Zinc oxide nanoparticles induce apoptosis by enhancement of autophagy via PI3K/Akt/mTOR inhibition

Ruchi Roy\textsuperscript{a,b}, Sunil Kumar Singh\textsuperscript{c}, L.K.S. Chauhan\textsuperscript{d}, Mukul Das\textsuperscript{a,b}, Anurag Tripathi\textsuperscript{a,**}, Premendra D. Dwivedi\textsuperscript{a,b,*}

\textsuperscript{a} Food, Drug and Chemical Toxicology Group, CSIR-Indian Institute of Toxicology Research (CSIR-IITR), M.G. Marg, Post Box No. 80, Lucknow 226001, India
\textsuperscript{b} Academy of Scientific and Innovative Research (AcSIR), New Delhi, India
\textsuperscript{c} Division of Parasitology, CSIR-Central Drug Research Institute, B.S. 10/1, Sector 10, Jankipuram Extension, Sitapur Road, Lucknow 226031, India
\textsuperscript{d} Electron Microscopy Facility, CSIR-Indian Institute of Toxicology Research, M.G. Marg, Post Box No. 80, Lucknow 226001, India

** Highlights

- ZNPs induced ROS generation whereas depleted antioxidant enzymes.
- ZNPs were found concentrated within autophagosomes and lysosomes.
- ZNPs followed extrinsic and intrinsic pathways of apoptosis in macrophages.
- Autophagy involved PI3K/pAKT/pmTOR cascade in ZNPs induced macrophages.
- NAC pre-treatment and LC3B-siRNA suppressed apoptosis and autophagy.

** Graphical Abstract

Schematic representation of the possible way of internalization of ZnO NPs into the macrophages and its after-effects.

** Abstract

Zinc oxide nanoparticles (ZnO NPs) induced macrophage cell death and its mechanism remains to be solved. Herein, we report that ZnO NPs induced ROS generation by depleting antioxidant enzymes, increasing lipid peroxidation and protein carbonyl contents in macrophages. The oxidative stress was induced by the inhibition of Nrf2 transcription factor release. ZnO NPs also activated the cleavage of apoptosis markers like caspases 3, 8 and 9, γH2AX activation and cleavage of poly (ADP-ribose) polymerase (PARP) that are known indicators of genotoxicity were found to be activated by following p53, p21/waf1 signaling. ZnO NPs increased the number of autophagosomes and autophagy marker proteins such as microtubule-associated protein 1 light chain 3 isoform II (MAP-LC3-II) and Beclin 1 after 0.5–24 h of treatment. Phosphorylated Akt, PI3K and mTOR were significantly decreased on ZnO NPs exposure. Moreover, the apoptotic and autophagic cell death could be inhibited on blocking of ROS generation by N-acetylcysteine (NAC) which demonstrated the critical role of ROS in both types of cell death. In addition, inhibition of LC3-II by siRNA-dependent knockdown attenuated the cleavage of caspase 3. This study demonstrates autophagy supports apoptosis on ZnO NPs exposure.

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1. Introduction

Zinc oxide nanoparticles (ZnO NPs) are widely used in many fields, such as rubber manufacture, cosmetics, pigments, food additives, medicine, chemical fiber and electronics industries. Because of the traditional concept that zinc oxide is non-toxic, the toxicological studies on ZnO NPs are far behind the speed of their application. In recent years, with the phenomenal increase in uses of ZnO NPs, associated health risks with its use is of concern.

Several studies have reported that the immune organs are the main sites for the deposition of nanoparticles after systemic exposure. It is highly probable that macrophages carry nanoparticles to the different organs, as their accumulation has been found in the phagolysosomes of tissue residing macrophages (Sadauskas et al., 2007; Adlersberg and Singer, 1973). Accumulation of ZnO NPs occurred in the liver, spleen, lungs, kidney and heart after single intraperitoneal administration (Li et al., 2012; Wang et al., 2008). Nanoparticles can travel from various entry routes such as dermal, oral and respiratory tract to other parts of the body. Macrophages unlike other cells are capable of efficient uptake of particles by phagocytosis and are present in many tissues as resident macrophages such as alveolar macrophages and Langerhans cells etc. ZnO NPs are widely added to the feed as additives. Ding et al. (2007) showed that the oral exposure to the low dosage of ZnO NPs in mice increased the phagocytic activity of mouse peritoneal macrophages. However, exposure to high doses of ZnO NPs induced the edema and degeneration of hepatocytes, inflammation of the pancreas and damage to the stomach and spleen (Wang et al., 2008).

There are several reports on the effect of different shapes and sizes on the toxicity of ZnO NPs (Lin et al., 2009; Deng et al., 2009; Yuan et al., 2010). Previously, we had reported that ZnO NPs can induce reactive oxygen species (ROS) generation and provoke modulation in immune responses (Roy et al., 2011, 2014). Most often, the harmful effects of ROS may be manifested by DNA damage, oxidations of poly-unaturated fatty acids in lipids and oxidations of amino acids in proteins (Limbach et al., 2007). Although, intra-cellular ROS had a significant correlation with cell viability but the link between ZnO NPs-induced cell death and oxidative stress is not well established in macrophages. Thus, in this study, we selected mouse primary peritoneal macrophages and investigated for oxidative damage to DNA, lipids and proteins of macrophages on ZnO NPs exposure. Further, we tried to find the evidences of autophagosome formation and its possible association with cell death. Autophagy is a key mechanism in various physiopathological processes including cell death and survival (Mizushima et al., 2008; Rubinszttein, 2006). Several reports have shown that autophagy not only enhances caspase-dependent cell death, but also required for it (Espert et al., 2006). In contrast, it has been shown that autophagy plays an important role in promoting cell survival against apoptosis. Macroparticles can induce dysfunction or overstimulation of autophagy pathway that may be further involved in the self cellular consumption and cytotoxicity. Defects in the autophagy pathway have been linked to a number of pathologies in humans such as chronic infection, muscular disorders, and cancer and neurodegenerative disease (Ravikumar et al., 2010).

Collectively, the aforesaid reports demonstrate that exposure to ZnO NPs affect macrophages and T cells mediated immune reactions. However, it is currently unclear whether cell survival mechanisms are influenced by ZnO NPs in macrophages or not. In light of the available evidences showing the impacts of ZnO NPs on the functionality of immune cells, the objective of the present study was to investigate the effect of ZnO NPs on autophagy and apoptosis cell death mechanisms as well as the possible association between them in macrophages exposed with ZnO NPs.

2. Materials and methods

2.1. Cell culture

Inbred strains of female Balb/c mice (8–10 weeks old) were sacrificed according to the guidelines for the care and use of the Animal Ethical Committee of Indian Institute of Toxicology Research, Lucknow, India. This study was carried out under the approval of the Institutional Ethics Committee (No. of approval-ITR/IAEC/47/11). Peritoneal exudate was collected from the peritoneal cavity of mice by injecting chilled RPMI 1640 medium and added to 96-well cell culture flat bottom plate. After 3 h of incubation in a CO2 incubator (5% CO2) at 37 °C, the non-adherent cells were removed by vigorous washing (three times) with warm RPMI 1640 medium. Furthermore, adhered cells were incubated overnight in RPMI 1640 medium supplemented with heat-inactivated 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in the humid air containing 5% CO2 to form macrophage monolayers. More than 95% of the adherent cell populations were macrophages as determined by morphometry and nonspecific esterase staining.

2.2. Transmission electron microscopy (TEM)

ZnO NPs powder was purchased from Sigma, USA and characterized for size and zeta-potential by transmission electron microscope (TEM) and dynamic light scattering (DLS). TEM result showed ZnO NPs were of ~50 nm and DLS size showed size distribution with an average diameter of 278.8 nm in culture media supplemented with 10% FBS. The zeta potential of ZnO NPs was ~11.5 mV in complete medium as shown in our previous study (Roy et al., 2014). Macrophages (2 × 10⁵ cells/well) were plated in 12-well cell culture plate. After overnight incubation, the cells were exposed to ZnO NPs (2.5 μg/ml) for 24 h. After exposure, the medium was aspirated and the cells were washed twice with 1× PBS. Cells were then centrifuged and the pellet was fixed with 2.5% glutaraldehyde and 2% paraformaldehyde. After washing with 0.1 M sodium cacodylate buffer the pellet was post fixed in 1% osmium tetroxide for 3 h. The fixed pellet was washed and dehydrated through increasing grades (30–100%) of acetone. The sample was infiltrated with Araldite™ resin overnight at room temperature and finally embedded in pure resin. The blocks were incubated at 60 °C for 72 h. After incubation, ultrathin sections were prepared using Reichert-Jung ultra microtome (Vienna, Austria). The sections were stained with uranyl acetate and Reynold’s lead citrate. To avoid confusions of any staining artifacts the grids were examined before and after staining under TEM (JEM-2100, JEOL Ltd., Tokyo, Japan) at 60 or 80 kV.

2.3. Assessment of necrosis and apoptosis

Apoptosis in macrophages was measured by using the annexin-V/propidium iodide (PI) apoptosis detection kit (BD Biosciences, San Diego, USA). Macrophages were incubated with 2.5 μg/ml of ZnO NPs for 0.5, 3, 6, 12, 24h and 10 mM N-acetylcysteine (NAC) pretreatment followed by ZnO NPs for 24h. After exposure, cells were trypsinized and centrifuged at 1000 rpm; the cell pellet was washed with PBS once and resuspended in 100 μl of binding buffer, then incubated with 2 μl annexin-V-FITC for 10 min, which was followed by staining with 1 μg/ml PI. Then, the samples were diluted by adding 400 μl binding and at least 10,000 cells were counted for each sample. The cell population of interest was gated on the basis of the forward and side-scatter properties. Vertical and horizontal lines were designed on the basis of autofluorescence of untreated control cells. Then different cell populations were identified, where FITC negative and PI negative were designated as viable cells; FITC positive and PI negative as early apoptotic cells; FITC positive and PI positive as late apoptotic cells or necrotic cells; and FITC negative and PI positive as necrotic cells. The data analysis was performed using BD FACS Diva software (Becton Dickinson, USA).

To analyze apoptotic cells by Hoechst labeling, both untreated and treated cells (1, 2.5, 5 or 10 μg/ml of ZnO NPs) were fixed with 4% formaldehyde followed by staining with Hoechst 33342 and then observed by confocal microscopy.

2.4. ROS measurement

In all 4 × 10⁵ cells/well were seeded into 96-well (black, flat bottom) cell culture plate. Cells were incubated with 10 μl/mL ZnO NPs for different time-points (0.5, 3, 6, 12 and 24h). For determination of specific free radical role in ZnO NPs-induced cell death, different specific scavengers against various free radicals such as 20mM mannitol and 20 mM ethanol for hydroxyl free radicals (Shen et al., 1997; Suthanthiran et al., 1984), 10 mM dimethyl formamide (DMF) for superoxide free radicals (Song and Yemen, 2002) and 40 mM L-Met for singlet oxygen (O²−) species (Berton et al., 1998) were added to macrophages for 0.5 h prior to 2.5 μg/ml of ZnO NPs exposure for 3 h. After incubation, the medium was taken out and cells were washed twice with 1× PBS. Thereafter, culture medium containing 2.7−dichlorodihydroflourorescin diacetate (H₂DCFDA) dye (20 μM) was added to cells. After 1 h, the plate was incubated for 0.5 h at 37 °C and the medium containing DCFDA was discarded. PBS (200 μl) was then added to each well and DCF fluorescence was recorded in a fluorimeter at excitation and emission wavelengths of 485 and 528 nm, respectively.
2.5. Oxidative stress markers

To study the time dependent effect of ZnO NPs on various oxidative stress markers, macrophages were treated with 2.5 μg/ml of ZnO NPs for 3, 6, 12 and 24 h. Reduced glutathione (GSH) content was assayed in the cells homogenate according to the method of Ellman (Ellman, 1959). Lipid peroxidation (LPO) in the cell homogenate was determined by the method of Packer and Joos (1980). Protein carbonyl content was measured in the homogenate according to the method of Levine et al. (Levine et al., 1999). Catalase activity was determined by the method of Sinha (Sinha, 1972). Glutathione reductase (GR), Glutathione peroxidase (GPx) and Glutathione S transferase (GST) activities were assayed according to the method of Moron et al. (Moron et al., 1979).

2.6. Detection of changes in mitochondrial membrane potential (MMP)

Mitochondrial membrane permeability was determined using the mitochondrial staining dye, Rhodamine 123. When the mitochondrial membrane potential collapses, the dye can no longer accumulate within the mitochondria and fluoresces green. Macrophages were treated with 2.5 μg/ml of ZnO NPs (0.5, 3, 6, 12 and 24 h) and 10 mM of NAC + 2.5 μg/ml of ZnO NPs treated groups in black bottom 96-well plate and were rinsed with PBS twice, stained with 0.3 μM Rhodamine-123 for 0.5 h at 37 °C after their incubations. Cells were rinsed with PBS twice, resuspended in 200 μl PBS, and immediately assessed by flow cytometry at 488 nm of excitation and emission of 535 nm to quantify the population of mitochondria with green fluorescence.

2.7. Determination of autophagosome formation

FITC tagged-LC3 antibody was used for detecting the formation of autophago-some by using Cyto-ID® autophagy detection kit (Enzo Life Sciences, Switzerland) as per the manufacturer’s protocol. Rapamycin (500 μM), an autophagy inducer and a known positive control of autophagy was added to cells for 16–18 h. The nucleus was stained with Hoechst 33342 stain. After the completion of staining, cells were washed twice with 1× assay buffer provided in the kit. Images of autophagic cells were taken by using a confocal microscope (TCS-SPE Confocal microscope, Mannheim, Germany) and fluorescence (autophagy flux) of total autophagic cells per group was read at FITC filter (Excitation 480 nm, Emission 530 nm) by using a fluorimeter.

2.8. Real Time PCR (RT-PCR) of Atg genes

RT-PCR analyses of Atg genes (Atg5, Atg10 and Atg12) in macrophages were carried out using gene-specific primers in control, 2.5 μg/ml of ZnO NPs (0.5, 3, 6, 12 and 24 h) and NAC + ZnO NPs treated groups. The oligonucleotide primers used were: Atg5: forward – primer-5′-AAGCTGTCTGCTCCGGACTCT-3′, reverse – primer-5′-TGAGAAGAACGCTTCTGGTACATCA-3′; Atg10: forward – primer-5′-GGCTCCAGTCTGGGAACTTGA-3′, reverse – primer-5′-TGCTGCATGCTCGACATCCT-3′; Atg12: forward – primer-5′-CATGCTTGAATCAAAAAGTTCC-3′, reverse – primer-5′-GGGAAAAGCCATTGCACTATAA-3′ and GAPDH: forward – primer-5′-TTACACCATGGAGACGGC-3′, reverse – primer-5′-GGCTGAGCTTGTGCTGATG-3′. Total RNA from cells were isolated with RNXzol®RT (Molecular Research Center, Inc., Cincinnati, OH, USA). cDNA from different groups were prepared by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Quantitative RT-PCR was carried out using a SYBER Green PCR master mix (Thermo Scientific, Waltham, MA, USA) according to the manufactures’ instructions. Amplification of housekeeping gene GAPDH was taken as an endogenous control. Normalized values were used for plotting the bar graphs.

2.9. Microtubule-associated protein 1 light chain 3-isofrom II (MAP-LC3-II)-siRNA transfection

Mouse MAP-LC3-II-siRNA was purchased from Santa Cruz Biotechnology. The siRNA was transfected into macrophages by using the transfection reagent (Santa Cruz Biotechnology, USA) and further treated with ZnO NPs for 24 h. The influence of autophagy on apoptosis was seen by blocking LC3 and then the cleavage of caspase 3 and LC-3 were identified.

2.10. Total cell lysate preparation

Macrophages were treated with 2.5 μg/ml of ZnO NPs (0.5, 3, 6, 12 and 24 h) and NAC + ZnO NPs. Following treatment to the cells, ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 100 mM Na3VO4, 0.5% NP–40, 1% Triton X–100, 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, pH 7.4) was added to the groups. Plates were placed on ice for 15 min and the lysates were collected in a microfuge tube. The lysates were cleared by centrifugation at 14,000 × g for 15 min at 4 °C and the supernatants (total cell lysate) were either used immediately or stored at −80 °C.

2.11. Western blotting

A forty microgram of protein was resolved in 10% SDS-PAGE and then elec-troblotted onto nitrocellulose membranes and incubated with different antibodies i.e. anti-H-Ras, anti-PI3K (BD Biosciences, USA) Bcl-2 (Abcam, USA); anti-nuclear factor-erythroid 2 (Nrf2), anti-p-mTOR, anti-γ-H2AX (Ser1339) (Santa Cruz, USA); anti-LC3 (Biogenex, USA); anti-p-Akt, anti-poly (ADP-ribose) polymerase (PARP), anti-p21, anti-p53, anti-Bax, anti-Becl-2, anti-cytochrome-c, anti-caspase 8, anti-caspase 9, anti-caspase 3 (Cell Signaling Technologies, Boston, MA). Immunoblots were detected with horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG using chemiluminescence kit and visualized by Versa Doc Imaging System (Bio- rad, CA, USA). To quantify equal loading, the membrane was reprobed with either anti-β-actin or anti-α-tubulin antibody. The density of each band was estimated by the Alpha Imager software. The β-actin was taken as an endogenous control and its respective densitometry values were used for normalizing different proteins expressions. Normalized values were indicated on the respective bands.

2.12. Statistical analysis

Data was expressed as mean ± SE and analyzed by Prism5.0 software using one-way and two-way ANOVA followed by Bonferroni intergroup comparison test. Correlation analysis was performed by determining the Pearson’s correlation coef-ficient (two-tailed) with a confidence level of 95% using SPSS software, p < 0.05 was considered as significant.

3. Results

3.1. Cell death induction by ZnO NPs

Cells were exposed to a series of concentrations of ZnO NPs (1, 2.5, 5 and 10 μg/ml) and cell death was analyzed by annexin/PI and Hoechst staining. Percentage of apoptotic cells death was calculated by adding the percentage of early apoptotic gated cells (annexin–V+ cells) and late apoptotic gated cells (annexin-V+/PI+). A significant increase (↑3.95%) in the apoptotic cells was observed with 2.5 μg/ml exposure to ZnO NPs (Fig. 1a) and whereas with 1, 5 and 10 μg/ml of ZnO NPs, the apoptotic cell death was 8.5, 7.35 and 7.15% observed respectively. In addition to this, a dose-dependent increase (1, 2.5, 5 and 10 μg/ml: 12.5, 13.6, 21.4 and 23.3% necrotic cells) in the percent of necrotic cells (PI+ stained) as well as nuclear alteration with chromatin condensation was observed (Fig. 1c).

3.2. ROS generation by ZnO NPs

Various nanoparticles are known inducer of ROS mediated cell death in several cell types. To probe ROS generation in macrophages after ZnO NPs exposure, H2DCFDA was used. After 3 h of exposure to 2.5 μg/ml of ZnO NPs, nearly two fold increase in ROS generation was observed over untreated control (Correlation coefficient, r = −0.145) (Fig. 1d). With the increase of concentration (1, 2.5, 5 and 10 μg/ml), it was found that ROS was significantly accumulated.

Apoptotic cell death and ROS generation were significantly observed at the dose of 2.5 μg/ml. Therefore, further experiments were performed on this concentration.

3.3. The effect of scavengers of ROS on ZnO NPs–induced ROS

ROS generation was enhanced at 2.5 μg/ml of ZnO NPs exposure therefore; determination of specific free radical role in ZnO NPs-induced cell death was identified on the same dose by using different specific scavengers such as mannitol, ethanol, DMF and tocopherol. We found that mannitol, ethanol and DMF provided a nearly complete reduction in ROS level which was induced by ZnO NPs (Fig. 1e) whereas tocopherol resulted in non-significant reduction in ROS.

3.4. Effect of ZnO NPs on oxidative stress markers in macrophages

Effect of ZnO NPs on oxidative stress markers in macrophages can be seen by a significant increase in LPO (2–4 fold) and protein
Fig. 1. Cell death mechanism and ROS generation. Macrophages were exposed to ZnO NPs (1, 2.5, 5 and 10 μg/ml for 24 h) and stained with (a) annexin V-FITC/PI before analyzed by flow cytometer. (b) Percent of total apoptotic cells represents the mean ± S.E.M. of five replicates. (c) Hoechst staining images of macrophages. Nuclear alteration and chromatin condensation are visible in ZnO NPs treated cells. (d) ROS generation on ZnO NPs treatment. Cells were treated with 2.5, 5 and 10 μg/ml ZnO NPs for 0.5, 1, 3, 6 and 12 h before incubation with 20 μM H2DCFDA and measured by fluorimeter. Percent increase in ROS production in treated cells was compared with untreated cells. Each reported value represents the mean ± S.E.M. of five replicates (*p < 0.05, compared with untreated control). (e) Effects of antioxidants (indicated in bar diagram as Tocopherol: T; Mannitol: M; Ethanol: E; and DMF: D) on ROS induced by ZnO NPs. Antioxidants were added 0.5 h prior to ZnO NPs exposure and ROS was measured after 24 h. Data in terms of percentage are presented as the mean ± SE of five values. (*p < 0.05, indicated significant compared with untreated control).
Fig. 2. Effect of ZnO NPs on oxidative stress markers in macrophages. Time-dependent effect of ZnO NPs on (a) LPO, (b) protein carbonyl, (c) GSH content, and activities of (d) GR, (e) GPx, (f) catalase and (g) GST of macrophages exposed for 3, 6, 12 and 24 h. Each value represents the mean ± SE of five values. *p < 0.05, significant with respect to control group.
carbonyl (~2 fold) along with a significant decrease in GSH content (one-third fold of control) on ZnO NPs exposure (Fig. 2a–c), while GPx and GST were significantly decreased and the activities of catalase and GR were gradually inhibited or reached to null at 24 h (Fig. 2d–g).

3.5. Effect of ZnO NPs on the oxidative stress mediated apoptotic signaling

Since Nrf2, a transcription factor plays a significant role in countering stress by inducing the expression of many other genes that are involved in antioxidant responses. We studied the effect of ZnO NPs on Nrf2 levels. As shown in Fig. 3a, ZnO NPs application resulted in enhancement of Nrf2 levels after 0.5 h but levels continued to decrease substantially after 3 h. Effect of ZnO NPs on the expression of p53 and p21/waf1 proteins in macrophages was also observed. Expression of these proteins was significantly enhanced after treatment of ZnO NPs for 0.5–12 h as compared to control. Further, ZnO NPs application for 0.5–24 h resulted in significant over expression of Bax along with reduction of Bcl2, which was associated with an increase in cytochrome c level in the cytoplasm (Fig. 3b). Caspase 8 cleavage was enhanced after 3 h of exposure of ZnO NPs to macrophages (Fig. 3c) while the expressions of cleaved caspase 9 and caspase 3 were enhanced maximally at 24 (Fig. 3d). Pretreatment of NAC reduced the cleavage of caspase 3, caspase 9 and caspase 8. Activation of caspase 3 resulted in PARP cleavage and γH2AX activation that was observed in a time-dependent manner after ZnO NPs application to macrophages (Fig. 3e) and these were also reduced on ROS inhibition by using NAC.

3.6. Antioxidant revert ROS induced cell death

In general, ROS induces autophagy and apoptosis therefore, we were interested to find effect of ROS on macrophages cell death. We pre-treated the macrophages with NAC, a well known inhibitor of ROS before ZnO NPs exposure and checked the percentage of apoptotic cells. Apoptotic cell death was significantly reduced from 14% to 5.4% and all over cell death decreased from 28% to 15% on treatment of NAC (Fig. 4a). This clearly suggested that cell death was induced as a result of ROS generation by ZnO NPs exposure.

Further, we performed experiment related to cell death which is loss of mitochondrial membrane potential (MMP). Remarkable loss of MMP was observed after exposure of ZnO NPs in a time-dependent manner (Fig. 4b). On pretreatment of NAC, MMP loss stopped and it was found comparable to control.

3.7. Internalization of ZnO NPs shown by TEM

Electron micrographs of macrophages exposed to ZnO NPs showed a significant cellular uptake of ZnO NPs (Fig. 5a). Multiple membrane foldings and plasma membrane (pseudopodial) extensions were fused together to engulf the ZnO NPs as indicated by the arrows. The nanoparticles detectable in the cytoplasmic region of macrophages showed accumulation of ZnO NPs within micron-sized vesicles resembling late endosomes and lysosomes, which are typical features of autophagosomes. In case of control, the membranous boundary was not at all folded and no vesicle was found there.

3.8. Autophagy induced by ZnO NPs

Next we examined whether ZnO NPs could induce autophagy or not. We incubated macrophages with ZnO NPs and found autophagosome accumulation (FITC+ LC3+ cells), a hallmark of autophagy after 0.5 h (Fig. 5b and c). Moreover we also assessed the expression of three other well known markers of autophagy namely Atg5, Atg10 and Atg12 genes in ZnO NPs treated cells and observed significant induction in their expression as compared to control when quantified by real time analysis (Fig. 6a). Also, at protein expression level, autophagy was confirmed by the increase in LC3-II and Beclin 1 levels in macrophages treated with ZnO NPs (Fig. 6b). Taken together, these results showed that ZnO NPs clearly induced autophagy in macrophages.

Further, the role of ROS in autophagy was determined by inhibiting ROS with NAC. We observed that NAC pre-treatment to ZnO NPs exposure leads to inhibition of Atg5, 10 and 12 mRNA expressions, LC3-II formation and Beclin 1 expression.

3.9. PI3K/AKT/mTOR signaling pathway activation by ZnO NPs for autophagy

To assess the role of PI3K and mTOR in ZnO NPs induced autophagy, we analyzed the phosphorylation levels of mTOR and PI3K by using western blotting and found that ZnO NPs reduced their expression in a time dependent manner (Fig. 6c). On treatment with ZnO NPs, level of phosphorylated Akt was significantly decreased. The effect of ZnO NPs on mTOR inhibition started after 6 h exposure. These results confirmed that ZnO NPs induced autophagy via PI3K/Akt/mTOR classical pathway in macrophages.

3.10. Influence of autophagy in apoptosis

Role of autophagy in induction of apoptosis remains unclear, and can have a protective or deleterious effect. Here, we examined the role of LC3 in the regulation of ZnO NPs-induced apoptosis and cell death. In LC3B-siRNA infected macrophages ZnO NPs displayed decreased formation of LC3-II in comparison to control (Fig. 6d). In LC3B-siRNA-treated macrophages, ZnO NPs treatment induced lower levels of apoptosis, as evidenced by the decreased cleavage of caspase-3.

4. Discussion

There are few reports concerning the cytotoxic effect of ZnO NPs on the immune cells. Previous studies showed that ZnO NPs exposure resulted in inflammatory responses and these were internalized in macrophages via caveolae mediated pathway (Roy et al., 2013, 2014). ZnO NPs have the potential to generate ROS under in vitro conditions that could be correlated to their potential to induce cellular inflammation under in vivo situations (Landsiedel et al., 2010; Ayres et al., 2008). Various studies have implicated intracellular oxidative stress as the cause of toxicity induced by ZnO NPs (Kim et al., 2010; Meyer et al., 2011; Yazdi et al., 2010). ZnO NPs induced ROS generation at 3 h could be due to sudden burden or exposure of NPs to metabolize these invaders or initiate the signaling required for balancing the intracellular homeostasis. Cells have adaptive and dynamic programs to create balance between generation and removal of ROS, hence for maintaining this balance Nrf2 transcription factor has been identified as the master regulator (Kensler et al., 2007). ZnO NPs suppressed Nrf2 that may cause significant depletion of antioxidant content and inhibition of anti-oxidant enzymes activity along with enhanced lipid peroxidation and protein carbonyls formation. It was found that DMF, ethanol and mannitol decreased the ROS generation which imply that superoxide ions and hydroxyl radicals were induced by ZnO NPs.

Exposure of ZnO NPs (2.5 μg/ml) caused significant enhancement in ROS generation at 3 h indicated that ROS generation following ZnO NPs exposure is an early event resulting in alterations of oxidative stress markers after 6 h (anti-oxidant and
Fig. 3. ZnO NPs induced oxidative stress, genotoxicity and apoptotic markers in macrophages. (a) Nrf2, p53 and p21/waf1 levels in 2.5 μg/ml ZnO NPs treated macrophages for 0.5, 3, 6, 12 and 24 h. (b) Bax, Bcl2, and cytochrome c levels in cytosolic fraction of macrophages. (c) Caspases 3, 8, and 9 cleavages in ZnO NPs (0.5, 3, 6, 12 and 24 h) and NAC + ZnO NPs treated macrophages. (d) γH2Ax activation and cleavage of PARP in ZnO NPs (0.5, 3, 6, 12 and 24 h) and NAC + ZnO NPs treated macrophages were analyzed by immunoblotting in whole cell lysates of macrophages.
anti-oxidant enzymes decreased while damage to lipid and protein increased. Our results were supported by a report where early ROS generation had later effect on oxidative enzymes (Kumar et al., 2011). Depletion in GR, catalase, GST and GPx activities after ZnO NPs exposure emphasized the occurrence of oxidative stress. Several studies suggested that enhanced free radicals may cause extensive damage to macromolecules of cell, i.e., DNA, protein and lipid, thus altering their structure and function (Das et al., 2005; Martin and Barrett, 2002; Gerloff et al., 2011). ZnO NPs exposure for 6–24 h caused significant DNA damage by enhancing cleavage of PARP following p53/p21 pathway and increasing expression of γH2Ax, suggesting its genotoxic and mutagenic potential. This finding was supported by the others studies, where ZnO NPs were considered to be genotoxic (Sharma et al., 2009, 2011). The toxic consequences might be due to decrease in mitochondrial membrane potential and presence of ZnO NPs in the nucleus. Cell death
Fig. 5. ZnO NPs trigger autophagy. (a) TEM images of autophagosomes and cellular structures in macrophages treated with ZnO NPs. Black arrows point to ZNP clusters. Autophagosome formations in ZNP treated cells as indicated by red arrows. High magnification view of a large autolysosome containing clusters of ZnO NPs and cellular debris. Nucleus of the treated cells also contain large numbers of dense ZnO NPs. (b) Detection of autophagic vacuoles in macrophages after different time points (control, 0.5, 3, 6, 12 and 24 h) treated cells and Rapamycin (positive control) treated cells. (c) Autophagic flux in macrophages on ZnO NPs exposure measured by staining with LC3–FITC antibody and assessed by fluorimeter.
Fig. 6. Quantitative mRNA levels of autophagy related genes. (a) Time-dependent upregulation of Atg10, Atg12 and Atg5 mRNA expression after ZnO NPs treatment (2.5 μg/ml for 0.5, 3, 6, 12, 24 h and NAC + ZnO NPs for 24 h). (b) Autophagic marker proteins (LC3-II and Beclin 1) in lysates of treated macrophages (2.5 μg/ml for 0.5, 3, 6, 12, 24 h and NAC + ZnO NPs for 24 h) assayed by western blotting. β-Actin was used as the loading control. All blots shown are representative of three independent experiments. (c) Effect of ZnO NPs on Ras-PI3K-mTOR-Akt signaling pathway. Cells were treated with ZnO NPs (2.5 μg/ml for 0.5, 3, 6, 12 and 24 h) and protein lysates were assayed for the expression of Ras-PI3K-pmTOR-Akt. (d) Effect of LC3-II-siRNA on LC3-II and cleavage of caspase 3 expressions in control, siRNA treated control, ZnO NPs treated and siRNA transfected followed by ZnO NPs treated groups.
due to mitochondrial collapse was well supported by couple of previous studies (Scherz-Shouval et al., 2007; Azad et al., 2009).

Up-regulation of pro-apoptotic protein Bax, down-regulation of Bcl-2 and release of cytochrome c indicated the occurrence of apoptosis due to ZnO NPs. Cytochrome c release in the cytoplasm activates apoptosis protease activating factor 1 (Apaf1), which leads to the activation of caspase 3, the execution caspase. Studies of Cecconi (1999) and Hsiao and Huang (2011) corroborated our findings. Subsequently, ZnO NPs activated caspase 3 cleaved PARP proteins (DNA repair enzyme in the cell and among the first target of executioner caspases). Cleavage of caspases 3, 8 and 9 indicated that ZnO NPs exposure followed both extrinsic and intrinsic pathways of apoptosis to ensure survival during death.

However, another cell death mechanism, autophagy also contributes to the abnormal ROS accumulation by selectively promoting the degradation of major enzymatic ROS scavengers which corroborate our results (Yu et al., 2006). Autophagy involves the de novo formation of small double-membrane-bound vesicles called autophagosomes within the cytoplasm that sequester cytosolic constituents. ZnO NPs-induced autophagosomes were observed in the levels of macrophages. These NPs induced the transcript levels of Atgs (Atg10, Atg12 and Atg5) that are required for the initiation of autophagy. Atg10 is essential to form Atg12 conjugation with Atg5 and this conjugate is necessary to form autophagic vesicles. Further, these NPs induced Atgs catalyzed the formation of microtubule-associated protein 1 light chain 3 (MAP-LC3-I) to MAP-LC3-II and release of (Atg6) Beclin 1. Both of these proteins are key activators and constituents of the autophagosome membrane formation (Geng and Klionsky, 2008; He and Klionsky, 2009). These results were in accordance with the activation of caspase-8, suggesting a regulatory role of LC3-II in extrinsic apoptosis activation (Chen et al., 2010).

Our results suggested that oxidative stress induced by ROS formation may lead to apoptosis, but whether these ROS were essential for autophagosome formation or not? To address this question, we tested the effect of NAC, a general antioxidant, on apoptosis and autophagosomes formation after ZnO NPs exposure. Addition of NAC to the growth medium before ZnO NPs exposure inhibited the apoptotic cell death, reduced the formation of LC3-II and abolished the formation of Beclin 1. Antioxidants are known to abolish the formation of autophagosomes and the consequent degradation of autophagy-related proteins (Scherz-Shouval et al., 2007).

To further understand the mechanism of autophagy induced by ZnO NPs, PI3K/mTOR/akt signaling pathway was explored that plays a critical role in the early stages of autophagosome formation (Petiot et al., 2000; Tassa et al., 2003). Initial activation of PI3K under stress condition may lead to rise in free radicals in the vicinity of mitochondria. Further, a significant reduction in p-Akt and p-mTOR expressions, downstream of classical PI3K/Akt/mTOR signaling pathway favored autophagy. To scrutinize the regulatory role of LC3-II over apoptosis, cleavage of caspase 3 was observed in LC3-II silenced macrophages. Inhibition of LC3-II reduced the cleavage of caspase 3. He and Klionsky (2009) reported that siRNA-dependent knockdown of LC3-II conferred protection against epithelial cell apoptosis. Thus, it seems that ROS production may be the reason of cell death and autophagy supported apoptosis on ZnO NPs exposure.

5. Conclusions

ZnO NPs exposure induced oxidative stress in macrophages initiated autophagy as well as apoptosis simultaneously. Formation of autophagosomes may be a cellular defense mechanism against oxidative stress. Moreover, autophagy induced by ZnO NPs followed PI3K/mTOR/Akt signaling cascade. Disruption in mechanism of cell death may hamper immune functions that may lead to serious diseases.

Conflict of interest

None of the authors have any conflict of interest to disclose.

Transparency document

The Transparency document associated with this article can be found in the online version.

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