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Participation of MAP Kinase p38 and IκB Kinase in Chromium (VI)-Induced NF-κB and AP-1 Activation

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Epidemiological studies demonstrate that environmental and occupational exposure of chromium(VI) [Cr(VI)] or Cr(VI)-containing particles can cause a number of human diseases, including inflammation and cancer. The biological mechanisms responsible for the initiation and progression of diseases resulting from exposure to Cr(VI) are not fully understood. The present studies evaluated the ability of Cr(IV) to induce activation of NF-κB and AP-1, two important transcription factors governing the expression of many early response genes involved in inflammation and carcinogenesis. The activation of NF-κB and AP-1 by Cr(IV) was dose dependent. Aspirin, a well-established antioxidant, substantially inhibited Cr(VI)-induced activation of both NF-κB and AP-1. SB202190, a specific inhibitor for p38, attenuated AP-1 activation induced by Cr(IV), whereas PD98059, a specific inhibitor for Erk, exhibited no effect on Cr(IV)-induced AP-1 activation. Blockage of NF-κB signaling pathway by a transient transfection of a dominant negative expressing vector for IκB kinase β resulted in inhibition of Cr(IV)-induced NF-κB, but not AP-1 activation. These data suggest that the activation of AP-1 or NF-κB by Cr(IV) is through the involvement of MAP kinase or IKK pathway, respectively.

KEY WORDS: Cr(VI), MAP kinase, NF-κB, AP-1

Chromium(VI) [Cr(VI)] compounds, widely used in industry, have been shown to have serious toxic and carcinogenic effects on humans.[1,2,26] Epidemiological investigations of industrially exposed chromium workers have identified chrome plating, chrome pigment manufacturing, leather tanning, and stainless steel production as sources of potential exposure to this metal. Because of its wide industrial application, environmental contamination is considered to be an additional source of human exposure to Cr(VI). Although Cr(VI) is now a well-established carcinogen, the mechanism of its carcinogenicity remains to be investigated.[1] Intensive studies in the past 2 decades have shown that reaction of Cr(VI) with certain cellular reductants generates reactive chromium intermediates, such as Cr(V) and Cr(IV). During this reduction process, reactive oxygen species (ROS) are generated. Thus, persistent oxidative stress may play a key role in mediating the expression of genes involved in inflammation and carcinogenesis.[14,17,21]

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Transcription factor NF-κB is considered to be an intracellular sensor of oxidative stress. NF-κB plays an important role in the transcriptional regulation of a number of genes, including those encoding cytokines, cell adhesion molecules, growth factors, and several anti-apoptotic proteins.[3] In resting cells, NF-κB remains in an inactive form in the cytoplasm by binding with its inhibitors, i.e., IκBα and IκBß. Exposure of cells to various extracellular stimuli, such as lipopolysaccharides, tumor necrosis factor-α, and environmental or occupational agents, results in the activation of IκB kinase (IKK) that phosphorylates IκBα or IκBß. Phosphorylated IκBα or IκBß is further ubiquitinated and degraded by ß-TrCP and proteasomes, respectively. This process leads to the activation and translocation of NF-κB into the nucleus, where it up-regulates the expression of genes involved in inflammatory or carcinogenic responses.

Another important transcription factor whose activity is stimulated by oxidative stress is AP-1, a dimeric, sequence-specific DNA binding protein composed of *jun* and *fos* proto-oncogene products.[19] Activation of AP-1 results in the over expression of *c-jun* and other proto-oncogenes. A number of mitogen activated protein kinases (MAPK) members participate in the activation of AP-1 hierarchically through divergent kinase cascades. MAPK, such as c-Jun-N-terminal kinase (JNK) and p38, are activated by a specific MAPK kinase (MAPKK) through phosphorylation of conserved threonine and tyrosine residues in JNK and p38. A MAPKK is activated by a specific MAPKK kinase (MAPKKK) through phosphorylation of conserved threonine and/or serine residues.

At present, there are only a few reports available that discuss the regulation of Cr(VI)-induced activation of NF-κB and AP-1 transcription factors.[18,27] Conclusions drawn from these reports concerning the activation of these two transcription factors by Cr(VI) are not straightforward. Both induction and inhibition of NF-κB and AP-1 activation by Cr(VI) have been reported.[26,29] Such contradictory data may result from the use of different experimental systems that varied in their response to Cr(VI). The present study was conducted to determine whether Cr(VI) is capable of activating NF-κB and AP-1 transcription factors in an in vitro cell culture system, and if so, how intracellular signal transduction systems participate in this process. The results of this study will support the goal to ultimately characterize which specific step of transcription factor activation was affected by Cr(VI) and provide a method for monitoring the early effects of Cr(VI).

Materials and Methods

Cells and Reagents. The mouse macrophage cell line RAW264.7 was purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in DMEM media (Sigma, St. Louis, MO) supplemented with 5% fetal bovine serum. Cr(VI) was from Aldrich Chem. Co. (Milwaukee, WI). The mouse JB6 fibroblast cell line stably co-transfected with an AP-1-dependent luciferase reporter gene and a NF-κB-dependent chloramphenicol acetyl transferase (CAT) reporter gene was described previously.[10] Aspirin was purchased from Sigma (St. Louis, MO) and dissolved in 0.05 M Tris-HCl to prepare a 1.0 M stock solution. The luciferase assay kit and mitogen-activated protein (MAP) kinase assay kits were from Promega (Madison, WI) and New England Biolabs (Beverly, MA), respectively. ECL Western blot detection reagents were from Amersham Life Science (Buckingham, England).

Electrophoretic Mobility Shift Assay (EMSA). For nuclear protein extraction, cells were harvested and resuspended in hypotonic buffer A (10 mM HEPES [pH 7.6], 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF), as previously described.[7] Cells were briefly incubated in buffer A for 10 min on ice, then vortexed for 10 sec. Nuclei were pelleted by centrifugation at 12,000 × g for 20 sec and resuspended in buffer C (20 mM HEPES [pH 7.6], 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) for 30 min on ice. The supernatants containing nuclear proteins were collected after centrifugation at 12,000 × g for 2 min and stored at –70°C. A ^{32}P-labeled double-stranded oligonucleotide containing κB sequence (5′-CAACGGCAGGGGAATTCCCCTCTCCTT-3′) was prepared according to previously published protocols.[5] Briefly, single-stranded DNA was synthesized using a Millipore Cyclone Plus automated synthesizer. To prepare double-stranded DNA, the first strand was annealed with a complementary decameric primer to its 3′-tail in annealing buffer (100 mM NaCl, 20 mM Tris [pH 7.5], 0.1 mM EDTA). The second strand was extended with DNA polymerase Klenow fragment in a reaction mixture containing 100 μCi [^{32}P] dCTP plus 5 mM dATP, dGTP and dTTP. For

EMSA, 4 μg of nuclear extract was mixed with the labeled double-stranded probe and incubated at room temperature for 30 min. The reaction solution was electrophorised on a native 5% polyacrylamide gel in 0.25 × TBE buffer for 2 to 3 h. The DNA binding proteins were visualized by autoradiography.

Reporter Gene Activity Assay. Cells containing NF-κB and AP-1 reporter genes were plated in 24-well tissue culture plates at 1×10^5 cells/well in DMEM medium supplied with 5% FBS for 24 hours and then cultured in 0.5% FBS for an additional 12 hours. The cells were treated with various doses of Cr(VI) with or without other agents as indicated in figures and figure legends. Cells in each well were then subjected to luciferase assay and CAT ELISA assay as described.[4]

Immunoblots. Whole cell extracts were mixed with 3 × SDS-PAGE sample buffer and then subjected to SDS-PAGE in 8–12% gels. The resolved proteins were transferred to a nitrocellulose membrane. Immunoblotting was performed by using antibodies against phospho-specific Erk, JNK, and p38 and antirabbit or antimouse IgG-horseradish peroxidase conjugates. The specific protein bands were visualized through enhanced chemiluminescence detection.

Results

The inflammatory and fibrogenic effects of environmental and occupational Cr(VI)-containing dusts have been previously reported.[12,22,26] Because NF-κB is a pivotal transcription factor governing the gene expression of many inflammatory mediators, activation of NF-κB by Cr(VI) was evaluated in a mouse macrophage cell line, RAW264.7 cells. As shown in Figure 1, compared to control (lane 1), Cr(VI) induced an appreciable activation of NF-κB in a dose-dependent fashion.

AP-1 is activated in response to environmental stresses such as agents that induce oxidative damage by increasing intracellular levels of ROS and those resulting in DNA damage.[9,25] It has been demonstrated that the activation of transcription factor AP-1 by extracellular stimuli requires the activation of several upstream kinases, including JNK, p38, and extracellular-signal-regulated kinases (Erk).[16] Figure 2 depicts that Cr(VI) induced activation of JNK and p38 in both a time- and dose-dependent manner, whereas no appreciable activation of Erk was observed in the same cells.

A cell line derived from JB6 mouse fibroblast stably transfected with a NF-κB-dependent CAT reporter gene construct and an AP-1-dependent luciferase construct was used to further investigate the functional activities of both NF-κB and AP-1 induction by Cr(VI). Figure 3 shows that Cr(VI) induced NF-κB-dependent CAT activity, which peaked at Cr(VI) concentration of 16 ng/ml, while declining at higher concentrations. AP-1-dependent luciferase activity, on the other hand, showed a dose-dependent increase at Cr(VI) concentrations from 4 to 1000 ng/ml.

A number of studies have indicated that the changes of intracellular redox status, such as the generation of $^{\bullet}OH$ and H_2O_2, are critical to Cr(VI)-induced pathogenesis.[8] To evaluate the contribution of reactive oxygen species (ROS) in Cr(VI)-induced NF-κB and AP-1 activation, the effect of aspirin, an antioxidant capable of scavenging $^{\bullet}OH$,[23,28] was studied. Previous results from our group indicate that the effective concentration of aspirin to inhibit silica-induced NF-κB activation and cycooxygenase II gene expression in the rat alveolar macrophage cell line NR8383 was 1.25–10 mM.[6] Consistent with this observation, Figure 4 shows that adding 5 mM aspirin resulted in a potent inhibition of both AP-1 (Figure 4A) and NF-κB (Figure 4B) reporter gene activity induced by Cr(VI). The cytotoxic effect of aspirin at this concentration was not observed (data not shown).

A number of extracellular stimuli can induce the activation of MAP kinases, but they exhibit different effects on different MAP kinases. For example, signals from the growth factors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), are known preferentially to induce the activation of Erk, whereas signals from the stress responses mainly activate JNK and p38.[16] By using specific inhibitors against Erk and p38, respectively, we examined the role of Erk and p38 in Cr(VI)-induced activation of both NF-κB and AP-1. As shown in Figure 5A, Cr(VI)-induced AP-1 activation was substantially inhibited in the presence of 2 μM SB202190, a specific inhibitor of p38 kinase. In contrast, a specific inhibitor for Erk kinase, PD98059, exhibited no inhibition of Cr(VI)-induced AP-1 activation. Both SB202190 and PD98059 exerted a marginal, insignificant inhibition of Cr(VI)-induced NF-κB activation (data not shown).

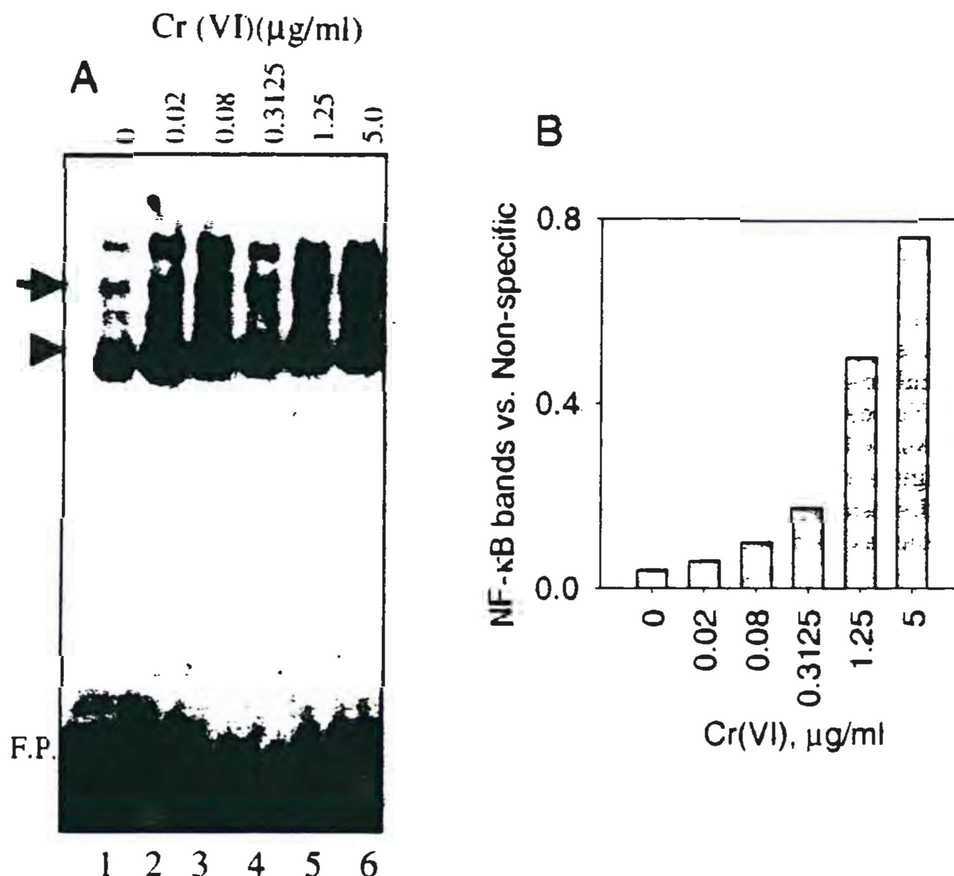


FIGURE 1. Activation of NF-κB by Cr(VI). A = RAW 264.7 cells were treated with various concentrations of Cr(VI) for 4 h. Preparation of nuclear proteins and EMSA were as described in "Materials and Methods." Arrow denotes NF-κB complexes and arrowhead denotes non-specific band, respectively. F. P., free probe. B = Normalization of Cr(VI)-induced NF-κB DNA binding activity in (A) by densitometry assay of NF-κB DNA binding bands verse non-specific bands.

It has been demonstrated that the activation of NF-κB by proinflammatory cytokines is controlled by sequential phosphorylation, ubiquitination, and degradation of its inhibitors, including IκBα and IκBß.[3,19] A kinase complex named IκB kinase signalsome (IKK) has been identified recently; it contains 2 crucial kinase subunits—IKK1 and IKK2—which are responsible for the phosphorylation of IκBα or IκBß.[19] To examine the role of IKK2 in Cr(VI)-induced NF-κB activation, an expression vector encoding dominant negative IKK2 (IKK-DN) was transfected into the cells containing AP-1 and NF-κB reporter genes. Figure 6B demonstrates that IKK-DN inhibited the activation of NF-κB induced by Cr(VI). In contrast, no effect of IKK-DN on Cr(VI)-induced AP-1 activation was observed (Figure 6A). These results indicate that IKK also participates in the processes of NF-κB activation induced by Cr(VI).

Discussion

Environmental and occupational exposure to Cr(VI) or Cr(VI)-containing particles can lead to various health problems.[20] The toxicity of Cr(VI) is largely based on the generation of ROS within tissues or cells, a process accountable for Cr(VI)-induced inflammation or carcinogenesis.[20] AP-1 and NF-κB, two crucial host transcription factors governing the expression of genes related to inflammatory mediators and oncogenes, have been reported to be sensitive to oxidative stress.[3,19] The results obtained from the present study show that

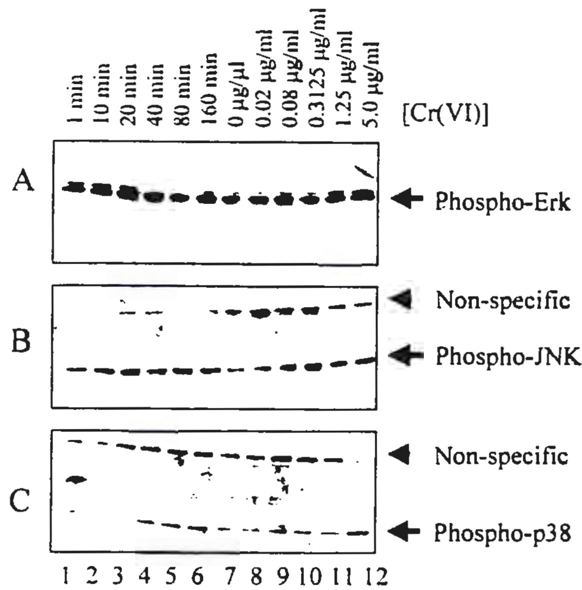


FIGURE 2. MAP kinase activation by Cr(VI). RAW264.7 cells were treated with 5000 ng/ml Cr (VI) for 0–160 min (lanes 1–6) or with a various concentration of Cr(VI) (lanes 7–12) for 60 min. MAP kinase activities for Erk (panel A), JNK (panel B), and p38 (panel C) were determined by using anti-phospho-specific JNK, Erk, and p38 antibodies. N.S., nonspecific bands.

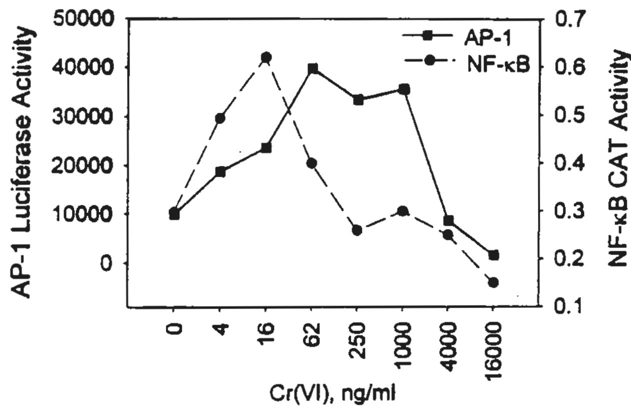
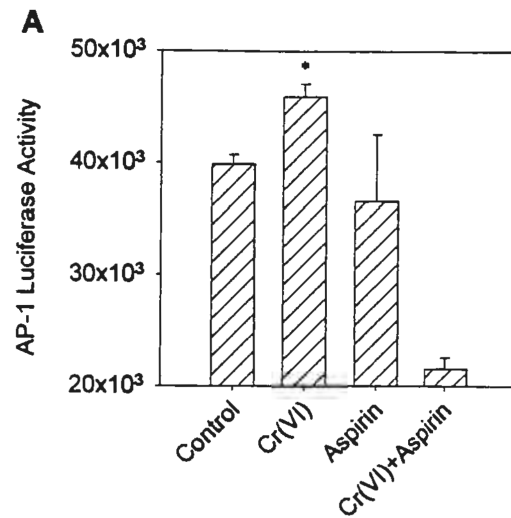


FIGURE 3. Functional activation of NF-κB and AP-1 by Cr(VI). JB6 cells, stably cotransfected with both NF-κB-dependent CAT reporter construct and AP-1-dependent luciferase reporter construct, were treated with various concentrations of Cr(VI) for 12 h. Cell lysates were prepared and subjected to AP-1 luciferase activity assay and NF-κB CAT ELISA assay as described in "Materials and Methods."



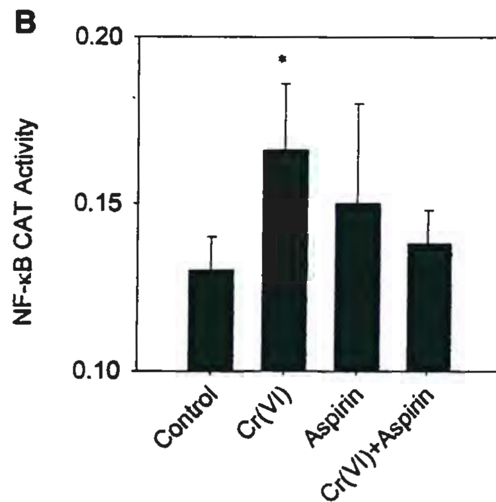


FIGURE 4. Effect of aspirin on Cr(VI)-induced AP-1 and NF-κB activation. JB6 cells, stably cotransfected with NF-κB and AP-1 reporter construct, were treated with 100 ng/ml of Cr(VI) in the absence or presence of 5 mM aspirin for 12 h. Cell lysates were prepared for AP-1-dependent luciferase assay and NF-κB-dependent CAT ELISA assay as described in "Materials and Methods." Values are means ± SE of 3 determinations. * indicates a significant increase from control ($p \leq 0.05$).

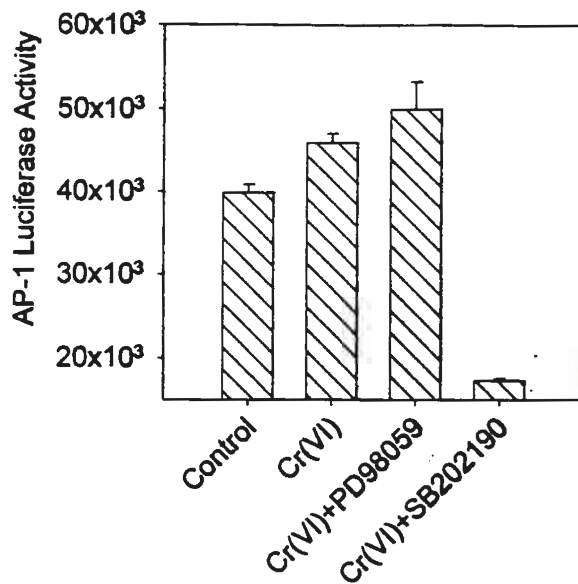


FIGURE 5. Effects of MAP kinase inhibitors on Cr(VI)-induced AP-1 activation. JB6 cells, cotransfected with AP-1 and NF-κB reporter genes, were treated with 100 ng/ml of Cr(VI) in the absence or presence of 12 μM Erk inhibitor, PD98059, or 2 μM p38 inhibitor, SB202190, for 12 h. Total cell lysates were used for AP-1-dependent luciferase assay.

(1) Cr(VI) is capable of inducing AP-1 and NF-κB activation in both a time- and a dose-dependent manner; (2) the induction of AP-1 by Cr(VI) is associated with phosphorylation of MAP kinase p38 and JNK, but not Erk; (3) aspirin, an antioxidant, inhibits the activation of AP-1 and NF-κB induced by Cr(VI); and (4) inhibition of p38 and IKK attenuates Cr(VI)-induced AP-1 and NF-κB, respectively. These results suggested that ROS may serve as a common upstream signal initiating the activation of both AP-1 and NF-κB in response to Cr(VI) stimulation, whereas p38 and IKK act as downstream executive kinases for the activation of AP-1 and NF-κB, respectively (Figure 7).

Cr(VI) is able to induce the generation of ROS through 1-electron intracellular reduction reaction.[8] Emerging evidence suggests that ROS function as a second messenger system in the context of cellular stimulations, such as Cr(VI) exposure. However, the specific targets of ROS have remained largely unclear. One potential target that can be directly effected by ROS may be the tyrosine phosphatases.[11] It has been demonstrated that most of the tyrosine phosphatases have a cys-

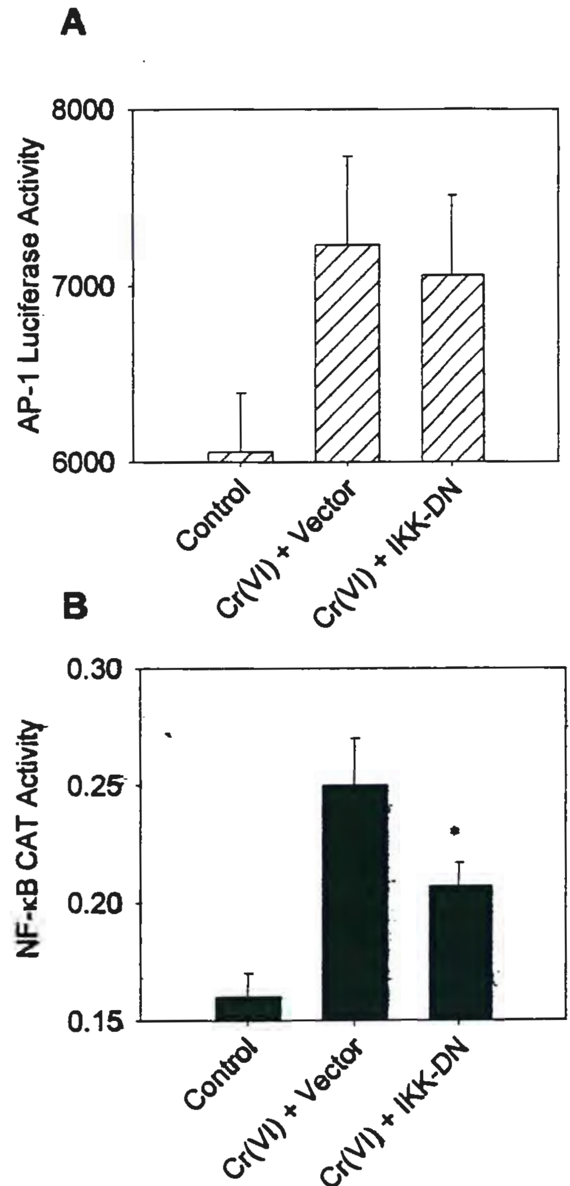


FIGURE 6. Inhibitory effect of dominant negative IKK on Cr(VI)-induced activation of NF-κB but not AP-1. JB6 cells transfected with AP-1 and NF-κB reporter genes were further transfected with an empty vector or expression vector for dominant negative IKK (IKK-DN) for 24 h. The cells were treated with 100 ng/ml of Cr(VI) for an additional 12 h followed by AP-1 (A) and NF-κB (B) reporter gene activity analysis as described in "Materials and Methods." Values are means ± SE of 3 determinations. * indicates a significant decrease from Cr(VI) + vector only ($P \leq 0.05$).

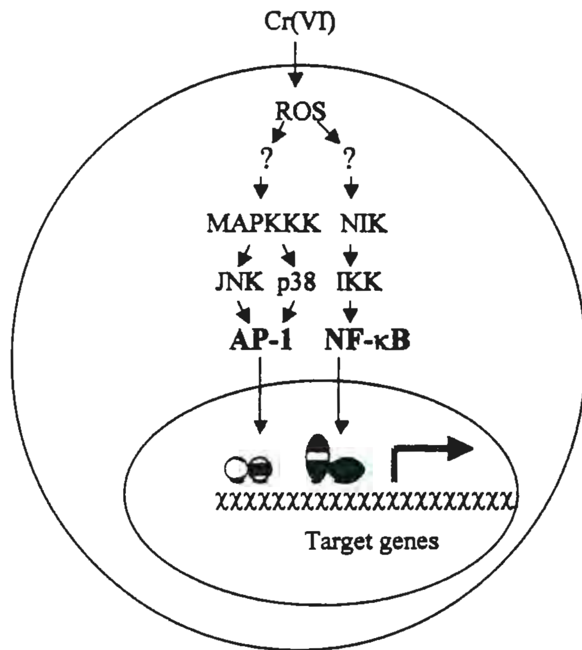


FIGURE 7. Possible signal transduction pathways in Cr(VI)-induced AP-1 and NF-κB activation.

teine residue in the active site that is essential for their biological activity. Modification of this cysteine residue by ROS results in the inactivation of these phosphatases. In this respect, it is important to note that several members of MAPKK are tyrosine and serine/therionine dual kinases.[13] Therefore, inactivation of tyrosine phosphatases will lead to elevation of the tyrosine kinase activity of MAPKK and, consequently, AP-1 activation. Although it is still unclear how ROS affect the signal transduction leading to the activation of NF-κB, a large number of investigations have shown that the activation of NF-κB induced by many extracellular stimuli can be inhibited by antioxidants, including aspirin, thiols, vitamin E, and dithiocarbamates.[3] Considering the fact that ROS neither facilitate the degradation of IκB proteins nor enhance the DNA binding of NF-κB,[15,24] the involvement of ROS in NF-κB activation must occur in a distal upstream signaling pathway or by circumventing the classic NF-κB activation pathway through an unidentified mechanism.

Understanding the signal transduction mechanisms of AP-1 and NF-κB activation may be important in designing specific and effective therapeutic strategies against diseases resulting from exposure to Cr(VI) or other oxidants. As shown in the present report, inhibitors toward MAP kinase p38 and IKK can effectively inhibit Cr(VI)-induced AP-1 and NF-κB, respectively. A more detailed and intensive study is being conducted in our laboratory to investigate the mechanisms of Cr(VI)-induced activation of transcription factors and their roles in mediating Cr(VI)-induced p53 activation, cell cycle regulation, transformation, and apoptosis.

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