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Zinc oxide nanoparticles delay human neutrophil apoptosis by a *de novo* protein synthesis-dependent and reactive oxygen species-independent mechanism

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**Highlights**

- ZnO nanoparticles are antiapoptotic for human neutrophils.
- ZnO nanoparticles do not increase ROS production in human neutrophils.
- ZnO nanoparticles induce *de novo* protein synthesis in human neutrophils.

**Abstract**

Inflammation is one of the major toxic effects reported in the literature following nanoparticle (NP) exposure. Knowing the importance of neutrophils to orchestrate inflammation, it is surprising that the direct role of NPs on neutrophil biology is poorly documented. Here, we investigated if ZnO NPs can alter neutrophil biology. We found that ZnO NPs increased the cell size, induced cell shape changes, activated phosphorylation events, enhanced cell spreading onto glass, but did not induce the generation of reactive oxygen species (ROS). Treatment of neutrophils with ZnO NPs markedly and significantly inhibited apoptosis and increased *de novo* protein synthesis, as demonstrated by gel electrophoresis of metabolically [35S]-labeled cells. Utilization of the protein synthesis inhibitor, cycloheximide, reversed such antiapoptotic effect. We conclude that ZnO NPs are activators of several human neutrophil functions and that they inhibit apoptosis by a *de novo* protein synthesis-dependent and ROS-independent mechanism. This is the first example that a NP acts on the neo-synthesis of polypeptides.

**Keywords**

- Inflammation;
1. Introduction

Nanomaterials are increasingly produced and the use of engineered nanoparticles (NPs) in a variety of sectors, including biomedical sciences, pharmaceutical industry, electronics, cosmetics, etc., is getting more and more common because the physicochemical properties of their small 1–100 nm size differ from standard bulk materials. Consequently the probability of exposure to NP has risen (Oberdorster et al., 2005). NPs could enter human body not only via inhalation, but also via ingestion and dermal exposure, reaching blood circulation. Therefore, it is crucial to evaluate the risk that NPs represent to human health. One of the major toxic effects of NPs frequently reported in the literature is inflammation. In in vivo model, airway NP exposure lead to an increased number of polymorphonuclear neutrophil (PMN) cells in lungs and bronchoalveolar lavages (Morimoto et al., 2011, Noel et al., 2012 and Roursgaard et al., 2011); PMN counts are used as biomarkers of inflammation.

The metal oxide group of NPs are among the families of NPs that generate increasing attention from the scientific community. One of the most ubiquitous NPs among metal-oxide NPs is zinc oxide (ZnO). Besides having great anti-microbial properties, ZnO NPs are widely used in a variety of compounds, including cosmetics, sunscreens and toothpastes, to name a few (Heng et al., 2011 and Vandebriel and De Jong, 2012). Despite the widespread use of ZnO NPs, its safety for humans is unknown and the intracellular regulatory mechanisms involved in ZnO NPs-induced cytotoxicity reported in a variety of cells are unclear (Heng et al., 2011, Sahu et al., 2013, Vandebriel and De Jong, 2012 and Wilhelmi et al., 2013). The direct interaction between NPs and PMNs is poorly documented. In recent few studies, we and others demonstrated that human neutrophil biology could be altered in response to NPs (Abrikossova et al., 2012, Babin
et al., 2013, Goncalves et al., 2010 and Goncalves and Girard, 2011). Among these, ZnO was found to induce degranulation in PMNs (Babin et al., 2013). Although it is relatively well known how PMNs can respond to pathogens, especially bacteria and fungi, as well as to a plethora of modulators like cytokines (Kato and Kitagawa, 2006), our knowledge regarding how PMNs react to NPs is unknown. Since the cytotoxicity and inflammatory properties of ZnO NPs appear to be cell type-dependent (Heng et al., 2011, Sahu et al., 2013, Vandebriel and De Jong, 2012 and Wilhelmi et al., 2013), the need to investigate how PMNs respond to these NPs is important. The aim of this study was to determine how ZnO NPs alter the biology of PMNs.

2. Materials and methods
2.1. Chemicals, agonists and antibodies
RPMI-1640, HEPES, penicillin/streptomycin (P/S); arsenic trioxide (As$_2$O$_3$ or ATO) and formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol 12-myristate 13-acetate (PMA) and cycloheximide (CHX) were purchased from Sigma–Aldrich, (Saint-Louis, Missouri); Recombinant human GM-CSF was purchased from Peprotech Inc. (Rocky Hill, NJ, USA).

2.2. Nanoparticles
The NPs used in this study are water-dispersible and non-aggregating zinc oxide (crystalline ZnO) carboxy-functionalized, in aqueous suspension stabilized by sodium polyacrylate (<20 nm particle size) with metal content of 97% for the ZnO NP suspension as determined by inductively coupled plasma (Vivenano, ONT). If not specified, ZnO NPs (referred hereafter simply as ZnO) were used at a concentration of 100 μg/ml, based on a previous study (Babin et al., 2013).

2.3. Transmission electron microscopy
The NP suspension obtained from the manufacturer was examined using a Hitachi H-7100 transmission electron microscope.

2.4. Characterization of ZnO
The classical *Limulus amebocyte* lysate (LAL) assay for determining endotoxin level is a method in which NPs could interfere (Neun and Dobrovolskaia, 2011). Therefore, for testing sterility, we incubated the ZnO suspension in Lysogeny broth agar plates for 48 h and observed the presence or absence of colonies. As a positive control, we used a suspension of voluntary contaminated TiO$_2$ NPs that we strictly reserved to perform this kind of analysis. Pictures of the plates were taken with a Motorola Atrix™ cell phone. The size distribution and zeta potential of ZnO were determined by dynamic light scattering using a Zetasizer Nano-ZS (model ZEN3600) from Malvern Instruments Inc. (Westborough, MA). Measurements were performed with NPs in suspension in HBSS, RPMI-1640 or RPMI-1640 + 10% human serum, according to the tested functions, at a concentration of 100 μg/ml and at 37 °C, the temperature at which the experiments were performed.

2.5. Neutrophil isolation

Neutrophils were isolated from venous blood of healthy consenting individuals according to institutionally approved procedures as previously described (Goncalves et al., 2010, Pelletier et al., 2002 and Pelletier et al., 2001). Cell viability was monitored by trypan blue exclusion and never exceed 3% and the purity (>98%) was verified by cytology from cytocentrifuged preparations colored Hema-3 stain set (Biochemical sciences Inc., Swedesboro, NJ).

2.6. Release of lactate dehydrogenase

Lactate dehydrogenase (LDH) release was measured using the colorimetric LDH cytotoxicity assay from ABCAM (Cambridge, MA).

2.7. Cell morphology of neutrophils

Microscopic observations were performed as previously reported (Girard et al., 1997 and Goncalves et al., 2010). Freshly isolated human PMNs ($10 \times 10^6$ cells/ml) were incubated at 37 °C in 5% CO$_2$ in 12-well plates during 24 h in the presence of buffer or 100 μg/ml ZnO. Morphological changes in cells were observed under light microscopy (400×), and photomicrographs were taken using a Nikon Eclipse TS100 camera.
2.8. Evaluation of cell size by flow cytometry

Freshly isolated human PMNs (10 × 10^6 cells/ml in RPMI-1640 HEPES-P/S) were treated with or without 100 μg/ml ZnO for 30 min and data for cell size (FSC-H) were acquired and analyzed by flow cytometry using a FACScan (BD Biosciences).

2.9. Expression of tyrosine phosphorylated proteins

Neutrophils (40 × 10^6 cells/ml in HBSS) were incubated for the indicated periods of time at 37 °C with buffer or 100 μg/ml ZnO in a final volume of 120 μl. Reactions were stopped by adding 120 μl 2× Laemmli’s sample buffer and the evaluation of the tyrosine phosphorylation events was performed essentially as previously described (Goncalves et al., 2010).

2.10. Neutrophil spreading onto glass

PMNs were incubated (10 × 10^6 cells/ml suspended in RPMI 1640 supplemented with 10% autologous serum) in 24-well plates at 37 °C in an atmosphere with 5% CO₂ in the presence of buffer or 100 μg/ml ZnO for 12 h (Girard et al., 1997). Then, 10 μl of the cell suspension were loaded onto a haemocytometer (Fisher Scientific; 0.100 mm deep; Hausser Bright-Line) and incubated for 5 min at 37 °C. Cells were then examined under a light microscope and recorded as spread (irregular shape) or nonspread (round shape). A minimum of 300 cells/experiment were counted and the results were expressed as percentage of spread cells.

2.11. Superoxide production

Superoxide (O₂⁻) production was monitored by colorimetric assay (reduction of cytochrome c) as previously published (Binet et al., 2006 and Simard et al., 2011). Briefly, neutrophils (1 × 10^6 cells/ml) were suspended in HBSS supplemented with 1.6 mM CaCl₂ with or without 10 μg/ml superoxide dismutase (SOD) with 130 μM ferrocytochrome c for 5–90 min at 37 °C in the presence of various concentrations of ZnO or PMA (10⁻⁷ M), used as a positive control. The reduction of cytochrome c was monitored at 550 nm and the concentration of O₂⁻ anions produced was calculated by the difference between corresponding wells with or without SOD using an extinction coefficient of 21.1.
2.12. Detection of intracellular ROS

Cells \((10 \times 10^6 \text{ cells/ml})\) were suspended in HBSS containing 10 μM CM-H2DCFDA for 15 min at 37 °C as previously published (Simard et al., 2011). Cells were then washed twice before being incubated in the presence of buffer or 100 μg/ml ZnO for 15, 30 and 60 min. PMA \((10^{-7} \text{ M})\) was used as a positive control. Fluorescence was recorded using a FACScan. ROS production was expressed as MFI.

2.13. Assessment of neutrophil apoptosis

PMNs \((10 \times 10^6 \text{ cells/ml in RPMI 1640-HEPES-P/S, supplemented with} 10\% \text{ heat-inactivated autologous serum})\) were treated with or without increasing concentrations of ZnO \((0.02–100 \mu \text{g/ml})\) for 24 h. Cytocentrifuged samples of PMNs were prepared, stained with the Hema-3 Stain staining kit according to the manufacturer’s instructions and processed as documented previously (Binet et al., 2006). In some experiments, PMNs were treated with buffer (ctrl), CHX (10 μg/ml) or with ZnO + CHX in order to evaluate the role of \textit{de novo} protein synthesis during apoptosis.

2.14. Metabolic labeling and \textit{de novo} protein synthesis assay

Cells \((10 \times 10^6 \text{ cells/ml in RPMI-1640 medium supplemented with} 10\% \text{ autologous serum})\) were metabolically labeled with 4.625 MBq of the Redivue Pro-Mix L-[35S] in vitro cell labeling mix (GE Healthcare, Baie d’Urfé, QC, Canada) in the presence or absence of 100 μg/ml ZnO, 65 ng/ml GM-CSF, 10 μg/ml cyclohexymide (CHX) or a mixture of both ZnO + CHX for 24 h, as previously described (Binet et al., 2006).

2.15. Statistical analyses

Statistical analyses were performed using repeated measures ANOVA (excepted for Fig. 4A, not repeated measures) and differences between groups were assessed using the Dunnett’s Multiple Comparison Test with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Statistical significance was established at \(p < 0.05\), and each experiment was performed and validated at least 3 times.

3. Results

3.1. Characterization of ZnO nanoparticles
As illustrated in Fig. 1A, the starting diameter of ZnO was close to the one indicated by the manufacturer (1–10 nm), but some NPs were larger, as shown in representative TEM images (see arrow). Table 1 summarized the characterization of the ZnO by DLS. While the diameter of ~50% of the NPs was ~7 nm in HBSS or RPMI-1640 at 37 °C, it increased to about 600 nm when serum was added to RPM I-1640, indicating the presence of aggregates. The zeta potential was relatively stable with a value of −6.9 ± 0.8 mV and −6.1 ± 1.4 mV in HBSS and RPMI-1640, respectively. Addition of serum in RPMI-1640 revealed a zeta potential of −9.5 ± 5.7 mV. Fig. 1B illustrates that ZnO are not contaminated (right panel) since no colonies were observed on plates after 48 h of incubation In contrast, colonies were observed in plates where the positive control was used (left panel, arrows).

Fig. 1.
Characterization of ZnO nanoparticles. An aliquot of the ZnO was taken directly from the bottle provided by the manufacturer (99% water, 1% sodium polyacrylate derivate) and (A) transmission electron microscopy or (B) sterility assay, were performed as described in Section 2. A, in general, the diameter of the NPs were close to that reported by the manufacturer (1–10 nm) but some were with a larger diameter (see arrow, diameter: 24.7 nm). B, the ZnO were not contaminated as evidenced by the absence of colonies after 48 h of incubation in Lysogeny broth agar plates (right panel) in contrast to the positive control (Pos ctrl) where some colonies are observable (arrows, left panel).

Table 1.
Size and zeta potential of ZnO NP.

<table>
<thead>
<tr>
<th></th>
<th>RPMI-1640</th>
<th>RPMI-1640 + 10% human serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (nm)</td>
<td>11.50 (53.4%)</td>
<td>80.1 (67.7%)</td>
</tr>
<tr>
<td></td>
<td>74.38 (26.1%)</td>
<td>366.6 (16.5%)</td>
</tr>
<tr>
<td></td>
<td>1.03 (20.6%)</td>
<td>11.8 (12.7%)</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>−9.1 ± 0.6</td>
<td>−16.0 ± 3.0</td>
</tr>
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</table>
3.2. Cell viability and activation of PMNs by ZnO

PMNs were treated with an increasing concentration of ZnO (0–200 μg/ml) for 24 h and at different periods of time (2, 4, 6, 12 and 24 h), cell viability was always greater than 97% except at 24 h with 200 μg/ml ZnO where ~5% of necrosis was observed (data not shown). Therefore, the concentration of 100 μg/ml was selected for the rest of the study. Consistent with that, we observed that ZnO did not increase LDH release (Fig. 2A). Of note, previous experiments revealed that ZnO did not interfere with the assay (data not shown). Next, we monitored cell shape by cytology after ZnO treatment as a marker of PMN activation. As illustrated in Fig. 2B, after 24 h, the cell shape remain relatively round or spherical in control cells (left panel) by opposition to the classical irregular shape observed in activated PMNs (right panel). This indicates that neutrophils are activated by ZnO whether the NPs enter the cells or not. We next measured cell size in ZnO-induced PMNs by flow cytometry and, as illustrated in Fig. 2C, ZnO increased the cell size when compared to control cells, suggesting that NPs are inside PMNs. To further support that PMNs are activated by ZnO, we next determined whether or not tyrosine phosphorylation events occurred. As illustrated in Fig. 2D, ZnO rapidly increased the level of tyrosine phosphorylation, as observed after 15 s. The maximum effect was observed after about 1 min of activation. The phosphorylation signal declined subsequently but still remained strong compared to control cells, confirming that PMNs are activated by ZnO. Measuring the PMNs ability to spread onto glass is an interesting way for evaluating a biological response in PMNs related to cytoskeletal activity (Girard et al., 1996a, Lee et al., 1993, Pelletier et al., 2001 and Whyte et al., 1993). As illustrated in Fig. 2E, after 12 h of incubation (optimal time point) the number of spreading cells was significantly increased by ZnO and, as expected, by the potent fMLP neutrophil agonist (Lee et al., 1993).
Fig. 2.

ZnO are not cytotoxic and activate PMNs. PMNs were incubated in the presence or absence (Ctrl) of 100 μg/ml of ZnO and cytotoxicity/necrosis was evaluated by the LDH assay (A) using the positive control (Pos) furnished by the manufacturer (Kit); cell shape changes (B) were monitored by optical microscopy as described in Section 2 for up to for 24 h (n = 6); cell size was assessed by flow cytometry (C); tyrosine phosphorylation events was performed by western blot using a pan anti-phosphotyrosine antibody (D, upper part) and the membranes were Coomassie blue stained at the end of the experiments to confirm equivalent loading proteins; and cell spreading was assessed as described in Section 2 using fMLP as a positive control (E). Results are means ± SD (n ≥ 3). *p < 0.05 vs Ctrl.

3.3. ZnO nanoparticles do not increase ROS production in human PMNs

We next evaluated the ability of ZnO to induce O$_2^-$ production after 30 min, a typical time point to observe O$_2^-$ production in neutrophils (Pelletier et al., 2001) and, as illustrated in Fig. 3A, ZnO, at a concentration of 10, 50 or 100 μg/ml, did not significantly increase O$_2^-$ production by human PMNs. However, cells were responsive since PMA, as expected, significantly increased the O$_2^-$ production (Pelletier et al., 2001). Next, we performed kinetic experiments in order to eliminate the possibility that ZnO act more rapidly or, conversely, require more time to increase O$_2^-$ levels. As illustrated
in Fig. 3B, O$_2^-$ concentrations did not significantly increase over time when compared to controls, even after 45–90 min, where the effect of PMA start to decline. We next investigated the possibility that ZnO increased the total ROS production in PMNs by flow cytometry using the CM-H2DCFDA probe and, as illustrated in Fig. 3C, ZnO, unlike PMA, had no significant effects of accumulation of intracellular ROS over time.

Fig. 3. ZnO do not induce ROS in neutrophils. PMNs were treated with buffer (Ctrl), PMA or increasing concentrations of ZnO for 30 min (A) or with buffer (○), PMA (●) or 100 μg/ml ZnO (■) for the indicated periods of time (B) and the superoxide (O$_2^-$) production was measured as described in Section 2. Production of total ROS was assessed by flow cytometry using the H2DCFDA probe as described in Section 2 (C). Inset in panel C illustrates a typical results plotted in the graph where a marked shift of fluorescence to the right is observed in response to PMA (arrow) but not in response to ZnO. Results are means ± SD (n = 3). *p < 0.05 vs Ctrl.
Fig. 4. ZnO delay neutrophil apoptosis by a de novo protein synthesis-dependent mechanism. (A) PMNs were incubated in the presence of buffer (Ctrl), 5 μM ATO, 65 ng/ml GM-CSF (GM) or the indicated concentrations of ZnO and apoptosis was assessed by cytology. Results are means ± SD (n ≥ 3). (B) PMNs were metabolically labeled as described in Section 2 and incubated with buffer (Ctrl), 65 ng/ml GM-CSF, 100 μg/ml ZnO, 10 μg/ml CHX or ZnO + CHX for 22 h and cell lysates were prepared for electrophoresis as described in Section 2. The left part is the corresponding Coomassie blue-stained gel of the autoradiograph illustrated in the right part ([35]S). Results are from one representative experiment out of three. (C) PMNs were incubated for 24 h with buffer (Ctrl), ZnO (100 μg/ml), CHX (10 μg/ml) or CHX + ZnO and apoptosis was assessed by cytology. Results are means ± SD (n = 3). *p < 0.05 vs Ctrl; #p < 0.05 vs corresponding bars.

3.4. ZnO nanoparticles inhibit human neutrophil apoptosis by a de novo protein synthesis-dependent mechanism
As illustrated in Fig. 4A, 50 and 100 μg/ml ZnO inhibited PMN apoptosis. As expected, ATO increased and GM-CSF (GM) inhibited significantly apoptosis (Pelletier et al., 2001). We next determined the apoptotic rate in ZnO-induced PMNs in the presence of cycloheximide (CHX), a potent inhibitor of protein synthesis (Binet et al., 2006, Bouchard et al., 2004 and Wagner et al., 2000). As illustrated in Fig. 4B, the ability of ZnO to inhibit spontaneous apoptosis was reversed when cells were treated with CHX + ZnO. Of note, CHX alone did not alter the basal apoptotic. We next investigated their capacity to stimulate de novo protein synthesis. As illustrated in Fig. 4C, ZnO can induce de novo synthesis of several polypeptides when compared to control cells. As expected, CHX drastically inhibited de novo protein synthesis when used alone and was found to reverse the effect of ZnO.

4. Discussion

The results of this study demonstrate that ZnO are human neutrophil activators, as evidenced by the capacity of these NPs to induce cell shape changes, tyrosine phosphorylation events and cell spreading. The ability of ZnO to induce cell shape changes, as well as cell spreading, fits well with our previous study where different NPs, including ZnO, were found to be able to induce actin polymerization, indicating cytoskeleton re-organization. However, ZnO do not induce the generation of ROS in human PMNs. This observation is in contrast with other studies reported in other cells where ZnO were found to induce oxidative stress in the human lung epithelial cell line BEAS-2B (Huang et al., 2010) and ROS-induced apoptosis in the human liver cell line HepG2 (Sharma et al., 2012). These effects were observed at a concentration ranging between 10 and 20 μg/ml ZnO. In our hand, no ROS production was observed at a concentration of ZnO ranging from 10 to 100 μg/ml (this report). Further, we found that ZnO delay or suppress PMN apoptosis. This is in contrast to previous studies indicating that ZnO induce apoptosis in cells other than PMNs (Heng et al., 2011, Sahu et al., 2013, Sharma et al., 2012, Vandebriel and De Jong, 2012 and Wilhelmi et al., 2013). Unlike the above studies, we used human cells of primary origin, PMNs, that are terminally non-dividing cells. Interestingly, the capacity of ZnO to inhibit PMN apoptosis was found to occur by a de novo protein synthesis-dependent mechanism, as for other
agents known to delay or suppress PMN apoptosis requiring continuous protein synthesis like the cytokines IL-4 (Girard et al., 1997), IL-13 (Girard et al., 1996b), IL-15 (Girard et al., 1996a), and other agents including sodium butyrate (Stringer et al., 1996) and dexamethasone (Cox and Austin, 1997). However, this do not ruled out the possibility that other pathways are involved, since we do not completely reversed the antiapoptotic activity with CHX.

Since a picture worth thousand words, it is clear from our results, showing that ZnO induced cell shape changes, that these NPs are PMN activators. Also, the results demonstrating that ZnO induced tyrosine phosphorylation events indicate that, at least, NPs react with cell membrane to activate some signaling events. These results are in agreement with others reporting that ZnO were found to induce phosphorylation of extracellular signal-regulated kinase (ERK) of MAPK pathways in human keratinocytes (Jeong et al., 2013). The increased of cell size observed by flow cytometry suggest also that ZnO react with cell membrane and penetrate inside cells as according to the technic of Suzuki et al. (2007) (Suzuki et al., 2007). In addition, our results are in agreement with a previous study using this same approach where ZnO were found to penetrate into human liver HepG2 cells (Sharma et al., 2012). Even if ZnO can activate human PMNs we cannot exclude the possibility that Zn\(^2+\) are released inside cells, which could be a potential mechanism of their toxicity. However, herein, we are interested in investigating the nanotoxicology of manufactured ZnO as they are. In addition, in this study, ZnO do not appear to be necrotic and/or cytotoxic for human PMNs at the tested concentrations. This is in line with our above observation that ZnO do not induce ROS generation in PMNs, a well-known potential toxic mechanism reported in other cells (Huang et al., 2010, Jeong et al., 2013, Sharma et al., 2012 and Vandebriel and De Jong, 2012).

To the best of our knowledge, this study is the first demonstrating that a NP, namely ZnO, can act on the biosynthesis of polypeptides, a major biological process involved in the majority of cellular functions. Further, instead of causing cellular toxicity through oxidative stress-induced apoptosis, we demonstrated here that ZnO suppress apoptosis in human neutrophils. However, depending on the context, an agent that suppresses PMN apoptosis could be seeing as a pro-inflammatory agent since, for example, this could lead to the persistence of PMNs at an inflammatory site.
perpetuating tissue damage instead of being eliminated by professional phagocytes for homeostasis. The fact they we recently demonstrated that ZnO can induce degranulation in human neutrophils, an oxygen-independent mechanism for host defense, is in line with the potential of inducing tissue damage. Therefore, we are aware that even if a given NP is not cytotoxic for human PMNs, this NP could possess pro-inflammatory activity.

In future, even if the data of this study further support the importance of investigating how human neutrophils respond to a given NP and that it is also mandatory to investigate several distinct functions before drawing any conclusion, determining the profile of cytokines and chemokines produced by ZnO-induced PMNs will help for better determining the potential pro-inflammatory effects of these NPs.

Conflict of Interest
The authors declare that there are no conflicts of interest.

Acknowledgments
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