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Role of Ras in metal-induced EGF receptor signaling and NF-κB activation in human airway epithelial cells

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Wu, Weidong, Ilona Jaspers, Wenli Zhang, Lee M. Graves, and James M. Samet. Role of Ras in metal-induced EGF receptor signaling and NF-κB activation in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 282: L1040–L1048, 2002; 10.1152/ajplung.00390.2001.—We showed previously that epithelial growth factor (EGF) receptor (EGFR) signaling is triggered by metallic compounds associated with ambient air particles. Specifically, we demonstrated that As, Zn, and V activated the EGFR tyrosine kinase and the downstream kinases MEK1/2 and ERK1/2. In this study, we examined the role of Ras in EGFR signaling and the nuclear factor-κB (NF-κB) activation pathway and the possible interaction between these two signaling pathways in a human airway epithelial cell line (BEAS-2B) exposed to As, V, or Zn ions. Each metal significantly increased Ras activity, and this effect was inhibited by the EGFR tyrosine kinase activity inhibitor PD-153035. Adenoviral-mediated overexpression of a dominant-negative mutant form of Ras(N17) significantly blocked MEK1/2 or ERK1/2 phosphorylation in As-, Zn-, or V-exposed BEAS-2B cells but caused little inhibition of V-, Zn- or EGF-induced EGFR tyrosine phosphorylation. This confirmed Ras as an important intermediate effector in EGFR signaling. Interestingly, V, but not As, Zn, or EGF, induced IκBα serine phosphorylation, IκBα breakdown, and NF-κB DNA binding. Moreover, PD-153035 and overexpression of Ras(N17) each significantly blocked V-induced IκBα breakdown and NF-κB activation, while inhibition of MEK activity with PD-98059 failed to do so. In summary, exposure to As, Zn, and V initiated EGFR signaling and Ras-dependent activation of MEK1/2 and ERK1/2, but only V induced Ras-dependent NF-κB nuclear translocation. EGFR signaling appears to cross talk with NF-κB signaling at the level of Ras, but additional signals appear necessary for NF-κB activation. Together, these data suggest that, in V-treated BEAS-2B cells, Ras-dependent signaling is essential, but not sufficient, for activation of NF-κB.

G proteins; epidermal growth factor receptor; nuclear factor-κB; mitogen-activated protein kinases; air pollution

THE RAS PROTEINS are the 21-kDa products of the Ras family of genes, which were first identified in retroviruses that trigger sarcoma-type tumors in rats (37). The importance of Ras proteins in growth regulation was recognized in the early 1980s, when it was discovered that ~30% of all human tumors contain an activating mutation in Ras (69). Ras activity is regulated through a GDP-GTP binding cycle, in which guanine nucleotide exchange factors (GEFs) promote the formation of active, GTP-bound Ras, while GTPase-activating proteins accelerate the formation of inactive, GDP-bound Ras (8). Increasing evidence has shown that Ras proteins act as a crucial switching station in intracellular signaling networks that regulate cell growth and differentiation. Ras activation is initiated by activated receptors with intrinsic or associated tyrosine kinase activity. Ras can also be activated through G protein-coupled receptors. Once activated, Ras proteins transmit signals to multiple downstream effectors, which mediate gene expression, remodeling of actin cytoskeleton, cell proliferation, survival, and transformation (69).

A major intracellular signaling pathway mediated by Ras is that initiated by the epidermal growth factor (EGF) receptor (EGFR). Altered regulation of EGFR signaling can lead to pleiotropic cellular responses, including mitogenesis or apoptosis, proliferation, oncogenic transformation, enhanced motility, protein secretion, and differentiation or dedifferentiation (62, 71). Generally, EGFR signaling begins with ligand binding, which leads to dimerization of EGFR monomers, activation of EGFR tyrosine kinase activity, autophosphorylation of EGFR tyrosine residues, and phosphorylation of downstream effectors. The best understood effector pathway is activation of Ras by the recruitment of son-of-sevenless (SOS) to the cell membrane via the adapter proteins Shc and/or Grb2. GTP-bound Ras is capable of interacting with downstream effectors such as the kinase Raf, which ultimately leads to the activation of mitogen-activated protein kinase (MAPK) and the corresponding cellular responses mentioned above (25). Our previous studies demonstrated that certain metals (As, Cu, V, or Zn) are able to activate the EGFR tyrosine kinase, MAPK kinase (MEK), and

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MAPK [extracellular signal-related kinase (ERK)] in human airway epithelial cells (58, 74). In preliminary studies, overexpression of dominant-negative Ras(N17) was observed to suppress the expression of interleukin (IL)-8 , IL-6, and cyclooxygenase-2 genes in BEAS-2B cells exposed to sodium metavanadate (+5) or vanadyl sulfate (+4) (unpublished observation). Because GTP-Ras is the putative activator of Raf-1 in the presence of certain cofactors (25), we have examined the role of active Ras in EGFR signaling induced by these metals.

As with signaling of Ras, nuclear factor-κB (NF-κB) has been found to play an important role in metal-induced gene expression of proinflammatory cytokines such as IL-6 and IL-8 in human airway epithelial cells (34, 51). NF-κB is a mammalian transcriptional activator that mediates the inducible expression of a wide variety of genes, including products involved in proinflammatory responses (15, 63). In most unstimulated cells, NF-κB exists in the cytoplasm mainly as a heterodimer composed of two subunits of 50 and 65 kDa and stays in an inactive form associated with inhibitory proteins termed IκBs, with IκBα being the predominant form (4, 50, 52, 72). NF-κB activation is induced by a variety of extracellular stimuli, which activate signal transduction pathways that target the NF-κB-IκB complex for disruption (28, 50). Degradation of IκB by the proteasome allows NF-κB to translocate into the nucleus, where it binds to specific response elements on the promoter region of certain genes and regulates their transcription (5, 28, 46). Reactive oxygen species (ROS) have been shown to activate NF-κB in rat lung epithelial cells in a Ras-dependent manner (32).

In this study, we explored the role of Ras in EGFR signaling and activation of NF-κB and the possible interaction between these two signaling pathways during exposure of human airway epithelial cells to the combustion-derived metals As, V, and Zn. We observed that all three metals significantly increased Ras activity and that Ras proved to be a crucial intermediate in signaling from the EGFR tyrosine kinase to the downstream kinases MEK1/2 and ERK1/2. Interestingly, of these three metals, only V caused IκBα degradation and NF-κB DNA binding, which was significantly suppressed by the EGFR tyrosine kinase activity inhibitor PD-153035 and overexpression of dominant-negative Ras(N17) (11). However, inhibition of MEK activity with PD-98059 failed to inhibit V-induced IκBα degradation and NF-κB activation, suggesting that V-induced activation of NF-κB is EGFR tyrosine kinase and Ras dependent, but MEK independent.

MATERIALS AND METHODS

Materials. American Chemical Society-grade metal salts were obtained from Alfa (Ward Hill, MA) or Sigma (St. Louis, MO); tissue culture medium, supplements, and supplies from Clonetics (San Diego, CA); SDS-PAGE supplies, such as molecular-mass standards, polyacrylamide, ready gels, and buffers, from Bio-Rad (Richmond, CA); [γ-^{32}P]ATP (6,000 Ci/mmol) from Amersham Pharmacia Biotech; specific anti-phospho MEK1/2 (Ser$^{217/221}$) and anti-IκBα (Ser^{32}) antibody from New England Biolabs (Boston, MA); protein A-Sepharose from Pharmacia Biotech (Uppsala, Sweden); specific anti-phospho ERK (Tyr^{204}) antibody, anti-IκBα antibody, agarose-conjugated anti-EGFR antibody, horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody p-Tyr HRP antibody from Santa Cruz Biotechnology (Santa Cruz, CA); and MEK activity inhibitor PD-98059 (3), EGFR kinase inhibitor PD-153035 (27, 29), and proteasome inhibitor MG-132 (17) from Calbiochem-Novabiochem (La Jolla, CA).

Cell culture and exposure. BEAS-2B (subclone S6) cells were obtained from Drs. Curtis Harris and John Lechner (National Institutes of Health). The BEAS-2B cell line was derived by transforming human bronchial cells with an adenovirus 12-simian virus 40 construct (53). BEAS-2B cells (*passages 70–80*) were grown to 90–100% confluence on tissue culture-treated Costar 6- or 12-well plates in keratinocyte basal medium (KBM) supplemented with 30 μg/ml bovine pituitary extract, 5 ng/ml human EGF, 500 ng/ml hydrocortisone, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, and 5 ng/ml insulin, as described previously (18, 49). Cells were placed in KBM (without supplements) for 20–22 h before treatment with metals. In some experiments, BEAS-2B cells were pretreated with 1 μM PD-153035 for 2 h or 20 μM MG-132 for 30 min before metal exposure.

A suspension of 100 mM sodium arsenite, vanadyl sulfate, and zinc sulfate was prepared in water and used as a stock for dilution into KBM, as described previously (58).

Western blotting. BEAS-2B cells treated with metals were lysed in RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS in PBS, pH 7.4) containing 0.1 mM vanadyl sulfate and protease inhibitors (0.5 mg/ml aprotinin, 0.5 mg/ml E-64, 0.5 mg/ml pepstatin, 0.5 mg/ml bestatin, 10 mg/ml chymostatin, and 0.1 mg/ml leupeptin) (58). After normalization for protein content, cell extracts were subjected to SDS-PAGE on 11% gradient polyacrylamide gels or 4–15% Tris·HCl ready gels (Bio-Rad) with a Tris-glycine-SDS buffer. Proteins were electroblotted onto a nitrocellulose membrane. The blots were blocked with 3% nonfat milk, washed briefly, incubated with phospho-specific MEK1/2, ERK1/2, or IκBα antibodies in 3% BSA at 4°C overnight, and then incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Bands were detected using chemiluminescence reagents and hypersensitive films, as described previously (58).

Immunoprecipitation. Confluent BEAS-2B cells were challenged with metals and lysed with RIPA buffer. The lysate supernatants were precleared with protein A-Sepharose and immunoprecipitated by incubation with agarose-conjugated anti-EGFR antibody for 1 h at 4°C. Immune complexes were washed twice with RIPA buffer and once with cold PBS. The immunoprecipitates were suspended with 25 μl of 4°C sample loading buffer (62.5 mM Tris·HCl, pH 6.8, 10% glycerol, 2% SDS, 0.7 M β-mercaptoethanol, and 0.05% bromphenol blue) and boiled for 5 min before separation on 4–15% Tris·HCl ready gels. The EGFR tyrosine phosphorylation bands were detected as described previously (74).

Ras activation assay. The Ras activation assay kit was purchased from Upstate Biotechnology (Lake Placid, NY). Ras activity was determined according to the supplier's instruction. Briefly, BEAS-2B cells treated with metals were lysed with 5× Mg^{2+} lysis/wash buffer (MLB: 125 mM HEPES, pH 7.5, 750 mM NaCl, 5% Igepal CA-630, 50 mM $MgCl_2$, 5 mM EDTA, and 10% glycerol). The lysates were precleared with glutathione agarose. Five microliters of a 50% slurry of Raf-1 Ras binding domain-agarose were incubated with 500–1,000 μg of cell lysate at 4°C for 30 min. The

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agarose was collected by centrifugation and washed with MLB three times and once with cold PBS and then boiled in 25 μl of reducing sample loading buffer. GTP-bound Ras protein was resolved by electrophoresis, transferred to nitrocellulose, and probed with a mouse monoclonal anti-Ras (clone Ras-10) antibody (1 μg/ml). Protein bands were visualized using a goat anti-mouse secondary antibody conjugated to HRP and a enhanced chemiluminescence detection system (74).

Infection with adenovirus. Ras(N17), a GDP-bound dominant-negative mutant, is used widely as an interfering mutant to assess Ras function in vivo (11, 66). Confluent BEAS-2B cells were infected with a multiplicity of infection of 100 plaque-forming units/cell nonrecombinant control adenovirus ad5CMV3 or recombinant inactive Ras(N17) (obtained from Dr. Craig Logsdon, University of Michigan) for 24 h (48). The infection medium was removed and replaced with KBM for another 24 h before challenge with metals. BEAS-2B cells were lysed, and the lysates were subjected to Western blotting or electrophoretic mobility shift assay (EMSA).

Separation of cytoplasmic and nuclear proteins. BEAS-2B cells treated with metals were lysed with cytoplasmic extract buffer [10 mM Tris·HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT)] containing protease inhibitors (34). Nonidet P-40 (0.1%) was added to the cell lysate, and the lysate was vortexed for 15 s. After centrifugation at 14,000 rpm for 30 s, the supernatant (cytoplasmic fraction) was transferred to another tube and boiled with sample loading buffer to detect the degradation of IκBα through Western blotting using rabbit κBα antibody (Santa Cruz Biotechnology). The nuclear pellets were resuspended with 100 μl of nuclear extract buffer (20 mM Tris·HCl, pH 8.0, 400 mM NaCl, 1.5 mM $MgCl_2$, 1.5 mM EDTA, 25% glycerol, and 1 mM DTT) containing protease inhibitors. After brief centrifugation, the supernatants containing the nuclear fraction were subjected to EMSA.

EMSA. EMSA was conducted as described previously (34). Briefly, an oligonucleotide probe (sequence from 5′ to 3′: GGCTGGGGATTCCCCATCT) for NF-κB binding site on myosin heavy chain class II gene was labeled by incubating 15 U T4 polynucleotide kinase (New England Biolabs, Beverly, MA), 100 ng double-stranded probe, and 100 μCi [γ-^{32}P]ATP at 37°C for 30 min. Unincorporated ^{32}P was removed using a desalting column (Nuc Trap, Stratagene, San Diego, CA), and DNA-protein binding reactions were performed for 10 min at room temperature in a mixture containing 2 μg nuclear extract, 1 μl labeled probe, 10 μl running buffer (10 mM Tris·HCl, pH 7.5, 50 mM NaCl, 2 mM EDTA, 1 mM DTT, and 5% glycerol), and 2 μg poly(dI-dC) (Roche Molecular Biochemicals). Samples were separated by electrophoresis through 4.5% nondenaturing polyacrylamide gels containing 0.5× Tris-borate-EDTA. Gels were dried, and radiolabeled species were autoradiographed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Activation of Ras in BEAS-2B cells exposed to metals. Ras has been reported to mediate its effects in part through activation of a cascade of kinases: Raf (c-Raf-1, A-Raf, and B-Raf), MEK (MAPK/ERK kinases 1 and 2), and ERK1/2 (21, 22). We previously showed that exposure to As, V, and Zn ions activates the MAPK cascade (MEK/ERK) in BEAS-2B cells (74). We therefore examined whether Ras plays a role in metal-induced activation of the MAPK cascade in BEAS-2B cells.

BEAS-2B cells grown to confluence were deprived of growth factors for 20–22 h and then challenged with 100 μM As, V, or Zn for 1 h. Ras activity was determined using a commercially available Ras activation assay kit, which is based on affinity precipitation of GTP-bound Ras with agarose-conjugated Raf-1-glutathione *S*-transferase. Western blotting using specific anti-Ras antibody showed that As, V, or Zn exposure induced a marked increase in levels of GTP-bound Ras compared with controls. As expected, EGF strongly activated Ras in BEAS-2B cells (Fig. 1).

Ras mediates metal-induced signaling from EGFR tyrosine kinase to MEK1/2. To determine whether Ras activation by metal exposure is dependent on EGFR signaling, we pretreated BEAS-2B cells for 2 h with vehicle alone or 1 μM PD-153035, a specific EGFR tyrosine kinase inhibitor (40), before exposure to As, V, or Zn and measured levels of GTP-bound Ras. As, V, Zn, and EGF each induced an increase in the active GTP-bound Ras formation in BEAS-2B cells (Fig. 2). PD-150335 markedly suppressed Ras activation in BEAS-2B cells exposed to As, V, or Zn, with no detectable change in Ras protein expression. PD-153035 also significantly blocked EGF-induced Ras activation. This implicated EGFR tyrosine kinase activity as a requirement for metal-induced Ras activation in BEAS-2B cells.

Our previous work suggested that EGFR tyrosine kinase is necessary for metal-induced MEK/ERK activation in BEAS-2B cells (74). Therefore, we next determined whether active Ras is a necessary intermediate in the transduction of signals initiated by exposure to As, V, or Zn through the EGFR and leading downstream to MEK/ERK. With the use of an adenoviral vector for dominant-negative Ras(N17), we were able

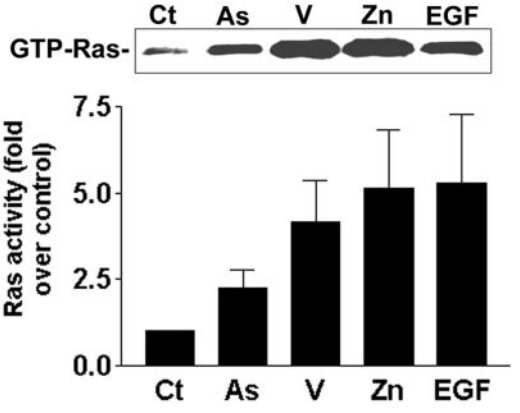

Fig. 1. Ras activation in BEAS-2B cells exposed to As, V, or Zn. Confluent BEAS-2B cells were depleted of growth factors for 24 h before stimulation with 100 μM As, Cu, V, or Zn for 60 min [epidermal growth factor (EGF) at 100 ng/ml was used as a positive control (Ct)] and lysed. Cell lysates were immunoprecipitated with Raf-1 Ras binding domain-conjugated agarose. Immunoprecipitates were subjected to 4–15% Tris·HCl ready gels. Membranes were blotted with anti-Ras $IgG2_{ak}$ overnight and then incubated with anti-mouse horseradish peroxidase-conjugated rabbit IgG. Results represent a summary of 3 separate experiments.

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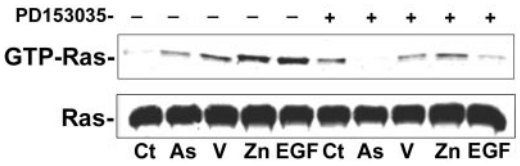

Fig. 2. The EGF receptor (EGFR) tyrosine kinase inhibitor PD-153035 blocked Ras activation in BEAS-2B cells exposed to As, V, or Zn. BEAS-2B cells pretreated with 1 μM PD-153035 for 2 h were challenged with metals at 100 μM for 60 min and lysed. Cell lysates were subjected to immunoprecipitation using Raf-1 Ras binding domain-conjugated agarose for detection of active Ras and 10% SDS-PAGE for detection of total Ras protein. GTP-bound Ras was detected as described in MATERIALS AND METHODS. Autoradiograph is representative of 3 separate experiments.

to determine the relationship between Ras and the MAPK cascade in these cells. To ensure that overexpression of recombinant inactive Ras(N17) takes place in infected BEAS-2B cells, the cells were infected with different multiplicities of infection of nonrecombinant control adenovirus ad5CMV3 or recombinant inactive Ras(N17). Expression of Ras protein was determined using a specific anti-Ras antibody. Similar to a previous report (48), Ras protein was overexpressed in BEAS-2B cells infected with adenovirus encoding a dominant-negative mutated form of Ras [Ras(N17)] compared with the cells infected with control adenovirus (Fig. 3*A*). Infected BEAS-2B cells were treated with 100 μM As, V, or Zn for 1 h. Phosphorylation of MEK1/2 or ERK1/2 was detected using phospho-specific MEK1/2 or ERK1/2 antibodies. Consistent with our previous reports (58, 74), Western blotting results showed that As, V, or Zn, as well as EGF, the potent inducer of MAPK phosphorylation, induced significant increases in MEK1/2 and ERK1/2 phosphorylation in BEAS-2B cells infected with the nonrecombinant control vector (Fig. 3, *B* and *C*). In contrast, in cells overexpressing Ras(N17), the phosphorylation of MEK1/2 and ERK1/2 induced by As, V, or Zn was significantly blocked. This was true of EGF-induced MEK1/2 and ERK1/2 phosphorylation as well. These data suggested that active Ras plays a pivotal role in metal-induced MEK/ERK activation in BEAS-2B cells.

Ras is believed to be downstream of the EGFR; therefore, Ras inhibition should have no inhibitory effect on metal- or EGF-induced EGFR tyrosine phosphorylation. To determine the specificity of the Ras(N17) blockage of MEK/ERK phosphorylation in BEAS-2B cells and whether active Ras is an important transducer from the EGFR to the MAPK cascade, the effect of Ras(N17) overexpression on EGFR tyrosine kinase phosphorylation was measured in cells treated with As, V, Zn, or EGF. As shown in Fig. 3*D*, As, V, and Zn ions or recombinant EGF induced pronounced phosphorylation of EGFR tyrosine kinase, which was not affected by Ras(N17) overexpression.

Ras(N17) blocked V-induced NF-κB DNA binding activity and IκBα degradation. Because previous studies showed that some metal ions can activate NF-κB, we next studied the role of Ras in NF-κB DNA binding activity in cells exposed to As, V, or Zn ions. The possible role of active Ras in metal-induced NF-κB DNA binding activity was tested in this study. BEAS-2B cells were infected with control or Ras(N17) adenovirus and challenged with metals. The cells were lysed, and nuclear proteins were extracted for EMSA. Interestingly, among As, V, and Zn, only V induced NF-κB DNA binding in BEAS-2B cells, while As, Zn, or EGF treatment did not induce an increase of NF-κB DNA binding activity relative to the vehicle control (Fig. 4*A*), which is consistent with our previous observations in primary human bronchial cells (34). Moreover, V-induced NF-κB DNA binding activity was suppressed by the Ras(N17) overexpression (Fig. 4*A*). The positive control tumor necrosis factor significantly enhanced NF-κB DNA binding activity (data not shown). Similar to our previous observations in primary human airway epithelial cells (33), supershift and lysis using specific antibodies against the p50, p65, and c-Rel compounds of the NF-κB binding complex identified the V-induced NF-κB DNA binding complex in BEAS-2B cells as the p65/p50 heterodimer (Fig. 4*B*). This suggested that Ras is required for V-induced NF-κB nuclear translocation in BEAS-2B cells.

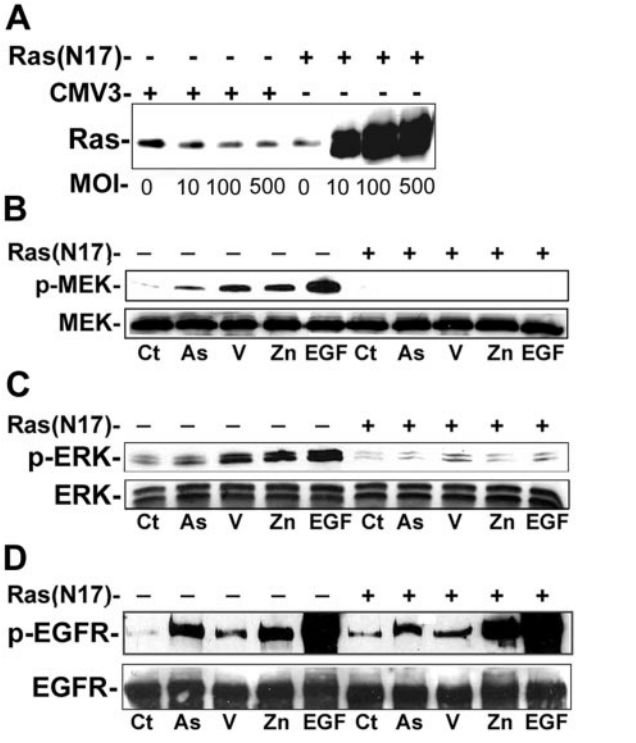

Fig. 3. Overexpression of dominant-negative Ras(N17) inhibited MEK and ERK, but not EGFR, tyrosine phosphorylation in BEAS cells exposed to As, V, or Zn. *A*: dose-dependent overexpression of Ras(N17) in BEAS-2B cells. BEAS-2B cells infected with 100 plaque-forming units/cell of Ras(N17) adenovirus were treated with 100 μM As, V, or Zn for 60 min (EGF at 100 ng/ml was used as a positive control). Cell lysates or immunoprecipitates were subjected to 4–15% Tris·HCl ready gels. Phospho MEK (p-MEK, *B*), ERK (p-ERK, *C*), or EGFR tyrosine (p-EGFR, *D*) was probed with corresponding phospho-specific antibodies, and then the membranes were stripped and blotted with anti-MEK, anti-ERK, or anti-EGFR antibodies, respectively. Autoradiographs are representative of 3 separate experiments.

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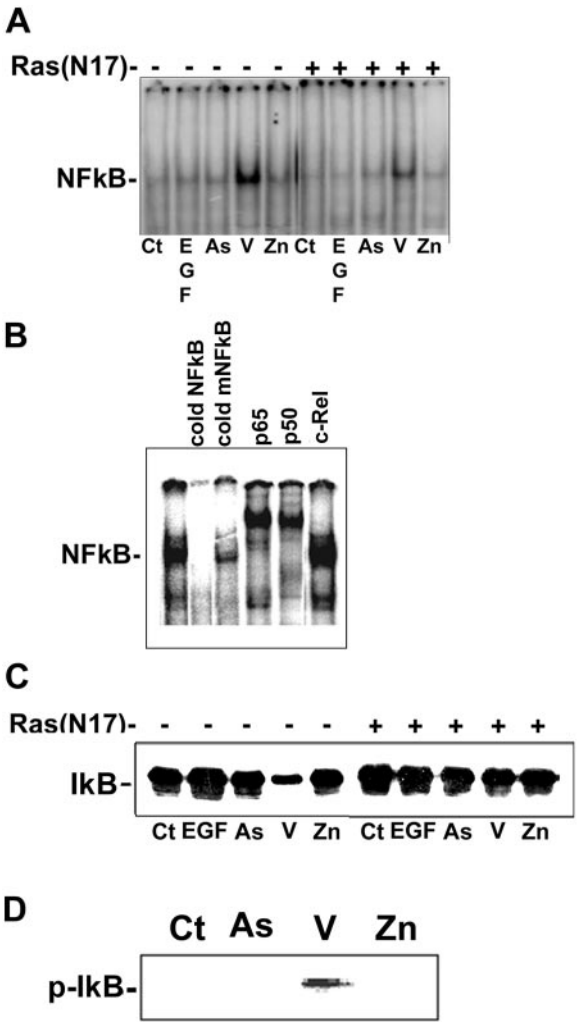

Fig. 4. Overexpression of dominant-negative Ras(N17) blocked nuclear factor-κB (NF-κB) DNA binding and IκB breakdown in BEAS-2B cells exposed to As, V, or Zn. BEAS cells infected with Ras(N17) adenovirus were treated with 100 μM As, V, or Zn for 60 min (EGF at 100 ng/ml was used as a positive control). *A*: nuclear proteins were analyzed for NF-κB DNA binding using electrophoretic mobility shift assay (EMSA). *B*: V-induced NF-κB DNA-binding complex was further identified by addition of 100-fold excess unlabeled wild-type and mutated NF-κB oligonucleotides and antibodies against p65, p50, and c-Rel. *C*: cytoplasmic proteins were analyzed for IκB breakdown by Western blotting. *D*: BEAS-2B cells were pretreated with MG-132 for 30 min and then stimulated with 100 μM As, V, or Zn for 60 min. Cytoplasmic protein fraction was separated by SDS-PAGE. Phosphorylated form of IκBα (p-IκB) at Ser^{32} was detected with phospho-specific antibody. Autoradiographs are representative of 3 separate experiments.

IκBα degradation is an event that precedes NF-κB nuclear translocation (46, 47). To confirm the above observation on NF-κB activation, IκBα levels were determined in BEAS-2B cells exposed to V. Western blotting using an anti-IκBα antibody showed only V-induced IκBα degradation in BEAS-2B cells, with no effect in cells exposed to As, Zn, or EGF. In addition, Ras(N17) overexpression significantly blocked V-induced IκBα degradation (Fig. 4*C*). The IκBα degradation process consists of a series of well-characterized steps (18). Inducible phosphorylation of IκB at Ser^{32} or Ser^{36} leads to the immediate recognition of the NH_2 terminus of IκBα by an E3 ubiquitin-protein ligase complex (39, 43, 75), which results in the polyubiquitinylation of IκB, leading to rapid degradation of IκBα by the 26S proteasome (17, 61). BEAS-2B cells were pretreated with the proteasome inhibitor MG-132, which prevents IκBα degradation via the 26S proteasome and, thereby, permits the accumulation of phosphorylated IκBα in stimulated cells (17). Pretreated cells were stimulated with As, V, or Zn, and the cytoplasmic protein fractions were separated by SDS-PAGE and immunoblotted with a phospho-specific antibody against the Ser^{32}-phosphorylated form of IκBα. As shown in Fig. 4*D*, only V induced increased phosphorylation of IκBα in BEAS-2B cells, which was consistent with the above observation that only V significantly induces IκBα degradation and NF-κB DNA binding activity.

Effect of an EGFR tyrosine kinase inhibitor, PD-153035, or an MEK activity inhibitor, PD-98059, on V-induced NF-κB activation in BEAS-2B cells. NF-κB DNA binding activity and IκBα phosphorylation and degradation suggested that only V induced NF-κB activation in BEAS-2B cells. Moreover, Ras is necessary for V-induced NF-κB activation. The EGFR is required for V-induced Ras activation. Therefore, the EGFR tyrosine kinase activity should also be necessary in V-induced NF-κB activation. We observed that the selective EGFR tyrosine kinase inhibitor PD-153035 suppressed V-induced IκBα degradation and NF-κB DNA binding activity in BEAS-2B cells (Fig. 5, *top panels*). Next, we tested whether MEK, a downstream kinase of Ras, could also contribute to V-induced NF-κB nuclear translocation in BEAS-2B cells. Confluent BEAS-2B cells were pretreated with the MEK activity inhibitor PD-98059 and then stimulated with V. Extracted nuclear proteins were then subjected to

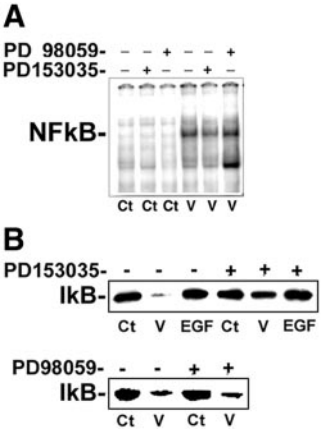

Fig. 5. EGFR tyrosine kinase activity inhibitor PD-153035, but not MEK activity inhibitor PD-98059, inhibited V-induced NF-κB DNA binding and IκBα breakdown in BEAS-2B cells. BEAS-2B cells pretreated with 1 μM PD-153035 for 2 h or 50 μM PD-98059 for 30 min were stimulated with 100 μM vanadyl sulfate or 100 ng/ml EGF for 60 min. *A*: nuclear proteins were analyzed for NF-κB DNA binding using EMSA. *B*: cytoplasmic proteins were analyzed for IκB breakdown by Western blotting using IκB antibody. Autoradiographs are representative of 3 separate experiments.

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EMSA for NF-κB activation. V induced IκBα degradation and NF-κB DNA binding. PD-98059 had no inhibitory effect on V-induced IκBα degradation and NF-κB DNA binding (Fig. 5, *bottom panels*), suggesting that MEK1/2 does not participate in V-induced NF-κB activation in BEAS-2B cells.

DISCUSSION

Ras is an important component of multiple signal transduction cascades (69). The data shown here demonstrate that combustion-derived metals such as As, V, and Zn induce Ras activation in human airway epithelial cells. Active Ras not only mediates signaling from the EGFR to the MAPK cascade in response to exposure to As, V, or Zn, but it also plays a pivotal role in V-induced NF-κB DNA binding. Although As, V, and Zn can each induce the activation of Ras, only V increases NF-κB activity, and it does so in a Ras-dependent manner, suggesting that, in BEAS-2B cells, Ras may be essential, but not sufficient, to induce NF-κB activity. Moreover, the MEK activity inhibitor PD-98059 failed to attenuate V-induced NF-κB DNA binding activity. These data suggest different mechanisms for signaling initiated by As, V, and Zn in human airway epithelial cells. The proposed mechanisms for metallic ion-induced intracellular signaling in BEAS-2B cells are depicted in Fig. 6.

V ions are known inhibitors of protein tyrosine phosphatases, including those that dephosphorylate signaling kinases (23). Zn (10, 70) and, to a lesser extent, As (14) ions are also reported inhibitors of certain tyrosine phosphatases. Part of the impetus for the work undertaken in this study is the aim to identify the initiating event(s) in metal ion-induced signaling. Our previous studies have suggested that dysregulation of protein tyrosine phosphate metabolism and inhibition of tyrosine phosphatases may be pivotal effects in this process (59, 60). We have shown that exposure to V and Zn, but not As, can cause profound inhibition of multiple tyrosine phosphatases in human airway epithelial cells (59). Thus the possibility exists that the effects of As, V, and Zn ions on signaling to NF-κB and/or MEK/ERK are secondary to an inhibitory effect on phosphatases that keeps these proteins in an inactive state in resting cells. In principle, the effect of phosphatase inhibition could occur at any or all levels in a signaling cascade. However, the data we present in this study, showing that As, V, and Zn each signal to MEK/ERK in a Ras-dependent manner, suggest that inhibition to tyrosine phosphatases, if required in the signaling induced by these metal ions, occurs upstream of Ras.

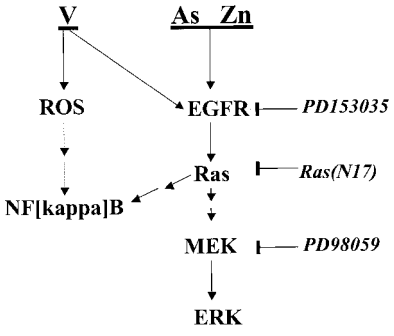

Fig. 6. Proposed mechanism for metal ion-induced EGFR signaling and NF-κB activation in BEAS-2B cells. As, V, and Zn ions each induce activation of EGFR, Ras, MEK, and ERK. Only V induces NF-κB activation, an event that bifurcates from MEK activation at the level of Ras.

An EGFR tyrosine kinase inhibitor significantly blocked metal-induced Ras activation in human airway epithelial cells, and dominant-negative Ras(N17) posed no detectable effect on EGFR tyrosine kinase activity. These data suggest that EGFR tyrosine kinase is required for Ras activation in BEAS-2B cells exposed to combustion-derived metals. The events involved in the signaling from EGFR to Ras were not examined in this study. It has been documented that Ras activation by the EGFR occurs through adapter proteins and GEFs (8). Furthermore, activation of Ras is responsible for metal-induced MEK and ERK phosphorylation in BEAS-2B cells exposed to metals. One question raised by these findings concerns the mechanism through which Ras transmits the signal from the EGFR to MEK and ERK. Our previous study demonstrated that MEK/ERK phosphorylation induced by As, V, or Zn occurred in a Raf-1-independent manner (74). Observations from mammalian cells indicate that the events downstream of Ras are more complex than simply activating the Raf kinases. Multiple Ras effectors have been recognized, such as $p120^{Ras}$ GAP, PI3KRIN1, AF6, and RalGDS (24, 30, 38, 55, 76). Additionally, MEK kinase (MEKK1), a large (196-kDa) protein, has been shown to be activated by low-molecular-weight GTP-binding proteins (Ras, Rac, and Cdc42) and activates MEK/ERK, JNK, and IκB kinases (44, 57, 78).

Interestingly, in addition to the pivotal role of Ras in the EGFR signaling in BEAS-2B cells exposed to combustion-derived metals, this study also shows that active Ras is necessary for V-induced NF-kB activation. As described previously (33), the crucial step in NF-κB activation is the signal-induced proteolytic degradation of IκB in the cytoplasm (47). IκB breakdown is initiated after the phosphorylation of its NH_2-terminal serine residues by IκB kinases such as IKK1 and IKK2 (7, 41, 47). V has been shown to induce IKK activity and increase IκBα phosphorylation and degradation and NF-κB nuclear translocation in different cell types, including human airway epithelial cells (16, 31, 33, 34). Several studies have suggested that IKK1 and IKK2 are themselves phosphorylated and activated by one or more upstream activating kinases such as MEKK1 and NF-κB-inducing kinase (6, 19, 42, 54, 67, 73, 79, 80), with NF-κB-inducing kinase displaying a preference for IKK1 and MEKK1 a preference for IKK2 (47). In addition, IKK2 activity was also found to be significantly elevated by V in mouse macrophages (16). Therefore, MEKK1, one of the downstream kinases of Ras, could be involved in V-induced IKK2 activation (16), further leading to IκBα breakdown and NF-κB

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nuclear translocation. Moreover, the MEK activity inhibitor PD-98059 had no detectable inhibitory effect on V-induced NF-κB activation in BEAS-2B cells, which implies that the upstream kinases of MEK, but not MEK itself, are responsible for V-induced NF-κB nuclear translocation. Transfection of transformed rat lung epithelial cells with a constitutively active MEKK1 construct or dominant-negative MEKK1 can significantly modulate oxidant-induced NF-κB activation (32). It is possible that MEKK1 was involved in the V-induced NF-κB activation in BEAS-2B cells. MEKK1 may therefore act as a bifurcation point in V-induced MEK/ERK activation and NF-κB nuclear translocation.

Ras-dependent NF-κB activation induced by diverse stimuli has been reported (32, 33, 40). It is intriguing that, although As, V, and Zn are able to induce Ras activation in BEAS-2B cells, only V induces NF-κB activation. In one study, Ras(N17) failed to block tumor necrosis factor-α-induced NF-κB activation in rat lung epithelial cells (32). Together, these observations suggest that active Ras is not sufficient for V-induced NF-κB DNA binding activity in BEAS-2B cells and that input from other signaling cascades or cofactors is necessary to activate NF-κB. In preliminary work, As and Zn were unable to induce oxidative stress in BEAS-2B cells. However, vanadyl sulfate (+4)- or vanadate (+5)-induced NF-κB nuclear translocation could be blocked by overexpression of catalase or the antioxidant *N*-acetyl-L-cysteine, respectively (33, 77), suggesting a crucial role for ROS in V-induced NF-κB activation. Additional evidence has shown that oxidant-induced NF-κB activation is Ras dependent in transformed rat lung epithelial cells (32). Therefore, it is possible that generation of ROS, which are induced by V, but not by As or Zn, contributes to the additional signals required for NF-κB activation (7). The mechanisms for synergism of Ras with ROS in V-induced NF-κB DNA binding in BEAS cells are not known. However, it has recently been demonstrated that the role of ROS in NF-κB activation is cell type or stimulus specific, and its role may be facilitatory, rather than causal (9).

In addition to its ability to generate ROS, other factors may also explain the effect of V on NF-κB DNA binding relative to that of As or Zn exposure. Arsenite has been reported to directly inhibit the activity of IKK and, thereby, limit the phosphorylation and degradation of IκBα (56). This is consistent with our data showing that As exposure of BEAS-2B cells does not lead to IκB degradation (34). Furthermore, As and other thiol-reactive metals, including Zn, have been found to inhibit NF-κB DNA binding through interactions with critical protein sulfhydryls (64).

In addition to Ras, members of the Ras superfamily of GTP-binding proteins such as Rho and Rac, also regulate a variety of signal transduction pathways in eukaryotic cells (20). It has recently been reported that, in certain circumstances, Rac is required for NF-κB activation (12). However, in our preliminary study, we did not observe Rac activation in BEAS-2B cells exposed to As, V, or Zn (data not shown).

Metal ions can be abundant in airborne particulate matter (PM) (1, 2, 45). It has been argued that metals contribute to PM-induced pulmonary inflammatory gene expression (13, 26, 34–36, 65, 68). The role of Ras in metal-induced EGF signaling and NF-κB activation in human airway epithelial cells may provide an important link in the elucidation of the mechanism of toxicity of PM inhalation.

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