Activation of MAPKs in human bronchial epithelial cells exposed to metals

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Samet, James M., Lee M. Graves, Jacqueline Quay, Lisa A. Dailey, Robert B. Devlin, Andrew J. Ghio, Weidong Wu, Philip A. Bromberg, and William Reed. Activation of MAPKs in human bronchial epithelial cells exposed to metals. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L551–L558, 1998.—We have previously shown that in vitro exposure to metallic compounds enhances expression of interleukin (IL)-6, IL-8, and tumor necrosis factor-α (TNF-α) in human bronchial epithelial cells. To characterize signaling pathways involved in metal-induced expression of inflammatory mediators and to identify metals that activate them, we studied the effects of As, Cr, Cu, Fe, Ni, V, and Zn on the mitogen-activated protein kinases (MAPK) extracellular receptor kinase (ERK), c-Jun NH2-terminal kinase (JNK), and P38 in BEAS cells. Noncytotoxic concentrations of As, V, and Zn induced a rapid phosphorylation of MAPK in BEAS cells. Activity assays confirmed marked activation of ERK, JNK, and P38 in BEAS cells exposed to As, V, and Zn. Cr and Cu exposure resulted in a relatively small activation of MAPK, whereas Fe and Ni did not activate MAPK under these conditions. Similarly, the transcription factors c-Jun and ATF-2, substrates of JNK and P38, respectively, were markedly phosphorylated in BEAS cells treated with As, Cr, Cu, V, and Zn. The same acute exposure to As, V, or Zn that activated MAPK was sufficient to induce a subsequent increase in IL-8 protein expression in BEAS cells. These data suggest that MAPK may mediate metal-induced expression of inflammatory proteins in human bronchial epithelial cells.

The MAPK pathways transduce signals that lead to diverse cellular responses, such as cell growth, differentiation, proliferation, apoptosis, and stress responses to environmental stimuli (for reviews see Refs. 16, 28, 33, 36, 53, 63, 67). Each of the three major MAPK pathways consists of a three-tiered cascade that includes a Ser/Thr MAPK that is phosphorylated by a dual-specificity Thr/Tyr MAPK kinase, which is, in turn, phosphorylated by a MAPK kinase kinase. The extracellular receptor kinase (ERK) pathway typically transduces growth factor signals that lead to cell differentiation or proliferation (33), whereas cytokines and stress signals (e.g., ultraviolet irradiation, heat, synthesis inhibitors) activate the c-Jun NH2-terminal kinase (JNK) and P38 pathways, resulting in stress responses, growth arrest, or apoptosis (2, 30, 45, 63, 67). Signaling through the MAPK pathways culminates in the phosphorylation-dependent activation of a variety of transcription factors that modulate cytokine gene expression (53, 57, 65).

The transcription factor c-Jun is a major phosphorylation target of JNK (7). c-Jun is a component of the activator protein-1 (AP-1) heterodimer that binds to the TRE/AP-1 DNA response element and regulates IL-6 and IL-8 gene expression (37). Similarly, ATF-2, a substrate for P38 and JNK, binds to AP-1 and CRE response elements and is involved in the expression of TNF-α (58). Similar to the MAPK pathways, the tran-
scriptional activities of c-jun and ATF-2 are induced by a broad range of stress signals, such as genotoxic agents, cytokines, and ultraviolet irradiation (23, 35, 66).

To characterize signaling pathways activated by metals in human bronchial epithelial cells and to identify metals that activate them, we examined the effects of soluble forms of the environmentally relevant metals As, Cr, Cu, Fe, Ni, V, and Zn on MAPK signaling, transcription factor activation, and IL-8 expression in the human bronchial epithelial cell line BEAS 2B (BEAS). We report here that acute exposure to As, Cr, Cu, V, or Zn results in activation of the ERK, J NK, and P38 MAPK pathways and induces the phosphorylation of the transcription factors c-jun and ATF-2 in BEAS cells.

MATERIALS AND METHODS

Reagents. Tissue culture medium, supplements, and supplies were obtained from Clonetics (San Diego, CA). SDS-PAGE supplies such as molecular-mass standards, polyacrylamide, and buffers were obtained from Bio-Rad (Richmond, CA). Bovine serum albumin (BSA), 2-mercaptoethanol, phospho-erbB2, phospho-ERK2, phospho-P38 (Thr180/Tyr182), anti-phospho-Elk1 (Ser383), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies were obtained from Sigma Chemical (St. Louis, MO). [γ-32P]ATP (7,000 Ci/mmol) was purchased from NEN (Wilmington, DE). Protein levels were quantified using a Coomassie blue reagent purchased from Bio-Rad. Stock NEN (Wilmington, DE). Protein levels were quantified using a Coomassie blue reagent purchased from Bio-Rad. Stock NEN (Wilmington, DE). Protein levels were quantified using a Coomassie blue reagent purchased from Bio-Rad. Stock NEN (Wilmington, DE).

MAPK activity assays. For the J NK, ERK2, and P38 activity assays, cells were lysed in a low-salt buffer containing 1% Triton X-100, 25 mM Tris, pH 7.5, 2 mM EGTA, 10% glycerol, 1 mM PMSF, 1 mM sodium metavanadate, 10 mM sodium fluoride, 1 µg/ml peptatin, and 1 µg/ml leupeptin. Lysates were loaded onto standard 11% SDS-polyacrylamide gels containing 250 µg/ml MBP, which was added to the gel just before polymerization. Samples were normalized for protein content before loading. Electrophoresed proteins were electrophoresed onto nitrocellulose (56), and the blots were blocked with 5% casein, washed briefly, and incubated overnight with the primary antibody in 3% BSA. HRP-conjugated goat anti-rabbit antibody was used as a secondary antibody. Bands were detected using chemiluminescence reagents and film as per the manufacturer’s instructions (New England Biolabs). In-gel kinase activity assay. Protein kinase activities in cell lysates fractionated by SDS-PAGE were measured as described by Wang and Erikson (60). Briefly, cells were lysed in a low-salt buffer containing 1% Triton X-100, 25 mM Tris, pH 7.5, 2 mM EGTA, 10% glycerol, 1 mM PMSF, 1 mM sodium metavanadate, 10 mM sodium fluoride, 1 µg/ml peptatin, and 1 µg/ml leupeptin. Lysates were loaded onto standard 11% SDS-polyacrylamide gels containing 250 µg/ml MBP, which was added to the gel just before polymerization. Samples were normalized for protein content before loading 50–100 µg of sample protein per well. After running, the gels were washed sequentially with 20% 2-propanol-50 mM Tris (pH 8.0), 50 mM Tris (pH 8.0)-0.05% 2-mercaptoethanol (buffer A), and 6 M guanidine hydrochloride in buffer A (two 30-min washes in each solution), followed by repeated washings in 0.04% Tween in buffer A overnight at 4°C. The phosphorylation reaction was carried out by adding 10 µl of cell lysates fractionated by SDS-PAGE and 250 µCi [γ-32P]ATP for 60 min at room temperature. The gel was then washed extensively overnight with 5% TCA-1% sodium pyrophosphate, dried, and exposed to film.

MAPK activity assays. For the J NK, ERK2, and P38 activity assays, cells were lysed in a low-salt buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 1% Triton X-100, 50 mM NaF, 10% glycerol, 1 mM sodium metavanadate, 4 µg/ml aprotinin, 20 µg/ml PMSF, 10 µg/ml leupeptin, and 1 µg/ml microcinystin, and ERK2 or P38 proteins were immunoprecipitated from 50–100 µg of cell lysate using agarose-conjugated anti-ERK2 or anti-P38 antibodies for 2 h at 4°C. The immunoprecipitates were then washed first with lysis buffer and then with a kinase buffer consisting of 20 mM HEPES (pH 8), 2 mM dithiothreitol (DTT), 100 µM EGTA, 5 mM MgCl2, 25 µM ATP, and 250 µCi [γ-32P]ATP for 60 min at room temperature. The gel was then washed extensively overnight with 5% TCA-1% sodium pyrophosphate, dried, and exposed to film.
post hoc test for multigroup comparisons. Were carried out using one-way ANOVA followed by Dunnett’s test. Molecular masses ranging from 40 to 110 kDa (Fig. 1).

Extracts from untreated BEAS cells showed a number of visible bands at varying molecular masses (Fig. 1). The strongest and most consistent increases in activity were evident as dark bands at 55–60 kDa in cells treated with As and in the range of 40–45 kDa in cells treated with V, Zn, and PMA. Less intense activation was seen in the 40- to 45-kDa band in cells exposed to As, Cr III, Cr VI, Cu, and Ni. Exposure to Fe appeared to have no effect on kinase activity relative to control levels (Fig. 1).

To survey the effect of acute metal exposure on kinase activity in BEAS cells, we used an in-gel activity assay to measure kinase activities in BEAS cells exposed to vehicle or 500 µM As, Cr III, Cr VI, Cu, V, or Zn. Cell extracts from untreated BEAS cells showed a number of bands corresponding to protein kinase activities of molecular masses ranging from 40 to 110 kDa (Fig. 1). Relative to unstimulated cells, treatment of BEAS cells with As, Cr, Cr, Cu, V, or Zn or with 100 nM PMA for 20 min resulted in a differential activation of kinases of varying molecular masses (Fig. 1). The strongest and most consistent increases in activity were evident as dark bands at 55–60 kDa in cells treated with As and in the range of 40–45 kDa in cells treated with V, Zn, and PMA. Less intense activation was seen in the 40- to 45-kDa band in cells exposed to As, Cr III, Cr VI, Cu, and Ni. Exposure to Fe appeared to have no effect on kinase activity relative to control levels (Fig. 1).

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The apparent molecular masses of the kinases activated by metal treatment of BEAS were consistent with that of the ERK1/2 (42–44 kDa), JNK (55 kDa), and, possibly, P38 (38 kDa) MAPKs. To determine whether exposure to metals ions can induce the activation of MAPKs, protein extracts from BEAS cells treated with 500 µM As, Cr III, Cr VI, Cu, Fe, Ni, V, or Zn ions or 100 nM PMA were subjected to SDS-PAGE followed by Western blotting using phosphospecific anti-ERK1/2, anti-JNK, and anti-P38 antibodies. As suggested by the in-gel kinase activity results, As, Cr III, Cr VI, V, Zn, and PMA induced phosphorylation of the MAPKs ERK2, JNK, and P38 (Fig. 2). Relative to the other metals, V was a potent activator of all three MAPKs. As, Cr III, Cr VI, Cu, and Zn were roughly equipotent in inducing phosphorylation of ERK2 (Fig. 2A). Because the phosphospecific antibody used for the ERK1/2 blot preferentially recognizes phospho-ERK2, an assessment of the phosphorylation of ERK2 (Fig. 2A). Because the phosphospecific antibody used for the ERK1/2 blot preferentially recognizes phospho-ERK2, an assessment of the phosphorylation of ERK2 was not possible. Zn appeared equivalent to V as a stimulus of JNK phosphorylation, whereas As, Cr III, Cr VI, Cu, and Ni had weaker effects (Fig. 2B). In the case of P38,
As, Cr III, Cr VI, and Zn were approximately as effective as V in inducing strong phosphorylation relative to unexposed cells (Fig. 2C). As predicted by the in-gel kinase data, treatment with Fe did not result in phosphorylation of MAPKs in BEAS cells (Fig. 2). Exposure to Ni produced generally weak and variable effects on MAPK phosphorylation (Fig. 2). PMA, used as a positive control in these studies, produced the expected stimulation of MAPK phosphorylation in BEAS cells (Fig. 2). Because Cr III and Cr VI appeared roughly equivalent in potency (Figs. 1 and 2), only Cr III, the predominant species in ambient air (24), was used in subsequent experiments.

To obtain a direct measure of the effect of the active metals on MAPK activities in bronchial epithelial cells, we measured the effect of BEAS cell exposure to 500 µM As, Cr, Cu, V, or Zn on immunoprecipitated ERK2, JNK, and P38 activities using exogenous substrates. As shown in Fig. 3 and in agreement with the in-gel kinase and Western blot data (Figs. 1 and 2), V was a potent activator of ERK2, JNK, and P38 activities (17.4 ± 3.8-, 9.1 ± 4.2-, and 3.3 ± 0.6-fold over controls, respectively). Similarly, As was an effective stimulus that induced the activation of all three MAPKs while producing the strongest effects on JNK (16.5 ± 4.1-fold increase) and P38 activities (3.9 ± 0.3-fold increase; Fig. 3). Exposure to Zn induced a clear increase in ERK2 activity (7.6 ± 3.3-fold) and a modest elevation in P38 activity (1.7 ± 0.3-fold; Fig. 3, A and C). In contradiction with the strong phosphorylation of JNK observed in the Western blots (Fig. 2B), the effect of Zn on JNK activity was only a modest (2.4 ± 1.1-fold) increase compared with the strong activation induced by As and V (Fig. 3B). As suggested by the in-gel kinase and Western blot findings (Figs. 1 and 2), treatment of BEAS cells with Cr or Cu induced minor activation of ERK2 and P38 relative to the effects induced by V exposure (Fig. 3).

To assess the potential effect of MAPK-mediated signaling on gene expression in human bronchial epithelial cells exposed to metals, we next determined the effect of treatment with As, Cr, Cu, V, or Zn on the activation of transcription factors in BEAS cells. Western blot analyses using phosphospecific antibodies showed that exposure to 500 µM As, Cr, Cu, V, or Zn induced phosphorylation of the transcription factors ATF-2 and c-jun in BEAS cells (Fig. 4). In keeping with the pattern established by the Western blots and activity assays, As, V, and Zn were the most effective in activating ATF-2 and c-jun, with smaller increases observed in BEAS cells treated with Cr and Cu (Fig. 4). Phosphorylation of the transcription factor Elk-1, a substrate of ERK, was not detectable in control or metal-stimulated BEAS cell protein extracts (data not shown).

To obtain a functional correlate for the acute metal-induced activation of MAPK and transcription factors, the effect of a transient (20-min) exposure to metals on IL-8 expression in BEAS cells was examined. BEAS cells were exposed to 500 µM vehicle, As, Cr, Cu, V, or Zn for 20 min, the cells were then washed, fresh medium was added, and the release of IL-8 into the medium was measured 6 and 24 h after stimulation. As shown in Fig. 5, the same exposure to As, V, or Zn that resulted in MAPK and transcription factor activation induced enhanced IL-8 expression in BEAS cells. This was evidenced by significant increases in IL-8 protein release detected at 6 h, which became significantly more pronounced 24 h after exposure to As, V, or Zn (Fig. 5). Although statistically significant, the effect of As on IL-8 protein synthesis was smaller (2.9 ± 0.87- and 2.3 ± 0.50-fold increase at 6 and 24 h, respectively) compared with that induced by V (7.4 ± 5.3- and 5.4 ± 3.6-fold) and Zn (10.2 ± 4.1- and 13.6 ± 7.2-fold).
DISCUSSION

These studies demonstrate that acute exposure to metals commonly found as ambient air contaminants can induce a rapid activation of three distinct MAPKs, result in the phosphorylation of MAPK-dependent transcription factors, and induce IL-8 expression in the human bronchial epithelial cell line BEAS. Specifically, our findings reveal a consistent pattern in which treatment of BEAS cells with V, As, or Zn was most effective in inducing pronounced MAPK activation, phosphorylation of ATF-2 and c-Jun, and elevated IL-8 release, whereas exposure to Cr and Cu produced generally weaker effects.

Because MAPK activity requires dual phosphorylation, the activation of ERK, JNK, and P38 by certain metals is evidence of phosphorylation of both Tyr and Ser/Thr residues in BEAS cells and thus implicates activation of the dual-specificity MAPK kinases located upstream of the MAPKs in the cascade (53, 55). Therefore, phosphorylation of ERK, JNK, and P38 may be secondary to the activation of their respective MAPK kinases, which, in turn, could imply activation of the next level of kinases, the MAPK kinase kinases. Moreover, the activation of the three MAPKs with consistent relative potency by each metal ion might suggest that the phosphorylation of ERK, JNK, and P38 was induced at a common point in the MAPK cascade. However, no single kinase that can phosphorylate ERK, JNK, and P38 has been described.

A second possible mechanism that may be responsible for metal-induced activation of MAPK in BEAS cells is inhibition of phosphatase activity. Pentavalent and tetravalent V ions are potent inhibitors of protein Tyr phosphatase activity (18) and have previously been shown to activate MAPKs in a variety of cell types (21, 41, 68). Moreover, we have previously shown that treatment of BEAS cells with a V-containing metallic mixture rapidly induces a persistent accumulation of protein Tyr phosphates through a mechanism that involves protein Tyr phosphatase inhibition (50). Similarly, trivalent As reportedly activates JNK and P38 in HeLa cells by inhibiting a dual-specificity Thr/Tyr phosphatase (5), and Zn has been shown to inhibit the receptor Tyr phosphatase HPTP beta (62). Thus the fact that the metals As, V, and Zn, which we report as the most potent activators of MAPKs, are known phosphatase inhibitors suggests that disruption of Tyr phosphate and, possibly, Ser/Thr phosphate homeostasis is a pivotal initiating event in metal-induced activation of MAPKs. Under such a scenario, basal levels of upstream kinase activity, when unopposed by phosphatase activity, would be sufficient to produce an accumulation of MAPK phosphorylation and thereby effect their activation. Additional work will be required to identify specific Tyr, Ser/Thr, or dual-specificity phosphatases in which inactivation increases MAPK activity in bronchial epithelial cells exposed to metals.

The metals Cu and Cr are not known to be phosphatase inhibitors. However, Cu is a transition metal capable of supporting redox cycling and generating reactive oxygen species such as $\text{H}_2\text{O}_2$ (22), which is a potent Tyr phosphatase inhibitor (54) and activator of MAPK (14). Similarly, hexavalent Cr is a potent oxidant and has been shown to activate ERK activity in
The activation of the distinct MAPKs ERK, J NK, and P38 in human bronchial epithelial cells exposed to metals may result in cellular responses such as growth, proliferation, apoptosis, and modulated inflammatory protein expression (30, 33). The expression of the cytokines IL-6, IL-8, and TNF-α has been shown to be regulated through signaling pathways that involve MAPKs (2, 25, 51) and activation of the transcription factors ATF-2 and c-jun (1, 6, 10, 37, 58, 59). Most strikingly, in the present study, the same brief exposure to As, V, or Zn that induces MAPK activation and transcription factor phosphorylation was sufficient to effect a subsequent increase in IL-8 expression in BEAS cells. This finding may have profound implications concerning the effectiveness of clearance mechanisms in protecting against metal-induced lung injury. Specifically, the finding that short-term exposure to metals, as might occur with metals leaching from a metal-laden particle deposited in the airway, can trigger subsequent IL-8 expression suggests that particle clearance may not occur sufficiently rapidly to prevent an inflammatory focus from developing.

Although no mechanistic correlation between them was established in this study, the temporal association between MAPK activation, transcription factor phosphorylation, and subsequent cytokine expression provides a strong circumstantial link between these events and suggests a mechanism for enhanced expression of proteins capable of mediating inflammatory pulmonary responses to inhaled metallic compounds in the airway.
METAL-INDUCED ACTIVATION OF MAPKs


