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Zinc induces chemokine and inflammatory cytokine release from human promonocytes

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ABSTRACT

Our previous studies found that zinc oxide (ZnO) particles induced expression of intercellular adhesion molecule-1 (ICAM-1) protein in vascular endothelial cells via NF-κB and that zinc ions dissolved from ZnO particles might play the major role in the process. This study aimed to determine if zinc ions could cause inflammatory responses in a human promonocytic leukemia cell line HL-CZ. Conditioned media from the zinc-treated HL-CZ cells induced ICAM-1 protein expression in human umbilical vein endothelial cells (HUVEC). Zinc treatment induced chemokine and inflammatory cytokine release from HL-CZ cells. Inhibition of NFκB activity by over-expression of IκBα in HL-CZ cells did not block the conditioned medium-induced ICAM-1 protein expression in HUVEC cells. Zinc treatment induced activation of multiple immune response-related transcription factors in HL-CZ cells. These results clearly show that zinc ions induce chemokine and inflammatory cytokine release from human promonocytes, accompanied with activation of multiple immune response-related transcription factors. Our *in vitro* evidence in the zinc-induced inflammatory responses of vascular cells provides a critical linkage between zinc exposure and pathogenesis of those inflammatory vascular diseases.

1. Introduction

Epidemiologic studies indicate that exposure to fine particulate matter (PM) in air pollution is associated with systemic inflammatory markers [1] as well as incidence of cardiovascular morbidity and mortality [2–4]. However, mechanisms behind this correlation remain largely unknown. When inhaled into the respiratory tract, small particles tend to rest in the deeper part of the lungs. Ultrafine particles [5–7] and, of particular relevance to the present study, their dissolved chemicals such as metal ions can penetrate the deepest part of the lungs and cross the pulmonary epithelial barrier into the bloodstream, directly exposing the vascular cells, such as monocytes and endothelial cells, to pollutants.

Analysis of zinc levels in suspended PM in air revealed that 82–93% of zinc was in the small PM_{10} particles [8]. 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 [9]. 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 [10]. ZnO particles induce cytotoxicity and apoptosis in mammalian cells [11,12] and the dissolved zinc ions seem to play the critical role in toxic effect of ZnO particles [13]. In our previous studies, ZnO particles induced ICAM-1 expression in vascular endothelial cells via an NFκB dependent pathway [14] and zinc ions alone were sufficient to induce similar levels of ICAM-1 expression as ZnO particles, suggesting that dissolved zinc ions might play the major role in inflammatory effect of ZnO particles on vascular endothelial cells [15]. These studies suggest that metal particle composition, or its dissolved metal ions, may determine the capability of metal oxide nanoparticles to induce inflammation in vascular endothelial cells.

Increasing evidence indicates that chronic obstructive pulmonary disease, asthma, and atherosclerosis are associated with systemic inflammatory cytokine changes. Various pathophysiological stimulators induce cytokine release, including modified LDL [16,17], free radicals [18], hemodynamic stress [19,20], and hypertension [21]. On the basis of our previous findings in vascular endothelial cells using ZnO particles, the present study aimed to determine if zinc could cause inflammatory responses in other vascular cells. We found that zinc induces chemokine and inflammatory cytokine release from human promonocytes possibly via activation of multiple immune response-related transcription factors.

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2. Materials and methods

2.1. Materials

$Zn(CH_3COO)_2$ (#370080250) was obtained from ACROS Organics (Geel, Belgium). Zinc preparation in this study was tested to be endotoxin-free by using an endotoxin inhibitor, polymyxin B, as previously described [22]. RayBio Human Cytokine Antibody Array 3 (#AAH-CYT-3) was obtained from RayBiotech, Inc. (Norcross, GA, USA). 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). Endothelial cell growth supplement (ECGS) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's phosphate-buffered saline (D-PBS), M199 medium, and RPMI 1640 medium were 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).

2.2. Construction of recombinant adenoviruses

Construction of recombinant AdEasy-GFP, AdEasy-IκBα, and AdV-NFκB-Luc has been previously described [14]. For construction of AdV-AP-1-Luc, a 2360-bp DNA fragment containing seven copies of the AP-1 response element, a TATA box, and a firefly luciferase gene in pAP-1-Luc (Stratagene, La Jolla, CA, USA), was amplified by PCR using pfu DNA polymerase. The amplified DNA fragment was digested by KpnI and SalI. After separation by agarose gel electrophoresis, the purified KpnI/SalI-digested DNA fragment was cloned into the KpnI/SalI-digested pACCMV.pLpA vector [23]. The function of the CMV promoter of the pACCMV.pLpA vector used here had been abolished. The recombinant adenovirus AdV-AP-1-Luc was generated by homologous recombination between the pJM17 plasmid [24] and the pACCMV.pLpA vector in 293 human embryo kidney cells. Construction of other recombinant adenoviruses (AdEasy-C/EBP-Luc, AdEasy-CRE-Luc, AdEasy-NFAT-Luc, AdEasy-SRE-Luc, and AdEasy-STAT-Luc) was generated using the AdEasy™ Adenoviral Vector System (Stratagene, La Jolla, CA, USA) (see Supplementary material in detail). The recombinant adenoviruses were purified and concentrated according to the manufacturer's instructions. General information of these immune response-related transcription factor-mediated luciferase reporter adenoviruses is summarized in Table 1.

2.3. Cells and treatments

Human promonocytic leukemia cell line HL-CZ (BCRC-60043), originally established by Dr. Wu-Tse Liu (National Yang-Ming University, Taipei, Taiwan) [25], were purchased from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) and were routinely cultured in RPMI 1640 medium. HUVEC cells were obtained by using collagenase digestion of umbilical veins [26] and were routinely cultured in M199 medium as previously described [27].

HL-CZ cells (8.5×10^6 cells per 100-mm dish) were left untreated or treated with $Zn(CH_3COO)_2$ as indicated for 6 h. Following treatments, conditioned media were dialyzed against D-PBS with stirring at 4 °C for 42 h, sterilized with a 0.45-μm syringe filter, and then was added with M199 medium (with 20% FBS and 30 μg/ml ECGS) at 1/1 ratio (v/v). HUVEC cells were treated with this conditioned medium/M199 mixtures for different time periods as indicated. Following the treatments, cell lysates were collected for immunoblot analyses.

In some cases requiring adenovirus infection, HL-CZ cells (3×10^6 cells per 100-mm dish) were first infected with AdEasy-GFP or AdEasy-IκBα at a multiplicity of infection (MOI) of 50 pfu/cell for 24 h. The infected cells were replaced with fresh RPMI 1640 medium and cultured for another 24 h for recovery. Hereafter, the cells were ready for the zinc treatments as just described.

2.4. Immunoblot analysis

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 β-glycerophosphate (0.2 mM). The cell lysates were subjected to SDS–PAGE and immunoblot analysis, as described previously [28]. The blots were probed with a primary antibody against ICAM-1, phosphor-IκBα (p-IκBα), IκBα, or actin. HRP-conjugated secondary antibodies. Protein bands in the membrane were visualized in an X-ray film by using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA, USA). The protein band intensity was quantified by densitometry scanning of X-ray films.

2.5. Analysis of cytokines in conditioned media from HL-CZ cells

Following zinc treatments of HL-CZ cells, conditioned media were dialyzed against D-PBS with stirring at 4 °C for 42 h and sterilized with a 0.45-μm syringe filter. Cytokines in conditioned medium were analyzed with the RayBio Human Cytokine Antibody Array 3 according to the manufacturer's instructions (see Supplementary material in detail). The kit provides a simple array format, and highly sensitive approach to simultaneously detect 42 cytokine expression levels from conditioned media.

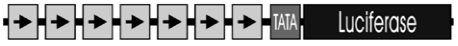
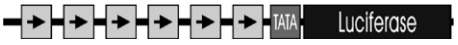
2.6. Immune response-related transcription factor-mediated luciferase reporter assay

To determine the activation of those immune response-related transcription factors, HL-CZ cells (1.0×10^4 cells per well in 96-well plates) were infected with one of the recombinant adenoviruses (AdV-AP-1-Luc, AdV-NFκB-Luc, AdEasy-SRE-Luc, AdEasy-NFAT-Luc, AdEasy-CRE-Luc, AdEasy-C/EBP-Luc, and AdEasy-STAT-Luc) (Table 1) at a MOI of 1 pfu/cell for 24 h. Following the adenovirus infection, the infected cells were replaced with fresh RPMI 1640 medium and cultured for another 24 h for recovery. Then, the infected cells were left untreated or treated with 150 μM $Zn(CH_3COO)_2$ for 6 h. Luciferase activity of each sample was determined using the Luciferase Assay System (Promega, Madison, WI), according to the manufacturer's instructions.

2.7. Statistics

Each experiment was performed independently at least three times. The statistical analysis was expressed using the mean ± standard deviation (SD) from each independent experiment. Induction of ICAM-1 protein expression in HUVEC cells by conditioned medium were examined by Student's *t*-tests with 2000 bootstrap samples. One-sample *t*-tests were used to determine the significant differences in induction of chemokine and inflammatory cytokine release from HL-CZ cells between the zinc-treated and untreated groups (test value = 1). 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).

Table 1
Immune response-related transcription factor-mediated luciferase reporter adenoviruses.

Recombinant adenoviruses	Transcription factors	Response element sequences (RES) [a] direction (5′ → 3′)	RES-driven luciferase constructs
AdV-AP-1-Luc	AP-1	TGACTAATGACTAATGACTAATGACTAA TGACTAATGACTAATGACTAA	
AdEasy-C/EBP-Luc	C/EBP	ATTGCGCAATATTGCGCAATATTGCGCAAT	
AdEasy-CRE-Luc	CREBP	AGCCTGACGTCAGAGAGCCTGACGTCAGAG AGCCTGACGTCAGAGAGCCTGACGTCAGAG AGCCTGACGTCAGAGAGCCTGACGTCAGAG AGCCTGACGTCAGAG	
AdEasy-NFAT-Luc	NFAT	ACGCCTTCTGTATGAAACAGTTTTTCCTCC ACGCCTTCTGTATGAAACAGTTTTTCCTCC ACGCCTTCTGTATGAAACAGTTTTTCCTCC	
AdV-NFκB-Luc	NFκB	GGGGACTTTCCGCTTGGGGACTTTCCGCT GGGGACTTTCCGCTGGGGACTTTCCGCT GGGGACTTTCCGC	
AdEasy-SRE-Luc	Elk1/SRF	CCATATTAGGACATCTAGGATGT CCATATTAGGACATCTAGGATGTCCATATTAGG AGCTAGCCCATATTAGGACATGCTAGGATGT CCATATTAGGAGCATCTAGGATGTCCCATATTAGG AC	
AdEasy-STAT-Luc	STAT	GGTTCCCGTAAATGCATCAGGTTCCCGTAAA TGCATCAGGTTCCCGTAAATGCATCAGG TTCCCGTAAATG	

[a] The response elements are marked by shading or underlining.

3. Results

3.1. Conditioned media from the zinc-treated HL-CZ cells induce ICAM-1 protein expression in HUVEC cells

Our previous study showed that zinc ions alone are sufficient to induce similar levels of ICAM-1 expression as ZnO particles, suggesting that the dissolved zinc ions play the major role in inflammatory effect of ZnO particles on vascular endothelial cells. In this study, we used HL-CZ cells, a human promonocytic leukemia cell line, and a soluble zinc compound $Zn(CH_3COO)_2$ to determine if zinc ions could cause inflammatory responses in this human promonocyte cell line. HL-CZ cells were treated with different concentrations of $Zn(CH_3COO)_2$ (0, 30, 50, 100, and 150 μM) for 6 h and then the conditioned media were collected. Then, HUVEC cells were treated with the conditioned media for 24 h. Following treatments, HUVEC cell lysates were collected for analysis of ICAM-1 protein expression with immunoblot. Results in Fig. 1A showed that levels of ICAM-1 protein expression in HUVEC cells were positively correlated with the $Zn(CH_3COO)_2$ concentrations used in HL-CZ treatments. The time-dependent ICAM-1 induction in HUVEC cells by the conditioned media from HL-CZ cells treated with 150 μM $Zn(CH_3COO)_2$ was also observed (Fig. 1B). The ICAM-1 induction could be up to 6–7 folds. These results suggested that zinc treatments might cause release of inflammatory cytokines from HL-CZ cells into culture medium and the released inflammatory cytokines were able to activate ICAM-1 expression in HUVEC cells.

3.2. Zinc treatments induce chemokine and inflammatory cytokine release from HL-CZ cells

Because a large number of cytokines have been characterized, it was complicated that how to effectively identify the expression profiles of multiple cytokines in conditioned medium. By using the RayBio Human Cytokine Antibody Array 3 for detection of secreted/active cytokines, we were able to simultaneously detect 42 cytokine levels in conditioned media. Results in Fig. 1 showed that conditioned media from HL-CZ cells treated with 150 μM $Zn(CH_3COO)_2$ caused the maximum level of ICAM-1 expression in HUVEC cells. Therefore, conditioned media by such zinc treatments were collected for cytokine analysis with the cytokine antibody array. As shown in Table 2, the zinc treatment induced significant releases of GRO-α, IL-6, IL-7, IL-8, and IL-10 by 3.98, 1.92, 1.72, 1.34, and 1.46 folds, respectively. Although, the array detects only 42 cytokines, the results clearly show that the zinc treatment causes significant releases of chemokines (e.g., GRO-α and IL-8), pro-inflammatory cytokines (e.g., IL-6 and IL-7), and anti-inflammatory cytokines (e.g., IL-10) from HL-CZ cells.

3.3. Inhibition of NFκB activity by over-expression of IκBα in HL-CZ cells does not block the conditioned medium-induced ICAM-1 expression in HUVEC cells

Because NFκB plays the major role in regulating the zinc-induced ICAM-1 expression in HUVEC cells [14], it was of importance to further ask if NFκB also mediates the zinc-induced inflammatory cytokine release from HL-CZ cells. By over-expression of IκBα in HL-CZ cells using an adenovirus-mediated expression system, we investigated whether the zinc treatment was able to induce IκBα phosphorylation in HL-CZ cells and whether overexpression of IκBα in HL-CZ cells could block the conditioned medium-induced ICAM-1 expression in HUVEC cells.

As shown in Fig. 2, in the un-infected and the AdEasy-GFP-infected controls, treatment of HL-CZ cells with 150 μM $Zn(CH_3COO)_2$ for 6 h induced degradation of endogenous IκBα by 63%; IκBα phosphorylation was barely detectable most likely due to the rapid polyubiquitination and subsequent degradation of phosphorylated IκBα by the 26S proteasome [29]. In the adenovirus-mediated overexpression of IκBα experiments, results indicated that inhibition of NFκB activity by over-expression of IκBα in HL-CZ cells did not block the conditioned medium-induced ICAM-1 protein expression in HUVEC cells; meanwhile, the zinc treatments did enhance phosphorylation of exogenous IκBα in HL-CZ cells. Because of the abundant IκBα expression by adenovirus

Table 2
Analysis of zinc effect on cytokine release from HL-CZ cells with RayBio Human Cytokine Antibody Array 3.

Cytokines	Induction fold (treated/untreated)			Mean	SD	*p* value for one-sample *t*-test (test value = 1)
	1st	2nd	3rd			
ENA-78	0.540	1.059	0.914	0.838	0.268	0.404
GCSF	1.027	1.009	0.797	0.944	0.128	0.530
GM-CSF	0.978	1.484	0.712	1.058	0.392	0.822
GRO	1.854	2.479	1.634	1.989	0.438	0.060
GRO-α	3.454	5.212	3.276	3.981	1.070	0.040*
I-309	1.054	1.186	0.871	1.037	0.158	0.725
IL-1α	0.799	1.271	0.836	0.969	0.262	0.855
IL-1β	0.992	1.052	0.975	1.006	0.040	0.812
IL-2	1.018	1.447	1.009	1.158	0.250	0.388
IL-3	0.550	0.987	1.046	0.861	0.271	0.468
IL-4	0.764	2.004	1.014	1.261	0.656	0.562
IL-5	0.812	1.735	0.795	1.114	0.538	0.749
IL-6	1.930	2.028	1.787	1.915	0.121	0.006**
IL-7	1.427	1.868	1.849	1.715	0.249	0.038*
IL-8	1.239	1.348	1.431	1.339	0.096	0.026*
IL-10	1.631	1.313	1.434	1.459	0.161	0.038*
IL-12 p40p70	0.613	0.987	0.913	0.838	0.198	0.292
IL-13	0.892	1.060	0.902	0.951	0.094	0.465
IL-15	0.708	1.198	0.998	0.968	0.246	0.843
INF-γ	0.904	1.001	0.979	0.961	0.051	0.319
MCP-1	1.449	1.223	1.013	1.228	0.218	0.211
MCP-2	1.005	1.461	1.121	1.196	0.237	0.289
MCP-3	0.540	1.503	0.749	0.931	0.507	0.835
MCSF	0.962	1.168	1.058	1.063	0.103	0.403
MDC	1.176	0.906	0.872	0.985	0.167	0.888
MIG	0.699	1.099	0.863	0.887	0.201	0.433
MIP-1δ	0.716	1.032	0.820	0.856	0.161	0.262
RANTES	1.031	0.997	0.862	0.963	0.089	0.551
SCF	0.951	0.864	0.794	0.870	0.079	0.103
SDF-1	0.678	0.918	0.844	0.813	0.123	0.119
TARC	0.724	0.988	1.011	0.908	0.159	0.422
TGF-β1	0.813	1.017	0.970	0.933	0.107	0.393
TNF-α	1.008	0.847	0.900	0.918	0.082	0.227
TNF-β	0.781	1.009	1.028	0.939	0.137	0.524
EGF	0.827	0.920	0.894	0.880	0.048	0.050
IGF-1	0.533	1.249	0.698	0.827	0.375	0.507
Angiogenin	1.014	0.965	0.690	0.890	0.175	0.388
Oncostatin M	0.886	0.976	0.761	0.874	0.108	0.181
Thrombopoietin	1.012	1.249	0.984	1.082	0.146	0.434
VEGF	1.361	1.817	1.243	1.474	0.303	0.114
PDGF BB	0.890	0.837	0.725	0.817	0.084	0.064
Leptin	0.998	1.026	0.922	0.982	0.054	0.621

* $p < 0.05$.
** $p < 0.01$.

system, we were able to detect the IκBα phosphorylation. These results suggest that inhibition of NFκB alone is not sufficient to completely block the inflammatory cytokine release from HL-CZ cells.

3.4. Zinc treatment induces activation of multiple immune response-related transcription factors in HL-CZ cells

On the basis of our present results, it was suggested that, in addition to NFκB, multiple immune response-related transcription factors might be involved in the zinc-induced cytokine release from HL-CZ cells. To verify this hypothesis, seven recombinant adenoviruses carrying a response element-driven luciferase reporter gene were established (Table 1). These immune response-related transcription factors include AP-1, C/EBP, CREBP, NFAT, NFκB, SRF, and STAT [30–32]; the activated transcription factors mediate luciferase expression via binding to their respective response elements. HL-CZ cells were infected with one of the recombinant adenoviruses and then were treated with 150 μM $Zn(CH_3COO)_2$ for 6 h. As shown in Fig. 3, the zinc treatment induced activation of AP-1, C/EBP, CREBP, NFAT, NFκB, SRF, and STAT by 1.18, 2.90, 2.46, 1.64, 4.27, 1.42, and 1.42 folds, respectively, in HL-CZ cells. Among them, C/EBP, CREBP, NFAT, NFκB, and SRF were significantly activated by the zinc treatment.

4. Discussion

Zinc is an essential trace element for animals and play important roles in regulation of immune function in humans [33]. However, excess zinc may also deregulate the homeostasis of immune system. Epidemiological studies revealed the association between elevated ambient air zinc and increased pediatric asthma morbidity [10]. Animal studies indicated that the ambient $PM_{2.5}$ samples with higher levels of metals, such as zinc, caused increases in the allergic respiratory disease in mice [34]. Our previous *in vitro* evidence revealed an important role for ZnO particle, or its dissolved zinc ions, in modulating inflammatory responses of vascular endothelial cells [14,15]. The present study further demonstrates that zinc ions induce chemokine and inflammatory cytokine release from vascular promonocytes, possibly, via activating multiple immune response-related transcription factors.

In the previous study, we evaluated vascular endothelial dysfunction by using ICAM-1 expression, an indicator for inflammatory response. ICAM-1, continuously present in low concentrations in

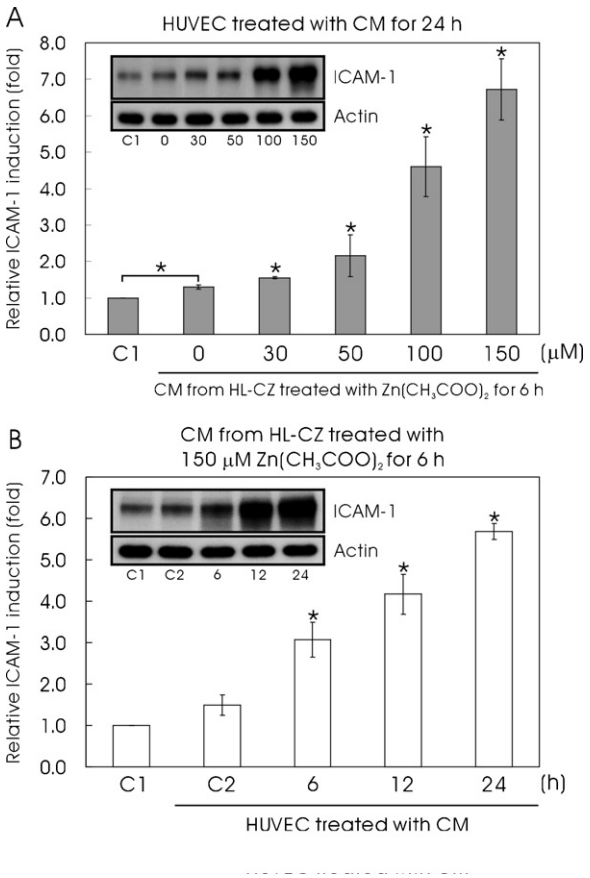


Fig. 1. Conditioned medium (CM) from the zinc-treated HL-CZ cells induces ICAM-1 protein expression in HUVEC cells. (A) HL-CZ cells were treated with different concentrations of $Zn(CH_3COO)_2$ (0, 30, 50, 100, and 150 μM) for 6 h and then the conditioned medium was collected. HUVEC cells were treated with the conditioned medium for 24 h. (B) HUVEC cells were also treated with the conditioned medium for 6, 12, and 24 h; the conditioned medium was collected from HL-CZ cells treated with 150 μM $Zn(CH_3COO)_2$ for 6 h. For C2 controls, HUVEC cells were treated with the conditioned medium for 24 h; the 6-h HL-CZ cultured medium with no zinc treatment were used as the conditioned medium. For C1 controls, HUVEC cells were cultured in M199 medium for 24 h. Following treatments, HUVEC cell lysates were analyzed for ICAM-1 and actin protein levels by immunoblot analysis. Representative immunoblots are shown (inserts). The protein levels of ICAM-1 and actin were quantified. The experiment was repeated three times. The data are expressed as relative ICAM-1 protein levels compared to that of the untreated control (C1) and are presented as means ± SD. Differences in ICAM-1 expression were examined by using Student's *t*-tests with 2000 bootstrap samples (for A, 0 *vs.* C1 and the other CM-treated groups *vs.* 0; for B, the other CM-treated groups *vs.* C2). $^{*}p < 0.05$.

the membranes of leukocytes and endothelial cells, is a ligand for LFA-1 (integrin), a receptor found on leukocytes [35]. Here, we showed that the conditioned medium from HL-CZ cells treated with 150 μM $Zn(CH_3COO)_2$ for 6 h caused a maximum level of ICAM-1 induction (Fig. 1). Therefore, we collected these conditioned media for analysis of cytokine secretion using the RayBio Human Cytokine Antibody Array 3. The kit detects secreted cytokines in conditioned medium that presents a more accurate reflection of active cytokine levels. Cytokines or other proinflammatory mediators induce adhesion molecule expression and facilitate leukocyte attachment to vascular endothelium via ICAM-1/LFA-1 binding [36,37]. The adhesion of monocytes to the arterial wall and their subsequent infiltration and differentiation into macrophages is a crucial step in the development of atherosclerosis.

Analysis of cytokines in conditioned media clearly showed that the zinc treatment induced chemokine and inflammatory cytokine release from HL-CZ cells. With the Cytokine Antibody Array, the GRO antibody detects CXCL1, CXCL2, and CXCL3; the

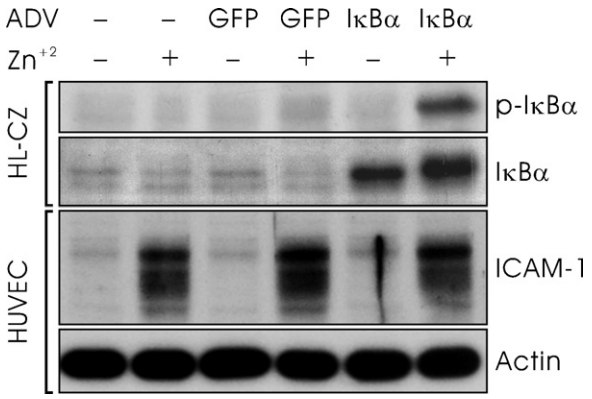


Fig. 2. Inhibition of NFκB activity by over-expression of IκBα in HL-CZ cells does not block the conditioned medium-induced ICAM-1 protein expression in HUVEC cells. HL-CZ cells were left uninfected (–) or infected with a recombinant adenovirus (ADV), AdEasy-IκBα (IκBα) or AdEasy-GFP (GFP). Cells were treated with 150 μM of $Zn(CH_3COO)_2$ for 6 h. Following treatments, both HL-CZ cell lysates and the conditioned media were collected. Then, HUVEC cells were treated with the conditioned media for 24 h and HUVEC cell lysates were collected. By using immunoblot analysis, IκBα expression (IκBα) and phosphorylation (p-IκBα) in HL-CZ cells and protein levels of ICAM-1 and actin in HUVEC cells were determined.

GRO-α antibody detects only CXCL1. The zinc treatment induced marked releases of GRO (CXCL1, CXCL2, and CXCL3) and GRO-α (CXCL1) by 1.99 and 3.98 folds, respectively (Table 2). Because GRO activity involves three CXC chemokines, it is suggested that GRO-α could be the major CXCL chemokine secreted by HL-CZ cells. GRO-α, initially isolated and characterized by its growth stimulatory activity on malignant melanoma cells, is a chemoattractant for neutrophils [38,39]. Recently, many new functions of GRO-α have been discovered and associated with atherosclerosis, angiogenesis, and many inflammatory conditions [40].

Moreover, the zinc treatment also induced significant releases of inflammatory cytokines, including IL-6, IL-7, IL-8, and IL-10. In addition to its known role in mediating the systemic acute-phase response, IL-6 plays multiple roles in initiating and sustaining vascular inflammation [41]. IL-7 has many roles in T cells, dendritic cells, and bone biology in humans and is involved in chronic inflammation linking stroma and adaptive immunity [42]. IL-8, or CXCL8, is also a CXC chemokine and bears the primary responsibility for the recruitment of monocytes and neutrophils, the signature

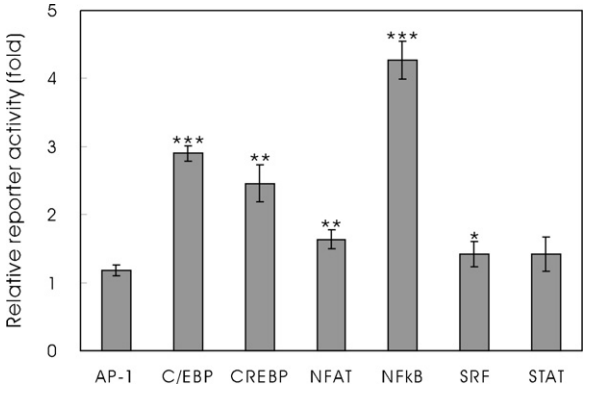


Fig. 3. Zinc treatment induces activation of multiple immune response-related transcription factors in HL-CZ cells. HL-CZ cells were infected with one recombinant virus carrying a transcription factor-mediated luciferase reporter gene. The infected cells were treated with 150 μM $Zn(CH_3COO)_2$ for 6 h. Following the treatments, luciferase activity of each sample was determined. The experiment was repeated three times. The data are expressed as relative reporter activity as compared to that of the untreated control and are presented as means ± SD. Differences in reporter activity (the zinc-treated *vs.* the zinc-untreated) of each transcription factor were examined by using one-sample *t*-tests. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

cells of acute inflammatory response [43]. IL-10, an important cytokine with anti-inflammatory properties, is produced by activated immune cells, in particular monocytes/macrophages and T cell subsets including Tr1, Treg, and Th1 cells [44]. On the basis of this information, the present results demonstrate the potential impacts of zinc exposure on disturbing homeostasis of inflammation via these inflammatory cytokines.

The major role of NFκB in regulation of zinc-induced ICAM-1 expression in vascular endothelial cells has been demonstrated [14]. However, results in Fig. 2 suggest that, in addition to NFκB, other immune response-related transcription factors may be also involved in zinc-induced chemokine/inflammatory cytokine release from HL-CZ cells. Indeed, among the seven immune response-related transcription factors tested, C/EBP, CREBP, NFAT, NFκB, and SRF were significantly activated (Fig. 3). Therefore, the zinc-induced inflammatory responses in promonocytes involve a more sophisticated signaling regulation than that in vascular endothelial cells. Moreover, the 6-h zinc treatment conditions were used in cytokine analyses and immune response-related transcription factor-mediated luciferase reporter assay; this short-term treatment was designed to avoid the potential secondary inflammatory responses. However, we could not rule out the possibility in the meantime. Thus, deciphering the time-course activation of inflammation-related signaling molecules and transcription factors in detail is needed in the following study.

The present study mainly dealt with the potential impacts on homeostasis of vascular immune system by those excess zinc exposures, especially from ambient particulate pollutants. In addition to vascular endothelial cells [14], here we further demonstrated that zinc induces chemokine and inflammatory cytokine release from human promonocytes. On the basis of this and our previous studies [14,15], the possible scenario of inflammatory responses induced by zinc from ambient particulate pollutants is described. First, fine particles tend to be 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 zinc and nickel) and thus may activate pulmonary inflammation. Third, ultrafine particles and dissolved metal ions are able to cross the pulmonary epithelial barrier and then into the bloodstream. Finally, direct exposure of vascular cells, including endothelial cells and circulating blood cells, to those proinflammatory metals ions, such as zinc ions in this study, elicits vascular inflammation. These studies provide new insight for understanding the mechanisms of those inflammatory diseases induced by ambient particulate pollutants.

5. Conclusions

Using human HL-CZ promonocytes as an *in vitro* system, this study reveals two important findings. Zinc treatment induces chemokine and inflammatory cytokine release from HL-CZ cells. The process involves activation of multiple immune response-related transcription factors, including C/EBP, CREBP, NFAT, NFκB, and SRF.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.09.035.

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