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Immunopharmacology and inflammation

Zinc oxide nanoparticles, a novel candidate for the treatment of allergic inflammatory diseases

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ABSTRACT

Zinc (Zn) is an essential trace metal for eukaryotes. The roles of Zn in the numerous physiological functions have been elucidated. Bamboo salt contains Zn that was shown to have anti-inflammatory effect and other health benefits. Nanoparticles of various types have found application in the biology, medicine, and physics. Here we synthesized tetrapod-like, zinc oxide nanoparticles (ZO-NP; diameter 200 nm, source of Zn) using a radio frequency thermal plasma system and investigated its effects on mast cell-mediated allergic inflammatory reactions. ZO-NP was found to inhibit the productions and mRNA expressions of inflammatory cytokines such as interleukin (IL)-1 β , IL-6, and tumor necrosis factor- α on the phorbol 12-myristate 13-acetate plus A23187 (PMACI)-stimulated human mast cell line, HMC-1 cells. In these stimulated cells, caspase-1 and nuclear factor- κ B activations were abolished by ZO-NP, and the expressions of receptor interacting protein2 (RIP2) and I κ B kinase β (IKK β) induced by PMACI were reduced. On the other hand, ZO-NP alone increased the expressions of RIP2 and IKK β in normal condition. ZO-NP inhibited the phosphorylation of extracellular signal-regulated protein kinase in the PMACI-stimulated HMC-1 cells. Furthermore, ZO-NP significantly inhibited passive cutaneous anaphylaxis activated by anti-dinitrophenyl IgE. These findings indicate that ZO-NP effectively ameliorates mast cell-mediated allergic inflammatory reaction, and suggest that ZO-NP be considered a potential therapeutic for the treatment of mast cell-mediated allergic diseases.

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1. Introduction

Mast cells reside in all mammalian tissues, and play important roles in the pathogenesis of inflammatory and autoimmune diseases (Heger et al., 2014). Actually, mast cells act as effector cells during early stage of allergic reactions, such as, life-threatening anaphylaxis, allergic rhinitis (hay fever), atopic dermatitis (eczema), and allergic asthma, and during later stage inflammatory response (El-Agamy, 2012; Galli and Tsai, 2012). Activation of mast cell induces degranulation and produces cytokines and chemokines (Galli and Tsai, 2012). Cytokines released by mast cells increase the proinflammatory cytokine production by resident cells. The release of histamine stimulated vessel permeabilization and promoted the migration of eosinophils, neutrophils, and macrophages into the inflammatory zone tissue. Thus the activation and degranulation of mast cells intensifies and extends the inflammatory response (Galli and Tsai, 2012).

Receptor interacting protein2 (RIP2) plays an important role in the regulation of immune response and inflammatory processes, and its signaling tightly linked with I κ B kinase β (IKK β), mitogen-activated protein kinases (MAPKs), nuclear factor- κ B, and caspase-1 signaling (Perkins, 2007; Song et al., 2012). Furthermore, IKK β activated by RIP2 triggers the phosphorylation and subsequent ubiquitination of inhibitory I κ B α protein, the separation of I κ B α and NF- κ B, and the degradation of I κ B α by proteasomes (Perkins, 2007). The NF- κ B so liberated is then translocated into the nucleus and binds to specific DNA sequences to trigger the syntheses of pro-inflammatory mediators, such as, interleukin(IL)-1 β , IL-6, IL-8, and tumor necrosis factor- α (TNF- α) (Song et al., 2012). NF- κ B is activated by MAPK family members, such as, extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) (Han et al., 2013). In addition, caspase-1 contributes to NF- κ B activation via the autocrine action of IL-1 β on cell surface receptors (Wu et al., 2009). Caspase-1 is also characterized by its ability to activate the inactive precursors of inflammatory cytokines (Yoo et al., 2002).

Zinc (Zn) is an essential trace metal for eukaryotes. Zn modulates the numerous physiological functions (Jansen et al., 2009; Maremanda et al., 2014). Bamboo salt contains Zn that was shown to have anti-inflammatory effect and other health benefits (Kim et

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al., 2012). Nanotechnology has been applied in biology, medicine, materials science, engineering, electronics, and environmental fields because of the unique properties of nanoparticles, for example, their superparamagnetic behaviors, small sizes, and great surface areas. Zinc oxide (ZnO) is source of Zn. ZnO decreased expressions of inflammatory genes (Hu et al., 2013; Ou et al., 2007). ZnO nanoparticles (ZO-NP) are used in a wide range of products, such as, in the cosmetics, food packaging, and imaging industries, and as antibacterial and antifungal agents (Roy et al., 2013, 2014; Sharma et al., 2012). In the present study, we synthesized ZO-NP using a radio frequency thermal plasma system and investigated their anti-inflammatory effects in phorbol 12-myristate 13-acetate plus A23187 (PMACI)-stimulated human mast cell line, HMC-1 cells.

2. Materials and methods

2.1. Materials

PMA, A23187, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), and other reagent were purchased from Sigma (St. Louis, MO, USA). Iacove's modified Dulbecco's medium (IMDM), and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Anti-human IL-1 β , IL-6, IL-8, and TNF- α , biotinylated anti-human IL-1 β , IL-6, IL-8, and TNF- α , recombinant human (rh) IL-1 β , IL-6, IL-8, and TNF- α were purchased from Pharmingen (San Diego, CA, USA). Antibodies (Abs) for IKK- β , RIP2, caspase-1, ERK, phosphorylated (p) ERK, p38, pp38, JNK, pJNK, NF- κ B, I κ B α , histone, and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The caspase assay kit was supplied by R&D Systems Inc. (Minneapolis, MN, USA).

2.2. ZO-NP preparation

As raw material, micron-sized powders of ZnO (particle size 1–10 μ m) were purchased from Yee Young Cerachem. Ltd. (Seoul, Korea). ZO-NP was obtained by treating raw powders in a commercial radio frequency (RF) thermal plasma system, consisting of a RF power supply, an induction torch (Tekna, PS-100) for generating the plasma, a synthesis reactor for reforming powder in the high thermal plasma, a cyclone for segregating bulky particles, a filtration unit for gathering the nanoparticles produced, and a powder feeder. The ZnO was reconstituted as a nanoparticles at an operating pressure of $\sim$ 89.6 kPa, a plate power levels of $\sim$ 140 kV A, and a feeding rate of 5 g/min. Argon and oxygen was used to produce the plasma flame at the following gas flow rates; central gas of 60 (Ar), sheath gas of 100 (Ar) and 100 (O₂), and quenching gas of 7200 (recycled gas).

2.3. Field emission scanning electron microscopy (FE-SEM), field emission transmission electron microscopy (FE-TEM), and X-ray diffraction (XRD)

Particle morphologies of ZnO and ZO-NP were examined by FE-SEM (S4800, Hitachi Ltd., Japan), FE-TEM (JEM-2200FS, JEOL, Japan), and Bio-TEM (H-7650, Hitachi Ltd., Japan). The FE-SEM was operated at acceleration voltages of 10 kV. FE-TEM or Bio-TEM samples were prepared by dispersing the ZO-NP in desired solvent (ethanol or 10% FBS in IMDM) and drying it on a Cu grid. XDR patterns in 2-theta range of 15° to 90° were obtained for both powders (D8Advance, Bruker AXS GmbH., Germany) using a copper K α radiation source (λ =1.5406 Å).

2.4. Cell culture

HMC-1 cells were grown in IMDM supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat inactivated FBS at 37 °C, 5% CO₂ and 95% humidity. Powdered ZO-NP and ZnO were prepared by dissolving with DMSO. Dilutions of ZO-NP and ZnO were made in 10% FBS in IMDM. Cells were pretreated with various concentrations of ZO-NP (0.01, 0.1, 1, or 10 μ g/ml) or ZnO (0.01, 0.1, 1, or 10 μ g/ml) for 1 h prior to PMACI stimulation

2.5. Enzyme-linked immunosorbent assay (ELISA)

Secreted IL-1 β , IL-6, IL-8, and TNF- α in culture supernatants were measured according to the manufacturer's specification (Pharmingen, San Diego, CA, USA).

2.6. MTT assay

HMC-1 cells (3×10^5 cells/ml) were treated with ZnO or ZO-NP, cultured in microplate wells for 8 h, and then incubated with 20 μ l of MTT solution (5 mg/ml) for an additional 4 h at 37 °C under 5% CO₂ and 95% air. Consecutively, 250 μ l of DMSO was added to extract the MTT formazan and the absorbance of each well at 540 nm was read by an automatic microplate reader.

2.7. RNA isolation and RT-PCR

Total RNA was isolated from HMC-1 according to the manufacturer's specifications using an easy-BLUE RNA extraction kit (iNtRON Biotech, Korea). Total RNA (2.0 μ g) was heated at 65 °C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37 °C using a cDNA synthesis kit. RT-PCR was carried out with 1 μ l of a cDNA mixture, in 20 μ l final volume with 2.5 mM MgCl₂, 200 mM dNTPs, 25 pM cytokine primers, and 2.5 U of TaqDNA polymerase in the reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9, and 0.1% Triton X-100). PCR was performed with the following primers for human IL-1 β (5' CCG GAT CCA TGG CAC CTG TAC GAT CA 3'; 5' GGG GTA CCT TAG GAA GAC ACA AAT TG 3'); human IL-6 (5' GAT GGA TGC TTC CAATCT GGAT 3'; 5' AGT TCT CCATAG AGA ACA ACA TA 3'); human TNF- α (5' CAC CAG CTG GTT ATC TCT CAG CTC 3'; 5' CGG GAC GTG GAG CTG GCC GAG GAG 3'); human GAPDH (5' CAA AAG GGT CAT CAT CTC TG 3'; 5' CCT GCT TCA CCA CCT TCT TG 3'). The annealing temperatures used were 50 °C for IL-1 β , 56 °C for IL-6, and 60 °C for TNF- α and GAPDH. Products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining.

2.8. Western blot analysis

For determine protein levels, stimulated cells were rinsed twice with ice-cold phosphate buffered saline (PBS) and then lysed in ice-cold lysis buffer (PBS containing 0.1% SDS, 1% triton and 1% deoxycholate). Cell lysates were separated through electrophoresis, the protein was transferred to nylon membranes by electrophoretic transfer. The membranes were blocked in 6% skim milk for 2 h, rinsed and incubated overnight at 4 °C with primary Abs. After three washes in PBS containing 0.05% Tween-20 (PBST), the membranes were incubated for 1 h with horse radish peroxidase-conjugated secondary Abs. After three washes in PBST, the protein bands were visualized by an enhanced chemiluminescence assay following the manufacturer's instructions.

2.9. Caspase-1 activity determination

The enzymatic activity of caspase-1 was assayed using a caspase-1 colorimetric assay kit according to the manufacturer's protocol. The lysed cells were centrifuged at 15,000 $\times$ g for 5 min.

The protein supernatant was incubated with 50 μ l reaction buffer and 5 μ l caspase substrate (WEHD-*p*-nitroaniline) at 37 °C for 2 h. The absorbance was measured using a plate reader at a wavelength of 405 nm. Equal amounts of the total protein from each lysate were quantified using a bicinchoninic acid protein quantification kit (Pierce, Rockford, IL, USA).

2.10. Passive cutaneous anaphylaxis (PCA)

The original stock of male ICR mice (4 weeks old) were purchased from the Dae-Han Experimental Animal Center (Eumsung, Republic of Korea), and the animals were maintained at the College of Korean Medicine, Kyung Hee University. Animal care and experimental procedures were performed under the approval of the animal care committee at Kyung Hee University

[Approval no. KHUASP (SE)-10-019]. The IgE-dependent cutaneous reaction was generated by sensitizing the skin with an intradermal injection of anti-DNP IgE followed 48 h later with an injection of DNP-HSA into the mice tail vein. ZO-NP was orally (10 mg/kg) or topically (10 μ g/site, dorsal skin) administered for 1 h. The mice ($n=6$) were sacrificed 40 min after the intravenous challenge. The amount of dye was determined colorimetrically method. The absorbent intensity of the extraction was measured at 620 nm in a spectrofluorometer, and the amount of dye was calculated with the Evans blue measuring-line.

2.11. Statistical analysis

Results were expressed as the mean $\pm$ standard deviation (S.D) of independent experiments, and statistical analyses were performed by

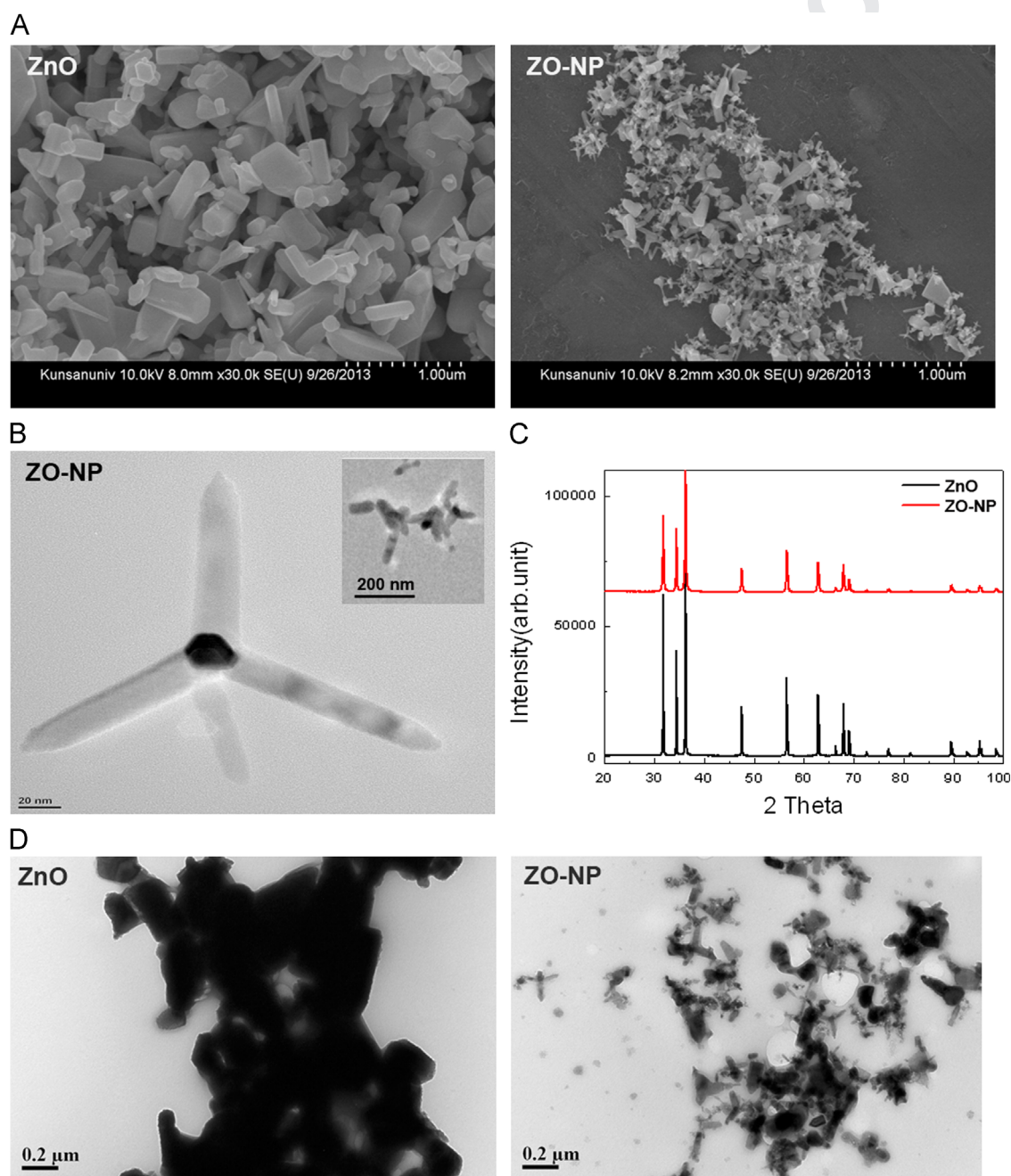


Fig. 1. Analysis of ZO-NP. (A) FE-SEM and (B) FE-TEM images of ZO-NP. (C) XRD patterns of ZnO and ZO-NP. (D) TEM images of ZO-NP and ZnO (10 μ g/ml) dissolved in IMDM containing 10% FBS.

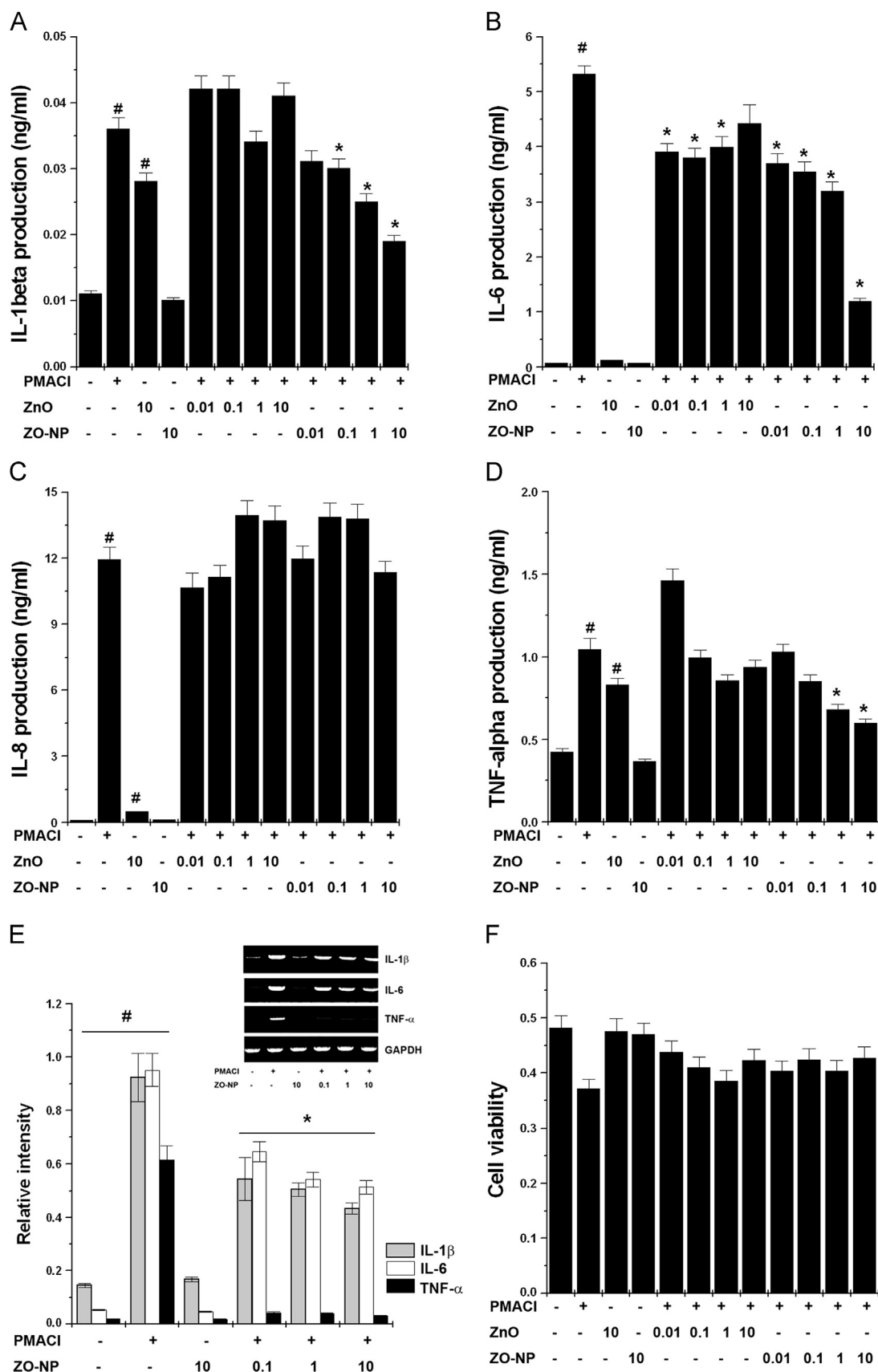


Fig. 2. Effects of ZO-NP on inflammatory cytokines and cell viability in HMC-1 cells. Cells were pretreated with various concentrations of ZO-NP (0.01, 0.1, 1, or 10 μ g/ml) or ZnO (0.01, 0.1, 1, or 10 μ g/ml) for 1 h prior to PMACI stimulation for 8 h. (A)–(D) Secreted cytokine levels in culture supernatants of cells were measured by ELISA. (E) Cells were pretreated with various concentrations of ZO-NP (0.1, 1, and 10 μ g/ml) or ZnO (10 μ g/ml) for 1 h prior to PMACI stimulation for 6 h. The total RNA was assayed by an RT-PCR analysis. The relative intensities were quantified by densitometry means IL-1 β /GAPDH, IL-6/GAPDH, and TNF- α /GAPDH. Each datum represents the mean $\pm$ S.D. of three independent experiments. (F) Cell viability was evaluated by an MTT assay. Data represent mean $\pm$ S.D. of three independent experiments. [#] P < 0.05: significantly different from unstimulated cells. ^{*} P < 0.05: significantly different from the PMACI-stimulated cells. PMACI, PMA plus A23187; ZO-NP, zinc oxide nanoparticles.

one-way analysis of variance with Tukey post hoc test to express the difference between groups. A value of $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Analysis of ZO-NP by FE-SEM, FE-TEM, and XRD

To investigate the physicochemical properties of ZO-NP used in the present study, we analyzed FE-SEM and FE-TEM images and confirmed the ZO-NP structure by XRD pattern. FE-SEM and FE-TEM images of ZO-NP are shown in Fig. 1A and B. The average particle size of ZO-NP was 200 nm and particles had a tetrapod shape (Fig. 1B). XRD peaks of ZnO were unchanged by RF thermal plasma processing, but the peak intensities of ZO-NP were lower than those of the ZnO due to the reduction in particle size (Fig. 1C). The size of ZO-NP or ZnO in IMDM containing 10% FBS at 10 $\mu\text{g}/\text{ml}$ concentration was also investigated by Bio-TEM. ZO-NP was about 200 nm and tetrapod-like shape and ZnO maintained large size more than ZO-NP (Fig. 1D).

3.2. Effect of ZO-NP on cytokine production and mRNA expression

We examined the effects of ZO-NP and ZnO on the production of IL-1 β , IL-6, IL-8, and TNF- α . PMACI stimulation significantly increased IL-1 β , IL-6, IL-8, and TNF- α compared with the media control (Fig. 2A–D, $P < 0.05$). Furthermore, these upregulation of IL-1 β , IL-6, and TNF- α by PMACI were significantly inhibited by ZO-NP (Fig. 2A, B, and D, $P < 0.05$). However, ZO-NP did not affect IL-8 protein levels induced by PMACI (Fig. 2C). ZnO only significantly inhibited PMACI-induced IL-6 production (Fig. 2B, $P < 0.05$). ZnO alone significantly increased IL-1 β , IL-8, and TNF- α production. In addition, we analyzed for IL-1 β , IL-6, and TNF- α mRNA levels by RT-PCR to further investigate the effect of ZO-NP on the modulation cytokine expression by PMACI. The mRNA expressions of IL-1 β , IL-6, and TNF- α in ZO-NP-treated cells were markedly lower than in the PMACI-treated group (Fig. 2E). We also examined the cell viability using a MTT assay, but no adverse effects were observed (Fig. 2F).

3.3. Effect of ZO-NP on PMACI-induced caspase-1 activation

Caspase-1 regulates cytokine production (Oh et al., 2011), and thus, to evaluate the regulatory effects of ZO-NP and ZnO on PMACI-induced caspase-1 activation, we measured caspase-1 activities using an assay kit. When cells were stimulated with PMACI, caspase-1 activity increased significantly. However, this increase in caspase-1 activity was inhibited by ZO-NP (Fig. 3A, $P < 0.05$). The expression of caspase-1 was assessed by Western blot analysis. In agreement with Fig. 3A data, ZO-NP decreased the activation of caspase-1 by PMACI (Fig. 3B), whereas, ZnO alone increased caspase-1 activation (Fig. 3).

3.4. Effect of ZO-NP on PMACI-induced NF- κB activation and I $\kappa\text{B}\alpha$ degradation

The expression of inflammatory cytokines is regulated by the transcription factor, NF- κB (Song et al., 2012). Reductions in cytokine mRNA expressions by ZO-NP are shown in Fig. 2E. Therefore, we examined the effect of ZO-NP on PMACI-induced NF- κB activation. In nuclear fractions, NF- κB upregulation by PMACI was diminished by ZO-NP (Fig. 4A and B). To determine whether this inhibitory effect of ZO-NP was due to its effects on I κB degradation, we investigated the cytoplasmic levels of I $\kappa\text{B}\alpha$ protein with a Western blot analysis. We found the degradation of

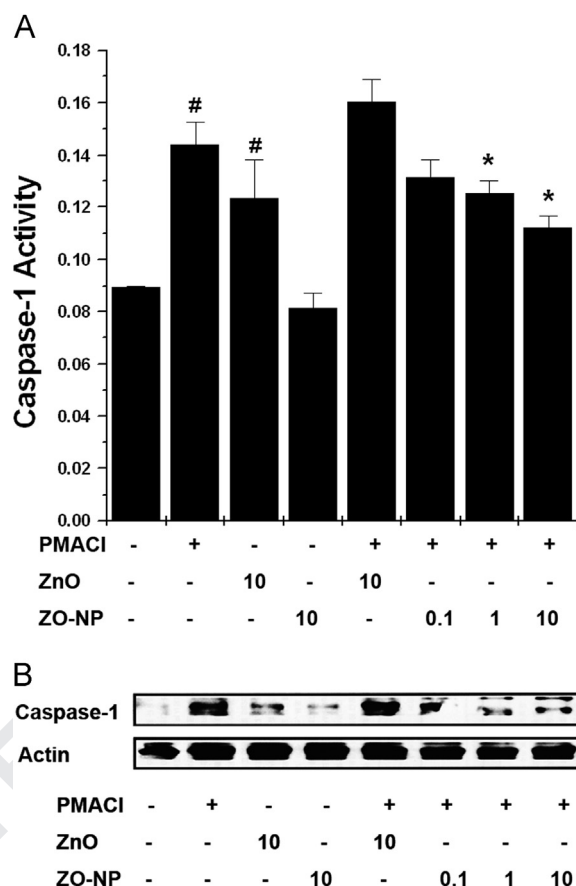


Fig. 3. Effect of ZO-NP on caspase-1 activation in HMC-1 cells. Cells were pretreated with various concentrations of ZO-NP (0.1, 1, or 10 $\mu\text{g}/\text{ml}$) or ZnO (10 $\mu\text{g}/\text{ml}$) for 1 h prior to PMACI stimulation for 2 h. (A) Enzymatic activities of caspase-1 were determined by a caspase-1 colorimetric assay. Data represent mean $\pm$ S.D. of three independent experiments. (B) The levels of caspase-1 were assayed by Western blot analysis. Results are representative of three independent experiments. # $P < 0.05$: significantly different from unstimulated cells. * $P < 0.05$: significantly different from the PMACI-stimulated cells. PMACI, PMA plus A23187; ZO-NP, zinc oxide nanoparticles.

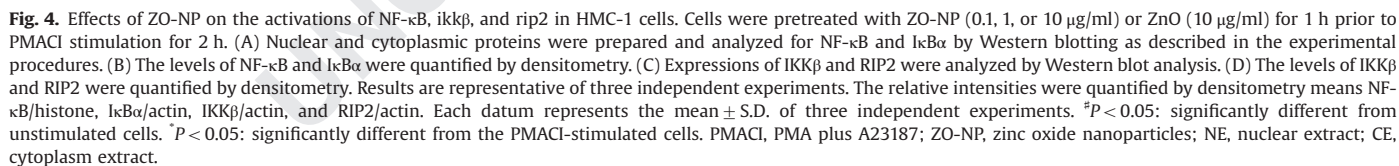
I $\kappa\text{B}\alpha$ in cytoplasm was inhibited by ZO-NP (Fig. 4A and B). In addition, ZnO also inhibited PMACI-induced NF- κB activation and I $\kappa\text{B}\alpha$ degradation (Fig. 4A).

3.5. Effects of ZO-NP on PMACI-induced *ikkb* and *rip2* activation

The IKK β and RIP2 signaling pathways play central roles in the activation of NF- κB and caspase-1. Thus, we investigated the effect of ZO-NP on the activations of these pathways. As shown in Fig. 4C and D, PMACI stimulation increased the activation of IKK β and RIP2, these activations were diminished by ZO-NP. But ZO-NP or ZnO alone increased the IKK β and RIP2 activation compared with the media control (Fig. 4C and D).

3.6. Effect of ZO-NP on PMACI-induced MAPKs phosphorylation

Cytokine expression and production by PMACI stimulation occurs via the activation of MAPKs (Han et al., 2013). To determine whether the anti-inflammatory activities of ZO-NP were mediated via MAPKs pathway regulation, Western blot analysis was performed for phosphorylated ERK, JNK, and p38. As indicated in Fig. 5, all three of these MAPKs were phosphorylated in PMACI-stimulated HMC-1 cells. ZO-NP diminished the phosphorylation of ERK in these cells (Fig. 5), but did not affect the phosphorylations of p38 and JNK (Fig. 5). Interestingly, in the absence of PMACI, both



Allergic inflammation is induced by a combination of early-phase immediate hypersensitivity and late-phase inflammation after allergen exposure (Galli and Tsai, 2012). Early-phase immediate hypersensitivity occurs within minutes of allergen exposure and is induced by the release of the preformed mediator from mast cells (Galli and Tsai, 2012; Oh et al., 2011). On the other hand, late-phase reactions are the result of proinflammatory cytokine production and the recruitment of immune cells, such as, neutrophils, basophils, eosinophils, macrophages, and mast cells to sites of inflammation (Ipp et al., 2014). For example, mast cells numbers are increased in atopic dermatitis, allergic rhinitis, and asthma (Enerbäck et al., 1986; Kneilling and Rocken, 2009; Laitinen et al., 1993), and proinflammatory cytokines (IL-1 β , IL-6,

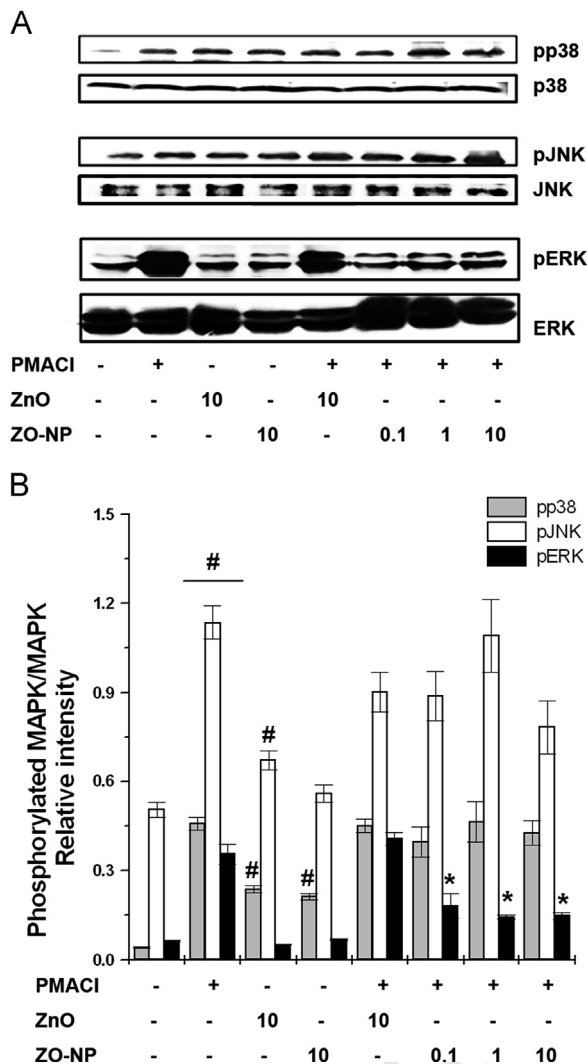


Fig. 5. Effects of ZO-NP on the phosphorylations of MAPKs in HMC-1 cells. Cells were pretreated with ZO-NP (0.1, 1, or 10 $\mu\text{g/ml}$) or ZnO (10 $\mu\text{g/ml}$) for 1 h prior to PMACI stimulation for 30 min. (A) Phosphorylated MAPKs levels were assayed by Western blot analysis. Results are representative of three independent experiments. (B) The levels of MAPKs expression were quantified by densitometry. PMACI, PMA plus A23187; ZO-NP, zinc oxide nanoparticles. * $P < 0.05$; significantly different from unstimulated cells. # $P < 0.05$; significantly different from the PMACI-stimulated cells. PMACI, PMA plus A23187; ZO-NP, zinc oxide nanoparticles.

IL-8, and TNF- α) released by mast cells increase the inflammatory process (Oh et al., 2011). The present study shows that ZO-NP significantly inhibits the productions and mRNA expressions of IL-1 β , IL-6, and TNF- α in activated HMC-1 cells. Thus, we hypothesize that ZO-NP can be helpful in preventing and treating mast cell-mediated inflammatory diseases.

Inflammatory cytokine expressions are dependent on the signal transductions of NF- κ B and MAPKs (Han et al., 2013; Song et al., 2012). When mast cells are stimulated with PMACI, MAPKs/NF- κ B signaling is activated and this leads to the over-expressions and release of many cytokines and chemokines, adhesion molecules, inflammatory mediators, and several matrix degrading enzymes (Oh et al., 2011, 2013; Marcu et al., 2010). MAPKs are important NF- κ B activating factors (Han et al., 2013), though it is also mediated by caspase-1 activation (Lamkanfi et al., 2006). Caspase-1 activation by PMACI induced the production of inflammatory cytokine in HMC-1 cells (Song et al., 2012). Caspase recruitment domain (CARD) of procaspase-1 has been reported to mediate NF- κ B activation by RIP2 via a CARD-CARD interaction

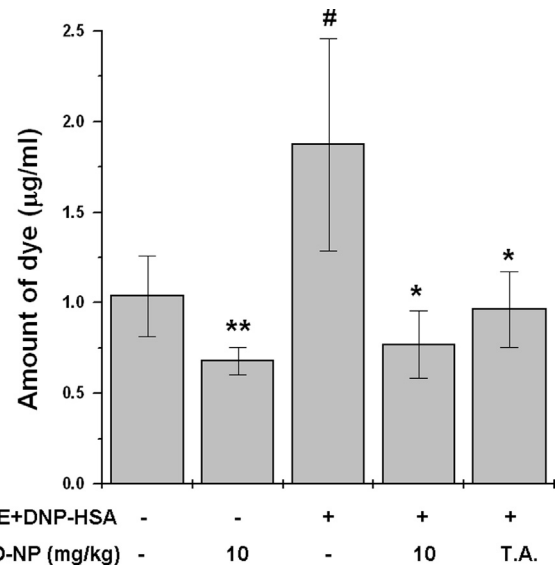


Fig. 6. Effect of ZO-NP on PCA reaction. The mice were injected intradermally with 100 ng of anti-DNP IgE. ZO-NP was administered orally (10 mg/kg) or applied topically (T.A, 10 $\mu\text{g/site}$) 1 h prior to the challenge with 1 mg/ml of antigen (DNP-HSA) containing 4% Evans blue (1:1) via the tail vein, after 48 h. $N = 6$, * $P < 0.05$; significantly different from blank, # $P < 0.05$; significantly different from the control value. ** $P < 0.01$; significantly different from blank.

(Kersse et al., 2011), and RIP2 was also found to induce the activations of MAPKs and IKK β pathways (Jiang and Chen, 2011). In another study, RIP2 mutants lacking functional kinase activity reduced the activation of ERK by TNF- α (Navas et al., 1999). Thus, it appears that targeting IKK β /RIP2 offers a novel means of modulating the expressions of NF- κ B regulated genes. In the present study, we found that ZO-NP reduced I κ B α degradation and prevented nuclear translocation of NF- κ B p65 and suppressed the activations of ERK, IKK β , and RIP2 in PMACI-stimulated HMC-1 cells. In addition, ZO-NP inhibited PMACI-induced caspase-1 activation. Therefore, our results show that the inhibitory effects of ZO-NP on the expressions and productions of proinflammatory cytokines are associated with the inhibitions of NF- κ B, caspase-1, ERK, IKK β , and RIP2 signaling pathways but does not involve p38/JNK signaling. On the other hand, ZO-NP alone increased the activations of IKK β and RIP2 compare with the media control. Sargeant et al. (2011) reported that zinc alone increased IL-8 production, while zinc treatment inhibited production of IL-8 in response to pathogens. Therefore, further work is needed to clarify the specific functions of ZO-NP in the activation of IKK β and RIP2 under normal conditions and pathogenic conditions such as PMACI stimulation.

Expression of inflammatory cytokines was regulated by various transcription factors such as NF- κ B, activated protein-1 (AP-1), NF-IL6, and hypoxia inducible factor-1 (Fiorini et al., 2000; Jeong et al., 2002, 2003). Various transcription factors bind to the various conserved sequences on promoter region of genes, respectively. The positions of these conserved sequences differ from gene to gene. ZnO decreased NF- κ B activation, while AP-1 was activated by ZnO (Sargeant et al., 2011). The NF- κ B negative feedback regulator A20 (zinc finger protein) is critical for the prevention of inflammation and autoimmunity and A20 deficiency dramatically increase the expressions of cytokines via the activation of NF- κ B (Heger et al., 2014). In the present study, ZnO did not increase NF- κ B activation, although ZnO up-regulates IL-1 β and TNF- α production in the absence of PMACI stimulation. ZnO inhibited PMACI-induced IL-6 production, NF- κ B activation and I κ B α degradation. Therefore, we postulate that ZnO inhibited the PMACI-induced IL-6 production via the inhibition of NF- κ B activation

whereas other cytokine production was increased by AP-1 or other transcription factors. However, further studies are needed to clarify the effect of ZnO in transcription mechanisms of mast cell-mediated inflammatory reactions.

Some authors have reported reactive oxygen species (ROS) and reactive nitrogen species produced by nanoparticles have cytotoxic effects and induce inflammatory responses by activating the PI3 kinase and MAPKs pathways (Heng et al., 2011; Roy et al., 2013). Jeong et al. (2013), reported ZnO nanoparticles (diameter 20 nm) internalized by endocytosis induced inflammatory response by activating the ROS/ERK/Egr-1 signal pathway in human keratinocytes. Sahu et al. (2014), reported that ZnO (approximately 5 μm) and ZnO nanoparticles (approximately 100 nm) increased release of inflammatory cytokines in THP-1 cells. ZnO nanoparticles (195 $\pm$ 17 nm in the medium) exhibit toxicity in lung, liver, skin, and brain (Chiang et al., 2012). However, others have reported that nanoparticles have anti-inflammatory effects (Niu et al., 2011; Rehman et al., 2012). Hu et al. (2013) found that ZnO decreased the expressions of genes associated with inflammation. Ou et al. (2007), suggested that ZnO reduced mast cell numbers and histamine release, and Klosterhalfen et al. (1996), observed that Zn^{2+} reduced levels of IL-1 β , IL-6, and TNF- α response to LPS. Oyarzun-Ampuero et al. (2012), reported that nanoparticles (221–729 nm) prevented mast cell degranulation. Pati et al. (2014), reported that ZnO nanoparticles (500 nm) suppressed the allergic inflammatory reactions in mice. Similarly in the present study, ZO-NP (about 200 nm in the medium) was found to inhibit mast cell-mediated inflammatory responses in HMC-1 cells. Meanwhile, inflammatory cytokines production and caspase-1 activity were up-regulated by ZnO alone. Chiang et al. (2012) reported that dissociation of ZnO results in destruction of cellular zinc homeostasis. The physical and chemical characteristics of nanoparticles are also quite different from those of their non-nano sized counterparts (Seabra et al., 2013; Seo and Hong, 2012). Tripathy et al. (2014), found that aggregation of nanoparticles influences cytotoxicity of macrophages and concentrations of nanoparticles play central roles in modulation of nanoparticles aggregation. In our study, high concentration of ZO-NP (100 $\mu\text{g}/\text{ml}$) exerts the cytotoxicity (data not shown) but low concentration of ZO-NP had no effect the cytotoxicity in HMC-1 cells. Xia et al. (2008), found that ZnO dissociation plays a crucial role in cytotoxicity and protein's shape, unfolding, and surface characteristics altered by nanoparticles affected cellular protein enzymatic and physiological activities. Previous studies reported that size and shape of nanoparticles, surfactants used for nanoparticles protection, medium, and experimental conditions can also affect cellular signal pathway (Lu et al., 2010; Xia et al., 2008). In this study, we found that ZO-NP reduced the PMACI-induced inflammatory reaction and the IgE-mediated PCA reaction, although the precise mechanism of ZO-NP and ZnO is not known at this time. Therefore, it is conceivable that ZO-NP inhibits the mast cell-mediated allergic reactions. However, further study will be needed to elucidate effect and mechanism of nanoparticles according to various experimental conditions in the allergic inflammatory reactions.

Furthermore, in the present study, whereas ZnO inhibited PMACI-induced IL-6 production and NF- κB activation, ZO-NP almost totally inhibited the up-regulation of inflammatory cytokines, the activation of NF- κB , caspase-1, IKK β , RIP2, and ERK by PMACI, and IgE-mediated PCA reaction, which indicates ZO-NP more effectively inhibits inflammatory reactions than ZnO.

5. Conclusion

Our results suggest the anti-inflammatory effects of ZO-NP are due to its suppression of the activation of RIP2, IKK β , and ERK and

to the subsequent inhibitions of NF- κB and caspase-1, and that these activities reduce the expressions and productions of inflammatory cytokines. ZO-NP also reduced PCA reaction. Our findings indicate that ZO-NP should be regarded a potential preventative and therapeutic agent for the treatment of mast cell-mediated allergic inflammatory diseases.

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