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ORIGINAL PAPER

ZnO nanoparticle induces apoptosis by ROS triggered mitochondrial pathway in human keratinocytes

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Abstract Zinc Oxide nanoparticles (ZnO NPs) in its small size with large reactive surfaces can lead to toxicological injury by generating reactive oxygen species (ROS) and oxidative stress. Recently, ZnO NPs were shown to play a role in acute or chronic toxicities with mammalian cells, where its mechanism of toxicity is not fully characterized yet. In this study, the potential mechanisms of ZnO NPs in inducing and increasing oxidative stress for causing cellular apoptosis were investigated with human keratinocytes. Indeed, ZnO NPs induced significant intracellular ROS and mitochondrial ROS productions. And it seemed that cellular ROS levels induced by ZnO NPs led to the dissipation of the mitochondrial membrane potentials and elicited the cellular apoptosis. The induced ROS production by ZnO NPs was blocked with chelator treatment, which inhibited the ZnO ion. The present study demonstrates that increased levels of ROS by ZnO NPs cause mitochondrial dysfunction and cellular apoptosis.

Keywords Zinc Oxide, Cytotoxicity, Reactive oxygen species, Keratinocyte, Nanoparticle

Nanoindustries had been closely related to the human life because of the development of nanotechnology. Especially, Zinc oxide nanoparticles (ZnO NPs) are the most diversely used nanomaterials such as in cosmetics, sunscreen and drug delivery[1]. The increased use of ZnO NPs in the human industry generated the importance of the health effect when ZnO NPs were exposed. ZnO NPs can lead the cellular toxicity by oxidative stress and reactive oxygen species (ROS) generation[2]. Also, the toxic effect of ROS induced by ZnO NPs is implicated with DNA damage and cellular apoptosis[2]. In many different cell systems, the production of ROS can generate apoptosis, senescence and inflammatory response[3,4].

Intracellular ROS are generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the mitochondria[5]. The increase of ROS generation in mitochondria causes an immediate damage of the inner mitochondrial membrane and induces a mitochondrial membrane potential disruption[6]. The diminution of the mitochondrial membrane potential leads to an exposure of cytochrome *c* into the intermembrane space, release of cytochrome *c* and cell death leading to apoptosis[7]. Cytochrome *c* is required for the caspase activation which is largely mediated by direct or indirect ROS action that triggers the apoptosis[8].

In this study, the oxidative stress and mitochondrial mediated apoptosis were investigated induced by ZnO NPs in human keratinocytes. We analyzed the cell death and oxidative stress generation by ZnO NPs exposure to elucidate the ROS generation by ZnO NPs in human keratinocytes. The production of apoptotic cells related with the mitochondrial membrane potential was also confirmed. The generation of ROS and mitochondrial mediated apoptosis by ZnO NPs were investigated by ZnO ion chelators.

Cytotoxicity of ZnO nanoparticles

In recent studies, various NPs have been reported to elicit various adverse cellular effects, including DNA damage, cellular apoptosis and inflammatory response

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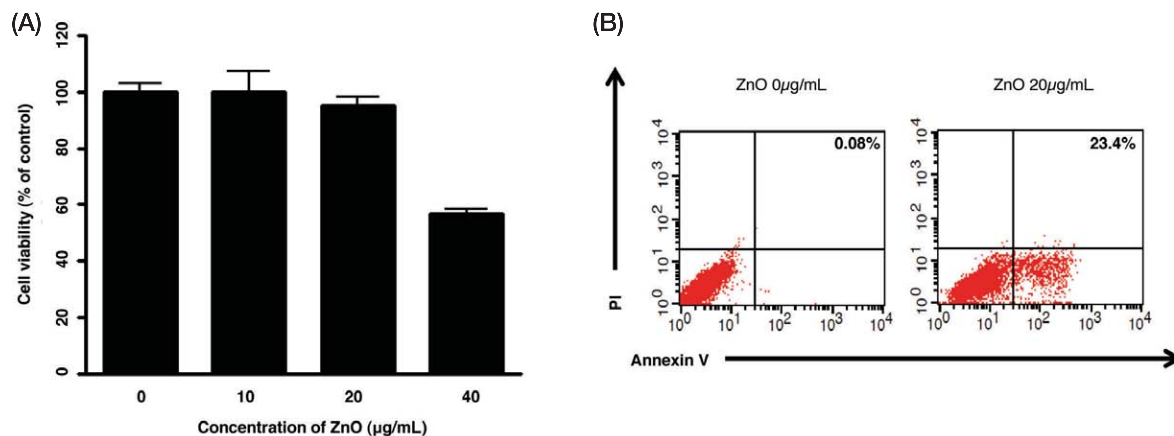

Figure 1. ZnO NPs induces keratinocytes apoptosis. (A) Cells were treated with various concentrations of ZnO-NPs for 24 h. Cell viability was measured by MTT assay after exposure to ZnO-NPs for 24 h in HaCaT keratinocytes cell line. Data are expressed as the percentage viability of cells exposed to ZnO-NPs relative to control cells and are mean ± SEM of triplicates ($P < 0.05$). (B) HaCaT cells were incubated with ZnO-NPs at the indicated doses for 24 h. Cells were stained with Annexin V and PI and analyzed by flow cytometry. Cell death was indicated at the top right corner of each plot.

in epidermal keratinocytes[2,9,10]. Based on those reports, the cytotoxicity of ZnO NPs in human keratinocyte HaCaT cells was measured by MTT assay to determine the effects of ZnO NPs on the cell viability. HaCaT cells were exposed by ZnO NPs for 24 h in different concentrations (0, 10, 20, 40 μg/mL). In ZnO NPs exposed HaCaT cells, the cell viability was maintained up to 20 μg/mL ZnO NPs (Figure 1A). At the concentration of 40 μg/mL ZnO NPs, the cell viability decreased to nearly 50%. As 20 μg/mL ZnO NPs did not cause cell death, this concentration was selected for subsequent experiments. ZnO NPs treated HaCaT cells were stained with Annexin V-FITC/PI and analyzed by flow cytometry to confirm the early apoptosis by ZnO NPs in HaCaT cells. Annexin positive cells were analyzed as a marker for apoptosis after exposure to ZnO NPs. ZnO NPs treated HaCaT cells apoptosis was induced in about 23.4% (Figure 1B). These results indicate that ZnO NPs induced cell apoptosis in HaCaT cells.

Increased oxidative stress and intracellular ROS level by ZnO NPs treatment in human keratinocytes

Recent studies showed that NPs can exert the ROS production in diverse cellular activities[11]. Thus, we attempted to determine whether ZnO NPs induce intracellular ROS production in HaCaT cells which could trigger the cellular apoptosis (Figure 2). The level of intracellular ROS production by ZnO-NPs was estimated using 2,7-dichlorofluorescein diacetate (DCF-DA) dye for cytosolic ROS and MitoSOX™ for mitochondrial ROS. 20 μg/mL 20 nm ZnO NPs were treated to HaCaT human keratinocytes for 6 h. No significant differences were observed according to differences in surface charge (data not shown). Cytosolic ROS and mitochondrial ROS levels were increased compared with the

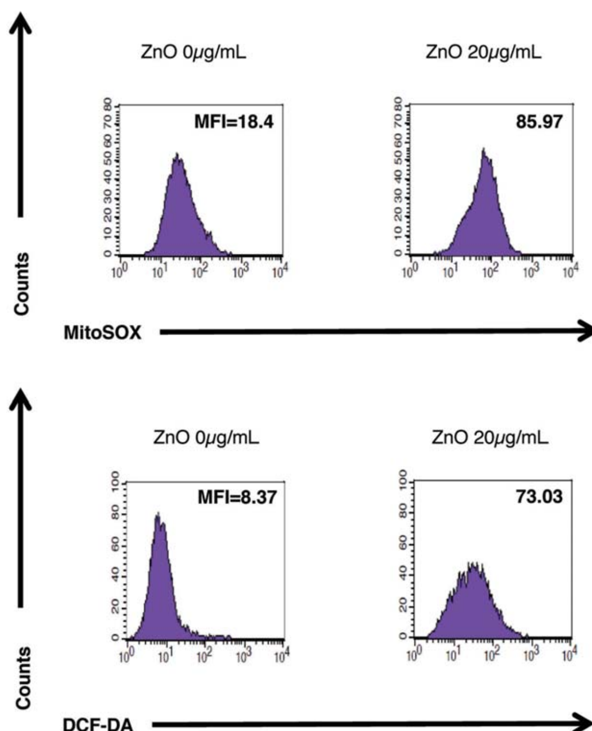

Figure 2. ZnO NPs induces ROS formation in keratinocytes. Flow cytometry analysis of mitochondrial superoxide and cytosolic ROS in HaCaT cells treated with or without 20 μg/mL of ZnO NPs for 6 h.

control. These results suggest that ZnO NPs generate the intracellular ROS in HaCaT keratinocytes.

Induction of mitochondrial membrane potential ($\Delta\Psi_m$) change by ZnO NPs exposure in human keratinocytes

Previous studies showed that $\Delta\Psi_m$ change can regulate cytochrome *c* release as a triggering factor of apoptosis[7]. There is a recent report that ZnO NPs induce oxidative damage and ROS-triggered mitochondria mediated apoptosis in human liver cells[12]. The cells were stained with JC-1 dye after ZnO NPs treatment to characterize if the ZnO NPs triggered ROS production induced $\Delta\Psi_m$ changes (Figure 3). As the healthy cells were detected as a red JC-1 aggregate, the apoptotic cells were detected in green JC-I monomers. The apoptotic cells which were shown as JC-1 monomers increased by 20 μg/mL ZnO NPs exposure. Compared to the control cells, apoptotic cells were depolarized in about 38.62%. The increase of the green fluorescence JC-1 monomers indicates a loss of $\Delta\Psi_m$ in HaCaT cells. These results indicate that the ZnO NPs exposure in HaCaT keratinocytes decreases the level of $\Delta\Psi_m$ which can induce apoptosis.

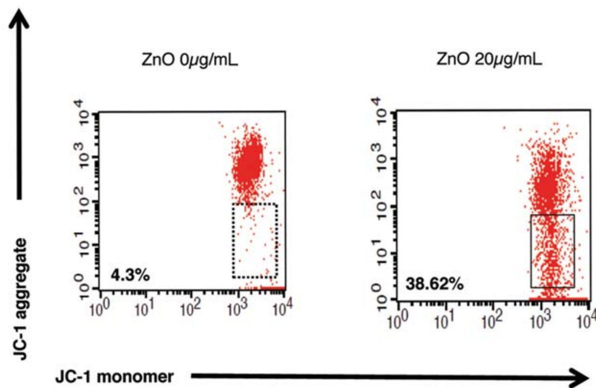

Figure 3. ZnO NPs decreases the level of $\Delta\Psi_m$ in keratinocytes. Flow cytometry analysis of mitochondrial membrane potential $\Delta\Psi_m$ in HaCaT cells treated with or without 20 μg/mL of ZnO NPs for 6 h.

ZnO NPs-induced ROS generation and mitochondrial mediated apoptosis

The recent study demonstrates the ZnO NPs dissolved to intracellular Zinc ion and results in cytotoxicity[13]. We analyzed the effects of ZnO NPs-induced Zinc ion for cytotoxicity using Zinc ion chelators (Figure 4A). The ROS production in HaCaT cells by ZnO NPs were about 2-fold increased. The ROS production was decreased by chelator exposure in ZnO NPs treated HaCaT cells. The ZnO NPs-induced ROS production was decreased to nearly basal level by chelators. If we compared the increase of JC-1 monomers in ZnO NPs treated cells, the JC-1 monomers were decreased down

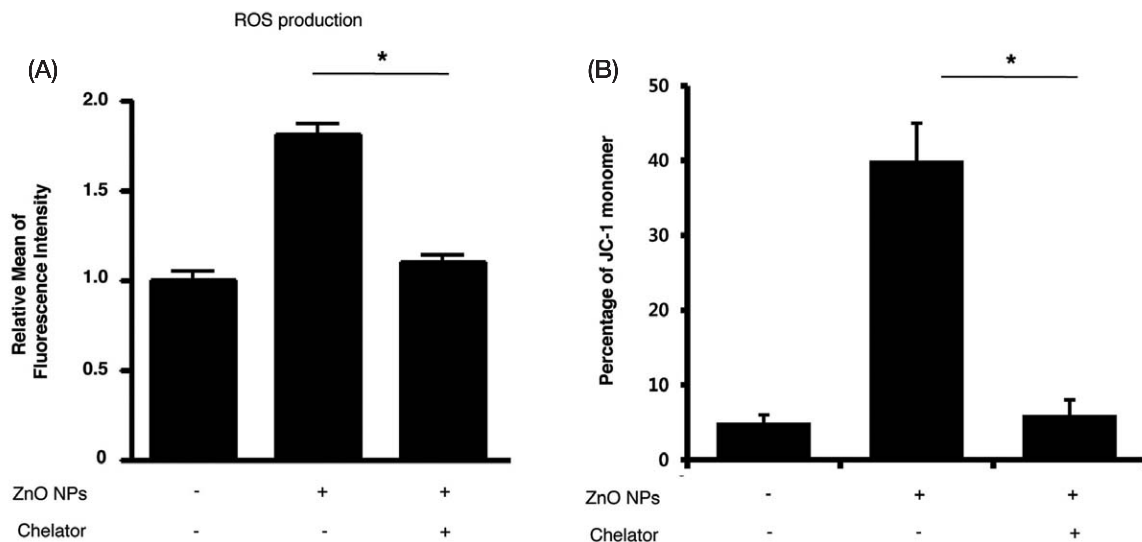

Figure 4. ZnO NPs-induced ROS generation and mitochondrial mediated apoptosis. (A) Flow cytometry analysis of cytosolic ROS in HaCaT cells treated with 20 μg/mL of ZnO NPs for 6 h. The bar graph represents the mean of fluorescence intensity of cytosol ROS by DCF-DA staining. Data are shown as mean ± SD. *indicates a statistically significant difference, compared to the control ($P < 0.05$). (B) Flow cytometry analysis of mitochondrial membrane potential $\Delta\Psi_m$ in HaCaT cells treated with or without 20 μg/mL of ZnO NPs for 6 h. The bar graph represents the mean of fluorescence intensity of mitochondrial membrane potential $\Delta\Psi_m$ by JC-1 staining. Data are shown as mean ± SD. *indicates a statistically significant difference, compared to the control ($P < 0.05$).

to the control level by chelating (Figure 4B). These findings suggest that ZnO NPs directly induce the ROS generation and mitochondrial mediated apoptosis.

Discussion

NPs are increasingly used material in diverse fields, particularly in sunscreen, cosmetics, medicals and food products[14]. Recent papers showed the mechanisms of NPs inducing apoptosis and cellular damage. ZnO NPs as the main component of sunscreen are one of the representative NPs. Despite the extensive use of ZnO NPs, ZnO NPs have been studied regarding its effects in human and its environmental hazardous effect.

The present study revealed that ZnO NPs induce apoptosis by mitochondrial membrane potential-mediated ROS production in human keratinocytes. The toxic effects of ZnO NPs on HaCaT human keratinocytes were analyzed by MTT assay and Annexin V-FITC/PI staining. As shown in Figure 1, an investigation of the effect of ZnO NPs on the cell viability after exposure indicates the cellular toxicity. We also investigated the intracellular ROS formation in accordance with the cell death and early apoptosis by ZnO NPs (Figure 2). Numerous reports highlighted the importance and the major role of ZnO NPs-induced ROS in the cytotoxicity[13,15]. Compared to the control group showed the intracellular ROS an increase after its exposure to ZNO NPs. The well-known ROS, superoxide and hydrogen peroxide, presenting the cytosolic and mitochondrial ROS were increased by the exposure to ZnO NPs on HaCaT human keratinocytes. The mitochondrial function was investigated according to the cytotoxicity and ROS generation by ZnO NPs. JC-1 monomers were confirmed in order to elucidate the ROS induced mitochondrial membrane potential, which arrived to apoptosis (Figure 3). Cytosolic JC-1 monomers significantly increased if they were exposed to ZnO NPs. The previous study showed that dissolved intracellular Zinc ion from ZnO NPs contributes to cellular toxicity[13]. Based on this report, we analyzed the effects of ZnO NPs-induced Zinc ion for cytotoxicity using Zinc ion chelators. The ZnO NPs-induced ROS production and JC-1 monomers were decreased to nearly basal level by Zinc ion chelators (Figure 4). These findings indicate the Zinc ion which was dissolved by ZnO NPs affects the ROS formation and apoptosis in HaCaT human keratinocytes.

In conclusion, this study demonstrated that ZnO NP induced ROS production and induces apoptosis according to mitochondrial change. Our study results may be helpful for safety evaluation and provide a basis for the manufacture of safe NPs.

Materials & Methods

ZnO nanoparticles

The 20 nm ZnO nanoparticle was purchased from Sumitomo (Osaka, Japan). Carboxylate-modified (+charged) ZnO NP was obtained by addition of 1% L-serine /HEPES solution (pH 6.2) and amine-modified (−charged) ZnO NP was obtained by addition of 1% citrate/HEPES solution (pH 7.3). ZnO NP which was charged (+) or (−) was vortexed for 1 min. To ensure an adequate dispersion, charged ZnO NP solutions were vortexed for 10 sec prior to the test.

Chemical reagents

NAC (N-Acetyl-L-cystein) (Sigma, St. Louis, USA) was used as ROS scavenger. 2′,7′-Dichlorofluorescein diacetate, DCF-DA (Invitrogen, Carlsbad, CA, USA) was used for cellular ROS detection. Annexin V-FITC and propidium iodide were purchased from BD (San Diego, USA). For the evaluation of the mitochondria membrane potential, JC-1 dye was purchased from Invitrogen (CA, USA). *N*,*N*,*N*,′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) (Sigma, St. Louis, USA) was used as ZnO chelator. TPEN was added to the cell culture media in a final concentration of 5 μM.

Cell culture

Human immortalized HaCaT keratinocyte cells (Cell Line Service; Eppelheim, Germany) was cultured in Dulbecco's modified eagle's medium (DMEM; Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone) with 1% penicillin and streptomycin (P/S; Gibco, Carlsbad, CA, USA) and was incubated in 5% CO_2 incubator at 37°C. HaCaT cells were cultured in a 60 mm cell culture dish in 10% FBS DMEM medium with 1% P/S for 24 h and then the cell culture medium was replaced with DMEM containing 0.5% FBS with 1% P/S for 24 h. After 24 h, HaCaT cells were treated with ZnO NP according to the various time and dose points. After ZnO NP stimulation, cells were collected with pre-chilled 1X PBS and analyzed.

MTT assay

To measure the cytotoxicity of ZnO NPs, we measured the cell number of keratinocytes. The number of keratinocytes was measured using MTT (MTT; sigma) according to the manufacturer's instructions. Briefly, confluent HaCaT cells grown on 96 well cell culture plate were treated with various concentrations of ZnO NPs for 24 h and then cells were incubated for 1 h at 37°C with 20 μL/well MTT (5 mg/mL). The amount of

purple formazan, which is directly proportional to the number of living cells, was evaluated by the measurement of the optical density at 590 nm in the DigiScan microplate reader (ASYS Hitech GmbH).

Detection of apoptosis by flow cytometric analysis

Apoptotic or necrotic cell levels were determined by flow cytometry after double staining with Annexin V-FITC and propidium iodide using an assay kit from BD (San Diego, USA). After 6 h incubation with ZnO NPs, the medium was removed and cells were washed twice with ice-cold PBS. After 20 min incubation with Annexin V-FITC and propidium iodide, cells were collected, washed with cold PBS and suspended in Annexin V-binding buffer in test tubes.

Measurement of mitochondrial membrane potential

To detect the mitochondrial membrane potential ($\Delta\Psi_m$), the fluorescent probe 5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolcarbocyanine iodide (JC-1; Molecular probes, Oregon, USA) was used. HaCaT cells exposed to ZnO NPs were harvested by trypsinization and washed with PBS three times. The cells were incubated with 5 μM JC-1 for 30 min at 37°C, washed with PBS and re-suspended in PBS at a concentration of 10^6 cells/mL. The fluorescence intensity was measured at an excitation wavelength of 490 nm and an emission wavelength of 590 nm using flow cytometry (FACSC alibur™, BD Bio-Sciences, San Jose, CA, USA).

ROS analysis

The level of intracellular ROS generation was estimated using 2,7-dichlorofluorescein diacetate (DCF-DA) dye. For intracellular ROS analysis, cells were treated with 10 μM DCF-DA (Invitrogen, Carlsbad, CA), incubated at 37°C for 20 min and washed by HBSS. Ultimately, cells were re-suspended and analyzed by flow cytometry.

Statistics

All data are expressed as mean values ± standard deviation (mean ± SD). Data were evaluated by two-way analysis of variance (ANOVA) for multiple comparisons. A *P*-value of less than 0.05 was considered significant.

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Conflict of Interest The authors declare no conflict of interest.

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