Zinc Oxide Nanoparticles Interfere With Zinc Ion Homeostasis to Cause Cytotoxicity

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The toxicological effects of zinc oxide nanoparticles (ZnO-NPs) are attracting increasing concern as the field of nanotechnology progresses. Although the literature suggests that toxicity of ZnO-NPs may be related to their dissolution, the mechanism for ZnO-NP perturbation of cytosolic zinc concentration ([Zn\(^2+\)]) homeostasis remains obscure. Using FluoZin-3 and RhodZin-3, this study investigated changes in both [Zn\(^2+\)], and mitochondrial free [Zn\(^2+\)] concentration ([Zn\(^2+\)]\(_m\)) under conditions of ZnO-NP treatment in vivo and in vitro. In human leukemia Jurkat cells and human lung carcinoma H1355 cells, ZnO-NP treatment resulted in an elevation of both [Zn\(^2+\)] and [Zn\(^2+\)]\(_m\). In H1355 cells, ZnO-NP treatment induced depolarization of mitochondrial membrane potential, as well as caspase-3 activation and lactic dehydrogenase (LDH) release. In our in vivo experiments, when rats were exposed to ZnO-NPs, higher [Zn\(^2+\)] and [Zn\(^2+\)]\(_m\) were recorded in both broncho-alveolar lavage (BAL) cells and white blood cells isolated from ZnO-NP-exposed rats, compared with high efficiency particulate air-filter–protected controls. BAL levels were also elevated in the BAL of ZnO-NP–exposed rats compared with controls. A mechanical toxicological pathway for ZnO-NP toxicity is suggested by these results: an elevation in [Zn\(^2+\)], resulting from ZnO-NP dissolution in the intracellular endosome; cytosolic Zn\(^2+\) sequestration by mitochondria; and elevated [Zn\(^2+\)]\(_m\) leading to mitochondrial dysfunction, caspase activation, and cell apoptosis. We conclude that exposure to ZnO-NPs interferes with the homeostasis of [Zn\(^2+\)] and that elevated [Zn\(^2+\)] results in cell apoptosis.

Key Words: zinc oxide nanoparticles; zinc ion; cytosolic zinc ion concentration; mitochondrial zinc ion concentration; mitochondria membrane potential; caspase-3.

The chemical compound zinc oxide (ZnO) is widely used in the manufacture of paints, coatings, and semiconductors. Exposure to ZnO is known to cause metal fume fever, characterized by dry cough, chills, aches, and fever. Metal fume fever is the most frequent acute respiratory ailment of welders (Blanc et al., 1993), and although exposure to respirable fumes of manganese and other metals have been implicated in producing symptoms related to metal fume fever, ZnO is the most common and best-characterized cause (Kelleher et al., 2000; Meo and Al-Khlaiwi, 2003). Inhalation of ZnO has been shown to obstruct pulmonary function in animals and to cause pulmonary impairment similar to that of metal fume fever in humans (Beckett et al., 2005; Fine et al., 1997). Exposure of human broncho-alveolar carcinoma A549 cells to zinc oxide nanoparticles (ZnO-NPs) exhibits stronger dose-dependent cytotoxicity than is seen with other metal oxides (Lin et al., 2009). NPs, with sizes below 100 nm, are extremely attractive materials for industrial use but have also brought new public health concerns because of their potential for negatively impacting human health and the fact that the mechanism for their action is unknown or less well-documented. Thus, a mechanistic toxicological pathway for ZnO-NP effects on human health remains obscure.

The toxicological effects of dissolution of nanosized metals have attracted numerous scientific studies. Solubility of oxide NPs, such as silicon dioxide, influences their cytotoxicity in mammalian cell lines (Brunner et al., 2006). Using recombinant bacteria as a zinc sensor, Heinlaan showed that the solubility of bulk ZnO, ZnO-NPs, and ZnSO\(_4\) was related to their toxicity (Heinlaan et al., 2008), and both ZnO and Zn\(^2+\) have been demonstrated to induce oxygen radical formation in human neutrophils (Lindahl et al., 1998). In 2008, Xia et al. (2008) measured the dissolution of ZnO in medium and buffer using Inductively Coupled Plasma-Mass Spectrometry to quantify Zn content and also measured intracellular ZnO and Zn\(^2+\) levels by confocal microscopy, quantifying elevations in cytosolic Ca\(^2+\) concentrations with the Ca\(^2+\)-sensitive fluorescent dye, fluo-4. They propose that oxidative stress occurs via Ca\(^2+\), causing collapse of mitochondria membrane potential in the affected cells. In 2010, George et al. proposed that decreasing the ZnO dissolution rate could reduce toxicity of ZnO by slowing Zn\(^2+\) release (George et al., 2010). Together, these previous studies show that Zn\(^2+\) is likely to play a critical role in the toxicity of ZnO-NPs. However, the precise mechanism

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for perturbation of cytosolic Zn\(^{2+}\) concentration ([Zn\(^{2+}\)]\(_c\)) homeostasis following exposure to ZnO-NPs remains unclear.

Some contradictory data have been reported. Chemical investigations using equilibrium dialysis have demonstrated slow dissolution of ZnO-NPs in an aqueous medium at pH 7.6 (Franklin et al., 2007). However, ZnO appears to react differently under different pH conditions, exhibiting extreme stability above pH 7.0, but much greater dissolution at lower pH. Differential toxic potency of ZnO-NPs compared with Zn\(^{2+}\) has been reported by Zhu in zebrafish embryos (Zhu et al., 2008, 2009). Thus, further study of [Zn\(^{2+}\)]\(_c\) homeostasis under conditions of ZnO-NP exposure is critical and will enable us to determine whether the toxicity of ZnO-NPs is, in fact, due to their dissolution.

Zinc is an essential element for humans but has toxic effects on mammalian cells at elevated concentrations. Zn\(^{2+}\) shares some common characteristics with Ca\(^{2+}\) and plays crucial roles in cell survival, exemplified by the fact that Zn\(^{2+}\) at low concentrations is vital for cellular metabolism and cell proliferation, yet at high concentrations Zn\(^{2+}\) can cause cell death. Mitochondria serve as checkpoints and amplifiers in cell death pathways. Elevation of [Zn\(^{2+}\)]\(_m\) can trigger breakdown of mitochondrial membrane potential, caspase activation, and cell apoptosis (Gazaryan et al., 2007; Link and von Jagow, 1995; Manev et al., 1997; Skulachev et al., 1967). Recent availability of fluorescent dyes sensitive to Zn\(^{2+}\) but not to Ca\(^{2+}\) has enhanced our ability to selectively measure these cations. FluoZin-3, highly selective for Zn\(^{2+}\), has been used to measure [Zn\(^{2+}\)]\(_m\) (Devinney et al., 2005; Gee et al., 2002), and RhodZin-3 has been shown to effectively localize to mitochondria and to selectively bind Zn\(^{2+}\); thus it can be used to detect changes in mitochondrial free Zn\(^{2+}\) concentration ([Zn\(^{2+}\)]\(_m\)) (Sensi et al., 2003b). Taking advantage of these tools, this study investigated both in vitro and in vivo changes in [Zn\(^{2+}\)]\(_m\) and [Zn\(^{2+}\)]\(_c\) following the treatment of two cultured human cell types (Jurkat and H1355) with ZnO-NPs or airway exposure (rats) to ZnO-NPs.

MATERIALS AND METHODS

Materials

Human leukemia Jurkat cells were purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan). Human lung carcinoma H1355 cells and human leukemia Jurkat cells were purchased from American Type Culture Center (Manassas, VA). Sprague-Dawley (SD) rats were purchased from BioLAS Co. (Taipei, Taiwan). ZnO-NPs of two different sizes (one < 50 nm with surface area 10.8 m\(^2\)/g and the other < 100 nm with surface area 15–25 m\(^2\)/g) were purchased from Sigma-Aldrich Co. (St Louis, MO) for use in the in vitro assays. Dulbecco’s Modified Eagle’s Medium, fetal bovine serum (FBS), Minimal Essential Medium, and RPMI 1640 medium were purchased from Gibco-BRL (Gaithersburg, MD). DiOC\(_2\) (3), FluoZin-3 AM, Newport-Green DCF diacetate, and RhodZin-3 AM were purchased from Invitrogen (Grand Island, NY). Phopholipids G122 was purchased from Omanolminum Inc. (Gaithersburg, MD). KCl, NaCl, and other salts were obtained from Merck KGaA (Darmstadt, Germany).

In Vitro Study

Cell culture. Human leukemia Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% FBS, pH 7.6, and were grown in a 5% CO\(_2\) incubator at 37°C (Harrett et al., 1998). Human lung carcinoma H1355 cells were cultured in RPMI 1640 medium supplemented with 5% FBS, pH 7.6, and were grown in a 5% CO\(_2\) incubator at 37°C (Lin et al., 2004). pH was maintained between 7.4 and 7.6 throughout the culture and treatment periods.

Characterization of NPs. For in vitro studies, stock suspensions (100 mM) of ZnO-NPs of the different size classes were prepared in ultrapure Milli-Q water, loading buffer (pH 7.6), and culture media (pH 7.6). Each suspension was sonicated with an ultrasonicator (Branson 5510) for 0.5 h prior to exposure. Each stock solution was then serially diluted to yield various concentrations. Size characterization of ZnO-NP samples was obtained by size distribution analysis of high-resolution transmission electron microscopy (TEM) images and scanning electron microscopy (SEM) images. TEM samples were prepared as 1 mM ZnO-NPs (size < 50 nm; 81.4 μg/ml in buffer, pH 7.5) suspension prepared as above. TEM images of ZnO-NP were taken on a TEM (Philips CM100). SEM samples were prepared as 1 mM ZnO-NPs suspension prepared as above, SEM images of ZnO-NP were taken on SEM (Nova NanoSEM 230). The size distribution and zeta potential of ZnO-NPs (size < 50 nm, 81.4 μg/ml, pH 7.63 and size < 100 nm, 81.4 μg/ml, pH 7.62) in RPMI medium were measured via dynamic light scattering (DLS, Malvern Zetasizer Nano-ZS, Malvern, U.K.) at 25°C.

Cytosolic free zinc concentration ([Zn\(^{2+}\)]\(_c\)) measurements. Jurkat cells were treated with ZnO-NPs (< 50 nm), having 100 μM particles added each day for three consecutive days (medium completely exchanged with each addition). H1355 cells were treated with ZnO-NPs (size < 50 nm) for 30 min. After treatment, cells (5 × 10\(^6\) cells/ml) were loaded with 10 μM FluoZin-3 or Newport-Green DCF at 37°C for 40 min. Cells were then washed twice with a loading buffer containing 150 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM MgCl\(_2\), 2.2 mM CaCl\(_2\), and 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid, pH 7.4. For Newport-Green DCF detection, fluorescence intensities were measured at 505 nm excitation and 535 nm emission and for FluoZin-3 at 494 nm excitation and 515 nm emission. All fluorescence measurements were taken using a fluorometer (SPEX, CM system). Cytosolic (intracellular) free zinc concentration ([Zn\(^{2+}\)]\(_c\)) was calculated using the manufacturer-recommended equation: [Zn\(^{2+}\)]\(_c\) = \(K_d\times(F_0-F_\text{min})/(F_\text{max} - F_0)\). A \(K_d\) value of 15 and 1000 μM Zn\(^{2+}\) was used for FluoZin-3 and Newport-Green DCF, respectively, were used.

Mitochondrial zinc concentration ([Zn\(^{2+}\)]\(_m\)) measurements. RhodZin-3 was used to detect [Zn\(^{2+}\)]\(_m\). Briefly, cells were loaded with 10 μM RhodZin-3 at 37°C for 40 min, then washed twice with pH 7.4 loading buffer. Fluorescence intensities were measured as above, at 550 nm excitation and 575 nm emission. [Zn\(^{2+}\)]\(_m\) was calculated using the equation: [Zn\(^{2+}\)]\(_m\) = \(K_d\times(F_0-F_\text{min})/(F_\text{max} - F_0)\). A \(K_d\) value of adding 0.01% digitonin and 0.1 mM Zn\(^{2+}\) to a cuvette at the end of each experiment; excess ethylene glycol tetraacetic acid (EGTA) was subsequently added to obtain F\(_{\text{min}}\) (minimal fluorescence intensity where no dye is bound by zinc ions). A \(K_d\) value of 15 and 1000 μM Zn\(^{2+}\) was used for FluoZin-3 and Newport-Green DCF, respectively, were used.

Lactic dehydrogenase release assay. The lactic dehydrogenase (LDH) release assay was modified from the study of Jiang et al. (2009). Briefly, cells were plated in 24-well plates on the day before the experiments. After treatment regime, supernatant containing LDH that has leaked from cells was collected from each well and set aside. Cells were then exposed to lysis buffer (9% Triton X-100) in order to obtain samples for intracellular LDH measurement. Following incubation for 30 min at room temperature, supernatants and cell lysates were separately subjected to the LDH assay. The extent of reduction of tetrazolium salt to a red formazan salt served as a measure of LDH activity. Absorbance was measured at 490 nm (A\(_{\text{max}}\)) with a reference wavelength of 690 nm. Results are expressed as percent released of total (supernatant – released LDH/[supernatant – released LDH + LDH from lysed cells]) × 100.

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**Mitochondrial membrane potential (ΔΨm) detection.** JC-1 staining was carried out according to the manufacturer’s instructions with modifications of Zhuang et al. (2008). Cells were collected, washed in PBS, and then incubated in 10 μg/ml JC-1 for 15 min at 37°C and washed again in PBS. Stained cells were analyzed by flow cytometry (Beckman, FC500).

**Zn-NP Generation and Rat Exposure (In Vivo Study)**

ZnO particles were produced by a particle-generation system. This system creates particles using an evaporation-coagulation mechanism, similar to the ZnO fume generator described by Fine et al. (1997). ZnO-NPs were generated from zinc powder heated to 570–600°C in a ceramic crucible furnace. ZnO vapor was produced by zinc vapor reacting with oxygen in high purity nitrogen gas. Before entering the major exposure chamber, ZnO vapors were mixed with filtered air in a series of cooling, humidifying, and diluting zones. Particle number, distribution, and concentration were monitored by a Scanning Mobility Particle Sizer and Condensation Particle Counter in the major exposure chamber (Fig. 7). For the rodent study, 8-week-old male SD rats were used after 1 week acclimatization to a 12-h light/dark cycle, 23°C ± 1°C temperature, 40±5% humidity. Animals were randomly separated into two groups (six each), the high efficiency particulate air (HEPA)-control and ZnO-NP exposure group. Rats were housed in cages having a front tube connected to the major exposure chamber, either with (control group) or without (treated group) a HEPA air-filter. Rats were sacrificed using pentobarbital (50 mg/kg) 24 h after ZnO-NP exposure. Broncho-alveolar lavage (BAL) fluid and peripheral blood were collected from each animal. Our study was approved by the Institutional Animal Care and Use Committee at National Taiwan University.

BAL fluid was collected by washing the airway with a phosphate-buffered saline solution (PBS, pH 7.4, 28 ml/kg body weight). Lavage fluid was used for trypsin blue cell counts by light microscopy to confirm cell viability. The collected BAL cells were loaded with Newport-Green DCF and FluoZin-3 to measure [Zn2+]i (as described above). LDH activity in lavage fluid was measured using the lactate dehydrogenase reagents and assay described above.

White blood cells (WBC) were prepared from peripheral blood samples. 20 ml lysis buffer (62.5 mM NH4Cl, 3.8 mM Tris base, and 2.2 mM EDTA, pH 7.2) was added to each 2 ml blood sample for 5–6 min, until the suspension became clear. Cells were twice in lysis buffer (centrifuged at 1000 × g, 5 min). Finally, cells were resuspended in loading buffer and loaded with RhodZin-3. The experiments measuring [Zn2+]i were carried out as described above.

**RESULTS**

**Particle Characterization**

The commercially purchased NPs used for in vitro studies were characterized using DLS, zeta potential, TEM, and SEM, as described. Particle size of ZnO-NP in buffer was determined by TEM and SEM (Fig. 1). Greater than 95% of the ZnO-NP in buffer was determined to be < 50 nm. The average of particle diameter was 22.3 ± 5.1 nm (n = 30) in TEM and 44.5 ± 13.5 nm in SEM. Distribution of particle size by intensity and volume using DLS is shown in Supplementary figures S1 and S2, respectively. Following suspension in medium, the ZnO-NPs (powder size < 50 nm) had an average diameter of 16.07 nm and zeta potential of −9.85 mV. Size distribution by intensity analysis demonstrated three peaks (diameter/intensity): 5.7 nm/30%, 25.2 nm/32%, and 255 nm/38%. For ZnO-NPs of < 100 nm suspended in medium, the average diameter was 90.70 nm and zeta potential −9.48 mV. Intensity analysis of size distribution again showed three peaks (diameter/intensity): 6.8 nm/13%, 60.9 nm/25%, peak three in 248 nm/61%. Thus, both preparations of ZnO-NP contained a high percentage of particles within nanometer range. The size distribution of ZnO vapor for the in vivo studies was characterized using a Scanning Mobility Particle Sizer and Condensation Particle Counter (Fig. 7). The size distribution of ZnO-NPs in three separate exposures was: 11–140 nm with peak at 32.4 nm; 7–139 nm with peak at 42.6 nm; and 8–143 nm with peak at 39.2 nm. All measurements show that the main particle sizes are distributed within nanometer range.

In order to prove our hypothesis that ZnO-NPs may dissolve into Zn2+ in endosomes, we measured the changes of [Zn2+]i...
under exposure to ZnO-NPs and we also observed the turbidity of ZnO-NPs solution in different pH (Supplementary figure S3). The ZnO-NPs solution changed from cloudy in pH 7.4 to clear in pH 5.5 (endosomal pH). We suggest that the disappeared particles were dissolved into zinc ion to support our hypothesis that ZnO-NPs may dissolve into Zn\(^{2+}\) in endosomal pH.

ZnO-NPs Induce an Elevation in [Zn\(^{2+}\)]\(_{c}\) and [Zn\(^{2+}\)]\(_{m}\) in Jurkat Cells

This study measured the effects of ZnO-NPs on [Zn\(^{2+}\)]\(_{c}\) in human leukemia Jurkat cells, as a model for circulatory cells. Figure 2 shows that the [Zn\(^{2+}\)]\(_{c}\) of ZnO-NP–treated cells was 20-fold higher than for untreated cells (p < 0.01). Since high [Zn\(^{2+}\)]\(_{c}\) may cause cell damage via Zn\(^{2+}\) flux from the cytosol to subcellular organelles such as mitochondria, we measured [Zn\(^{2+}\)]\(_{m}\) with RhodZin-3, a selective dye for [Zn\(^{2+}\)]\(_{m}\) in order to determine whether this occurs. Figure 2B shows that [Zn\(^{2+}\)]\(_{m}\) levels for treated cells were 7.0-fold higher than for untreated cells (p < 0.05).

ZnO-NPs Induce an Elevation in [Zn\(^{2+}\)]\(_{c}\) and [Zn\(^{2+}\)]\(_{m}\) in H1355 Cells

In human lung epithelial H1355 cells, ZnO-NP treatment resulted in elevation of [Zn\(^{2+}\)]\(_{c}\) and [Zn\(^{2+}\)]\(_{m}\) in a dose-dependent manner (Fig. 3). Using Newport-Green DCF in Figure 3A, treatment with 1mM (81 \(\mu\)g/ml) ZnO-NPs (size < 50 nm) elevated [Zn\(^{2+}\)]\(_{c}\), 28.8-fold compared with untreated cells. Smaller ZnO-NPs (size < 50 nm) resulted in a greater rise in [Zn\(^{2+}\)]\(_{c}\) than ZnO-NPs of larger (< 100 nm) size (data not shown). At 100\(\mu\)M (8.1 \(\mu\)g/ml), both < 50 and < 100 nm particle size classes resulted in significantly elevated [Zn\(^{2+}\)]\(_{c}\) (p < 0.01) but induced only a 2.5- and 1.5-fold elevation, respectively. Figure 3B shows the changes in [Zn\(^{2+}\)]\(_{c}\) measured using FluoZin-3, a highly Zn\(^{2+}\)-sensitive dye. Pretreatment with ZnO-NPs induced an increase in [Zn\(^{2+}\)]\(_{m}\). At 10\(\mu\)M (0.8 \(\mu\)g/ml), ZnO-NPs (< 50 nm) significantly elevated [Zn\(^{2+}\)]\(_{c}\), 5.8-fold over controls (Fig. 3B; p < 0.05). The images of either FluoZin-3 or Newport-Green DCF show that the cytosolic fluorescences were at a higher level in ZnO-NPs–treated cells compared with untreated (Supplementary figure S4).

Changes in [Zn\(^{2+}\)]\(_{m}\) were measured using RhodZin-3. Data is shown in Figure 3C. Pretreatment with ZnO-NPs induced elevation not only of [Zn\(^{2+}\)]\(_{c}\), but also of [Zn\(^{2+}\)]\(_{m}\). At 10\(\mu\)M (0.8 \(\mu\)g/ml) ZnO-NPs, [Zn\(^{2+}\)]\(_{m}\) was 2.4-fold higher in treated compared with untreated cells (p < 0.05). At 1mM (81 \(\mu\)g/ml), [Zn\(^{2+}\)]\(_{m}\) increased 6.6-fold (size < 50 nm, p < 0.01) and 6.5-fold (size < 100 nm, p = 0.12, data not shown) compared with untreated controls.

ZnO-NPs Decrease \(\Psi\)\(_{m}\) in H1355 Cells

Influx of cations from the cytosol to mitochondria can change the \(\Psi\)\(_{m}\) and cause a sudden increase in mitochondrial membrane permeability, leading to release of cytochrome c and triggering the intrinsic apoptosis pathway (Brookes et al., 2004; Kroemer, 1999). Depolarization of \(\Psi\)\(_{m}\) was examined using the fluorescent dye JC-1. CCCP, a proton ionophore, causing depolarization of \(\Psi\)\(_{m}\) was used as a positive control. Figure 4A shows the change in \(\Psi\)\(_{m}\); red fluorescence decreased in ZnO-NP pretreated cells. Figure 4B shows that 200\(\mu\)M (16 \(\mu\)g/ml) ZnO-NPs caused a significant decrease in \(\Psi\)\(_{m}\) (p < 0.05). One millimolar (81 \(\mu\)g/ml) ZnO-NPs decreased \(\Psi\)\(_{m}\) to 53.8 \(\pm\) 16.2% of untreated control. In order to understand the impact of mitochondrial dysfunction on the cells, LDH release and caspase-3 activities were measured in the following experiments.
ZnO-NPs Reduce Viability of H1355 Cells

In H1355 cells, LDH release was increased following ZnO-NP treatment in a dose-dependent and time-dependent manner (Fig. 5). Hundred micromolar (8.1 μg/ml) ZnO-NPs, < 50 nm, induced significant LDH release ($p < 0.05$). Three-day treatment with ZnO-NPs caused higher LDH release than 1-day treatments at concentrations above 10 μM (0.8 μg/ml). Smaller particles (< 50 nm, Fig. 5A) induced higher LDH release than larger ones (< 100 nm, Fig. 5B), especially at 81 μg/ml ZnO-NPs. In order to classify the toxicity of ZnO-NP, the cytotoxicity of bulk ZnO and ZnCl₂ were investigated. Bulk ZnO induced no significant change at the concentration from 0.081 to 81 μg/ml. In the case of ZnCl₂, significant effects only occurred at 1 mM (Fig. 5C).

In order to confirm involvement of the intrinsic apoptosis pathway in H1355 cells, we used Phophilux G1D2 to measure the effect of ZnO-NP treatment on caspase-3 activation. The proportion of cells having caspase-3 activity was greater in ZnO-NP–treated cells compared with controls (Fig. 6). At 81-μg/ml treatment, there was an 11.2-fold increase in caspase-3 activity.

**Elevated [Zn$^{2+}$]$_c$ in BAL and WBC From Rats Exposed to ZnO-NPs**

In experiments measuring the effects of exposure to ZnO-NPs in SD rats, [Zn$^{2+}$]$_c$ in BAL cells were determined. Following exposure, BAL cells were collected and loaded with Newport-Green DCF for measurement of [Zn$^{2+}$]$_c$ (Fig. 8A). [Zn$^{2+}$]$_c$ in BAL cells from exposed rats was 2.8-fold higher than for HEPA-control animals ($p < 0.05$). FluoZin-3 measurements confirmed the finding of [Zn$^{2+}$]$_c$ increase in BAL cells from exposed rats (Fig. 8B), showing a 1.5-fold elevation compared with controls. Similarly, [Zn$^{2+}$]$_c$ of WBC from ZnO-NP–exposed rats was elevated 5.9-fold above HEPA-controls (Fig. 8C; $p < 0.05$). RhodZin-3 measurement of [Zn$^{2+}$]$_m$ in rat WBC, showed a 1.1-fold higher level in experimental animals compared with HEPA-controls (Fig. 8D).

**LDH Release in Rat BAL Fluid Is Elevated Following Exposure to ZnO-NPs**

LDH activity was measured in BAL fluid from exposed and control rats. In the low-dose group, animals were exposed to ZnO-NPs of 34 nm at $5.3 \times 10^5$ particles/cm$^3$ for 5 h; in the high-dose group, rats were exposed to 25 nm particles at $4.1 \times 10^6$ particles/cm$^3$ for 10 h. Figure 9 shows that LDH was elevated in BAL fluid from all exposed rats. LDH activity was 4.1 and 6.9-fold higher in low- and high-exposure animals, respectively, compared with their HEPA-controls. The sub-G1 level of BAL cells collected from exposure rats was 1.88 ± 0.29 folds of BAL cells collected from HEPA-control rats.

The Kruskal-Wallis analysis shows that the LDH release level was significantly related to the surface area of ZnO-NPs with an $R^2$ value of 0.42, $p < 0.01$; significantly related to the mass of ZnO-NPs with an $R^2$ value of 0.41, $p < 0.01$; and significantly related to the particle number concentration of ZnO-NPs with an $R^2$ value of 0.36, $p < 0.01$.

**DISCUSSION**

Based on our in vitro and in vivo data, we propose a mechanical pathway of ZnO-NP exposure leading to cell damage via dissolution into zinc ions, as shown in Figure 10. This proposed pathway is divided into two phases, reflecting cellular compartmentalization and changes in cytosol (phase I) and in mitochondria (phase II) during ZnO-NP invasion. Phase
I begins with exposure to ZnO-NPs, leading to a rise in $[\text{Zn}^{2+}]_c$, which, in turn, leads to phase II, where a consequent rise in $[\text{Zn}^{2+}]_m$ results in cell death.

In phase I, ZnO-NPs are engulfed by cells via phagocytosis or endocytosis, resulting in intracellular ZnO-NPs that are present principally in vesicles. During intracellular membrane trafficking, endocytic vesicles fuse with preendosomes to produce endosomes. When ZnO-NPs are present in endosomes (pH approximately 5.5), the particles are gradually dissolved under acid conditions. Rise in $[\text{Zn}^{2+}]_c$ results from leakage of $\text{Zn}^{2+}$ at high concentrations from endosomes.

Our data have characterized phase I of this hypothetical mechanical pathway. The in vitro evidence shown in Figures 2 and 3 document elevation of $[\text{Zn}^{2+}]_c$ in cultured lymphocytes and lung cells following exposure to ZnO-NPs. The in vivo data shown in Figure 8 shows the dramatic changes in $[\text{Zn}^{2+}]_c$ for both BAL and WBC from rats exposed to ZnO-NPs, compared with HEPA-controls. Both our data, and that of others, strongly supports the existence of the phase I mechanical pathway. It is well known that ZnO dissolves under acid conditions. Intracellular vesicles maintain a lower pH via the $\text{H}^+$ pump in the vesicular membrane, and intracellular endosomes have a pH close to 5. The dissolution rate of ZnO at pH 5 (endosomal) will therefore be higher than at either pH 7.2 (cytosolic) or 7.4 (extracellular). In 2008, Xia et al. (2008) measured labeled ZnO-NPs present in intracellular vesicles and reported data consistent with our proposal of endocytosis of ZnO-NPs in phase I. Transient receptor potential channels are $\text{Zn}^{2+}$-permeable cation channels that play a critical role in cellular $\text{Zn}^{2+}$-induced injury (Inoue et al., 2010; Tu et al., 2010). These channels could provide a mechanism for $\text{Zn}^{2+}$ flux in phase I. Considering all of the reported data, both here and elsewhere, we predict the existence of the phase I toxicological pathway.

The phase II pathway begins with elevation of $[\text{Zn}^{2+}]_m$ and culminates in cell death. Elevation of $[\text{Zn}^{2+}]_c$ is likely counteracted by mitochondrial sequestration of $\text{Zn}^{2+}$ in order to maintain homeostasis.
to maintain a cytosolic dynamic homeostasis. Mitochondrial uptake of accumulated cytosolic Zn$^{2+}$ leads to an elevation in \([Zn^{2+}]_m\) and consequent depolarization of \(\Psi_m\) and \(\Delta\Psi_m\) loss, triggering the intrinsic apoptosis pathway. The mechanical toxicological pathway of ZnO-NPs, characterized in this study as phase II, encompasses the ZnO-NP–induced elevation in \([Zn^{2+}]_m\), loss of \(\Delta\Psi_m\), activation of caspase-3, and release of LDH. Figures 2 and 3 show ZnO-NP–induced elevation in \([Zn^{2+}]_m\) for Jurkat and H1355 cells, respectively. In addition to this in vitro evidence, we have obtained in vivo data using our rat model. In Figure 8, it can be seen that WBC isolated from ZnO-NP–exposed rats had higher \([Zn^{2+}]_m\) than HEPA-controls. In cultured human lung cells, loss of \(\Delta\Psi_m\), activation of caspase-3, and cell death data are shown in Figures 4–6, respectively. Additional data from other laboratories concerning the effects of Zn$^{2+}$ on the permeability of mitochondrial membranes support our proposed pathway. In 1967, Brierley and Knight (1967) observed Zn$^{2+}$ uptake by mitochondria, and in other studies, Zn$^{2+}$ was shown to interfere with the tricarboxylic acid cycle and with the mitochondrial electron transfer chain.
transport chain (Dineley et al., 2003; Link and von Jagow, 1995; Skulachev et al., 1967). In 1998, Lindahl observed that Zn$^{2+}$ bound to and stimulated PLA2, causing oxygen radical generation (Lindahl et al., 1998). Loss of Nm induced by Zn$^{2+}$ was measured by Dinely et al. (2002, 2003), and the same laboratory has also shown that mitochondrial generation of reactive oxygen species (ROS) can be induced by an elevation in \([Zn^{2+}]_c\) (Dineley et al., 2005). In addition, Zn$^{2+}$ at high concentration has been shown to cause DNA fragmentation and to activate caspase-3 (Jimenez Del Rio and Velez-Pardo, 2004; Watjen et al., 2002). Taken together, our data and that of other laboratories give solid support to the proposed steps in phase II of our mechanical toxicological pathway for ZnO-NPs.

Cations play vital roles in mitochondria, where they are known to modulate ATP synthesis and ROS generation. The singular importance of Ca$^{2+}$ is documented by numerous reports and reviews (Feissner et al., 2009). Zn$^{2+}$ shares some common characteristics with Ca$^{2+}$ as a divalent cation and has been shown to act as a signal regulating neuronal function and to play a role in mitochondrial function (Capasso et al., 2005; Feissner et al., 2009; Pavon et al., 2009; Sensi et al., 1999; Xia et al., 2004). Rapid Zn$^{2+}$ influx triggers ROS generation, loss, mitochondrial dysfunction, caspase activation, and apoptosis (Dineley et al., 2005; Donadelli et al., 2009; Sensi et al., 2000, 2003a; Sensi and Jeng, 2004; Zhang et al., 2004). Our data also show that elevation in \([Zn^{2+}]_c\) and \([Zn^{2+}]_m\) is coupled with mitochondrial dysfunction, caspase activation, and eventual cell death. We postulate that elevation in \([Zn^{2+}]_c\) induced by ZnO-NP may well trigger subsequent cell damage.

The known roles of Zn$^{2+}$ in the cytotoxicity of ZnO-NPs have been noted above. Kelleher et al. (2000) in 2000 postulated that increase in Zn$^{2+}$ may be the critical step in stimulating oxygen radical generation by cells exposed to ZnO in the circulation. ZnO-NPs have been shown to decrease mitochondrial function (Jeng and Swanson, 2006), and Luo et al. (2009) recently observed that Zn$^{2+}$ is significantly associated with cell damage induced by ZnO. Also in 2009, Deng et al. (2009) documented the cytotoxicity of ZnO and Zn$^{2+}$ and suggested that the toxicity of both was likely via Zn$^{2+}$. Our study clearly shows that \([Zn^{2+}]_c\) increases in a dose-dependent fashion following ZnO-NP exposure in both cultured human cell lines and in BAL cells from ZnO-NP–exposed rats. Together, these data strongly suggest that ZnO-NP toxicity results from the effects of their dissolution to Zn$^{2+}$ ions, and the data reported here give further evidence that ZnO-NPs can interfere with \([Zn^{2+}]_c\) homeostasis and lead to cell damage and death.

FIG. 8. Measurements of \([Zn^{2+}]_c\) and \([Zn^{2+}]_m\) in rat BAL cells and WBC. Rats were either exposed to ZnO-NPs (n = 6) or were under HEPA filter-control (n = 6). The exposure concentration was 5.6 mg/m$^3$ containing $9.2 \times 10^5$ particles/cm$^3$. Following exposure, BAL cells and WBC were collected. Panel A: \([Zn^{2+}]_c\) measurements of rat BAL cells with Newport-Green DCF. Panel B: \([Zn^{2+}]_c\) measurements of rat BAL cells was measured using FluoZin-3. Panel C: \([Zn^{2+}]_c\) measurements of rat WBC using Newport-Green DCF. Panel D: \([Zn^{2+}]_m\) measurements of rat WBC using RhodZin-3. Data are expressed as mean ± SD. *: \(p < 0.05\) significant difference between ZnO-NP–exposed and HEPA-control rats.

FIG. 9. ZnO-NP exposure elevated LDH content in rat BAL fluid. Rats were exposed to ZnO-NPs (high dose [n = 6]: 25 nm size at $4.1 \times 10^6$ particle numbers/cm$^3$, 23.2 mg/m$^3$ for 10 h; low dose [n = 6]: 34 nm size at $5.3 \times 10^5$ particle numbers/cm$^3$, 6.6 mg/m$^3$ for 5 h; and HEPA-control [n = 6]), and BAL cells were collected. The data are expressed as mean ± SD. *: \(p < 0.05\) significant difference between BAL fluids from ZnO-NP–exposed and HEPA-control rats.
The Zn$^{2+}$ fluorescent dyes FluoZin-3 and RhodZin-3 have high sensitivity to Zn$^{2+}$ but not to Ca$^{2+}$ (Devinney et al., 2005; Gee et al., 2002; Sensi et al., 2003b), whereas Ca$^{2+}$-sensitive fluorescent dyes are widely known to bind not only Ca$^{2+}$ but also other metal ions, including Zn$^{2+}$ (Devinney et al., 2005; Dineley, 2007; Haase et al., 2009). However, calcium ion sensitive dye Fura-2 has a 100-fold higher affinity for Zn$^{2+}$ over Ca$^{2+}$ (Grynkiewicz et al., 1985), and Fluo-4 shows greater than 10-fold higher affinity for Zn$^{2+}$ (Haase et al., 2009). By using FluoZin-3 and RhodZin-3, our measurements of changes in [Zn$^{2+}$]$_c$ and [Zn$^{2+}$]$_m$ give an accurate picture of Zn$^{2+}$ homeostasis and effectively eliminate possible Ca$^{2+}$-dye binding. Our data show that elevated [Zn$^{2+}$]$_c$ and [Zn$^{2+}$]$_m$ play a critical role in activation of caspase-3 and leads to cell apoptosis and LDH release. We conclude that elevation in [Zn$^{2+}$]$_c$ is the critical event in the toxicological process of ZnO-NPs. Our study elucidates a mechanical toxicological pathway for ZnO-NP inhalation. Phase I initiates with endocytosis of ZnO-NPs, their dissolution in endosomes and a consequent increase in [Zn$^{2+}$]$_c$. In phase II, mitochondria sequester excess cytosolic Zn$^{2+}$ to result a rise in [Zn$^{2+}$]$_m$. High [Zn$^{2+}$]$_m$ induces mitochondrial membrane potential collapse, activating caspase-3 and resulting in cell apoptosis.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/

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