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Vanadate Induction of NF-κB Involves IκB Kinase β and SAPK/ERK Kinase 1 in Macrophages*

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The present studies investigated the signaling pathways of vanadate, a vanadium ion with +5 oxidation state, to activate NF-κB transcription factor, a pivotal regulator of inflammatory responses. Treatment of macrophages with vanadate results in the activation of both NF-κB and c-Jun N-terminal kinase (JNK). The activity of a recently identified cellular kinase, IκB kinase-β (IKKβ), was significantly elevated concomitant with the increased degradation of IκBα and enhanced NF-κB activity in cells exposed to vanadate. To determine whether the IKK pathway and JNK pathway are interconnected or bifurcate upon vanadate stimulation, cells were transfected with either a kinase inactive form of IKKβ or a kinase inactive form of SAPK/ERK kinase 1 (SEK1). Inactive IKKβ was able to block vanadate-induced degradation of IκBα, yet it was unable to influence the activation of JNK by vanadate. Conversely, blockage of JNK activation by transfection of a kinase-inactive form of SEK1 resulted in partially inhibition of vanadate-induced IκBα degradation. Both vanadate-induced degradation of IκBα and activation of JNK were potently inhibited by pretreatment of cells with *N*-acetylcysteine or dimercaprol. These results demonstrate that early activation of stress kinases or change of cellular redox states plays a key role in vanadate-induced activation of NF-κB and JNK.

Epidemiological studies have demonstrated that the inhalation of environmental or occupational airborne particulate matter (PM)[1] results in an increased incidence of cardiopulmonary disorders and lung cancer (1–4). Yet our understanding about biological mechanisms and the initiation and progression of disease as a result of exposure to PM is still primitive and fragmentary. The genetic program that mediates cellular reaction following the inhalation of environmental or occupational airborne PM involves the transient expression of select transcription units, including those that encode interleukin-1, interleukin-2, interleukin-6, interleukin-8, tumor necrosis factor-α (TNF-α) and several cell adhesion molecules (5). Emerging evidence suggests that this process is initiated through a pre-existed genetic switch, NF-κB transcription factor, that stimulates the κB-enhancer sequences present in the promoters of genes involved in the inflammatory response and the control of cell death or transformation (6, 7). In resting cells, NF-κB is sequestered in the cytoplasm in an inactive form by a group of inhibitory proteins including IκBα, IκBβ, and IκBϵ. In response to extracellular stimuli, IκB is phosphorylated and proteolytically degraded, resulting in the release of active NF-κB that translocates to the nucleus. Both IκB kinase (IKK) and mitogen-activated protein kinase may be actively involved in signal-induced phosphorylation of IκB and subsequent activation of NF-κB (8, 9). The question, however, that still remains is whether these two kinase pathways act independently or have a mutual dependence on each other. It is also an important issue to resolve whether certain NF-κB-inducers use one of the two pathways preferentially. It is known that both NF-κB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1) can directly phosphorylate and activate IKKα and IKKβ, two components of the IKK complex. However, co-expression studies demonstrate that NK-κB-inducing kinase can phosphorylate IKKα at Ser^{176}, but weakly phosphorylates IKKβ, whereas MEKK1 is apparently more potent in the phosphorylation of IKKβ (8–10).

Chemical evidence demonstrates that most of environmental or occupational PMs contain various trace metal ions, including vanadium, chromium, arsenite, zinc, iron, and nickel (11, 12). It has been hypothesized that metal ions may contribute to the pathological effects of inhaled PM. In this report, we characterized signaling pathways of NF-κB transcription factor, a pivotal regulator of inflammatory and carcinogenic responses, activated by vanadate, a vanadium ion with +5 oxidation state abundant in PM from combustion of fossil fuels and other air pollutants. We demonstrated that both IKK and SAPK/ERK kinase 1 (SEK1), an intermediate kinase within the MEKK1 to c-Jun N-terminal kinase (JNK) cascade, are involved in vanadate-induced NF-κB activation.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The mouse macrophage cell line RAW264.7 from American Type Culture Collection (ATCC, Manassas, VA) was cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 5% fetal bovine serum. Vanadyl sulfate trihydrate (V(IV)) and sodium metavanadate (V(V)) were purchased from Aldrich (Milwaukee, WI). *N*-Acetylcysteine (NAC), dimercaprol (BAL), and electrophoresis reagents were from Sigma. The luciferase assay kit and JNK assay kit were from Promega (Madison, WI) and New England Biolabs (Beverly, MA), respectively. ECL Western blotting detection reagents were from Amersham Pharmacia Biotech. All antibodies against NF-κB family

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[1] The abbreviations used are: PM, particulate matter; IKK, IκB kinase; NF-κB, nuclear factor-κB; JNK, c-Jun N-terminal kinase; MEKK1, mitogen-activated protein kinase/ERK kinase kinase-1; SEK1, stress-activated protein kinase/ERK kinase-1; TNF, tumor necrosis factor; NAC, N-acetylcysteine; BAL, dimercaprol; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase.

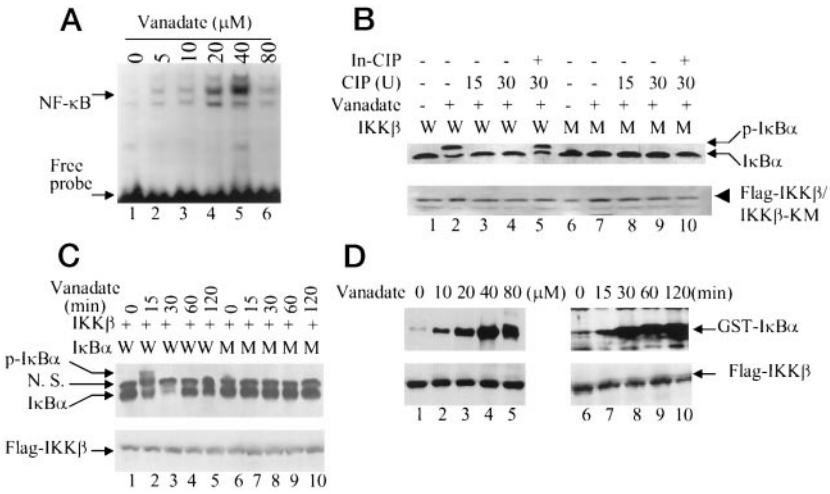

FIG. 1. **Vanadate induces IKKβ kinase activity.** *A,* mouse macrophage cell line RAW 264.7 cells were treated with 0–80 μM vanadate as indicated for 2 h. Nuclear extracts were prepared and subjected to gel shift assay with a radiolabeled double-stranded κB oligonucleotide. The *arrows* on the *left side* of the panel indicate the NF-κB binding complex and free probe, respectively. *B,* macrophages (5×10^5) were transfected with 2 μg of wild type IKKβ (*W*) or its kinase inactive mutant (*M*). After 40 h of transfection, cells were treated with 30 μM vanadate for 30 min. Cytosolic extracts were fractionated by a 12-cm long SDS-PAGE (10% gel) and subjected to immunoblot with the IκBα antibody C-21 (*upper panel*). The upper phosphorylated (*p-IκBα*) and the lower non-phosphorylated IκBα are noted with *arrows*. To verify that the *upper band* of IκBα was phosphorylation, the cytosolic extracts (30 μg) from vanadate-treated cells were preincubated with calf intestinal phosphatase (CIP) without (*lanes 3, 4, 8,* and *9*) or with a mixture of phosphatase inhibitors (*In-CIP*: 10 mM β-glycerophosphate and 10 mM sodium fluoride, *lanes 5* and *10*) at 30 °C for 30 min. The expression of transfected exogenous IKKβ or IKKβ-KM was determined by immunoblot with anti-flag antibody (*lower panel*). *C,* IKKβ-transfected macrophages (5×10^5) were co-transfected with 2 μg of wild type (*W*) IκBα or its mutant (*M*) in which Ser^{32} and Ser^{36} were substituted with alanines and subjected to phosphorylation and degradation assay of exogenous IκBα (*upper panel*). The *lower panel* shows equal expression of exogenous IKKβ. *D,* IKKβ transfected macrophages were treated with 0–80 μM vanadate for 30 min (dose response, *lanes 1–5*) or treated with 30 μM vanadate for 0–120 min (time course, *lanes 6–10*). Cell lysates were prepared and immunoprecipitated with IKKβ-specific antibody (H-470) and subjected to *in vitro* IKKβ kinase activity assay using full-length GST-IκBα as substrate (*upper panel*). Identical lysates were also subjected to immunoblot assay for the expression of IKKβ (*lower panel*).

members, IκBα, IKKα, and IKKβ, were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-flag monoclonal antibody was from Sigma. Mouse anti-phosphotyrosine monoclonal antibody 4G10 and goat anti-mouse IgG horseradish peroxidase conjugate were purchased from Upstate Biotechnology (Lake Placid, NY).

Expressing Plasmids—pCR-Flag-IKKβ, pCR-Flag-IKKβ-KM(K44A), pcDNA3-Flag-SEK1 (Ala), and pcDNA3-Flag-JNK(APF) were gifts from Hiroyasu Nakano (Juntendo University, Japan) (10), and Roger Davis (University of Massachusetts) (13), respectively. An expression vector for wild type IκBα (pCMV-IκBα) was described previously (14). To generate an expression vector for IκBα that is resistant to phosphorylation and degradation, wild type expression vector, pCMV-IκBα, was subjected to site-directed mutation using a QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA), resulting in the mutation of S32A and S36A (S32A/S36A).

Reporter Gene Activity Assay—Macrophages were plated in 6-well tissue culture plates at 5×10^5 cells/well for 2 days. The cells were transfected with indicated expression vectors using the DEAE-dextran method (15). The total amount of plasmid DNA was made constant by adding respective amounts of empty vector plasmids to transfection mixtures. Cells in each well were incubated with 1 ml of transfection mixtures for 2 h in serum-free medium. Twenty to 40 h after transfection, cells were subjected to the respective treatment as indicated under "Results." Luciferase activity was determined and normalized relative to the β-galactosidase activity.

Kinase Activity Assay—IKK activity assay was performed by the methods of Geleziunas *et al.* (16) with minor modifications. Briefly, macrophages, transfected with pCR-IKKβ, were treated with vanadate and lysed in a lysis buffer containing 1% Nonidet P-40, 250 mM NaCl, 50 mM HEPES (pH 7.4), 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, aprotinin (10 μg/ml), and leupeptin (10 μg/ml). After centrifugation of the lysate at 16,000 × g for 20 min at 4 °C, the supernatant was incubated with anti-IKKβ antibody H-470 with rotation for 4 h at 4 °C, followed by the addition of 20 μl of Protein A-Agarose and incubation at 4 °C for additional 2 h. The immunoprecipitate was collected by centrifugation at 2,000 × g and washed three times with lysis buffer and two times with kinase buffer containing 20 mM HEPES (pH 7.4), 20 mM β-glycerophosphate, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 2 mM sodium fluoride, and 1 mM dithiothreitol. For kinase reaction, the immunoprecipitate was incubated in 20 μl of kinase buffer supplemented with 5 μCi of [γ-^{32}P]ATP and 1 μg of GST-IκBα(FL) (Santa Cruz Biotechnology) for 30 min at 30 °C. The reaction was stopped by addition of SDS sample buffer. The samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) which was then dried and subjected to autoradiography. The activity of JNK was determined using a SAPK/JNK assay kit following the procedures provided by manufacturer.

Western Blotting—Whole cell extracts were mixed with 3 × SDS-PAGE sample buffer and then subjected to SDS-PAGE in 10% gels. The resolved proteins were transferred to a nitrocellulose membrane. Western blotting was performed as described previously (15) using antibodies against IκBα, IKKβ, Flag, phosphotyrosine, and anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugates. For stabilizing and detecting the slightly slower mobility of the phosphorylated IκBα, macrophages were pretreated with 40 μM lactacystin for 2 h and subjected to SDS-PAGE using a 12-cm long minigel instead of the 7-cm long minigels used normally. The specific protein bands were visualized through enhanced chemiluminescence detection.

RESULTS

Vanadate Activation of NF-κB Requires IKKβ—Recent studies have demonstrated that a multisubunit IKK complex, containing two interactive catalytic components, termed IKKα and IKKβ, that mediate specific phosphorylation of IκBα at Ser^{32} and Ser^{36}, is required for the activation of NF-κB in response to stimulation by HTLV-1 Tax protein and cytokines (16–19). In this regard, we reasoned that the induction of NF-κB by vanadate (Fig. 1*A*) might also be through the activation of IKK. To test this hypothesis, we examined the effect of vanadate on IKKβ activity by analysis of intracellular kinase activity and *in vitro* kinase reaction. We chose to examine the IKKβ activity in cells transfected with an expression vector encoding wild type IKKβ because of a failure to detect the phosphorylation of IκBα and the endogenous IKK activity in non-transfected cells. When cells were pretreated with lactacystin, a relatively specific inhibitor for proteasome (20), to block the rapid degradation of phosphorylated endogenous IκBα protein, an upshifted phosphorylated IκBα band could be easily detected in the cells treated with vanadate (Fig. 1*B, lane 2, upper panel*). Incubation of this cellular extract with 15 to 30 units of calf intestine

alkaline phosphatase resulted in the disappearance of this slowly migrating IκBα band (Fig. 1*B, lanes 3* and *4, upper panel*). This phosphorylated IκBα band could not be induced by vanadate in cells transfected with a kinase inactive IKKβ, IKKβ-KM (Fig. 1*B, lanes 6–10, upper panel*). The *lower panel* in Fig. 1*B* shows the equal expression of exogenous IKKβ (*lanes 1–5*) or IKKβ-KM (*lanes 6–10*). Since it is possible that vanadate can induce other cellular kinases to phosphorylate IκBα, it is important to determine whether the vanadate-induced kinase activity is specific for the phosphorylation of Ser^{32}/Ser^{36} on the IκBα molecule. Thus, IKKβ-transfected macrophages were co-transfected with an expression vector for a wild type IκBα or its mutated form in which Ser^{32} and Ser^{36} were substituted with alanines. The *upper panel* in Fig. 1*C* demonstrated that IKKβ was able to induce phosphorylation (*lane 2*) and degradation (*lane 3*) of wild type IκBα in cells treated with vanadate for 15–30 min. However, no phosphorylation and degradation of S32A/S36A mutated IκBα was observed (Fig. 1*C, lanes 6–10*). By the use of a rabbit polyclonal antibody that could immunoprecipitate IKKβ from the cells treated with vanadate and the use of a GST-IκBα fusion protein which served as a substrate, the IKKβ activity was observed by an *in vitro* kinase activity assay. As shown in Fig. 1*D*, IKKβ activity was significantly elevated in the immunoprecipitates from cells treated with increasing concentrations of vanadate (Fig. 1*D, lanes 1–5, upper panel*). *Lanes 6–10* indicated a time course induction of IKKβ kinase activity in cells treated with 30 μM vanadate.

Kinase Inactive IKKβ Suppresses Vanadate-induced NF-κB—Since above studies clearly demonstrated that vanadate induction of NF-κB was through the activation of IKKβ kinase activity, the effects of a kinase inactive form of IKKβ (IKKβ-KM) on vanadate-induced NF-κB activation was evaluated. While co-transfection of macrophages (5×10^5 cells) with wild type IKKβ (0 to 4 μg) and a 2 × NF-κB luciferase reporter (0.5 μg) resulted in a marginal increase of vanadate-induced luciferase activity, co-transfection of macrophages with increasing concentrations of IKKβ-KM inhibited vanadate-induced reporter gene activity in a concentration-dependent manner (Fig. 2*A*). The inhibition of IKKβ-KM on vanadate-induced NF-κB activation was further verified by Western blot analysis for vanadate-induced degradation of IκBα, an intracellular inhibitor for NF-κB transcription factor (Fig. 2*B*). IκBα degradation was induced by vanadate treatment for 20 min (Fig. 2*B, lane 3*). A peak degradation of IκBα induced by vanadate was at 40 min (Fig. 2*B, lane 4*). After 80 min treatment of macrophages with vanadate, resynthesized IκBα was observed (Fig. 2*B, lane 5*). IKKβ-KM (*lanes 11–15*), but not empty vector (*lanes 1–5*) or wild type IKKβ (*lanes 6–10*), potently inhibited IκBα degradation induced by vanadate. Transfection of cells with a wild type IKKβ resulted in a more rapid degradation of IκBα induced by vanadate (Fig. 2*B*, comparing *lanes 6–10* with *lanes 1–5*).

Vanadate Is a Potent Inducer of c-Jun N-terminal Kinase—Emerging evidence suggested that JNK was activated by a spectrum of stimuli similar to those that cause NF-κB activation, and that MEKK1, an upstream kinase for JNK, was capable of regulating IKK activity and NF-κB activation (8, 10, 21–24). We next examined whether vanadate was also able to induce the activation of JNK. Lysates of untreated and vanadate-treated cells were assayed for kinase activity with N-terminal c-Jun-GST fusion protein as substrate for JNK. While marginal basal JNK activity was detected in non-stimulated cells, elevated JNK activation was observed in a dose-dependent manner in cells treated with increasing concentrations of vanadate (Fig. 3*A*). Time course studies indicated that the activation of NF-κB by vanadate was transient with a maximum activation at 1 h by a gel shift assay (Fig. 3*B*). To examine whether similar kinetics existed in the induction of JNK by vanadate, a time course study was performed. Note that within 30 min after treatment of cells with vanadate, the cells began to exhibit elevated JNK activity. The maximum induction of JNK activity occurred after a 2–4 h treatment of cells with vanadate. A small decrease in the levels of JNK activation was then observed at 8 h of exposure and was followed by a further drop at 10 h of stimulation (Fig. 3*C* and data not shown). These

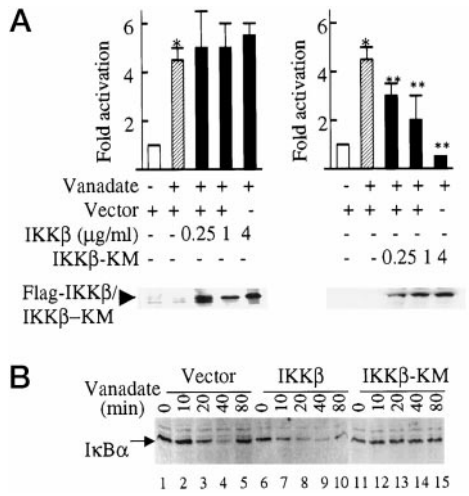

FIG. 2. **A kinase inactive form of IKKβ blocks activation of NF-κB by vanadate.** *A,* macrophages (5×10^5) were transfected with a 2 × NF-κB luciferase reporter (0.5 μg) along with various doses of IKKβ (*left panel*) or IKKβ-KM (*right panel*) for 40 h followed by the treatment of cells with 30 μM vanadate for additional 18 h. In addition to luciferase reporter, total amount of the DNAs was made up to 4 μg by adding pCR-3 vector. Luciferase activity was normalized per β-galactosidase values. Fold induction over basal level is shown. Each column represents an average value ± S.E. of three experiments (each in duplicate). *, $p < 0.002$ as compared with cells cultured in the absence of vanadate. **, $p < 0.05$ as compared with cells transfected with empty vector and treated with vanadate. The *bottom panels* show expression of transfected exogenous IKKβ or IKKβ-KM. *B,* macrophages were transfected with 2 μg of pCR-3 vector, IKKβ, or IKKβ-KM for 40 h and treated with 30 μM vanadate for an additional 0–80 min as indicated. Immunoblot was performed with IκBα antibody (C-21) to determine the degradation of IκBα.

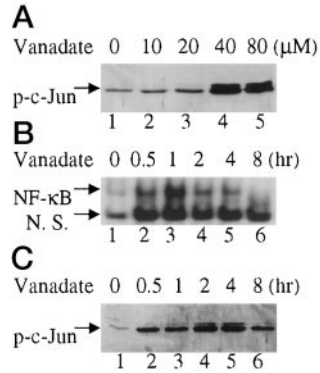

FIG. 3. **Vanadate induces JNK activation.** *A,* lysates of macrophages treated with various concentrations of vanadate for 4 h were prepared and subjected to *in vitro* kinase assay for JNK activity using GST-c-Jun (1–89) fusion protein as substrate for JNK. The JNK activity was represented by the phosphorylation of GST-c-Jun (*p-c-Jun*). *B,* time course study of vanadate-induced NF-κB activation. Macrophages were treated with 30 μM vanadate for different time periods as indicated. Nuclear extracts were prepared and subjected to gel shift assay with a radiolabeled double-stranded κB oligonucleotide. *Arrows* on the *left side* of this panel indicate the NF-κB binding complexes and nonspecific bands (*N.S.*). *C,* lysates of macrophages treated with vanadate as indicated in *B* were subjected to *in vitro* JNK kinase assay.

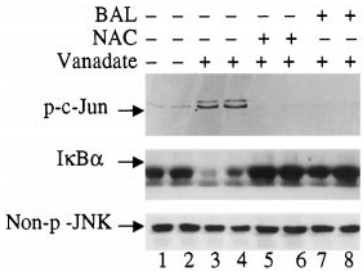

FIG. 4. **Effects of NAC and BAL on the activation of JNK and degradation of IκBα.** Macrophages were incubated in control medium or in the presence of 20 mM NAC or 100 μM BAL for 2 h prior to the treatment with 30 μM vanadate. Cells were harvested 30 min after vanadate treatment. Cell lysates were prepared and divided into three parts for *in vitro* JNK kinase assay (*top panel*), IκBα immunoblot (*middle panel*), and JNK immunoblot (*bottom panel*).

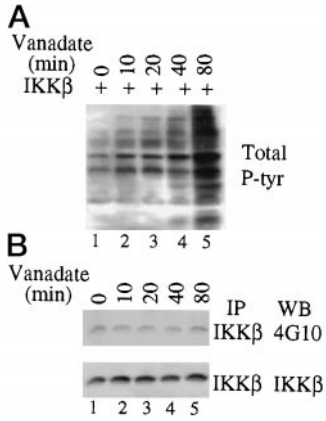

FIG. 5. **Vanadate is unable to induce IKKβ tyrosine phosphorylation.** *A,* macrophages were transfected with 2 μg of IKKβ for 40 h and incubated with 30 μM vanadate for an additional 0–80 min. Cell lysates were then prepared and fractionated on 10% SDS-PAGE for an anti-phosphotyrosine immunoblot using the anti-phosphotyrosine (*P-tyr*) antibody (4G10). *B,* macrophages were treated with the same condition as indicated in *A*. Cellular lysates were subjected to immunoprecipitation (*IP*) with IKKβ antibody (H-470) and then fractionated on 10% SDS-PAGE. Western blot (*WB*) analyses were performed with the anti-phosphotyrosine antibody 4G10 (*upper panel*) or the same IKKβ antibody (*lower panel*) used in immunoprecipitation.

results suggest that the induction of JNK by vanadate is more persistent than that vanadate induction of NF-κB (Fig. 3*C*).

Thiol Compounds Block Vanadate-induced Activation of JNK and Degradation of IκBα—The pathological effects triggered by vanadium ions are thought to be due to its capacity to change the intracellular redox states. Within tissue, vanadate is reduced to vanadyl by intracellular reducing agents such as glutathione (25) that will result in the depletion of GSH. On the other hand, electron spin resonance spectroscopy (ESR) spin trapping revealed that an one-electron reduction of vanadate occurs in the presence of NAD(P)H with the reduction of molecular oxygen to hydrogen peroxide, which subsequently reacts with vanadyl to generate hydroxyl free radicals and cause lipid peroxidation (26). To examine whether the induction of JNK and NF-κB by vanadate involves the alteration of GSH levels, cells were treated with 20 mM NAC for 2 h prior to exposure to 30 μM vanadate. As indicated in Fig. 4, pretreatment with NAC inhibited the activation of JNK (*top panel*) and degradation of IκBα protein (*middle panel*) induced by vanadate (Fig. 4, *lanes 5* and *6*). Similarly, addition of 2,3-dimercaptopropanol (British Anti-Lewisite, BAL) to protect the intracellular –SH groups abolished the induction of JNK and degradation of IκBα by vanadate (Fig. 4, *lanes 7* and *8*). By themselves, both NAC and BAL had no effect on the activation of JNK and NF-κB (data not shown). The expression of JNK protein was not affected by these treatments (Fig. 4, *bottom panel*). Thus, the alteration of the intracellular level of thiols, has a potent effect on the induction of JNK and NF-κB by vanadate.

Vanadate Is Unable to Induce Tyrosine Phosphorylation on IKKβ—It has been recognized that certain kinds of vanadium compounds, including perxovanadium and sodium oxodiperoxovanadate, are protein tyrosine phosphatase inhibitors and are able to activate Syk kinase and Src family kinases through increasing tyrosine phosphorylation (27, 28). The involvement of tyrosine phosphorylation of IκBα has also been demonstrated in oxidation- and pervanadate-induced NF-κB activation (29). In this context, we next investigated whether a similar mechanism was occurring in the activation of IKK by vanadate. Vanadate treatment of cells transfected with an expression vector for wild type IKKβ resulted in an accumulation of tyrosine-phosphorylated total cellular protein (Fig. 5*A*). However, an anti-phosphotyrosine antibody 4G10 blot of immunoprecipitated IKKβ revealed no appreciable change of phosphotyrosine levels on IKKβ throughout the time course (Fig. 5*B, upper panel*). In addition, we were unable to detect a change in tyrosine phosphorylation of IκBα protein (data not shown).

Activation of JNK Is Independent of the Pathway of NF-κB in Response to Vanadate—Well established evidence has suggested that the activation of NF-κB and JNK are through separate signaling pathways in response to TNFα and Epstein-Barr virus-encoded latent membrane protein 1 (8, 30, 31). Nevertheless, both pathways may utilize certain common signaling molecules (21, 23, 32) as summarized in Fig. 6*A*. To determine whether inhibition of vanadate-induced NF-κB signaling pathways could interfere with the JNK pathway, cells were treated with a proteasome inhibitor, lactacystin, transfected with a kinase inactive form IKKβ (IKKβ-KM) or a degradation resistant IκBα(S32A/S36A). As shown in Fig. 6*B*, treatment of cells with lactacystin completely blocked the degradation of IκBα induced by vanadate (Fig. 6*B, upper panel*). Lysates from identical cell cultures were also subjected to a JNK activity assay. Lactacystin was unable to inhibit vanadate-induced JNK activation. In contrast, treatment of cells with lactacystin resulted in an increase of vanadate-induced JNK activity (Fig. 6*B, lanes 6–10, lower panel*). Since other cellular biological processes may also be affected by the proteasome inhibitor, we next used IKKβ-KM and degradation resistant IκBα (S32A/S36A) to block the NF-κB pathway specifically. In the cells transfected with IKKβ-KM, vanadate-induced degradation of IκBα was abolished as expected (Fig. 6*C, lanes 5–10, panel 1*). However, no influence of IKKβ-KM on JNK activation was observed (Fig. 6*C, lanes 6–10, panel 3*). It was also observed that degradation resistant IκBα (S32A/S36A) failed to affect the activation of JNK by vanadate (Fig. 6*C, lanes 11–15, panel 3*). The equivalent experiment to measure the degradation of endogenous IκBα induced by vanadate in IκBα (S32A/S36A)-transfected cells was also performed. However, we could not detect endogenous IκBα degradation because of the presence of exogenous degradation resistant IκBα bands that obscure the bands of endogenous native IκBα.

Interrupting JNK Pathway Partially Reduces NF-κB Activation—The involvement of MEKK1 in NF-κB signaling suggested that the JNK pathway may integrate into the activation pathway for NF-κB under certain circumstances. Therefore, we subsequently examined the effect of a kinase inactive form of SEK1, SEK1-KM, on vanadate-induced IKKβ activity and IκBα degradation. As shown in Fig. 7*A*, treatment of cells with vanadate resulted in the elevation of IKKβ activity. While wild type SEK1 marginally enhanced vanadate-induced IKKβ ac-

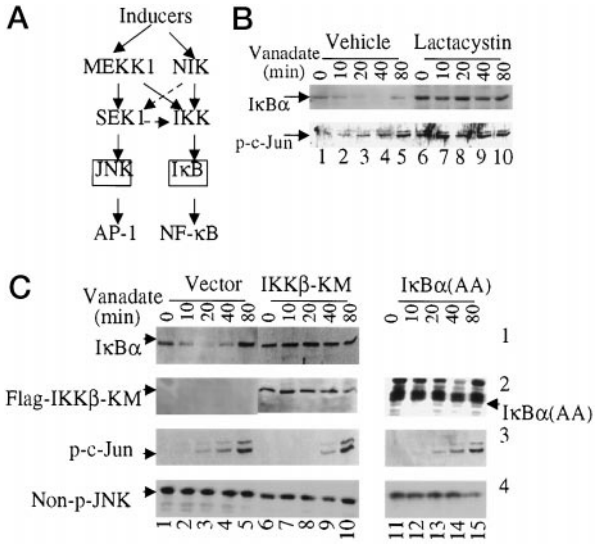

FIG. 6. **Vanadate-induced JNK is independent from NF-κB signaling.** *A,* potential interconnection may occur between the signaling pathways leading to JNK and NF-κB activation. Inducers such as cytokines and environmental or occupational particulate matter activate both MEKK1 for the JNK pathway leading to the activation of AP-1 transcription factor and the NK-κB-inducing kinase pathway leading to the activation of NF-κB. The *dashed arrows* indicate points in these signaling cascades that require further confirmation. *B,* macrophages were treated with vehicle (Me_2SO, *lanes 1–5*) or 40 μM lactacystin dissolved in Me_2SO for 2 h and then treated with 30 μM vanadate for the indicated time. Cellular lysates were used for IκBα immunoblot (*upper panel*) or *in vitro* JNK activity assay (*lower panel*). *C,* macrophages were transfected with 2 μg of pCR-3 vector, IKKβ-KM, or IκBα (S32A/S36A) for 40 h and then treated with 30 μM vanadate for an additional 0–80 min. Cellular lysates were prepared and used for IκBα immunoblot (*panel 1*) and *in vitro* JNK activity assay (*panel 2*). The expression of exogenous IKKβ, IκBα(AA) (*panel 3*) and endogenous JNK (*panel 4*) was determined using antibodies against Flag, IκBα, and JNK, respectively.

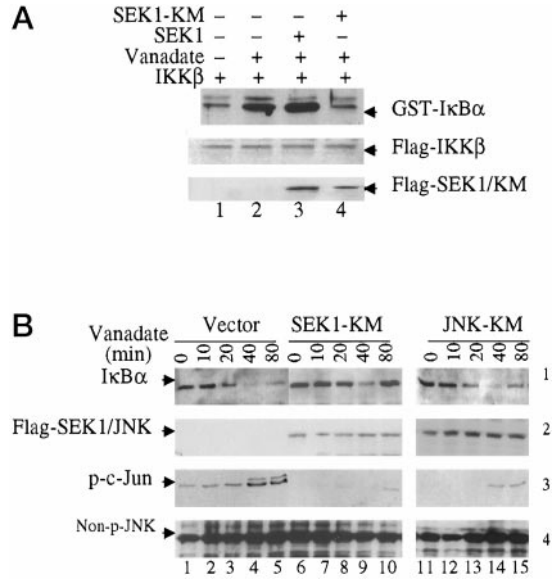

FIG. 7. **Kinase inactive SEK1 interferes with vanadate-induced activation of NF-κB.** *A,* macrophages were co-transfected with 2 μg of IKKβ and 2 μg of pCR-3 vector (*lanes 1* and *2*), wild type SEK1 (*lane 3*), and kinase inactive SEK1, SEK1-KM (*lane 4*) for 40 h. Cells were further treated with control medium (*lane 1*) or 30 μM vanadate (*lanes 2–4*) for an additional 1 h. Cell lysates were then immunoprecipitated with IKKβ antibody (H-470) and subjected to *in vitro* kinase assay in the presence of full-length GST-IκBα as substrate (*top panel*). The *middle* and *bottom panels* show the expression of exogenous IKKβ, SEK1, or SEK1-KM. *B,* macrophages were transfected with 2 μg of pCR-3 vector (*lanes 1–5*), SEK1-KM (*lanes 6–10*), and JNK-KM (*lanes 11–15*), respectively, for 40 h and further treated with 30 μM vanadate for an additional 0–80 min. Cellular lysates were then prepared and used for IκBα immunoblot (*panel 1*) and *in vitro* JNK activity assay (*panel 3*). The *panels 2* and *4* show the expression of exogenous SEK1/JNK and endogenous JNK, respectively.

tivity, overexpression of SEK1-KM significantly decreased vanadate-induced activities of IKKβ (Fig. 7*A, lane 4, top panel*). The expression of transfected exogenous IKKβ, SEK1, and SEK1-KM was shown in the *middle* and *bottom panels,* respectively. Transfection of cells with SEK1-KM resulted in a significant inhibition of JNK activation by vanadate (Fig. 7*B, lanes 6–10, panel 3*). Western blot analysis indicated that blocking the activation of JNK by transfection of SEK1-KM led to a partial inhibition of vanadate-induced IκBα degradation (Fig. 7*B, lanes 6–10, panel 1*). We also examined the effect of a kinase inactive form of JNK, JNK-KM, on the activation of NF-κB. Transient transfection JNK-KM reduced the JNK activation by vanadate (Fig. 7*B, lanes 11–15, panel 3*). This transfection, however, had no effect on vanadate-induced IκBα degradation (Fig. 7*B, lanes 11–15, panel 1*).

DISCUSSION

We provide evidence in the present study that vanadate, one of the most common forms of vanadium found in tissues or cells after exposure (33, 34), is capable of inducing the activation of NF-κB and JNK. Transfection studies demonstrated that the activation of NF-κB by vanadate required IKKβ. Kinase activity assays suggested that vanadate was a potent activator of IKKβ kinase activity (Fig. 1, *B-D*). We did not determine the IKKα activity in vanadate-treated cells in the present study. It is possible that vanadate was able to activate IKKα as well, despite the fact that IKKβ appears to be much more potent and specific than IKKα for the signaling pathway leading to the activation of NF-κB (21, 35). Our IKK kinase activity assay relies on immunoprecipitation of transfected IKKβ. IKKβ is in favor of forming a heterodimer with IKKα *in vivo*. It was also possible that some endogenous IKKα was pulled-down together with IKKβ and composed part of kinase activity in this kinase activity assay. Vanadate can also activate JNK as demonstrated in present study. It will be interesting, therefore, to examine the relationship between NF-κB and JNK pathways in vanadate-exposed cells. Inhibition of NF-κB activation by IKKβ-KM or degradation resistant IκBα (S32A/S36A) failed to influence the activation of JNK by vanadate suggesting that the JNK activation can be independent from NF-κB signaling (Fig. 6, *B* and *C*). In contrast, inhibition of JNK signaling pathway by a kinase inactive form of SEK1, SEK1-KM, but not JNK-KM, did exhibit a detectable inhibition of vanadate-induced IKKβ activity, IκBα degradation and NF-κB activation (Fig. 7). This result suggests that the involvement of JNK pathway in the activation of NF-κB occurred upstream or at the same level of SEK1. At present the detailed molecular mechanism by which SEK1 is involved in NF-κB signaling remains to be determined.

Even though there are some similarities between NF-κB and JNK activation by a variety of extracellular stimuli, the signaling pathways for the activation of NF-κB and JNK are thought to be separate responses. Important evidence to support this is provided by the study of TNF type 1 receptor-mediated activation of NF-κB and JNK (30). Over-expression of a catalytically inactive MEKK1 (K432M) to block JNK activation by TNF or TRAF2 did not prevent NF-κB activation. Likewise, overexpression of the phosphorylation-defective IκBα (S32A/S36A) mutant to block NF-κB activation had no effect on JNK activation. A similar observation was achieved in the study of Epstein-Barr virus-encoded latent membrane protein 1-induced activation of NF-κB and JNK (31). Nevertheless, a possible convergence may occur for both signaling axes under

different circumstances (Fig. 6*A*). The first report to indicate this interconnection between NF-κB and JNK signaling is based on the observation that the activation of a purified IKK complex required MEKK1 (22). Blockage of MEKK1 was able to inhibit TNF- or HTLV-1 Tax-induced activation of NF-κB reporter gene (23, 32, 36). The observation that a 70-kDa MEKK1 catalytic subunit is associated with the IKK complex provided plausible evidence to support this notion (19). Our finding that vanadate activates both NF-κB and JNK, and the involvement of SEK1 in vanadate-induced NF-κB signaling further supports the possible connection between NF-κB and JNK pathways.

It is noteworthy that some reports suggest that several vanadium compounds exert an inhibitory effect on protein tyrosine phosphatase and thereby increase the levels of tyrosine phosphorylation on total cellular proteins (27, 28). Studies by Krejsa and his co-workers (27) showed that IκBα was tyrosine phosphorylated in cells treated with stabilized peroxovanadium compound pV (phen) and suggested that this process contributed to the activation of NF-κB by vanadium. Yet it is not clear whether tyrosine phosphorylation is involved in the activation of NF-κB and JNK by vanadate in the present study. First, we found the tyrosine phosphorylation level was very marginal early in the exposure of cells to vanadate (Fig. 5*A, lanes 2–4*). Second, IκBα degradation or JNK activation induced by vanadate occurred within 10 to 30 min (Fig. 2*B* and Fig. 3*C*) or earlier (data not shown). However, an appreciable increase of tyrosine phosphorylation of total cellular protein was achieved 80 min after cells were treated with vanadate. Finally, we could not identify any change of tyrosine phosphorylation on IKKβ (Fig. 5*B*) or IκBα (data not shown). The discrepancy between our results and these of others may be simply due to the use of different forms of vanadium and different cell types. Indeed, early studies have shown that vanadate slightly inhibited or had no effect on many of the mammalian protein tyrosine phosphatases tested (37, 38). In addition, since exogenously added vanadate, after permeating into the cell interior, is efficiently reduced to vanadyl ion, it is possible that this form of vanadium exhibits an inhibitory effect on receptor tyrosine kinases (39).

How does vanadate activate cellular kinase cascades that lead to the activation of NF-κB and JNK? A simplified interpretation of our results is that this activation might be through the change of cellular redox states, since oxidant free radicals have been considered a common denominator involved in environmental insult-triggered diseases (40). To support this, treatment of cells with NAC to elevate intracellular thiol-containing molecules, such as GSH, abolished vanadate-induced JNK activation and IκBα degradation. It is known that many kinases or phosphatases contain redox-sensitive cysteines. Oxidation of these cysteines will result in the activation or inhibition of these kinases or phosphatases (41). The exact role of free radicals in the regulation of kinase activity is being investigated in our as well as other laboratories.

There is ample evidence that activation of JNK leading to the elevation of AP-1 activity is linked to the malignant transformation of cells (42). On the other hand, activation of IKK leading to the increased NF-κB activity is mainly associated with inflammation (6–9). The situation will be worse if both JNK and IKK are activated at the same time. A certain degree of additive or synergistic effects may occur between these two signaling axes. This is true in the diseases related to vanadium exposure, in which both mutagenicity and inflammation induced by vanadate have been reported (11, 43–45). Regarding environmental or occupational PM, in addition to vanadium, other metals and metal-metal interactions may also have some impact on overall PM-induced biological changes. A more detailed biological study is needed to determine the role of vanadium-baring PM from the cellular to molecular level in order to provide the mechanistic information necessary for risk assessment.

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Vanadate Induction of NF-κB Involves IκB Kinase β and SAPK/ERK Kinase 1 in Macrophages

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