺ in single cells and organelles over time. We found that at the ALI, intracellular Zn²⁺ values peaked 3 h post exposure and decayed to normal values by 12 h, while in submerged cultures, intracellular Zn²⁺ values continued to increase over time. The lowest toxic NP dose at the ALI generated peak intracellular Zn²⁺ values that were nearly three-folds lower than the peak values generated by the lowest toxic dose of NPs in submerged cultures, and eight-folds lower than the peak values generated by the lowest toxic dose of ZnSO₄ or Zn²⁺. At the ALI, the majority of intracellular Zn²⁺ was found in endosomes and lysosomes as early as 1 h post exposure. In contrast, the majority of intracellular Zn²⁺ following exposures to ZnSO₄ was found in other larger vesicles, with less than 10% in endosomes and lysosomes. Together, our observations indicate that low but critical levels of intracellular Zn²⁺ have to be reached, concentrated specifically in endosomes and lysosomes, for toxicity to occur, and point to the focal dissolution of the NPs in the cellular environment and the accumulation of the ions specifically in endosomes and lysosomes as the processes underlying the potent toxicity of airborne ZnO NPs.

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Declaration of interest

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Keywords

Airborne nanoparticles; air–liquid interface; endosomes; FluoZin-3; intracellular Zn²⁺; lysosomes

Introduction

Zinc oxide (ZnO) nanoparticles (NPs) carry physicochemical properties that are attractive for a wide array of applications ranging from UV absorption in sunscreen and textile to photovoltaic and novel nanomechanical devices (Ji & Ye, 2008; Wang, 2004; Wang et al., 2007). At the same time, *in-vitro* and *in-vivo* studies have shown that exposures to ZnO NPs induce toxicity in several cell types and animal models. *In-vivo* studies using intratracheal instillation and inhalation of ZnO NPs in the rat showed lung inflammatory and cytotoxic responses (Cho et al., 2010; Sayes et al., 2007; Warheit et al., 2009). These responses resembled “metal fume fever” in human – a condition associated with an increase in lung proinflammatory cytokines and polymorphonuclear leukocytes induced by exposures to ZnO fumes (Kuschner et al., 1995). *In-vitro* studies in bronchial and alveolar epithelial cell lines, exposed to ZnO NPs in solution, reported oxidative stress and inflammatory responses, DNA damage and cell death (Hsiao & Huang, 2011; Huang et al., 2010; Karlsson et al., 2008; Wu et al., 2010; Xia et al., 2008).

One of the major routes of exposure to airborne NPs is through the respiratory tract. *In-vivo* and modeling studies have shown that airborne NPs are likely to be deposited in the alveolar region (Donaldson et al., 2008; Mercer et al., 2010; Oberdorster et al., 2005). However, the majority of *in-vitro* studies characterizing ZnO NP toxicity were conducted in submerged cell cultures, where the NPs were administered suspended in aqueous solution or growth media. While ZnO NPs are relatively stable at neutral pH (Franklin et al., 2007; Moos et al., 2010; Xia et al., 2011), they are readily dissolved in cell culture media, with 80% dissolution achieved by 3 h (Xia et al., 2008) or less (Buerki-Thurnherr et al., 2013). As such, cells are exposed in submerged cultures to a mixture of dissolved zinc ions as well as NPs, making it difficult to dissociate the toxicity and processes induced by the intact NPs from those induced by the dissolved ions in the exposure solution to better understand airborne ZnO NP toxicity.

The toxicity of the dissolved zinc ions has been demonstrated *in vitro* and *in vivo*. Iron doping of ZnO NPs, which slows the dissolution rate, was shown to decrease the level of toxicity induced in cultured BEAS-2B cells and zebrafish embryos, as well as by inhalation in the rat, when compared with undoped particles (George et al., 2010; Xia et al., 2011). The use of Zn chelation, together with exposures to ZnO NPs with varying degrees of solubility, also showed clear relationships between dissolution and toxicity in cultured Jurkat cells (Buerki-Thurnherr et al., 2013). *In-vitro* studies using ZnSO₄ and ZnCl₂, which are readily dissolved in solution to generate zinc ions, showed significant cellular injury, inflammation and cytotoxicity in several cell types (Kim et al., 2010; Lin et al., 2009; Sharma et al., 2012). However, ZnSO₄ was shown to induce toxicity at Zn²⁺ concentrations that were much higher than the Zn²⁺ concentrations shed by toxic NP doses (Lin et al., 2009), implicating the intact NPs in toxicity. Furthermore, intratracheal instillation of ZnO NPs was found to

induce long-term inflammation, including eosinophils and neutrophils recruitment, while the supernatant, containing only dissolved Zn^{2+} , induced a mild and transient neutrophilic inflammation Cho et al. (2012a). These observations suggest distinct mechanisms of toxicity or potency for the intact NPs and the dissolved ions. In support of this view, our recent work showed striking differences in the dynamics of reactive oxygen species (ROS) generation following exposure of alveolar epithelial cells to aerosolized ZnO NPs at the air–liquid interface (ALI), when compared with cells exposed to the NPs when suspended in growth media (Xie et al., 2012). Likewise, distinct patterns of interleukin (IL)-8 and hemeoxygenase-1 (HO-1) gene expression were observed in A549 cells exposed to aerosolized ZnO NPs at the ALI versus exposure to NP suspension in growth media (Lenz et al., 2009).

The dissolution of intact NPs inside acidic organelles has been suggested to underlie, at least in part, ZnO NP toxicity (Cho et al., 2011; Gilbert et al., 2012; Xia et al., 2008). However, a quantitative understanding of the contribution, intracellular dynamics and fate of the dissolved ions to airborne NP toxicity is still missing. To dissociate the contribution of ions in the exposure solution from the contribution of ions generated by airborne NPs in the cellular environment, we exposed alveolar type II epithelial cells (C10) to precise cellular doses of aerosolized ZnO NP at the ALI, and quantified the levels of intracellular zinc ions generated by the lowest toxic NP dose using a highly selective fluorescent indicator for Zn^{2+} . This approach allowed us to identify the lowest intracellular Zn^{2+} concentrations required to induce toxicity. Together with fluorescent probes for distinct organelles, we also quantified the content of Zn^{2+} in distinct organelles over time. By comparing these measurements with those we made in cells exposed to NPs or to ZnSO_4 in submerged cultures, we were able to pin-point the precise intracellular accumulation dynamics and fate of intracellular zinc ions originated from exposures to airborne NPs in the cellular environment and better understand the mechanisms underlying airborne ZnO NP toxicity.

Materials and methods

Reagents

Propidium iodide (PI), Hoechst 33258 and RPMI growth medium were purchased from Life Technologies (Carlsbad, CA). Cyto Tox 96[®] non-radioactive cytotoxicity assay and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) were purchased from Promega (Madison, WI). Fetal bovine serum (FBS) was purchased from ATCC (Manassas, VA). L-Glutamine, penicillin and streptomycin were purchased from Sigma (St. Louis, MO). Millipore (Billerica, MA) water was sterilized using 0.22 μm cellulose nitrate filter (Corning, Corning, NY). Rab5-RFP, Lamp1-RFP, Lamp1-GFP Cell Light Reagents and LysoTracker Green were purchased from Life Technologies (Carlsbad, CA).

Cell culture

A non-tumorigenic alveolar type II epithelial cell line derived from mouse lung (C10) was used in this study. One of the main roles of this cell type is to produce the surfactants that prevent the alveolar collapse during expiration. We have previously confirmed the formation

of microvilli in these cells (Orr et al., 2007, 2009) and the expression of surfactant proteins A and D, providing a microenvironment that mimics the environment *in vivo* (Xie et al., 2012). The cells were grown in RPMI growth medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. For cultures at the ALI, 1×10^5 cells were seeded on cell culture membrane inserts (BD Falcon, transparent polyethylene terephthalate (PET) membranes, 4.2 cm² effective growth area, 0.4 µm pore size, $2.0 \pm 0.2 \times 10^6$ pores/cm²) with 3 mL growth medium on the basal side and 1.3 mL on the apical side. The cells were kept under submerged conditions for about 3 d until full confluence was reached. Cultures at the ALI were achieved by removing the apical growth medium and incubating at 37 °C and 95% relative humidity in a 5% CO₂ incubator for an additional 24 h before exposure to the NPs. For exposures in submerged cultures, 3×10^5 cells were seeded on cell culture treated 35 mm plastic dishes (BD Falcon, 11.78 cm² effective growth area). The cells were kept in submerged cultures for about 3 d until full confluence was reached, after which the cells were covered with 1.3 mL growth media and exposed to the NPs suspended in solution.

NP characterization

ZnO NPs (25 nm primary particle diameter) were kindly provided by Dr. Jeffrey Zink (University of California, Los Angeles) under the National Institute of Environmental Health Sciences (NIEHS) Nanotechnology Environmental Health and Safety consortium effort (Xia et al., 2013). The NPs were tested and found negative for endotoxin contamination (Xia et al., 2013). For some of the experiments, the NPs were labeled with Alexa Fluor 546 (Molecular Probes, Eugene, OR), as described below. NP size distribution was previously characterized by placing electron microscopy (EM) grids randomly over the cell monolayer before exposure. By analyzing the EM images, a median diameter of 117 nm ($\sigma = 2.17$) was found for all NP doses at the ALI, and 115 nm ($\sigma = 2.43$) was found for low NP doses in submerged cultures, with larger diameters for higher NP doses (Xie et al., 2012, Figures 2A and B and 3). NP geometry was also previously characterized using transmission EM (Xie et al., 2012, Figure 2C). Hydrodynamic size distribution and zeta potential were measured for both unlabeled and Alexa 546-labeled ZnO NPs using Zeta PALS (Brookhaven Instruments, Holtsville, NY). Size distribution measurements were done in PBS containing 5% FBS for aerosolized NP exposures at the ALI, and in complete RPMI culture medium for exposures in submerged cultures. Dynamic light scattering measurements were weighted by particle number and showed an average size distribution of 86.13 ± 8.3 nm for ZnO particles in aerosolized solution and 73.78 ± 1.6 nm in RPMI growth medium containing FBS. Fluorescently labeled NPs showed an average size distribution of 85.62 ± 5.33 nm in RPMI growth medium containing FBS. Zeta potential showed -8.78 ± 1.18 mV for unlabeled particles, and -37.05 ± 0.82 mV for Alexa 546-labeled particles. Characterization of NP dissolution dynamics under the experimental parameters used in this study was done using inductively coupled plasma-mass spectrometry (ICP-MS) as described below.

Fluorescence tagging of the NPs

NPs were labeled with Alexa Fluor 546 (Molecular Probes), as previously described (Orr et al., 2007). Briefly, ZnO NPs were suspended in Millipore water at 10 mg/mL and probe

sonicated. The particles were placed into isopropanol by dialysis, then centrifuged and resuspended in dimethoxyethane (DME, Aldrich, St. Louis, MO). Alexa Fluor 546 carboxylic acid, succinimidyl ester dye (Invitrogen, Carlsbad, CA) was coupled to aminopropyltriethoxysilane (APTES, Aldrich) by addition to dimethylformamide (DMF, Aldrich) at 4.5 mM and by overnight reaction at ambient temperature. The 546 dye/APTES complex was added to a suspension of ZnO particles in DME and heated to 85 °C in a round bottom flask under nitrogen and stirring overnight. The suspension was cooled and excess dye complex was removed by repeated centrifugation at 15 000 rpm for 20 min and washings in ethanol and PBS. No or minimal detachment of dye molecules occurred before the NPs entered the cells, as determined by the lack of red fluorescent signals in cells exposed to supernatants that were generated from fluorescent NP suspensions at a toxic NP dose after 24 h incubation at 37 °C (Figure S4).

The ALI exposure system

The system for aerosolized NP exposure was described previously (Xie et al., 2012). Briefly, the system consists of three main components: (1) a vibrating membrane nebulizer (Aeroneb® Lab nebulizer system, Aerogen, Galway, Ireland) that generates aerosolized NPs; (2) Vitrocell® exposure chambers (Waldkirch, Germany), where the aerosolized NPs are deposited on the cells; and (3) an air flow system that distributes the aerosolized NPs through the system at a highly controlled rate. Synthetic breathing air (Air Liquid USA Inc., Houston, TX) was directed at 1 L/min into a water bubbler for humidification. From there, the air was transported to the nebulizer for generating the aerosol, which was directed into the three exposure chambers at 10 mL/min for 10 min using a vacuum pump and a flow regulator. While these parameters do not reflect a realistic exposure scenario, they were optimal for achieving a precise NPs dose while maintaining cell viability, which declined with increasing deposition time or flow rate. Each exposure chamber was designed to expose one membrane insert carrying cells at the ALI to the aerosolized NPs. Deposited NP dose was quantified with ng/cm² sensitivity using a quartz crystal microbalance, as described below. The exposure chambers were kept at 32 °C using circulating water supplied by a water bath, while the whole system was encased in a heated incubation chamber to keep the entering air flow at 32 °C. Following 10 min exposure sessions, the cells were returned to the incubator until assayed. It was found that cell death increased with increasing exposure time, and airflow rate beyond 10 mL/min accelerated this process. Therefore, the minimal exposure time required to achieve the dose range used in this study, under 10 mL/min flow rate, was chosen.

Quantify cellular dose at the ALI using quartz crystal microbalance (QCM)

Sensor plates were placed in each exposure chamber instead of the membranes carrying the cells, and NP suspensions at the concentrations listed above were used to generate the aerosolized NPs. These concentrations were then plotted against the NP dose that reached the sensor plate, as measured by the QCM in µg/cm² (Figure 1). The resulting standard curves were used to determine the actual cellular dose achieved by the respective concentrations of the NP suspensions used for each experiment.

Preparation of NP suspensions

NPs were suspended in sterile water (2 mg/mL) and bath-sonicated (Branson 1210 Ultrasonic Cleaner, Danbury, CT) at room temperature (RT) for 10 min, followed by 2×60 s probe sonication (Misonix Sonicator 3000, Farmingdale, NY) at 15 W on ice, with a 15 s rest between pulses. For the ALI exposures, the NP solution was diluted to concentrations of 0, 0.05, 0.20, 0.30, 0.50, 1.00 and 1.50 mg/mL and placed in the nebulizer for 10 min exposure sessions. The final solutions included 5% FBS to minimize differences in particle properties presented to the cells at the ALI and in submerged cultures. Following exposure, serum was removed as it interferes with FluoZin-3 labeling, and cells were incubated at the ALI for the designated time intervals. For exposures in submerged cultures, NP concentrations (0, 5, 10, 15, 30, 45, 60, 75 $\mu\text{g/mL}$) were achieved by diluting the NP solution in RPMI growth medium containing 10% FBS. NP suspensions and dilutions were prepared right before exposure. The particle suspension was added to the cells in growth media for 1 h incubation at 37 °C, after which the NP solution was replaced with serum free growth media to allow FluoZin-3 tagging and incubation continued for the designated time points.

Characterization of ZnO NPs dissolution using inductively coupled plasma-mass spectrometer (ICP-MS)

Samples were digested with ~2 mL of 70% double distilled nitric acid (GFS Chemicals, Inc. Columbus, OH) over night until clear. Aliquots were diluted to 2% nitric acid, and total zinc was quantified using an Agilent 7500 CE (Santa Clara, CA, USA) ICP-MS. ^{66}Zn was quantified in normal mode using ^{45}Sc and ^{115}In (2 ng/mL) as internal standards. Additionally, ^{68}Zn was also monitored. Three rinses with 2% nitric acid between runs were used to minimize carryover. Quantification was accomplished using a linear model fit to an external calibration curve (VHG Labs, Inc., Manchester, NH) with internal standard correction. DI water and complete RPMI samples were spiked with Zn standard for which recoveries were close to 100%. Limits of quantitation for zinc were ~1 ng/mL for samples diluted in 2% nitric acid.

To quantify the dissolution dynamics of ZnO NPs, 10 $\mu\text{g/mL}$ NP suspensions were prepared in complete RPMI following the protocol listed in the above section. Following particle dispersion by sonication, the NP suspension was split in 2 mL plastic vials and incubated at 37 °C for designated time intervals. Preparing and sonicating the working suspensions from the NP stock took 5 min, as such the shortest incubation time was 5 min. At the end of the incubation interval, the plastic vials containing the NP suspensions were centrifuged for 20 min at 4 °C and 20 000 g after which 1 mL of the supernatant was carefully removed and transferred to ICP sample vials. ZnO NPs dissolution reached saturation within 30 min (Figure 2).

The ratios of particulate to dissolved Zn in each exposure system were measured for a series of ZnO NP concentrations suspended in RPMI (for exposures in submerged cultures) and in PBS containing 5% FBS (for the ALI exposures). Samples were incubated at 37 °C for 1 h or 10 min, for exposures in submerged cultures or at the ALI, respectively, followed by centrifugation and analysis of the supernatant containing dissolved Zn. For suspensions used

in submerged cultures, the ratios of particulate to dissolved Zn varied across NP concentrations (Figure S1A and B), where less than 80% of the Zn was in a particulate form for concentrations lower than 45 µg/mL. For all but the lowest concentrations used to generate the aerosolized NPs at the ALI, more than 95% of the Zn was present in a particulate form (Figure S1-C and D).

Toxicity assessment

Cyto Tox 96® non-radioactive cytotoxicity assay was used to quantify lactate dehydrogenase (LDH) release at 24 h post exposure through conversion of a tetrazolium salt (INT) into a red formazan product. For positive control, lysis solution (0.9% triton X-100) in RPMI growth medium was added to the apical side of control cells for 60 min of incubation at 37 °C. For negative control, fresh RPMI medium was used. At 24 h post exposure, 100 µL from the apical (in submerged cultures) or basal (at the ALI) media was collected, centrifuged for 10 min at 2000 g in a bench top centrifuge, and 50 µL of the supernatant was placed in 96 multiwell plates to which the substrate mix was added (50 µL). Following a 30 min incubation at RT under light protection, stop solution was added (50 µL), and the formazan absorbance was measured at 490 nm using a microplate reader with multiple reads per well. For the time series assessment of membrane integrity, 50 µL from the apical or basal media was collected at the end of the exposure time-points (1, 3, 6, 9, 12 and 24 h) and processed as above. Similar time-matched control samples were prepared for all exposure conditions. The LDH release for NP exposure was normalized to the time-matched control.

Cell proliferation

CellTiter 96 AQueous non-radioactive cell proliferation assay was used to determine cell viability 24 h post exposure through the reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) into aqueous soluble formazan by dehydrogenase enzymes found in metabolically active cells. The MTS solution, in RPMI growth media (1:10 dilution), was added to the apical side of the cells for 1 h incubation at 37 °C. Formazan absorbance was measured at 490 nm using a microplate reader (Spectra Max Plus 384, Molecular Devices, Sunnyvale, CA) with multiple reads per well. The mean absorbance of negative control, where cells were exposed to solution or aerosol containing no NPs, was established as 100% cellular viability. The absorbance of cells exposed to the NPs was measured in at least five membrane inserts for each dose, and significant decrease relative to negative control indicated a decrease in cell proliferation or viability.

Intracellular Zn²⁺ measurements

FluoZin-3 was used to detect free intracellular Zn²⁺ in live cells. Following exposure to ZnO NPs or ZnSO₄, cells were incubated with 1 µM FluoZin-3 in RPMI for 30 min at 37 °C, after which the FluoZin-3 solution was replaced with fresh media and incubation continued for an additional 30 min. After FluoZin-3 staining the cells were either directly imaged using fluorescence microscopy as described below, or trypsinized and resuspended in 1 mL RPMI for flow-cytometry measurements using the Influx Flow-cytometer/Cell Sorter (BD Biosciences, Seattle, WA). Forward and side scatter were used to gate out cellular debris and the 488 nm Argon laser was used to excite FluoZin-3 while measuring emission at 520/15

nm. Gating and median calculations from 30 000 cells were done using Flow Jo software (Tree Star, Ashland, OR). Median fluorescence values were calculated for each treatment to quantify the relative levels of free cytosolic Zn^{2+} , and results obtained for different experiments were normalized to the same control value.

Live cell fluorescence imaging and image analysis

C10 cells were plated on cell culture inserts for ALI exposures or 35mm culture dishes for submerged culture exposures. Upon reaching 70% confluence, the cells were transiently transfected with either endosomal (Rab5-RFP) or lysosomal (Lamp1-RFP) fluorescent protein markers using the CellLight Reagents (Life Technologies, Eugene, OR). Twenty-four hours later, the cells were transferred to serum free media and exposed to aerosolized ZnO NPs at the ALI or NP suspension as well as to ZnSO_4 in submerged cultures. At the end of the incubation time-points, the cells were labeled with 1 μM FluoZin-3 and imaged alive using Zeiss LSM 710 upright confocal microscope with 63 $\times$ water immersion objective lens. FluoZin-3 was imaged using the 488 nm line of an Argon laser (green channel) and the Rab5-RFP-positive or Lamp1-RFP-positive vesicles were imaged using the 561 nm DPSS laser (red channel). The two channels were imaged sequentially using fast frame switching with an average pixel dwell time of 1 μs and 1.5 Hz scan rate. For each channel, optical sections covering the entire z range of the cell monolayer were acquired and the sampling density in x, y and z was optimized to satisfy the Nyquist criterion. No fluorescence bleed through was detected between the two emission channels (Figure S5). The transmitted light image was used to delineate the cell contour.

Image analysis

Image analysis was done in Volocity 3D Image Analysis software (Perkin-Elmer, Waltham, MA). For each cell, two populations of intracellular vesicles were identified – FluoZin-3 positive vesicles (green channel – population A) and Rab5-RFP positive vesicles (red channel – population B). Size and coordinates were registered for both populations as well as the Zn^{2+} content per vesicle for the FluoZin-3 positive vesicles (measured as the integrated fluorescence intensity in the green channel). Three-dimensional object based co-localization was used to identify the subset of the FluoZin-3 positive vesicles that were also positive for Rab5-RFP (subset A_E). Finally the percentage of free cytosolic Zn^{2+} incorporated into the early endosome was calculated as the ratio between total green fluorescence in the subset A_E and total green fluorescence in A. A similar analysis was performed for Lamp1-RFP to determine the percent free cytosolic Zn^{2+} incorporated into the lysosomes.

Imaging fluorescent ZnO NPs in lysosomes

Lysosomal integrity was evaluated by comparing the degree of co-localization between fluorescent NPs and Lamp1-GFP and the degree of co-localization between the NPs and LysoTracker, an acidophilic dye that accumulates only in intact acidic lysosomes. Cells were plated on membrane inserts for exposures at the ALI and on 35mm culture dishes for exposures in submerged cultures. Upon reaching 70% confluence, the cells were transiently transfected with Lamp1-GFP. Twenty-four hours later, the cells were exposed to Alexa Fluor 546-labeled ZnO NPs. At the end of the incubation time, the cells were imaged live

using Zeiss LSM 710 upright confocal microscope with 63 × water immersion objective lens. Lamp1-GFP was imaged using the 488 nm line of an Argon laser (green channel) and the fluorescent NPs were imaged using the 561 nm DPSS laser (red channel). In parallel, non-transfected cells were exposed to the NPs as described above followed by their incubated with 1 μM LysoTracker Green for 5 min at 37 °C. The cells were imaged alive, where LysoTracker Green was imaged using the 488 nm line of an Argon laser (green channel). No fluorescence bleed through was detected between the two emission channels (Figure S5).

Statistical analysis

Data is presented as mean ± standard error. The two-tailed Student's t test was used to compare two group means. Analysis of variance (ANOVA) followed by *post hoc* analysis was performed to compare the significance of differences between multiple (3) groups. For all statistical analyses, *p* values of less than 0.05 were considered significant.

Results

Intracellular Zn²⁺ dynamics is fundamentally different in cells exposed to aerosolized NPs at the ALI or to NP suspension in submerged cultures

Intracellular Zn²⁺ was imaged and quantified using FluoZin-3, a highly selective fluorescent Zn²⁺ indicator. The labeled cells were imaged alive at increasing time points post exposure at the ALI or in submerged cultures using confocal fluorescence microscopy. Figure 3 shows an example of images of cells exposed to aerosolized NPs at the ALI, where a marked increase in intracellular Zn²⁺ (green) was noticed at 3 h post exposure. However, to accurately quantify intracellular Zn²⁺ over time, flow cytometry was used. The median values, generated from 30 000 analyzed cells per membrane insert or dish, were calculated from three inserts or dishes in independent experiments, conducted on at least two different days, for each time point. The values for cells exposed to aerosolized NPs at the ALI are plotted in Figure 4(A), and the values for cells exposed to NP suspension in growth media are plotted in Figure 4(C). Cells at the ALI were exposed to a toxic NP dose of 6.0 μg/cm², showing a significant membrane damage as early as 3 h post exposure, as measured by LDH release (Figure 4B). The significant increase in membrane damage correlated with a 3 h peak in intracellular Zn²⁺, which decayed to normal values by 12 h post exposure (Figure 4A). In contrast, cells exposed in submerged cultures to a toxic dose of NP suspension (45 μg/mL/1 h) showed a significant increase in intracellular Zn²⁺ that continued to grow over time (Figure 4C). The increase in intracellular Zn²⁺ over time occurred even though the NP suspension was replaced after 1 h to resemble the exposure condition at the ALI, where no increase in NP dose occurred over time. Ideally, the NP solution would be replaced after 10 min, the time used to achieve final deposited dose at the ALI, but such a short exposure time in submerged cultures would require very high NP concentrations, where large NP aggregates are formed (Xie et al., 2012). Membrane damage was measured by LDH release, showing a significant increase as early as 6 h post exposure (Figure 4D). The values of intracellular Zn²⁺ at the ALI and in submerged cultures are plotted in Figure 5 using the same scale, showing the fundamental differences in the dynamics of intracellular Zn²⁺ in the two exposure systems.

The lowest toxic NP dose at the ALI generates intracellular Zn^{2+} at a level that is several folds lower than the levels generated by the lowest toxic doses of NPs or ions in submerged cultures

Flow cytometry was used to measure intracellular Zn^{2+} levels over a range of NP doses to establish the relationships between toxicity, determined at 24 h by LDH release (Figure 6) and MTS viability assays (Figure S2), and peak intracellular Zn^{2+} , quantified in FluoZin-3 intensity units (Figure 6). Peak intracellular Zn^{2+} at the ALI was measured 3 h post exposure and peak intracellular Zn^{2+} in submerged cultures was measured 24 h post exposure (Figure 5, arrows). A minimum of three membrane inserts or dishes in independent experiments, conducted on at least two different days, were analyzed for each dose. We found that the lowest dose of aerosolized NPs that induced detectable toxicity at the ALI was $4.13 \mu\text{g}/\text{cm}^2$, as determined by the LDH release assay (Figure 6B, arrow) and confirmed by the MTS viability assay (Figure S2-A). This dose correlated with a significant increase in peak intracellular Zn^{2+} value of 28.8 ± 1.2 FluoZin-3 intensity units (Figure 6A, arrow). The lowest dose of NP suspension that induced detectable membrane damage and cell death in submerged cells was $30 \mu\text{g}/\text{mL}/1 \text{ h}$ (Figure 6D, arrow, and Figure S2-B), which correlated with a significant increase in peak intracellular Zn^{2+} value of 82.1 ± 16.1 FluoZin-3 intensity units (Figure 6C, arrow), nearly three-folds higher peak value than the value found at the ALI. The lowest toxic dose of ZnSO_4 in submerged cultures, which readily dissolved to generate free ions, was $25 \mu\text{M}$ (Figure 6F, arrow, and Figure S2-C). This dose correlated with a significant increase in peak intracellular Zn^{2+} of 233.9 ± 8.2 intensity units (Figure 6E, arrow), nearly eight-folds higher than the peak value at the ALI. The peak intracellular Zn^{2+} values correlating with the lowest toxic dose in each exposure system are listed in Table 1. Interestingly, for ZnSO_4 exposure in submerged cultures, a significant increase in peak intracellular Zn^{2+} was detected even in response to a non-toxic dose of $20 \mu\text{M}$.

The majority of intracellular zinc ions following exposure at the ALI are found in endosomes and lysosomes, whereas the majority of the ions following exposure to ZnSO_4 in submerged cultures are found in other vesicles

The cells were transfected with fluorescent protein markers, specific for endosomes or lysosomes, to identify the intracellular fate of the dissolved ions and quantify their accumulation in specific compartments. Rab5-RFP was used to detect endosomes and Lamp1-RFP was used to detect lysosomes. Fluorescent protein markers, rather than fluorescent antibodies, were used because FluoZin-3 tagging can be done only in live cells. The transfected cells were exposed to aerosolized ZnO NPs at the ALI, and at the end of the incubation periods, the cells were labeled with FluoZin-3 for detecting and quantifying intracellular Zn^{2+} . The cells were then imaged alive using confocal fluorescence microscopy, and a minimum of 15 cells for each time point and vesicle type were analyzed using 3D co-localization analysis software to quantify Zn^{2+} accumulation in the endosomes or lysosomes. Figure 7(A) shows an example of images taken 1 h post exposure at the ALI, where a significant co-localization (yellow) between endosomes (red) and Zn^{2+} (green) is detected. However, much less co-localization is detected between lysosomes (Figure 7B, red) and Zn^{2+} (green) at 1 h post exposure. At 3 h post exposure, a significant co-localization is detected between the lysosomes (Figure 7D, red) and Zn^{2+} (green), while less co-localization is detected between endosomes and Zn^{2+} (Figure 7C) at this time point.

Quantitative analysis of the images showed that at 1 h post exposure, $44.6 \pm 3.7\%$ of intracellular Zn^{2+} was found in endosomes and $19.7 \pm 3.4\%$ was found in lysosomes (Figure 7E). In contrast, at 3 h post exposure, $41.7 \pm 3.5\%$ of intracellular Zn^{2+} was found in lysosomes, while $24.3 \pm 3.2\%$ was found in endosomes. It is worth noting that this analysis could include only those vesicles expressing fluorescent proteins. It is likely that additional non-fluorescent endosomes and lysosomes also contained Zn^{2+} , partially accounting for the ~35% intracellular Zn^{2+} not showing co-localization with either endosomes or lysosomes in our analysis.

A similar analysis was done in cells exposed to ZnSO_4 in submerged cultures to identify and quantify the intracellular fate of dissolved ions shed in the exposure solution, rather than in the cellular environment. Figure 8 shows representative images, where minimal or no co-localization between endosomes (red) and Zn^{2+} (green) is detected at 1 h (Figure 8A) or 3 h (Figure 8C) post exposure. Likewise, minimal co-localization is detected between lysosomes and Zn^{2+} at 1 h (Figure 8B) or 3 h (Figure 8D) post exposure. Instead, intracellular Zn^{2+} (green) appears in other larger vesicles or vacuoles, as can be clearly seen in the enlarged area (Figure 8D, inset). Quantitative analysis of the images showed that only $2.2 \pm 0.5\%$ of intracellular Zn^{2+} was found in endosomes, and $6.4 \pm 0.8\%$ was found in lysosomes at 1 h post exposure (Figure 8E). Likewise, only $5.5 \pm 1.0\%$ of intracellular Zn^{2+} was found in endosomes, and $6.0 \pm 1.0\%$ was found in lysosomes at 3 h post exposure. When cells were exposed to ZnO NPs in submerged conditions (Figure S3), the percent of Zn^{2+} found in endosomes and lysosomes at 3 h post exposure was 39.6 ± 4.6 , lower than the fraction found at the ALI (66.0 ± 6.5) but higher than the fraction found ZnSO_4 -exposed cells (11.5 ± 2.0) at the same time point.

Because the ratio of particulates to ions at NP concentrations used in submerged cultures varied with NP concentration (Figure S1-B), the percent NPs found in endosomes/lysosomes in submerged cultures is likely to change with NP dose.

The acidic environment in the lysosomes is disrupted by the NPs or their dissolved ions

LysoTracker, an acidophilic fluorescent dye that accumulates in acidic vesicles, was used to determine whether the presence of the NPs or their dissolved ions disrupts the acidic environment in the lysosomes. Such disruption is expected to decrease the degree of co-localization between LysoTracker and fluorescent NPs when compared with the degree of co-localization between the NPs and Lamp1-GFP, which is a lysosomal marker that is independent of the lysosomal environment. Cells transfected with Lamp1-GFP were exposed to ZnO NPs tagged with Alexa Fluor 546. Figure 9 shows examples of images of cells exposed to the NPs at the ALI (Figure 9A) and in submerged cultures (Figure 9C). A significant co-localization was detected between Lamp1-GFP (green) and the fluorescent NPs (red) in both exposure systems. In contrast, cells exposed to fluorescent NPs followed by staining with LysoTracker showed no co-localization between the NPs and the acidophilic dye, as demonstrated in Figure 9(B and D). Quantitative analysis of 16 cells in each exposure system showed that $37.8 \pm 4.1\%$ of the total NPs inside the cells co-localized with Lamp1-GFP at the ALI, and $48.9 \pm 4.4\%$ of the total NPs co-localized with Lamp1-GFP in submerged cultures (Figure 9E). In contrast, only $3.1 \pm 1.0\%$ of the total NPs inside

the cells at the ALI and $5.2 \pm 1.0\%$ of the total NPs in submerged cultures co-localized with LysoTracker, as found by analyzing 19 cells for each exposure system.

Discussion

The main new finding to emerge from this work is that low but critical levels of intracellular Zn^{2+} have to be reached, concentrated specifically in endosomes and lysosomes, for toxicity to occur, and that the focal dissolution of airborne ZnO NPs in the cellular environment and the accumulation of the ions specifically in endosomes and lysosomes underlie the potent toxicity induced by these airborne NPs. While relatively low intracellular Zn^{2+} levels were generated by the lowest toxic NP dose at the ALI, the levels generated by the lowest toxic doses of NPs or Zn^{2+} in submerged cultures were several folds higher. The majority of intracellular zinc ions originated from airborne NP exposure at the ALI was found in endosomes and lysosomes, while the majority of intracellular ions following exposures to ZnSO_4 in solution was found in other large vesicles or vacuoles, together implicating endosome and lysosome vulnerability in airborne ZnO NP toxicity.

The role of NP dissolution in ZnO NP toxicity was demonstrated before, but a quantitative understanding of the underlying dynamic processes was still missing. One of the main challenges in obtaining such understanding is the high rate of ZnO NP dissolution in growth media (Figure 2) (Buerki-Thurnherr et al., 2013; Xia et al., 2008, 2011). To overcome this challenge and dissociate the toxicity and processes induced by the intact NPs from those induced by the dissolved ions in the exposure solution, we quantified the accumulation dynamics of intracellular zinc ions in cells exposed to airborne ZnO NPs at the ALI in relationships to their induced toxicity. By comparing these measurements with those made in cells exposed to NP suspension or ions in submerged cultures, we gained a quantitative understanding of the role of NP dissolution in toxicity induced by airborne NPs.

By quantifying the level of intracellular Zn^{2+} over time using FluoZin-3, we found fundamental differences in their accumulation dynamics following exposures at the ALI versus exposures in submerged cultures (Figures 4 and 5). Exposure to ZnO NP suspension in submerged cultures resulted in a slow onset, biphasic linear increase in intracellular Zn^{2+} (Figure 4C). In contrast, exposure to aerosolized NPs at the ALI resulted in a faster, transient increase in intracellular Zn^{2+} that peaked at 3 h post exposure (Figure 4A). This difference could be partially explained by the differences in NP deposition dynamics on the cell surface. The final deposited, cellular dose at the ALI was reached within 10 min, whereas the deposited cellular dose in submerged cells likely continued to increase through the incubation time. Although we replaced the NP solution in submerged cultures with fresh growth media after 1 h incubation for the reasons mentioned below, the cells continued to be submerged in solution that allowed additional NP dissolution to occur through the incubation periods. The NP solution was replaced after 1 h to achieve a defined cellular dose of deposited particles in a relatively short time period. Ideally, the NP solution would be replaced after 10 min incubation time to achieve similar exposure parameters as those used at the ALI. However, to achieve a significant deposited dose on cells in solution in such a short time would require the exposure to a very high NP concentration, where large aggregates were formed and fell out of solution.

We found that relatively low levels of intracellular Zn^{2+} were required to induce detectable toxicity, if the source of the ions was limited to dissolution of intact ZnO NPs in the cellular environment, achieved by exposures to aerosolized NPs at the ALI (Figure 6A and B and Table 1). The possibility that some NP dissolution occurs near the cell membrane before internalization cannot be excluded. However, such dissolution is likely to occur focally, spatially limited to the area around the NP. This view is supported by the observation that the vast majority of intracellular ions following exposures at the ALI were found in endosomes and lysosomes (Figure 7E), whereas exposures to ions dispersed in the solution led to their accumulation mostly in large vacuoles and other vesicles (Figure 8). In support of this view are also observations made by Gilbert et al. (2012), where x-ray and atomic force microscopy were used to demonstrate the internalization of intact ZnO NPs and their dissolution in the intracellular environment.

The goal of the ALI exposure was to introduce intact NPs with no or minimal exposure to ions to mimic *in vivo* exposures to airborne NPs, while ZnSO_4 exposures in submerged cultures provided information about the toxicity of the ions in the absence of NPs as well as their unique intracellular distribution. Although ZnO NPs are unlikely to be dissolved in the air or be fully dissolved before reaching the cell surface, an attempt to expose the cells to aerosolized ZnSO_4 solutions at the ALI was taken. However, no membrane damage or cell death was observed even at mM ZnSO_4 concentrations (data is not shown), indicating that fundamental modifications in the ALI setup and exposure parameters, used for aerosolized NP exposures, were required for aerosolized ZnSO_4 exposures.

In contrast to the low levels of intracellular Zn^{2+} generated by the lowest toxic NP dose at the ALI, nearly three-folds higher levels of intracellular Zn^{2+} were generated by the lowest toxic dose in cells exposed to the NPs in suspension, and more than eight-folds higher levels were generated in submerged cells exposed to the lowest toxic dose of ZnSO_4 or only ions in solution (Figure 6 and Table 1). Using fluorescent proteins specific for the endosomes or lysosomes we quantified intracellular Zn^{2+} in these compartments over time using live cell fluorescence imaging (Figures 7 and 8). At the ALI, nearly half of the intracellular Zn^{2+} was found in the endosomes as early as 1 h post exposure (Figure 7E), indicating that a significant NP dissolution takes place in the early phases of the endocytic pathway, possibly starting at the cell membrane and continuing in the endosomes. By 3 h post exposure, the majority of intracellular Zn^{2+} was found in the lysosomes, consistent with the natural progression of endosomes merging with the lysosomal compartment. Although the sum of intracellular Zn^{2+} fractions in the endosomes and lysosomes did not change significantly over time (Figure 7E), additional dissolution of intact NPs reaching the lysosomes likely occurred, as the total intracellular Zn^{2+} at the ALI increased between 1 and 3 h post exposure (Figure 4A). Interestingly, high levels of intracellular Zn^{2+} were found in ZnSO_4 -exposed cells even at non-toxic doses, which were more than three times higher than the levels found in cells exposed to toxic doses of NPs at the ALI. The majority of intracellular Zn^{2+} in ZnSO_4 -exposed cells was found in vesicles or vacuoles other than endosomes or lysosomes, with only a small fraction (~11%) reaching the endosomes and lysosomes in response to a toxic dose (Figure 8E). Together, our observations suggest that a low but

critical level of intracellular Zn^{2+} has to be reached, concentrated specifically in endosomes and lysosomes, before toxicity is induced.

Another study (Lenz et al., 2009), where human alveolar epithelial-like cell line (A549) was exposed to ZnO NPs at the ALI, reported a significant increase in IL-8 and HO-1 mRNA expression at doses as low as $2.0 \mu\text{g}/\text{cm}^2$. In our ALI studies, the lowest dose to induce detectable LDH release and decrease in viability was $4.13 \mu\text{g}/\text{cm}^2$. This difference is expected as the expression of pro-inflammatory or oxidative stress response genes is likely to increase before membrane damage or cell death takes place. Considering that no significant increase in intracellular Zn^{2+} was observed in our studies at $2.0 \mu\text{g}/\text{cm}^2$, the increase in the expression of these genes is likely to be induced in response to the intact NPs. Zinc ions play essential structural and catalytic roles in multiple proteins (Auld, 2001). A specialized group of proteins containing reversible zinc-binding motifs play a role in transcriptional regulation (transcription factor MTF-1) or in the regulation of zinc uptake (Laity & Andrews, 2007; Lucarelli et al., 2007). Zinc also plays an important role in the immune system (Rink & Haase, 2007). Consequently, intracellular zinc concentrations are highly regulated through a multitier process. A dedicated class of transport proteins mediates zinc trafficking across the cell membrane (Cousins et al., 2006; Eide, 2006), while metallothionein, an abundant cysteine-rich protein, acts as a zinc buffer by binding free intracellular zinc with high affinity (Krezel & Maret 2007). In addition, a specialized class of cytoplasmic vesicles – zinosomes – functions as a zinc storage system by reversibly store mM concentrations of zinc (Wellenreuther et al., 2009). This multitier control of zinc homeostasis confers the ability to adapt to a wide range of extracellular Zn^{2+} concentrations. However, the focal internalization and accumulation of free zinc in endosomes and lysosomes, which we observed at the ALI, is likely to bypass the homeostatic machinery and induce irreversible damage to proteins and subcellular structures that are found along this endocytic pathway. In contrast, the high levels of free zinc found mostly in larger vesicles, possibly zinosomes (Könczöl et al., 2012), after exposure to ZnSO_4 are likely to be better controlled as part of the homeostatic machinery and therefore less toxic even at much higher concentrations.

The lysosome is an acidic vesicle that contains numerous acidic hydrolases – glycosidases, phosphatases, proteases, nucleases, peptidases, sulphatases and lipases – that collectively are capable of degrading all cellular components (de Duve, 1983). As such, the lysosome serves as the main degradation factory in cells, receiving cargo from phagosomes, endosomes and autophagosomes. The release of lysosomal acidic hydrolases through the lysosomal membrane permeabilization (LMP) is a known component of the apoptotic pathway (Johansson et al., 2010; Kroemer & Jaattela, 2005). It was shown that increased oxidative stress can trigger a rise in the free zinc levels in the cytosol and lysosomes of cultured neurons and astrocytes, resulting in LMP and cell death (Lee & Koh, 2010). In addition, changes in the chemical environment of the lysosome can disrupt the catalytic properties of lysosomal enzymes inhibiting the lysosome degradation functions (Soura et al., 2012). Using Lamp1-GFP to tag the lysosomes independently of fluctuations in their acidic environment, we found clear co-localization of fluorescence-tagged ZnO NPs and lysosomes both at the ALI and in submerged cultures (Figure 9A and C). In contrast, minimal co-localization was found between the fluorescent NPs and LysoTracker, an acidophilic

lysosomal marker, in both exposure systems (Figure 9B and D), even though many LysoTracker-tagged vesicles were detected. Since the NPs clearly reached the lysosomes, as indicated by their co-localization with Lamp-1, these observations point to the disruption of the acidic environment inside the lysosomes, which prevented the accumulation of LysoTracker in these vesicles. It is possible that the NPs were dissolved completely or partially at earlier stages in the endocytic pathway and the observed dye molecules were already detached from the NPs. Therefore, both the NPs and the dissolved ions are likely to have a role in disrupting the acidic lysosomal environment. These observations are supported by previous studies attempting to link ZnO NP exposure to lysosomal dysfunction (Cho et al., 2011; Hwang et al., 2008; Lee & Koh, 2010; Xia et al., 2008).

Dissolution and generation of free metal ions has been known to play an important role in metal oxide NP toxicity, with a clear correlation between solubility and toxicity (Cho et al., 2012b). For example, the toxicity of both nickel and copper oxide NPs was found to be proportional to the dissolution rate, with faster dissolving particles exhibiting higher toxicity in cultured A549 cells (Horie et al., 2009; Karlsson et al., 2008). Likewise, soluble chromium oxide NPs were found to induce severe cytotoxicity in comparison with the insoluble and chemically stable micro scale particles in the same cell system (Horie et al., 2013). Multiple studies indicate that silver ions play a major role in Ag NP toxicity. Inhibiting Ag oxidation, which is a prerequisite for Ag⁺ release, resulted in a 20-fold decrease in Ag NP associated toxicity (Xiu et al., 2011). Similarly, complexing Ag⁺ with a thiol ligand was found to decrease significantly the induced toxicity (Bar-Ilan et al., 2009; Kittler et al., 2009). At the same time, the presence of metal oxide NPs in the exposure solution tend to exhibit higher levels of toxicity compared with ionic solution obtained from salts or dissolved NPs (AshaRani et al., 2009; Horie et al., 2009, 2013; Karlsson et al., 2008; Kawata et al., 2009). Our studies provide a possible new understanding of these observations, made with other metal and metal oxide NPs, where the focal dissolution of the NPs and accumulation of the ions specifically in endosomes and lysosomes might underlie the potent NP toxicity when compared with the dissolved ions.

Conclusions

By quantifying the accumulation dynamics and fate of intracellular zinc ions in cells exposed to airborne ZnO NPs at the ALI, we were able to dissociate the toxicity and processes induced by exposures to intact NPs from those induced by exposures to dissolved ions in the exposure solution. By comparing these measurements with those made in cells exposed to NP suspension or ions in submerged cultures, we gained a quantitative understanding of the role of NP dissolution in toxicity induced by airborne NPs. We quantified the level of intracellular Zn²⁺ over time using FluoZin-3 and found fundamental differences in the accumulation dynamics of the ions following exposures to aerosolized NPs at the ALI versus exposures to NP suspension in submerged cultures. At the ALI, intracellular Zn²⁺ values peaked at 3 h and decayed to normal values by 12 h post exposures, while in submerged cultures, intracellular Zn²⁺ values continued to increase over time. We found that relatively low levels of intracellular Zn²⁺ were generated by the lowest toxic NP dose at the ALI. In contrast, nearly three-folds higher levels of intracellular Zn²⁺ were generated by the lowest toxic dose of NP suspension, and more than eight-folds higher

levels were generated by the lowest toxic dose of ZnSO_4 in submerged cultures. Using fluorescent protein markers specific for the endosomes or lysosomes we quantified intracellular Zn^{2+} in these compartments over time. At the ALI, nearly half of the intracellular Zn^{2+} was found in the endosomes as early as 1 h post exposure, indicating that a significant NP dissolution takes place in the early phases of the endocytic pathway. In contrast, only a small fraction of intracellular Zn^{2+} was found in endosomes and lysosomes following exposure to ZnSO_4 in submerged cultures. These observations suggest that a low but critical level of intracellular Zn^{2+} has to be reached, concentrated specifically in endosomes and lysosomes, for toxicity to occur. Together, our observations point to the focal dissolution of airborne ZnO NPs in the cellular environment and the internalization and accumulation of the ions specifically in endosomes and lysosomes as the processes underlying the potent toxicity of these airborne NPs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A standard curve used to determine deposited cellular dose of aerosolized NPs at the ALI. Deposited NP doses were measured using QCM in $\mu\text{g}/\text{cm}^2$ and plotted against the initial NP concentrations that were used to generate the aerosolized NPs. The values from three independent measurements for each initial NP concentration were averaged and plotted.

Figure 2. ZnO NP dissolution dynamics was measured at 10 µg/mL NP concentration in RPMI growth medium using ICP-MS. NP dissolution reached saturation within 30 min. Note that the earliest time point was 5 min due to the time it took to prepare the NP suspensions from the dry form.

Figure 3.

Examples of fluorescence images of live alveolar type II epithelial cells, grown at the ALI, taken at different time points post exposure to aerosolized NPs. Intracellular Zn²⁺ (green) was labeled with FluoZin-3 (green) and the nuclei were labeled with DAPI (blue). A marked increase in intracellular Zn²⁺ is noticed at 3 h post exposure.

Figure 4.

Quantitative analysis of intracellular Zn^{2+} at increasing time points post exposure to aerosolized NPs at the ALI (A) and to NP suspension in submerged cultures (C) was done by measuring fluorescence intensity of FluoZin-3 in thousands of cells per time point using flow-cytometry. Cells at the ALI were exposed to a toxic deposited NP dose of $6 \mu\text{g}/\text{cm}^2$ and membrane integrity was evaluated at the same time points post exposure by measuring LDH release into the basal growth media (B). Likewise, cells in submerged cultures were exposed to a toxic dose of NP suspension ($45 \mu\text{g}/\text{mL}/1 \text{ h}$) and membrane damage was evaluated by the release of LDH into the growth media (D). A fundamental difference in intracellular Zn^{2+} dynamics is observed between cells exposed to aerosolized NPs at the ALI versus cells exposed to NP suspension in submerged cultures. Significant increase over time-matched controls is indicated by: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Figure 5.

Intracellular Zn^{2+} values in cells exposed to aerosolized NPs at the ALI (solid line) and to NP suspension in submerged cultures (dashed line) are plotted using the same fluorescence intensity scale, showing the fundamental differences in the dynamics of intracellular Zn^{2+} in the two exposure systems. Over 24 h, the peak value in intracellular Zn^{2+} at the ALI is reached 3 h post exposure (arrow), while the peak value in submerged cultures is reached 24 h post exposure (arrow).

Figure 6.

Dose–response relationships for toxicity, determined at 24 h by LDH release, and peak intracellular Zn²⁺, quantified in FluoZin-3 intensity units, were measured in cells exposed to aerosolized NPs at the ALI (A, B) and cells exposed to NP suspension (C, D) or ZnSO₄ (E, F) in submerged cultures. The lowest dose that induces a significant increase in LDH release and the corresponding peak intracellular Zn²⁺ value are marked by the arrows for each exposure system. The peak intracellular Zn²⁺ value generated by the lowest toxic dose at the ALI (28.8 ± 1.2 FluoZin-3 intensity units) is several folds lower than the peak values generated by the lowest toxic dose of NPs or ZnSO₄ in submerged cultures (82.1 ± 16.1 and 233.9 ± 8.2 intensity units, respectively). Significant increase over time-matched controls is indicated by: * $p < 0.05$, ** $p < 0.01$.

Figure 7.

The presence of zinc ions in endosomes or lysosomes was detected and quantified at 1 and 3 h post exposure to aerosolized NPs at the ALI (6 μg/cm²) using live cell fluorescence microscopy. Examples of images for each treatment are presented in A–D, where the cell edges and nuclei are marked by the solid line and the areas enlarged in the insets are marked by the dashed line. Cells expressing the endosomal fluorescent protein marker – Rab5-RFP, show a significant co-localization (yellow) between intracellular Zn²⁺ (green) and endosomes (red) at 1 h (A), but less co-localization at 3 h (C) post exposure. Cells

expressing the lysosomal fluorescent protein marker – Lamp1-RFP, show less co-localization between intracellular Zn^{2+} (green) and lysosomes (red) at 1 h (B), but a significant co-localization (yellow) at 3 h (D) post exposure. A quantitative 3D co-localization analysis is summarized in E. The fractions of intracellular Zn^{2+} found in endosomes (dashed bars) or lysosomes (solid bars) are plotted for 1 and 3 h post exposure, showing that the majority of intracellular zinc ions following exposure at the ALI are found in endosomes and lysosomes.

Figure 8.

The intracellular fate of zinc ions was studied at 1 and 3 h post exposure to 30 µM ZnSO₄ in submerged cultures using live cell fluorescence microscopy. Representative overlaid fluorescence and DIC images of cells expressing Rab5-RFP to mark the endosomes (A, C), and Lamp1-RFP to mark the lysosomes (B, D) show minimal or no co-localization between intracellular Zn²⁺ (green) and these vesicles (red) at 1 and 3 h post exposure. Instead, intracellular Zn²⁺ is accumulated in other larger vesicles or vacuoles, as clearly shown in the inset, where the area marked by the dashed line in D is enlarged. A quantitative 3D co-

localization analysis is summarized in E, where the fractions of intracellular Zn^{2+} found in endosomes (dashed bars) or lysosomes (solid bars) are plotted for 1 and 3 h post exposure, showing that the majority of intracellular zinc ions following exposure to ZnSO_4 in submerged cultures are found in other vesicle types.

Figure 9.

LysoTracker, an acidophilic fluorescent dye, was used to determine whether the presence of the NPs or their dissolved ions disrupt the acidic environment in the lysosomes by comparing the degree of co-localization between LysoTracker or Lamp1-GFP and fluorescent NPs. Cell samples were exposed to ZnO NPs labeled with Alexa 546 at the ALI ($3.01 \mu\text{g}/\text{cm}^2$) or in submerged cultures ($40 \mu\text{g}/\text{mL}$) and incubated overnight. Representative fluorescence images are shown in A–D, where the cell edges and the nuclei are marked by the solid line and the areas enlarged in the insets are marked by the dashed line. Only the cells of interest are marked and fluorescent signals outside the delineated cell area come from

fluorescent NPs in neighboring cells. A significant co-localization is detected between the fluorescent NPs (red) and Lamp-GFP (green) at the ALI (A) and in submerged cultures (C). In contrast, minimal or no co-localization is detected between the NPs (red) and LysoTracker (green) at the ALI (B) and in submerged cultures (D). A quantitative 3D co-localization analysis is summarized in E, where the fractions from the total NPs inside the cells that co-localized with Lamp1-GFP (dashed bars) or with LysoTracker (solid bars) are plotted. The significant co-localization with Lamp1-GFP indicates that about 40–50% of the NPs reach the lysosomes, either intact or dissolved. However, the minimal co-localization with LysoTracker indicates that the presence of the NPs or their ions prevented the accumulation of the acidophilic dye in these vesicles?.

Table 1

Summary of peak intracellular Zn^{2+} values generated by the lowest toxic dose in each exposure system.

Exposure system	Peak intracellular Zn^{2+} generated by the lowest toxic dose (FluoZin-3 intensity)
Aerosolized ZnO NPs at the ALI	28.8 ± 1.2
ZnO NPs in submerged cultures	82.1 ± 16.1
ZnSO_4 in submerged cultures	233.9 ± 8.2