Zn\textsuperscript{2+}-induced IL-8 expression involves AP-1, JNK, and ERK activities in human airway epithelial cells

Yu-Mee Kim, William Reed, Weidong Wu, Philip A. Bromberg, Lee M. Graves, and James M. Samet

1Department of Environmental Sciences and Engineering, 2Center for Environmental Medicine, Asthma, and Lung Biology, 3Department of Pharmacology, University of North Carolina, Chapel Hill; and 4Human Studies Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina

Submitted 10 November 2005; accepted in final form 20 December 2005.

EXPOSURE TO ZINC-LADEN PARTICULATE MATTER (PM) in ambient and occupational settings has been associated with adverse health effects (4, 13, 14, 27, 42). Numerous studies have indicated that atmospheric zinc is ubiquitously detected in ambient air samples collected from a variety of places and is likely derived from industrial operations and emissions and surface wear (1, 6, 7, 15, 28, 29, 41, 53). Recently, an epidemiological study reported that zinc in the ambient air is strongly associated with the prevalence of asthma (4). Inhalation of zinc oxide particles produced during welding induces metal fume fever, an inflammatory syndrome involving multisystemic symptoms and pulmonary inflammation (9, 13, 14).

Furthermore, animal and human studies have shown that the inhalation of zinc induces adverse pulmonary and systemic health effects, such as acute pulmonary inflammatory responses and hematological changes (5, 13, 14, 30, 31, 44).

Interleukin (IL)-8 is a pivotal chemotactic cytokine in the human lung that is responsible for the recruitment of neutrophils and macrophages to the site of inflammation. Elevated levels of IL-8 are associated with several pathophysiological states in the lung, including bronchoconstriction, edema, and neutrophilia (39, 55). In humans, the airway epithelium is a major source of IL-8. Levels of IL-8 expression by human airway epithelial cells (HAEC) increase in response to ambient PM and PM components such as zinc (12, 19, 21, 26, 49, 60).

The level of IL-8 expression is under the control of multiple signaling cascades in HAEC, including NF-\textkappaB and the MAPK pathways ERK, JNK, and p38. MAPK regulation of IL-8 expression is mediated through the activation of the transcription factors Fos, Jun, and activating transcription factor, which are categorized as activating protein (AP)-1 family proteins (24, 36, 47, 48).

The MAPKs have previously been described as regulators of the AP-1 transcription factor and linked to IL-8 gene expression (48). Fos proteins including c-Fos, FosB, and Fra1 are known to be phosphorylated by ERK on serine and/or threonine residues in the COOH-terminal domain (40, 47). JNK is well characterized as a specific kinase that binds and phosphorylates Jun proteins including c-Jun, JunB, and JunD (47). Several lines of evidence indicate that phosphorylation of c-Jun and c-Fos by MAPK contributes to both the abundance and activity of AP-1 proteins. Phosphorylation of c-Jun on Ser\textsuperscript{73} and Ser\textsuperscript{73} by JNK increases the stability of c-Jun by reducing its ubiquitination and degradation. In addition, it increases the transactivating potential, enhancing AP-1-dependent transcriptional activity (24). In the case of c-Fos, ERK-dependent phosphorylation of c-Fos in the COOH-terminal domain results in a marked increase in its transactivating potential (40).

Zinc exposure has been reported to activate several MAPKs and their associated downstream transcription factors in human airway epithelial cells (49, 58). Levels of MAPK phosphorylation are regulated by the kinase activity of the dual-specificity kinases known as the MAPK kinases (MAPKKs) and the dephosphorylating activity of the MAPK phosphatases (MKPs) (8). In contrast to MAPKKs, MKPs negatively regulate MAPK


Yu-Mee Kim, William Reed, Weidong Wu, Philip A. Bromberg, Lee M. Graves, and James M. Samet.

104 Mason Farm Rd., US EPA, Human Studies Facility, Chapel Hill, NC, 27514 (e-mail: Samet_James@epa.gov).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
activity by dephosphorylating tyrosine, serine/threonine, or both tyrosine and threonine (dual specificity) residues of the TXY motif in MAPK. The activity of tyrosine phosphatases can be inhibited by exposure to a number of extracellular stimuli including vanadium, arsenite, and oxidative stress in a various cell types (3, 34, 50, 51). Exposure to zinc has also been shown to inhibit phosphatase activity in various cell types, including HAEC (38, 50).

The underlying mechanisms responsible for Zn\(^{2+}\)-induced expression of IL-8 remain to be understood. In this study, we examined the molecular signaling of Zn\(^{2+}\)-induced IL-8 expression in HAEC. Herein, we report that ERK, JNK, and AP-1 are responsible for Zn\(^{2+}\)-induced IL-8 expression in HAEC. In addition, Zn\(^{2+}\) inhibits the activities of ERK- and JNK-directed phosphatases. These results suggest that Zn\(^{2+}\)-induced inhibition of phosphatase activity contributes to the molecular signaling events, including MAPK and AP-1 activation, that lead to Zn\(^{2+}\)-induced IL-8 expression.

MATERIALS AND METHODS

Reagents. Common laboratory chemicals including BSA and 2-mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, MO). SDS-PAGE supplies were obtained from Bio-Rad (Richmond, CA). Kinase inhibitors, such as PD-98059 (2’-amino-3’-methoxy-flavone), PD-153035 [4-[(3-bromophenyl)amino]-6,7-dimethoxy-quinoxaline], SP-600125 [anthera(19,9-dipyrazol-6H)-one], and SB-203580 [4-(4-fluorophenyl)-2-(4-methylsulfanylphenyl)-5-(4-pyridyl) 1H-imidazole] were purchased from Calbiochem (San Diego, CA). Specific anti-phospho-JNK (Thr\(^{183}/\text{Tyr}^{185}\)), anti-phospho-p38 (Thr\(^{180}/\text{Tyr}^{185}\)), anti-phospho-ERK (Thr\(^{202}/\text{Tyr}^{204}\)), anti-phospho-c-Jun (Ser\(^{63}\) and Ser\(^{73}\)) anti-ERK, anti-JNK, anti-p38, and anti-c-Jun were obtained from Cell Signaling Technology (Beverly, MA). Anti-phospho-c-Fos (Thr\(^{232}\)) antibody was purchased from Biosource International (Lake Placid, NY).

Cell culture. BEAS-2B (subclone S6) cells were obtained from the Human Studies Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and maintained in keratinocyte growth medium (Cambrex Bioproducts, Clonetics Division, San Diego, CA). This cell line was derived by transformation of human airway epithelial cells with an adi12-SV40 adenovirus construct (46).

IL-8 expression analyses. BEAS-2B cells were lysed in a buffer containing guanidine isothiocyanate and purified using an RNeasy kit according to manufacturer’s instructions (Qiagen, Valencia, CA). Total RNA (100 ng), 0.5 mM NTP (Pharmacia, Piscataway, NJ), 5 μM random hexaoligonucleotide primers (Pharmacia, NJ), 10 U/μl RNase inhibitor (Promega, CA), and 10 U/μl Moloney murine leukemia virus RT (GIBCO-BRL Life Technologies) were incubated in a 40°C water bath for 1 h in 50 μl of 1× PCR buffer to synthesize first-strand cDNAs. The reverse transcription was inactivated by heating at 92°C for 5 min. Quantitative PCR of IL-8 and glyceraldehyde-hyde-3-phosphate dehydrogenase (GAPDH) specimen cDNA and standard cDNA was performed in a 50-μl final volume mixture containing TaqMan master mix (Perkin-Elmer, CA), 1.25 μM probe, 3 μM forward primer, and 3 μM reverse primer. The probe anneals to the template between the two primers. This probe contains both a fluorescence reporter dye at the 5’-end (6-carboxyfluorescein: emission λ\(_{\text{em}} = 518\) nm) and a quencher dye at the 3’-end (6-carboxytetramethyl rhodamine: emission λ\(_{\text{em}} = 582\) nm). During polymerization, the probe is degraded by the 5’-3’ exonuclease activity of the Taq DNA polymerase, and the fluorescence is detected by a laser in the sequence detector (TaqMan ABI Prism 7700 Sequence Detector System; Perkin-Elmer, CA). Thermal cycler parameters include 2 min at 50°C, 10 min at 95°C, and 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The abundance of IL-8 and GAPDH transcripts in first-strand cDNAs was measured by TaqMan quantitative PCR using the oligonucleotide primers and probes previously described (20, 21), TaqMan Universal MasterMix and an Applied Biosystems 7700 Sequence Detection System.

Release of IL-8 protein into the cell culture supernatant was assayed with a human IL-8 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Western blotting. Preparation of cytoplasmic and nuclear extracts from BEAS-2B cells for nuclear protein detection was performed as described (20, 21). Cytoplasmic extraction buffer (CEB) consisted of 10 mM Tris·HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM DTT, and protease inhibitors (PI; 1 mM Pefabloc, 50 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 40 μg/ml bestatin, 3 μg/ml E-64, and 100 μg/ml chymostatin; all purchased from Boehringer Mannheim Roche Applied Science, Indianapolis, IN). Cells were scraped into CEB, transferred into a microcentrifuge tube, and placed on ice for 15 min. Nonidet P-40 (NP-40; Sigma-Aldrich, MO) was added to a final concentration of 0.1% and vortexed for 10 s. Nuclei were pelleted by centrifugation at 15,000 g for 30 s; we repeated this after washing the nuclei with CEB. The supernatant was removed, and the nuclei were incubated for 10 min on ice in a nuclear extraction buffer (consisting of 20 mM Tris·HCl, pH 8.0, 400 mM NaCl, 1.5 mM MgCl\(_2\), 1.5 mM EDTA, and 1 mM DTT, 25%) with PI to extract nuclear proteins. After brief centrifugation, the supernatants, containing the nuclear proteins, were stored at −80°C until ready for use.

Gel electrophoresis followed by Western blotting was performed essentially as described by Laemmli (32) and Towbin et al. (56a). Cells were lysed with radioimmunoprecipitation assay lysis buffer containing 1% NP-40, 0.5% deoxycholate, and 0.1% SDS in PBS at pH 7.4, phosphatase inhibitor cocktail set I and II, and protease inhibitor cocktail set III purchased from Calbiochem (San Diego, CA). We normalized each sample for a protein content of 50–100 μg before loading it on a SDS-PAGE gel. Lysates were mixed with one volume of reducing SDS-PAGE loading buffer containing 0.125 M Tris (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.05% bromphenol blue. The samples were heated for 1 min at 90°C, run on 10% SDS-PAGE gels with prestained molecular weight markers (Bio-Rad, CA) in Tris-glycine-SDS buffer (Bio-Rad), and electrobotted on nitrocellulose membranes (Bio-Rad). The blots were blocked with 5% nonfat milk and incubated with primary antibodies. HRP-conjugated goat anti-rabbit antibody was used as a secondary antibody. Protein bands on the membrane were detected with the enhanced chemiluminescence detection solution (Amersham Biosciences, Piscataway, NJ) and exposed on high-performance chemiluminescence film (Amersham Biosciences). The bands were quantified by a Kodak electrophoresis documentation and analysis system (Rochester, NY).

Promoter-reporter activity assays. An IL-8 promoter-reporter cassette was generated that was composed of the transcription-silencing sequence (bases 2,193–2,689) from pNF-κBIC (Stratagene) upstream of a 1.46-kb fragment of the IL-8 gene (~1,410 to +54) followed by the coding sequence of enhanced green fluorescent protein (EGFP) and an SV40 polyadenylation sequence. This cassette was inserted in the multicloning site of pShuttle (Clontech, Palo Alto, CA). A recombinant plasmid, psh-IL8pro-EGFP, bearing the desired sequence was used by the University of North Carolina (UNC) Vector Core Facility to generate a recombinant adenovirus.

Wild-type IL-8 promoter activity was assayed using the recombinant adenoviral IL-8 promoter-reporter vector, AdIL-8proEGFP, previously described (26). Mutant IL-8 promoter-reporter adenovirus having an inactivated AP-1 response element was generated as fol-
low. The AP-1 response element of the IL-8 promoter (bases -126 to -120) was changed from TGACTCA to TatCTCA by site-directed mutagenesis of psh-IL8pro-EGFP, the wild-type IL-8-EGFP promoter reporter adenoviral shuttle vector (26). Adenoviruses carrying the mutant promoter-reporter gene were generated by the UNC Vector Core Facility.

Transduction of cells with adenovirus was as described previously (26). In brief, BEAS-2B cells grown to 80% confluency in 24-well plates were transduced with AdIL-8-pro-EGFP at a multiplicity of infection of 500 focus-forming units/cell for 8 h. The recovery period of 24 h was followed by the challenge with 50 μM ZnSO4 or TNF-α (10 ng/ml). IL-8 promoter activity was measured as follows: cells were put in suspension by brief treatment with trypsin-EDTA at 37°C (10 ng/ml). IL-8 promoter activity was measured as follows: cells were put in suspension by brief treatment with trypsin-EDTA at 37°C and assessed for mean EGFP fluorescence intensity using a FACSsort flow cytometer (Becton Dickinson, San Jose, CA).

Phosphatase activity assays. We used a nonradioactive method for determining phosphatase activity in whole cell lysates. Recombinant, phosphorylated substrates of phosphatases including phospho-ERK or phospho-JNK were dephosphorylated by the activity of phosphatases in cell lysates and detected by Western blotting using anti-phospho-ERK or anti-phospho-JNK were dephosphorylated by the activity of phosphatases in cell lysates and detected by Western blotting using anti-phospho-ERK or anti-phospho-JNK antibodies. To measure ERK- or JNK-directed phosphatase activity in the cells, BEAS-2B cells were exposed to media, or 50 μM Zn2+ or V4+, for 30 min, and were lysed in low-salt buffer containing 100 mM HEPES, 0.2% NP-40 (Sigma-Aldrich), and PI cocktail (Calbiochem) for the ERK-directed phosphatase activity assay or in high-salt lysis buffer containing 20 mM Tris·HCl (pH 8.0), 400 mM NaCl, 1.5 mM MgCl2 PI cocktail, and 0.2% NP-40 for the JNK-directed phosphatase activity assay. We diluted 20 or 150 μg of whole cell lysate for ERK- or JNK-directed phosphatase activity assay, respectively, into a total volume of 30 or 50 μl in phosphate assay buffer (350 mM sucrose, 20 mM HEPES, and 150 mM KCl). We added 10 ng of recombinant, phosphorylated GST-tagged ERK2 or 6xHis-tagged JNK2 (Upstate Biotechnology, Lake Placid, NY) to each sample, and the reactions were incubated at 37°C for 30 min. To measure the activity of recombinant MKP-1 (Upstate) under 50 μM Zn2+ or V4+, 50 units of recombinant MKP-1 were diluted to a total volume of 30 μl in phosphatase assay buffer and reacted with 50 μM Zn2+ or V4+ at 37°C for 5 min. We added 10 ng of recombinant phosphorylated ERK or JNK to each sample and subjected it to further reaction at 37°C for 30 min. We stopped the reactions by adding 10 μl of Laemmli buffer and boiling for 5 min. Western blotting was performed to detect phosphorylated ERK or JNK.

Statistical analysis. Data are presented as means ± SE of at least three separate experiments. Data comparisons were carried out by one-way ANOVAs followed by Dunnett’s posttest and two-tailed Student’s t-test.

RESULTS

Zn2+ exposure upregulates IL-8 expression in human airway epithelial cells. To ascertain the effect of an acute, noncytotoxic exposure to soluble Zn2+ ions on HAEC, BEAS-2B cells were grown to subconfluence in 24-well plates and exposed to 50 μM ZnSO4 for 20 h. This exposure dose and duration were found to have no effect on cell viability (data not shown). Exposure of BEAS-2B cells to Zn2+ for 12 h significantly induced IL-8 protein release into the cell culture medium in a dose-dependent manner. Exposure to 15 or 50 μM Zn2+ induced IL-8 protein release by 1.6- and 4.6-fold, respectively (Fig. 1A). Similarly, Zn2+ induced IL-8 mRNA expression was dose dependent. Exposure of BEAS-2B cells to 15, 50, and 100 μM Zn2+ for 3 h induced IL-8 mRNA expression ~2-, 7-, and 10-fold, respectively (Fig. 1B). Figure 1C shows the time course of IL-8 mRNA expression in BEAS-2B cells exposed to 50 μM Zn2+. Increases in Zn2+-induced IL-8 mRNA expression reached statistical significance as early as 2 h and continued to increase up to 4 h of exposure.

Fig. 1. Zn2+ induces interleukin (IL)-8 mRNA and protein expression in human airway epithelial cells (BEAS-2B). A: BEAS-2B cells were treated with 15 or 50 μM Zn2+ for 12 h. The concentration of IL-8 protein in the culture supernatant was determined by ELISA and expressed as the fold increase over control. B: BEAS-2B cells were treated with 15–100 μM Zn2+ for 3 h. Levels of IL-8 mRNA were analyzed by real-time RT-PCR. IL-8 mRNA induction was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels and expressed as the fold increase over control. C: BEAS-2B cells were treated with 50 μM Zn2+ up to 4 h. IL-8 mRNA induction was normalized to GAPDH levels and expressed as the fold increase over control. D: BEAS-2B cells were infected with an adenoviral IL-8-enhanced green fluorescent protein (EGFP) promoter reporter vector and challenged with Zn2+ (50 μM) or tumor necrosis factor (TNF)-α (10 ng/ml) for 12 h. IL-8 promoter activity (mean EGFP fluorescence intensity/cell) was determined by flow cytometry. Data represent means ± SE of 3 independent experiments. *P < 0.05 compared with control (Ct).
To determine whether Zn\(^{2+}\)-induced IL-8 expression is transcriptionally regulated, IL-8 promoter activity was analyzed in BEAS-2B cells infected with an adenoviral IL-8 promoter EGFP reporter before exposure to Zn\(^{2+}\). The level of EGFP in BEAS-2B cells increased 2.4-fold in response to 50 μM Zn\(^{2+}\), indicating that Zn\(^{2+}\) exposure increased IL-8 promoter activity in BEAS-2B cells (Fig. 1D). In comparison, TNF-α induced a 3.9-fold increase in IL-8 promoter activity in BEAS-2B cells.

Zn\(^{2+}\) exposure induces MAPK phosphorylation in BEAS-2B cells. To determine whether Zn\(^{2+}\) increases the phosphorylation of MAPKs in BEAS-2B cells, we exposed the cells to 50 μM Zn\(^{2+}\) for 15 min–2 h and analyzed levels of phosphorylated MAPK by Western blotting. We observed that exposure of BEAS-2B cells to 50 μM Zn\(^{2+}\) induced a rapid and marked phosphorylation of the MAPKs ERK, JNK, and p38 at Thr\(^{202}/\)Tyr\(^{204}\), phospho-JNK(Thr\(^{183}/\)Tyr\(^{185}\)), and Thr\(^{180}/\)Tyr\(^{182}\) respectively, which was detectable as early as 15 min and continued to increase through 2 h of continuous exposure (Fig. 2, A–C).

Zn\(^{2+}\)-induced IL-8 expression involves ERK and JNK activation. To examine the role of Zn\(^{2+}\)-induced MAPK activation in the upregulation of IL-8 expression in BEAS-2B cells exposed to Zn\(^{2+}\), we administered kinase activity inhibitors of MEK, JNK, and p38 MAPK for 30 min before exposure of BEAS-2B to 50 μM Zn\(^{2+}\). The MEK activity inhibitor PD-98059 reduced Zn\(^{2+}\)-induced IL-8 mRNA and protein increases ~30 and 60%, respectively (Fig. 3, A and B). The inhibitor of JNK activity SP-600125 partially blunted IL-8 mRNA and protein levels 30 and 40%, respectively, in BEAS-2B cells treated with Zn\(^{2+}\) (Fig. 3, C and D). In contrast, the p38 MAPK inhibitor SB-203580 did not prevent Zn\(^{2+}\)-induced IL-8 expression at either the mRNA or protein levels (Fig. 3, E and F). These data suggested that Zn\(^{2+}\)-induced ERK and JNK activities partially contribute to Zn\(^{2+}\)-induced IL-8 expression in BEAS-2B cells.

The human IL-8 promoter contains a number of response elements whose activity is regulated by the interaction with transcription factors that include NF-κB, AP-1, and NF-IL-6. ERK and JNK are upstream regulators of transcription factors c-Fos and c-Jun, which together comprise the AP-1 heterodimer. We examined the effect of Zn\(^{2+}\) on AP-1-dependent IL-8 expression by measuring the phosphorylation of c-Fos and c-Jun following exposure of BEAS-2B cells to Zn\(^{2+}\). Western blotting of nuclear extracts showed that treatment with 50 μM Zn\(^{2+}\) for 0.5–4 h results in clear increases in levels of both phospho-c-Fos (Thr\(^{357}\)) and phospho-c-Jun (Ser\(^{63}/\)Ser\(^{73}\)) in BEAS-2B cells compared with untreated controls. The time course of the induction of c-Fos and c-Jun phosphorylation was in keeping with that observed for Zn\(^{2+}\)-induced phosphorylation of ERK and JNK (Fig. 4).

To examine directly the role of AP-1 in Zn\(^{2+}\)-induced IL-8 expression, we transfected BEAS-2B cells with either a wild-type IL-8 promoter reporter construct or an IL-8 promoter reporter construct in which the AP-1 response element was mutated. As Fig. 5 shows, Zn\(^{2+}\) or TNF-α treatment increased IL-8 promoter activities ~1.8- and 3.4-fold, respectively, in BEAS-2B cells. Mutation of the AP-1 response element resulted in a reduction in Zn\(^{2+}\)- and TNF-α-induced IL-8 promoter activities (Fig. 5). These data suggested that Zn\(^{2+}\)-induced IL-8 expression is at least partially dependent on AP-1 activity in BEAS-2B cells.

Zn\(^{2+}\) exposure inhibits ERK and JNK dephosphorylation in BEAS-2B cells. We next focused on identifying the mechanism that underlies Zn\(^{2+}\)-induced phosphorylation of signaling molecules that leads to IL-8 expression. On the basis of the fact that Zn\(^{2+}\) is a known inhibitor of tyrosine phosphatases, we hypothesized that Zn\(^{2+}\)-induced phosphorylation of MAPKs is the result of unopposed baseline MAPKK activity. To test this hypothesis in the present study, we examined the effect of Zn\(^{2+}\) exposure on ERK- or JNK-directed phosphatase activity in BEAS-2B cells.

We first measured the rate of dephosphorylation of recombinant phospho-ERK or phospho-JNK by lysates prepared from BEAS-2B cells that were exposed to 50 μM Zn\(^{2+}\) or V\(^{4+}\) for 30 min. As shown in Fig. 6, Zn\(^{2+}\) or V\(^{4+}\) exposure to BEAS-2B cells for 30 min significantly retarded the dephosphorylation rate of recombinant phospho-ERK (Fig. 6A) or JNK (Fig. 6B). In our experiments, V\(^{4+}\) was used as a reference protein tyrosine phosphatase (PTP) inhibitor for comparison with the effects of Zn\(^{2+}\).

**DISCUSSION**

Studies have shown that Zn\(^{2+}\) induces the expression of IL-8, a pivotal inflammatory mediator in the human lung that may contribute to the adverse health effects of PM inhalation (1, 13, 30). However, heretofore, there have been no mechanistic studies investigating the underlying signaling events that lead to Zn\(^{2+}\)-induced IL-8 expression. In the present study, we demonstrate that Zn\(^{2+}\) induces IL-8 expression through a...
Fig. 3. ERK and JNK activities are involved in IL-8 mRNA and protein expression induced by Zn\(^{2+}\) exposure. Cells pretreated with DMSO (vehicle), ERK inhibitor (PD-98059, 10 \(\mu\)M), JNK inhibitor (SP-600125, 10 \(\mu\)M), or p38 inhibitor (SB-203580, 10 \(\mu\)M) for 30 min were incubated with Zn\(^{2+}\) (50 \(\mu\)M) for 4 h (mRNA) or 12 h (protein). IL-8 mRNA levels were normalized to levels of GAPDH. Data are expressed as the fold increase over control; shown are means \(\pm\) SE of 3 independent experiments. Significance from untreated controls (*) and significance from Zn\(^{2+}\)-treated IL-8 level (#), \(P < 0.05\) compared with control.

Fig. 4. Zn\(^{2+}\) induced nuclear phosphorylation of activating protein (AP)-1 family proteins, c-Fos (A) and c-Jun (B), in BEAS-2B cells. Nuclear extracts from BEAS-2B cells that had been treated with 50 \(\mu\)M Zn\(^{2+}\) for 30 min–4 h were separated by SDS-PAGE and immunoblotted with c-Fos, phospho-c-Fos(Thr232), c-Jun, or phospho-c-Jun(Ser63 and Ser73) antibodies.

Fig. 5. Zn\(^{2+}\)-induced IL-8 promoter activity depends on the AP-1 response element of the IL-8 promoter. BEAS-2B cells were infected with either a wild-type (WT) adenoviral IL-8 promoter EGFP construct or the same vector with the AP-1 response element mutated. After a 24-h recovery period, the cells were challenged with Zn\(^{2+}\) (50 \(\mu\)M) or TNF-\(\alpha\) (10 ng/ml) for 10 h. IL-8 promoter activity (mean EGFP fluorescence intensity/cell) was determined by flow cytometry. Data shown represent means \(\pm\) SE of 3 independent experiments. Significance from untreated controls (*) and significance from Zn\(^{2+}\)-treated IL-8 level (#), \(P < 0.05\) compared with control.
mechanism that involves ERK, JNK, and AP-1 activation in BEAS-2B cells. We also report that the initiating mechanism following Zn$^{2+}$/H11001 exposure may be an inhibition of ERK- and JNK-directed phosphatase activity in these cells (Fig. 7). Studies in our laboratory support the relevance of the BEAS-2B cells line as a model for the study of the effects of Zn$^{2+}$/H11001 on signaling events leading to IL-8 expression in primary human airway epithelial cells (Y.-M. Kim, unpublished observations) (58, 59, 61).

The MAPKs are important transducers of extracellular stimuli to the nucleus. The mechanisms of MAPK-mediated gene expression include the modulation of transcription factor activity and the regulation of mRNA stability (18, 56). IL-8 is one of the genes reported to be regulated by MAPKs (35, 48). Stimulation with Zn$^{2+}$, as shown in this study, or with nickel, as reported elsewhere (2), has been demonstrated to involve the ERK and JNK pathways. p38 is involved in IL-8 expression in response to hyperosmolarity, diesel particles, or ultrafine carbon particles (16, 17, 26). However, inhibition of p38 activity did not block IL-8 expression in response to zinc in this study or to nickel in a previous study (2). This may reflect cell type- or stimulus-dependent differences in the role of p38 in cytokine expression.

AP-1, comprising Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fraf1, and Fra2) family members, plays a central role in regulating gene transcription involved in cell proliferation and differentiation, apoptosis, inflammation, and the immune response. Various toxic stimuli including PM, ultrafine particles, diesel extracts, arsenite, tobacco smoke, and hydrogen peroxide have been reported to activate AP-1 proteins and increase its transactivating potential and binding to DNA (3, 11, 23, 33, 43, 52). In this study, we demonstrate that the AP-1 family proteins c-Jun and c-Fos are phosphorylated in response to Zn$^{2+}$ exposure. These data are consistent with the finding that Zn$^{2+}$-induced IL-8 expression is dependent on the presence of a functional AP-1 response element in the IL-8 promoter.

In addition to AP-1, other transcription factors such as NF-κB and NF-IL-6 are important transcription factors activating the IL-8 promoter (45, 48). In a separate study, we have observed that Zn$^{2+}$ induces the activity of a reporter construct governed by a 5X tandem repeat of the NF-κB response element in BEAS-2B cells, suggesting that NF-κB might be involved in Zn$^{2+}$-induced IL-8 promoter activity (Y.-M. Kim, unpublished observations). Therefore, Zn$^{2+}$-induced IL-8 promoter activity appears to be activated by the coordination of a number of transcription factors including AP-1 and NF-κB.

Intracellular levels of phosphorylated MAPKs are regulated by the opposing activities of the MAPKKs and the MAPK phosphatases. MAPKKs including MEK1/2, MKK4/7, and MKK3/6 phosphorylate TXY motifs in the activation loops of ERK, JNK, and p38, respectively. On the other hand, MAPK phosphatases negatively regulate MAPK activity by dephosphorylating tyrosine, serine/threonine, or both tyrosine and threonine (dual specificity) residues of TXY motif in MAPK. The tyrosine-specific MKPs include PRP-SL, STEP, and HePTP, whereas serine/threonine-specific MKPs are PP2A and PP2C. Dual-specificity MKPs include MKP-1, MKP-2, hVH3/B23, MKP-3, MKP-X, MKP-4, MKP-5, hVH-5/M3/6, and...
PAC-1, and MKP-6. MKPs differ in terms of substrate specificity, tissue distribution, subcellular localization, and target gene regulation (8, 22, 51, 57).

In the present study, we demonstrate that Zn$^{2+}$ inhibits the activities of endogenous phosphatases directed at phospho-ERK or phospho-JNK in BEAS-2B cells. These data suggest the existence of specific phosphatases that normally function to dephosphorylate ERK or JNK and lose their activities upon exposure of BEAS-2B cells to Zn$^{2+}$. One potential phosphatase involved in this effect is MKP-1, a dual-specificity MPK that is known to dephosphorylate MAPKs including ERK and JNK (8, 10, 54). The expression of MKP-1 induced by glucocorticoids at the promoter level has been proposed as a mechanism for inactivation of ERK1/2 and subsequent anti-inflammatory effects of glucocorticoids (25). We have observed that MKP-1 is expressed in BEAS-2B cells and demonstrated that the activity of recombinant MKP-1 is inhibited by Zn$^{2+}$ and V$^{4+}$ in vitro, suggesting that Zn$^{2+}$ inhibits the activities of ERK- or JNK-directed phosphatases such as MKP-1 in BEAS-2B cells (Y.-M. Kim, unpublished observations). Additional studies will be required to identify the specific PTPs the inhibition of which leads to the activation of MAPKs and the expression of IL-8 in response to Zn$^{2+}$ treatment.

Zn$^{2+}$ has been reported to inhibit PTPs possibly through the binding to the thiol group of the essential catalytic cysteine residue in the phosphatase active site (37, 38). Most MKPs have an essential cysteine residue in the catalytic domain that might be the target of Zn$^{2+}$-induced inhibition (8, 22). Therefore, it is possible to speculate that thiol binding of Zn$^{2+}$ is the mechanism of Zn$^{2+}$-induced inhibition of ERK or JNK-directed phosphatase activity, leading to unopposed MAPKK activity, an accumulation of phospho-ERK and phospho-JNK, and ensuing activation of AP-1-dependent IL-8 expression in human airway epithelial cells.

In summary, we show here that Zn$^{2+}$-induced IL-8 expression involves ERK, JNK, and AP-1 activities in HAEC. In addition, we show that Zn$^{2+}$ induces the phosphorylation and activation of ERK and JNK, likely through the inhibition of ERK- and JNK-directed phosphatase activities. Our data suggest a mechanism for the initiation of signaling processes that underlie adverse responses induced by exposure to PM constituents.

ACKNOWLEDGMENTS

We acknowledge Dr. R. Jude Samulski and the UNC Vector Core for preparation of the AdL8-puro-EGFP and AdL8-8MAP-1pro-EGFP.

DISCLOSURES

The research described herein has been reviewed by the National Health and Environmental Effects Research Laboratory and has been approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. EPA, nor does mention of trade names constitute endorsement of recommendation for use.

REFERENCES


