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# Zinc Ions Induce Inflammatory Responses in Vascular Endothelial Cells

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**Abstract** Using one particulate zinc oxide (ZnO) and two soluble zinc compounds ($Zn(NO_3)_2$ and $Zn(CH_3COO)_2$), we aimed to clarify if zinc ions ($Zn^{2+}$), like particulate ZnO, caused inflammatory responses in vascular endothelial cells. Treatments of human umbilical vein endothelial cells (HUVECs) with 368.6 μM of each zinc compound caused marked increases in IκBα phosphorylation and intercellular adhesion molecule-1 (ICAM-1) expression. Treatments with $Zn(CH_3COO)_2$ (50–350 μM) induced a dose-dependent ICAM-1 expression. These results show that $Zn^{2+}$ alone is sufficient to induce similar levels of ICAM-1 expression as ZnO particles, suggesting that dissolved $Zn^{2+}$ may play the major role in inflammatory effect of ZnO particles on vascular endothelial cells.

**Keywords** Zinc · Vascular endothelium · Inflammation · Adhesion molecules · ICAM-1

Atherosclerosis is thought to be a chronic inflammatory disease of the vessel wall (Ross 1999). Endothelial dysfunction is a critical early step in atherogenesis (Ross 1999; Sattar 2004). Thus, inflammation and endothelial dysfunction have been shown to intimately link to the pathogenesis of vascular disease (Sattar 2004). Analysis of zinc levels in suspended particulate matter in air revealed that 82%–93% of zinc was in the small $PM_{10}$ particles (Vadjic et al. 2010). Studies in mothers subjected to cigarette smoking or air pollution showed that both cigarette smoking and air pollution contributed to the increased levels of placental zinc (Sorkun et al. 2007). A previous study in ambient air zinc levels and health care utilization for asthma revealed the association between elevated ambient air zinc and increased pediatric asthma morbidity (Hirshon et al. 2008). Our previous study reveals that ZnO particles induce expression of intercellular adhesion molecule-1 (ICAM-1) protein, an indicator of vascular endothelium inflammation, in vascular endothelial cells via NF-κB signaling (Tsou et al. 2010), suggesting the possible involvement of ZnO exposure in the development of vascular inflammatory disease. In addition, ZnO particles induce cytotoxicity and apoptosis in mammalian cells (Gojova et al. 2007; Xia et al. 2008) and the dissolved $Zn^{2+}$ seems to play the critical role in toxic effect of ZnO particles (Song et al. 2010). Because inflammation is observed with not only zinc particles but also with other zinc compounds, the zinc-mediated mechanism of inflammation remains unclear. Therefore, one particulate ZnO and two soluble zinc compounds, $Zn(NO_3)_2$ and $Zn(CH_3COO)_2$, were used to determine whether inorganic zinc was sufficient to produce inflammation by studying ICAM-1 expression in HUVEC cells. The present study showed that zinc was found sufficient to induce inflammation since all the three zinc compounds were able to upregulate ICAM-1 expression in HUVEC cells.

Szu-Ching Yeh and Feng-Yuan Tsai contributed equally.

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## Materials and Methods

ZnO particles (50 nm) (#677450) were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). $Zn(NO_3)_2 \cdot 6H_2O$ (#62042150) was obtained from Showa Chemical Industry Co., Ltd. (Tokyo, Japan). $Zn(CH_3COO)_2$ (#370080250) was obtained from ACROS Organics (Geel, Belgium). Rabbit polyclonal antibodies against ICAM-1 (sc-7891) and IκBα (sc-847) and a goat polyclonal antibody against p-IκBα (sc-7977) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A mouse monoclonal antibody against actin (MAB1501) was purchased from Chemicon Int. Inc. (Temecula, CA, USA). Dulbecco's phosphate-buffered saline (DPBS) was obtained from Gibco (Grand Island, NY, USA). M199 medium was obtained from Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT, USA). Penicillin (10,000 units/mL)/streptomycin (10,000 μg/mL) solution was obtained from Invitrogen Corp. (Carlsbad, CA, USA). Gentamycin sulfate was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). HUVECs were obtained by collagenase digestion of umbilical veins, as described previously in detail (Tsou et al. 2005).

Following treatments, cells were lysed in ice-cold RIPA buffer (50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.25% sodium deoxycholate) containing PMSF (2 mM), aprotinin (2 μg/mL), leupeptin (2 μg/mL), NaF (2 mM), $Na_3VO_4$ (2 mM), and $\beta$-glycerophosphate (0.2 mM). After centrifugation at 14,000*g* at 4°C for 30 min, protein concentration in supernatants was quantified using Bradford method (Bio-Rad) and 25–30 μg of protein per lane was subjected to SDS–PAGE and immunoblot analysis, as described previously (Tsou et al. 2004). The blots were probed with a primary antibody against *p*-IκBα, IκBα, ICAM-1, or actin. HRP-conjugated secondary antibodies and Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA, USA) were used to reveal specific protein bands. Protein band intensities were quantified by densitometry.

Each experiment was performed independently at least three times. The statistical analysis was expressed using the mean ± standard error (SE) from each independent experiment. To avoid statistical bias, nonparametric tests of Mann-Whiney *U* and Jonckheere-Terpstra tests were used to determine statistical significance of the differences between experimental groups. Differences were considered statistically significant when $p < 0.05$. Analyses were carried out using the Statistical Package for Social Sciences (SPSS) version 12.0 (SPSS Inc., Chicago, IL, USA).

## Results and Discussion

We have previously demonstrated the inflammatory effects of ZnO particles on vascular endothelial cells (Tsou et al. 2010). Treatments of HUVECs with ZnO (≤45 μg/mL) were performed to determine the expression of ICAM-1 protein, an indicator of vascular endothelium inflammation, revealing that ZnO particles induced a dose-dependent increase in ICAM-1 expression via NF-κB signaling. In the present study, we further asked if $Zn^{2+}$ alone is sufficient to induce similar levels of ICAM-1 expression as ZnO particles. One particulate zinc compound ZnO (50 nm in size) and two soluble zinc compounds—$Zn(NO_3)_2$ and $Zn(CH_3COO)_2$—were used in this study. Following treatments of HUVECs with an equal molarity of $Zn^{2+}$ (368.6 μM, i.e., 30 μg/mL for ZnO) for 24 h, IκBα phosphorylation and ICAM-1 protein levels were determined with immunoblot analysis. As shown in Fig. 1a, all the zinc treatments caused marked increases in IκBα phosphorylation and ICAM-1 expression. Quantitative densitometry analysis showed that the zinc-induced ICAM-1 expression was about 3 to fourfold induction as compared to the control with statistical significances using Mann-Whiney *U* tests ($p = 0.037$). Moreover, results from treatments of HUVECs with increasing levels of $Zn(CH_3COO)_2$ for 24 h showed that $Zn^{2+}$ induced ICAM-1 protein expression in a dose-dependent manner (Fig. 1b). A significant increase of ICAM-1 was observed when $Zn^{2+}$ concentration was increased from 200 to 350 μM ($p = 0.006$, using the Jonckheere-Terpstra test).

A previous study in ambient air zinc levels and health care utilization for asthma revealed the association between elevated ambient air zinc and increased pediatric asthma morbidity (Hirshon et al. 2008). Mechanistic studies with several different types of cells have demonstrated the in vitro evidence for the induction of inflammation responses by zinc. Our previous study revealed an important role of ZnO particles in modulating the inflammatory responses of vascular endothelial cells via NF-κB signaling (Tsou et al. 2010). A recent study further showed that ZnO particles caused increases in IL-8 secretion from cultured primary human nasal mucosa cells (Hackenberg et al. 2011). Because of the critical role of dissolved $Zn^{2+}$ in the ZnO-induced cytotoxicity (Song et al. 2010), we further asked if $Zn^{2+}$ alone is sufficient to induce similar levels of ICAM-1 expression as ZnO particles. Indeed, here we demonstrate that $Zn^{2+}$ alone is sufficient to induce inflammatory responses in vascular endothelial cells.

Although zinc is an essential trace element for animals and play important roles in regulation of immune function in humans (Prasad 2008), excess zinc can be harmful. Epidemiological studies revealed the association between

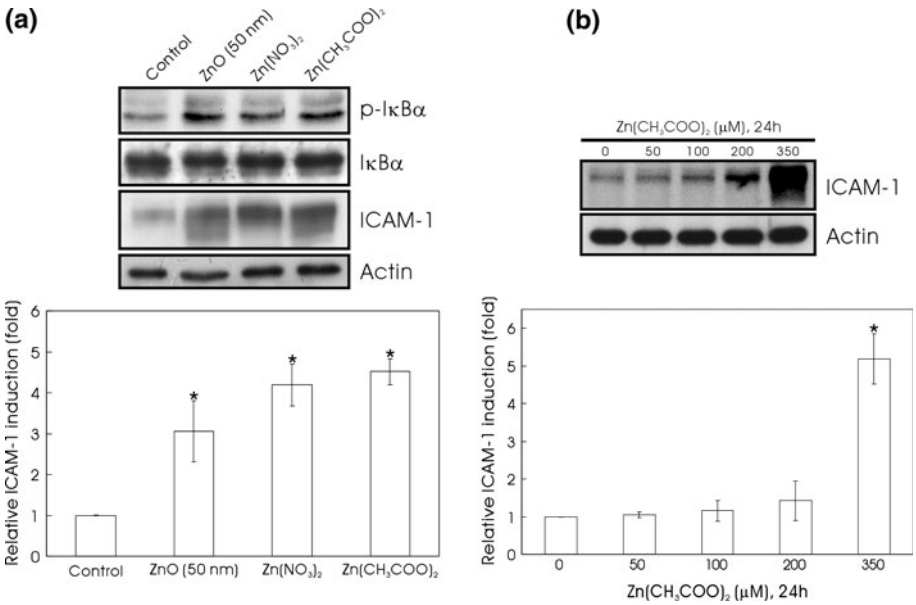


**Fig. 1** Zinc effect on IκBα phosphorylation and ICAM-1 expression in vascular endothelial cells. **a** Both particulate ZnO and soluble zinc compounds induce IκBα phosphorylation and ICAM-1 protein expression. HUVECs were left untreated or treated with 368.6 μM of ZnO (50 nm), $Zn(NO_3)_2$, or $Zn(CH_3COO)_2$ for 24 h. Following treatments, the cell lysates were analyzed for phosphorylated IκBα (p-IκBα), IκBα, ICAM-1, and actin protein levels by immunoblot analysis. Representative immunoblot results were shown (*upper panel*). The protein levels of ICAM-1 and actin were quantified. Differences in ICAM-1 expression between the three zinc compounds and the untreated control were examined by using Mann-Whiney *U* tests (*bottom panel*). **b** $Zn^{2+}$ induces ICAM-1 protein expression in a dose-dependent manner. Cells were left untreated or treated with 50, 100, 200, or 350 μM $Zn(CH_3COO)_2$ for 24 h. Following treatments, the cell lysates were analyzed for ICAM-1 and actin protein levels by immunoblot analysis. Representative immunoblot results were shown (*upper panel*). The protein levels of ICAM-1 and actin were quantified (*bottom panel*). The data are presented as means ± SD (n = 3) and are expressed as relative ICAM-1 protein levels compared to that of the untreated control. * $p < 0.05$ versus the untreated control

elevated ambient air zinc and increased pediatric asthma-related morbidity (Hirshon et al. 2008). Animal studies indicated that the ambient PM2.5 samples with higher levels of metals, such as zinc, caused increases in the allergic respiratory disease in mice (Gavett et al. 2003). Our in vitro evidence showed an important role of ZnO particles or $Zn^{2+}$ in modulating the inflammatory responses of vascular endothelial cells. Therefore, we asked how these results provide new insight for understanding the mechanisms of those inflammatory diseases induced by ambient particulate pollutants. First, it is well accepted that fine particles are readily trapped in the deeper pulmonary alveoli through inhalation. Second, in situ decomposition of the trapped particles results in a local increase of metal ions (such as $Zn^{2+}$) and thus may activate inflammatory responses around the particles. Third, ultrafine particles and dissolved metal ions are able to cross the pulmonary epithelial barrier into the bloodstream. Fourth, of particular relevance to the present study is that the direct exposure of vascular endothelium to metal ions such as $Zn^{2+}$ induces the marker of inflammation, which is ICAM-1 in the present study. Using HUVECs as an in vitro system, this study reveals that $Zn^{2+}$ play the major role in ICAM-1 expression of vascular endothelial cells in response to ZnO particle treatments.

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## References

Gavett SH, Haykal-Coates N, Copeland LB, Heinrich J, Gilmour MI (2003) Metal composition of ambient PM2.5 influences severity of allergic airways disease in mice. Environ Health Perspect 111(12):1471–1477

Gojova A, Guo B, Kota RS, Rutledge JC, Kennedy IM, Barakat AI (2007) Induction of inflammation in vascular endothelial cells by metal oxide nanoparticles: effect of particle composition. Environ Health Perspect 115(3):403–409

Hackenberg S, Scherzed A, Technau A, Kessler M, Froelich K, Ginzkey C, Koehler C, Burghartz M, Hagen R, Kleinsasser N (2011) Cytotoxic, genotoxic and pro-inflammatory effects of zinc oxide nanoparticles in human nasal mucosa cells in vitro. Toxicology in Vitro, Corrected Proof (in press)

Hirshon JM, Shardell M, Alles S, Powell JL, Squibb K, Ondov J, Blaisdell CJ (2008) Elevated ambient air zinc increases pediatric asthma morbidity. Environ Health Perspect 116(6):826–831

Prasad AS (2008) Zinc in human health: effect of zinc on immune cells. Mol Med 14(5–6):353–357

Ross R (1999) Atherosclerosis–an inflammatory disease. N Engl J Med 340(2):115–126

Sattar N (2004) Inflammation and endothelial dysfunction: intimate companions in the pathogenesis of vascular disease? Clin Sci (Lond) 106(5):443–445

Song W, Zhang J, Guo J, Zhang J, Ding F, Li L, Sun Z (2010) Role of the dissolved zinc ion and reactive oxygen species in cytotoxicity of ZnO nanoparticles. Toxicol Lett 199(3):389–397

Sorkun HC, Bir F, Akbulut M, Divrikli U, Erken G, Demirhan H, Duzcan E, Elci L, Celik I, Yozgatli U (2007) The effects of air pollution and smoking on placental cadmium, zinc concentration and metallothionein expression. Toxicology 238(1):15–22

Tsou TC, Yeh SC, Tsai FY, Chang LW (2004) The protective role of intracellular GSH status in the arsenite-induced vascular endothelial dysfunction. Chem Res Toxicol 17(2):208–217

Tsou TC, Yeh SC, Tsai EM, Tsai FY, Chao HR, Chang LW (2005) Arsenite enhances tumor necrosis factor-alpha-induced expression of vascular cell adhesion molecule-1. Toxicol Appl Pharmacol 209(1):10–18

Tsou TC, Yeh SC, Tsai FY, Lin HJ, Cheng TJ, Chao HR, Tai LA (2010) Zinc oxide particles induce inflammatory responses in vascular endothelial cells via NF-$\kappa$B signaling. J Hazard Mater 183(1–3):182–188

Vadjic V, Zuzul S, Pehnec G (2010) Zinc levels in suspended particulate matter in Zagreb air. Bull Environ Contam Toxicol 85(6):628–631

Xia T, Kovochich M, Liong M, Madler L, Gilbert B, Shi H, Yeh JI, Zink JI, Nel AE (2008) Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. ACS Nano 2(10): 2121–2134