Chromium Induces a Persistent Activation of Mitogen-activated Protein Kinases by a Redox-sensitive Mechanism in H4 Rat Hepatoma Cells

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ABSTRACT

Chromium is an important industrial metal, an environmental pollutant, and a human carcinogen. To investigate the mechanisms of chromium-induced carcinogenesis, activation of mitogen-activated protein (MAP) kinases ERK1 and ERK2 was examined in rat hepatoma cells following exposure to hexavalent chromium (Cr(VI)). Cr(VI) was found to activate both forms of MAP kinase in a dose- and time-dependent manner. Furthermore, unlike phorbol 12-myristate 13-acetate, which induced a transient activation of MAP kinases, Cr(VI) caused persistent activation of these enzymes. Furthermore, unlike phorbol 12-myristate 13-acetate, the ability of chromium to activate MAP kinases was found to be independent of PKC since chromium-induced MAP kinase activation occurred in PKC-depleted cells. Stimulation of ERK1 and ERK2 was associated with the ability of Cr(VI) to increase cellular peroxide levels as determined using the H_{2}O_{2}-sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate and flow cytometry. Furthermore, the activation of these kinases by chromium was enhanced in cells treated with the glutathione-depleting agent, l-buthionine-[S,R]-sulfoximine, and attenuated in cells pretreated with an agent that elevates cytosolic levels of glutathione (i.e., N-acetyl-L-cysteine). The ability of chromium to modulate MAP kinase activity in this manner suggests a mechanism of chromium-induced carcinogenesis that involves the persistent stimulation of cellular regulatory pathways.

INTRODUCTION

Previously, we had shown that exposure of mammalian cells to Cr(VI) induces tyrosine phosphorylation of numerous cellular proteins and alters the pattern of phosphoproteins produced in response to growth factors (1). Specific types of phosphoproteins affected by chromium were determined to be subsets of phosphatidylinositol 3'-kinase-binding proteins that were distributed throughout the soluble and particulate fraction of cells. Binding of certain phosphoproteins to phosphatidylinositol 3'-kinase is known to stimulate the activity of this enzyme, resulting in the generation of distal mediators of signal transduction (2). Therefore, Cr(VI) may mediate its effects on cells by modulating the activity of various signaling molecules. This possibility prompted us to examine the effect of chromium on other specific proteins/enzymes that function as key regulators of cellular signaling. One such family of regulatory molecules is the serine/threonine kinases known as MAP kinases. Apparently, MAP kinases function as convergence points through which disparate agents mediate common effects on growth and differentiation (3, 4).

For example, the PKC agonist, PMA, as well as numerous peptide growth factors are known to activate MAP kinases (5). Although the effect of PMA on MAP kinase activity has been determined to be PKC dependent (6), the kinetics of MAP kinase activation in cells by some peptide growth factors are not influenced by PKC depletion (7). These observations suggest that there are numerous pathways by which MAP kinases can become activated. A MAP kinase-activating pathway that has been characterized in considerable detail is a signaling cascade mediated by Ras (reviewed in Ref. 3). Positive modulators of Ras-action (i.e., growth factor receptor kinases) induce the activation of a serine/threonine kinase known as Raf-1 (8). The Raf-1-mediated phosphorylation of a kinase immediately proximal to the MAP kinases, known as MEK, leads to its activation (9). This kinase then phosphorylates specific tyrosine and threonine residues on the MAP kinases, stimulating their serine kinase activity (10). Other growth-signaling proteins are capable of activating MEK (11, 12), further increasing the possible pathways by which MAP kinases can become activated. Following activation, MAP kinases then modify both cytosolic and nuclear substrates by serine/threonine-specific phosphorylation (13-15). Of particular note are the nuclear substrates of MAP kinases, Elk-1 and SAP-1 (16, 17). Following phosphorylation, these factors form ternary complexes with serum response factor, SRF, and serum reaction element, SRE, in the promoter region of the c-fos gene (18). The resulting induction of c-fos and the subsequent influence of the gene product on the expression of specific growth-related gene represents a distal segment of numerous mitogenic signaling pathways that is mediated through MAP kinase.

Because MAP kinases appear to be essential for transducing regulatory signals, the effect of chromium on MAP kinase activity was explored. The present studies demonstrate that Cr(VI) induces a persistent activation of the MAP kinases ERK1 and ERK2 that is associated with the ability of this metal to generate cellular peroxides. Although the ability of Cr(VI) to produce various types of DNA lesions has supported the notion that this element functions as an initiating agent in the carcinogenic process (19, 20), the results of the present study suggests epigenetic mechanisms by which chromium can modulate the growth/differentiation state of cells and potentially influence the development of tumors.

MATERIALS AND METHODS

Cell Culture and Treatment. H4-II-E rat hepatoma cells (American Type Culture Collection, Rockville, MD) were grown in Swim’s 77 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 2 mM L-glutamine, 0.05 mM L-cysteine, 20% v/v horse serum, 5% v/v fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Confluent plates (10 cm) of cells were deprived of serum for 24 h prior to treatment. The cells were then treated with various agents as indicated. Trivalent chromium (i.e., Cr(III), CrCl_{3}) and the hexavalent form of this metal (i.e., Cr(VI)), K_{2}Cr_{2}O_{7}, were from Aldrich Chemical Company, Inc. (Milwaukee, WI). Throughout the text, the concentration of hexavalent chromium is presented as [Cr(VI)], such that 100 μM Cr(VI) is equivalent to 50 μM K_{2}Cr_{2}O_{7}. PMA was purchased from Leinco Technologies (St. Louis, MO). The treatment of cells with the specific agents was conducted in serum-free growth medium. It must be noted that stock solutions of chromium were made in PBS immediately prior to use. Furthermore, the addition of chromium was not found to significantly alter the pH of the serum-free growth medium. In some experiments, cells were pretreated...
with agents known to decrease (BSO; 10 μM for 8 h) or increase (NAC; 10 mM for 24 h) cellular glutathione levels. Treatment with these agents was conducted in serum-containing growth medium. These agents were then washed from the cells (2 × 10 ml serum-free medium) prior to further treatment with either Cr(VI) or PMA as described above. For harvesting, plates of cells were washed twice with 10 ml of ice-cold PBS and an extraction buffer (750 μl) containing 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 150 mM NaCl, 2 mM Na₂VO₃, 1% NP-40, 10 μg/ml each of aprotinin and leupeptin (Sigma), and 1 mM phenylmethylsulfonyl fluoride (Sigma) was added to each plate. The cells were removed from the plate using a rubber spatula and transferred to a microfuge tube. The cell homogenates were then mixed vigorously for 1 min, and the insoluble material was removed by centrifugation at 13,000 × g for 10 min at 4°C. Aliquots of the supernatants, matched for proteins according to standard procedures (21), were added to an equal volume of 2× sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 40 mM DTT] and placed in a boiling water bath for 5 min. Proteins in the samples were separated using SDS-PAGE and immunoblotted (see below).

Electrophoresis and Immunoblotting. Solubilized proteins in crude cell extracts were analyzed using SDS-PAGE according to the method of Laemmli (Ref. 22; 10% polyacrylamide in the separating gel). Proteins separated by the gel system were transferred electrophoretically to nitrocellulose (Trans-Blot transfer medium, 0.45 μm; Bio-Rad Laboratories, Hercules, CA). To visualize MAP kinases, the membranes were first incubated for 2 h with a blocking buffer containing 50 mM Tris-HCl (pH 8.0) and 5% nonfat dry milk. 0.1% Tween-20, and 150 mM NaCl. The blots were then probed with a MAP kinase (ERK1 and ERK2)-specific monoclonal antibody (Zymed Laboratories, Inc., South San Francisco, CA) for 1 h with the blocking buffer without milk. The blots were then washed four times, and antibody reactions were detected using horseradish peroxidase-conjugated sheep anti-mouse IgG and a chemiluminescent detection system (ECL; Amersham, Arlington Heights, IL).

In-Gel MAP Kinase Renaturation Assay. In-gel kinase assays were conducted using the method described by Samuels et al. (23). Briefly, proteins were resolved on minigels containing 10% polyacrylamide and 0.5 mg/ml MBP (Sigma) in the separating gel buffer. Following electrophoresis, the gels were washed twice with 200 ml of a buffer containing 50 mM Tris-HCl (pH 8.0) and 20% isopropanol to remove SDS. The gels were then incubated with 100 ml of 50 mM Tris-HCl (pH 8.0) and 5 mM 2-mercaptoethanol and subsequently incubated twice for 30 min each in 100 ml of 50 mM Tris-HCl (pH 8.0), 6 μg guanidine HCl, and 5 mM 2-mercaptoethanol. The proteins in the gel were then renatured by incubation with 250 ml of a buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, and 0.04% Tween-20 (Sigma) for 1 h. The gels were washed twice with 200 ml of a buffer containing 50 mM Tris-HCl (pH 8.0) and 20% isopropanol. The kinase assays were conducted by incubating the gel with 25 ml of 10 mM HEPES (pH 8.0), 0.1 mM EGTA, 5 mM MgCl₂, 2 mM DTT, 25 μM ATP, and 25 μCi of [γ-32P]ATP for 1 h at 25°C. The gels were then extensively washed with (6 × 100 ml) of 5% trichloroacetic acid (w/v) containing 1% sodium PO₄. Dried gels were exposed to Kodak X-OMAT film for 24 h at ~70°C prior to development.

Measurement of Intracellular Peroxides. Peroxide production by H4 cells was monitored using the H₂O₂-sensitive fluorescent probe DCFH-DA (Molecular Probes, Eugene, OR) as described previously (24). The specificity of this dye for H₂O₂ and cellular peroxides is described elsewhere (25). For peroxide determination, cells were removed from the plate with 0.2 ml EDTA-PBS, washed, and resuspended in PBS (10⁶ cells/ml). The samples were then adjusted to 10 μM DCFH-DA and incubated in a 37°C shaking water bath for 15 min. Sets of samples were further treated without or with 100 μM Cr(VI) or 160 mM PMA for 20 min prior to flow cytometric analysis. Stained samples of H4 cells were analyzed using an EPICS Profile flow cytometer (Coulter Electronics, Inc., Hialeah, FL) equipped with an Ormochrome 25 mW argon laser emitting at 488 nm with 15 mW of power. Green fluorescence signals were collected using 550-nm long-pass dichroic and 525-nm band pass filters. The histograms generated in these experiments were analyzed using EPICS CytoLogic Software (Version 2.0; Coulter Electronics, Inc.). In separate experiments, Cr(VI) was not found to directly oxidize the peroxide-specific dye, DCFH-DA, or its de-esterified analogue in cell-free buffers (data not shown).

Measurement of Cellular GSH. For GSH determinations, H4 cells, treated as indicated above, were removed from the plate with 0.2 ml EDTA-PBS and washed 2× with PBS (25°C). Equal aliquots of each sample were then transferred into two separate tubes. One aliquot was treated with the GSH-specific dye, MBCL (Molecular Probes; 40 μM for 15 min at 25°C; Ref. 26). The remaining aliquot of cells was treated with the thiol-alkylation agent NEM (200 μM for 15 min at 25°C) prior to staining with MBCL as described above. Since NEM specifically alkylates cellular thiols, including GSH, and precludes the interaction of MBCL with GSH, this sample was used to determine the background fluorescence of stained cells. Cellular fluorescence was measured using an EPICS 753 flow cytometer (Coulter Electronics, Inc.) equipped with an adjustable laser using 150 mW of power from the UV laser line. Integrated fluorescence signals (log) were collected through a 380-nm laser blocking filter and a 450-nm long pass dichroic filter. Cellular debris was excluded from fluorescence analysis using forward angle and 90° light scatter (log) gates. As observed for other rodent cell types, MBCL staining of H4 hepatocytes attained maximal levels following a 10-min incubation (data not shown). Furthermore, MBCL staining was found to reflect cellular GSH levels as confirmed by standardized high-performance liquid chromatography-based methods (27).

RESULTS

Cr(VI) Activates MAP Kinases in Transformed Rat Hepatocytes. In these studies, we examined the activation of MAP kinases ERK1 and ERK2 by Western blot analysis in which the activated (i.e., phosphorylated) kinases exhibit retarded mobility in SDS-PAGE. In some experiments, activation of the kinases was verified by the in-gel kinase renaturation assay using MBP as the substrate. The addition of Cr(VI) to serum-deprived H4 hepatoma cells induced a dose-dependent activation of ERK1 and ERK2 at concentrations as low as 10 μM (Fig. 1, A and B). The ability of chromium to activate these MAP kinases was specific for the hexavalent form of the element since inorganic, trivalent chromium was ineffective. Cr(VI) treatment resulted in a gradual time-dependent activation of ERK1 and ERK2, with initial activation observed within 10 min of treatment and maximal effect occurring within 1 h (Fig. 1C). Interestingly, activation of the MAP kinases by Cr(VI) was found to persist for at least 8 h. This is in contrast to many peptide growth factors as well as agents such as PMA (see below), which induce a transient activation of MAP kinases (7). Since Cr(VI)-induced activation of these kinases was not apparently subject to down-regulation, we next exposed the cells to low doses of Cr(VI) for prolonged periods of time. In these studies, we were able to detect activation of the ERK2 form of MAP kinase in H4 cells following a 16-h treatment with Cr(VI) concentrations as low as 0.3 μM, whereas the activation of both forms of the kinase was observed at 3 μM Cr(VI) (Fig. 1D). The results indicate that chronic treatment with low concentrations of Cr(VI) can lead to a persistent activation of these key regulatory kinases. It is important to note that cellular viability was routinely determined by flow cytometric analysis of propidium iodide-stained samples using standard protocols (28). Throughout our studies, the activation of ERK1 and ERK2 occurred at doses of Cr(VI) that do not cause significant cell death (data not shown).

The Ability of Cr(VI) to Activate MAP Kinases Is PKC Independent. In previous studies, we demonstrated that Cr(VI) induces a pattern of phosphotyrosine-containing proteins in H4 cells that is similar to the pattern generated in response to the tumor-promoting agent, PMA (1). Furthermore, unlike PMA-mediated phosphorylations, those induced by Cr(VI) were determined to be independent of PKC activity. In the current studies, a comparison of Cr(VI) and PMA-mediated activation of MAP kinases was conducted and the influence of PKC on Cr(VI)-induced kinase activation was examined. We found that treatment of H4 cells with PMA alone activated ERK1 and ERK2 within 5 min (Fig. 2A). However, in contrast to Cr(VI), activation of the kinases by PMA was transient, reaching maximal levels within 30 min after the addition and returning to basal levels within 4 h (compare Fig. 2A to Fig. 1C). In other studies, plates of H4
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Fig. 1. Dose- and time-dependent effects of Cr(VI) on ERK1 and ERK2 activation. A. serum-deprived rat hepatoma cells were treated with increasing concentrations of Cr(VI) for 1 h. Soluble proteins in crude cell extracts were resolved using SDS-PAGE and immunoblotted using MAP kinase-specific antibodies. Proteins reacting with the antibodies were visualized using peroxidase-conjugated sheep antimouse secondary antibodies and a chemiluminescent detection system as described in "Materials and Methods." Decreased gel mobility of ERKI (M, 44,000) and ERK2 (M, 42,000) indicates activation (i.e., phosphorylation) of these MAP kinases (*). B, MAP kinase activity in Cr(VI)-treated cells was determined using the in-gel kinase assay (see "Materials and Methods"). Phosphorylation of immobilized MBP reflects increased MAP kinase activity. Positions of ERK1 and ERK2 were confirmed on duplicate immunoblots (data not shown). C. cells were treated with 100 µM Cr(VI) for increasing periods of time as indicated. The activation of ERK1 and ERK2 was determined on immunoblots as described above. D, serum-deprived H4 cells were treated without or with low levels of Cr(VI) for 16 h. Data are presented as densitometric scans of X-ray film developed from regions of immunoblots containing ERK1 and ERK2. Expected positions of activated forms of the kinases are indicated (arrows).

cell were treated for prolonged periods with amounts of PMA known to deplete cellular PKC levels (1). The cells were then treated with either Cr(VI) (100 µM for 1 h) or additional PMA (1 µg/ml for 30 min), and activation of the MAP kinases was determined. During the prolonged exposure to PMA, ERK1 and ERK2 become stimulated and then return to baseline levels within 2 h (Fig. 2A). In these pretreated cells, additional PMA treatment failed to activate the MAP kinases (Fig. 2B, Lane 6), demonstrating that the cells were refractory to PMA stimulation following down-regulation of PKC. Interestingly, Cr(VI), unlike PMA, activated ERK1 and ERK2 in PKC-depleted cells, indicating that the ability of Cr(VI) to activate these kinases is not dependent on the isoforms of PKC that are down-regulated during

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CHROMIUM-INDUCED ACTIVATION OF MAP KINASES

A

PMA

(160 nM)

TIME (min) 0 1 2 5 10 30 60 120 240 480

[•*] ERK1

[•*] ERK2

B

Pretreatment:
PMA (160 nM, 16 h) - - - + + +

Fig. 2. Cr(VI)-induced activation of MAP kinases in PKC-depleted cells. A. serum-deprived H4 rat hepatoma cells were treated with PMA (160 nM) for increasing periods of time. Cell extracts were then subjected to Western blotting with MAP kinase-specific antibodies, and activation of ERK1 and ERK2 was determined on immunoblots as described in Fig. 1. B. cellular levels of PKC were down-regulated in H4 cells by prolonged incubation with PMA (Lanes 4–6; see Ref. 1 for PKC-specific immunoblots confirming PKC down-regulation in H4 cells by extended PMA exposure). The cells were then treated with either Cr(VI) (100 μM for 1 h) or additional PMA (1 μg/ml for 30 min). Activation of ERK1 and ERK2 in cell extracts was determined on immunoblots as described in Fig. 1. Results were compared to sets of plates not receiving chronic PMA pretreatment (Lanes 1–3). Data are representative of three independent experiments.

chronic PMA exposure (Fig. 2B, Lane 5). Since the time course and PKC dependence of ERK1 and ERK2 activation by Cr(VI) and PMA are dissimilar, the results suggest that these agents affect the activation of MAP kinases by distinct mechanisms.

Cr(VI)-mediated Activation of MAP Kinases Is Associated with the Peroxidative Capacity of This Element and Is Sensitive to Cellular Redox Potential. Chromium induces reactive oxygen species in cells by characterized mechanisms (29, 30). This is of significance to our studies since intracellular peroxides are known to mediate tyrosine phosphorylation of specific regulatory proteins (31, 32). Therefore, it is possible that the peroxidative effects of Cr(VI) may stimulate signal transduction pathways, resulting in the activation of MAP kinases. Using the peroxide-sensitive fluorescent probe DCFH-DA and flow cytometry, we found that hexavalent chromium, but not the trivalent form of this metal, is an effective inducer of peroxide generation in H4 cells, with 100 μM Cr(VI) resulting in an approximately 3.5-fold increase in cellular peroxides relative to control basal levels (Fig. 3). Furthermore, the dose-dependent production of cellular peroxides by Cr(VI) was correlated with Cr(VI)-induced ERK1 and ERK2 activation (compare Fig. 3 to Fig. 1A). If, in fact, the activation of these kinases by Cr(VI) is mediated by peroxides or other reactive oxygen species, then ERK1 and ERK2 activation by this element should be sensitive to the redox status of the cell. This possibility was examined by pretreatment with agents known to alter the cellular redox potential. Here we characterized the effect of Cr(VI) on ERK1 and ERK2 activity in cells pretreated with BSO, an agent that depletes cellular stores of glutathione by inhibiting GSH synthesis (33). In these studies, relative cellular levels of glutathione were determined using the GSH-specific fluorescent probe, MBCL, and flow cytometry. We found that an 8-h treatment of H4 cells with 10 μM BSO decreased cellular GSH levels 70% relative to untreated cells.

Fig. 3. Effects of chromium on cellular peroxides. H4 cells were incubated with the peroxide-sensitive dye, DCFH-DA (10 μM) for 15 min and then treated with increasing concentrations of either Cr(VI) or Cr(III) for an additional 20 min. Peroxide production in treated cells was then quantified using flow cytometry. The position of each data point on the Y axis represents the average mean relative (peroxide-dependent) fluorescence of three independently prepared samples. The SD for all points on the graph was <3%. Control cells had a mean relative fluorescence of 1.22 (data point not shown). Similar results were obtained in four separate experiments.
Fig. 4. Effects of BSO and NAC on cellular GSH levels, Cr(VI)-induced peroxide production, and MAP kinase activation. Plates of H4 cells were treated with either the glutathione-depleting agent, BSO (10 μM for 8 h) or the glutathione precursor, NAC (10 mM for 24 h) in serum-containing medium. A, GSH levels were determined in control H4 cells (A/7) and cells pretreated with either BSO (A///Ì) or NAC (A/Vi) using the GSH-specific dye MBCL and flow cytometry. In these studies, NEM-treated cells were used to determine background dye binding (A/). B, basal, Cr(VI)- or PMA-induced peroxide levels were also determined in these cells using DCFH-DA and flow cytometry as described in Fig. 3. C, MAP kinase activity in H4 cells pretreated without or with BSO (10 μM for 8 h) was determined following treatment with increasing concentrations of Cr(VI), as indicated. D, MAP kinase activation by either Cr(VI) (100 μM for 1 h) or PMA (160 nM for 30 min) was determined in cells treated without (Lanes 1-3) or with NAC (Lanes 4-6).

Fig. 4A). Cells treated with the GSH-depleting agent alone produced higher basal levels of peroxides compared to control cultures (Fig. 4B). Furthermore, these cells displayed basal ERK1 and ERK2 activity that was significantly higher than control levels (Fig. 4C). Apparently, the elevated basal peroxide production of BSO-treated cells has the ability to modulate the phosphorylation of various key proteins in signal transduction that results in the activation of MAP kinases. As expected, Cr(VI) was more effective at activating MAP kinases in GSH-depleted cells, compared to cells with normal levels of GSH. The increased ability of Cr(VI) to activate MAP kinase in GSH-depleted cells was associated with a corresponding increase (2.7-fold) in Cr(VI)-induced peroxide production in these cells relative to control. In other studies, Cr(VI)-induced activation of MAP kinases was examined in cells that were pretreated with the GSH precursor, NAC (34, 35). Here, NAC treatment (10 mM for 24 h) increased cellular levels of GSH 5-fold, relative to control (Fig. 4A). NAC pretreatment also inhibited the ability of Cr(VI) to generate peroxides as well as to activate ERK1 and ERK2. (Fig. 4, B and D.
This lag period was found to represent the time required for NAC to inhibit Cr(VI)-mediated activation of these kinases was delayed approximately 2 h (data not shown). This lag period was found to represent the time required for NAC to affect de novo synthesis of GSH. In these studies, NAC treatment induced an increase in cellular GSH levels that was initially observed following a 2-h incubation and extending over a 24-h period. It was following this lag period (i.e., the period between the point of NAC addition and the detection of significant increases in cellular GSH levels) that the effect of Cr(VI) on ERK1 and ERK2 activation was attenuated. These results, together with those from studies involving BSO (described above), suggest that activation of MAP kinases by Cr(VI) occurs by a mechanism that is sensitive to the redox status of the cell. In these studies, activation of ERK1 and ERK2 by Cr(VI) could also be differentiated from activation by PMA. For example, activation of these kinases in H4 cells by PMA was not associated with increases in cellular peroxides and was not significantly influenced by elevated cellular GSH levels (Fig. 4, B and D).

**DISCUSSION**

In the current study, we demonstrate that Cr(VI) activates MAP kinases ERK1 and ERK2, which are key regulatory kinases common to many cellular signaling cascades. The activation of these kinases by Cr(VI) was found to be dependent on the ability of this element to generate cellular peroxides as evidenced by the fact that Cr(VI)-induced activation of the MAP kinases was sensitive to the redox status of the cell. Experimental manipulation of cellular glutathione levels had a dramatic effect on the ability of Cr(VI) to induce activation of ERK1 and ERK2. The depletion of cellular GSH levels was associated with a concomitant decrease in the concentration of Cr(VI) required to activate these kinases. In contrast, the elevation of GSH levels was found to prevent ERK1 and ERK2 activation by Cr(VI). GSH serves as a major component of cellular antioxidant defense mechanisms and is known to scavenge reactive oxygen intermediates and prevent peroxide-mediated sulfhydryl oxidation (36). Therefore, these observations suggest that oxidant-mediated modification of a positive modulator of ERK1 and ERK2 by Cr(VI)-induced reactive oxygen species may result in the observed activation of these kinases. The ability of oxidants to stimulate other signaling cascades have been associated with the ability of these agents to oxidize critical thiols on components of signal transduction pathways (31, 37-39). Interestingly, Ras, a positive modulator of MAP kinase, is thought to be a direct target of reactive free radicals (40). Oxidants such as H2O2 and nitric oxide increase the signaling capacity of this molecule in vitro by mechanisms that are thought to involve modification of critical thiols (41). Therefore, it is possible that this redox-sensing signaling molecule mediates the activation of MAP kinase in response to Cr(VI). However, there are numerous pathways by which MAP kinases can become activated. The specific pathway/signaling molecule modulated by oxidation in this manner, which would result in the subsequent activation of MAP kinases, is not currently known.

In the present studies, Cr(VI)-mediated ERK1 and ERK2 activation was distinct from activation by PMA. Although Cr(VI) induced a persistent activation of these MAP kinases that was associated with the ability of this agent to generate cellular peroxides, the effect of PMA was transient and not peroxide dependent. Furthermore, Cr(VI), unlike PMA, activated MAP kinases in a PKC-independent manner. Interestingly, other investigators have reported that the stimulatory effect of PMA on MAP kinase activity was dependent on the ability of this agent to induce reactive oxygen intermediates (42). However, these studies were conducted using NIH-3T3 cells, which are known to produce elevated levels of peroxides in response to PMA. Since cellular peroxides can indeed activate MAP kinase, it is likely that PMA-induced peroxides contribute to kinase activation in PMA-treated NIH-3T3 cells. In the current studies using H4 hepatocytes, we clearly demonstrate a PMA-mediated activation of MAP kinases that is neither associated with peroxide production nor significantly influenced by the redox status of the cell.

The activation of MAP kinases by reactive oxygen intermediates has been observed in other cell systems. For example, it has been demonstrated that the reactive oxygen species generated by neutrophils during bacterial killing may be responsible for a redox-dependent regulation of cellular signaling pathways, including the MAP kinase cascade (43). Supposedly, these oxidant-stimulated kinases may be responsible for mediating an appropriate response of granulocytes to invading organisms. In other studies, reactive oxygen intermediates generated within NIH-3T3 cells exposed to X-irradiation were also found to stimulate MAP kinase activity (42). It has been suggested that the activation of MAP kinases in this situation represents a cellular response to radiation. In the current study, we suggest that persistent MAP kinase activation by Cr(VI)-induced reactive oxygen species may contribute to the mechanism of toxicity of this element and its ability to cause cancer. The constitutive activation of positive modulators of MAP kinase function (i.e., Ras, Src, Mos, Raf, MEK, and others) as well as the overexpression of downstream proto-oncogenic components of the MAP kinase cascade (i.e., c-fos) have been linked to the development of the transformed phenotype (44-48). Therefore, the persistent effect of Cr(VI) on the activity of the MAP kinases ERK1 and ERK2 may reflect a molecular mechanism by which this element can alter the growth and differentiation state of cells in a manner that would promote the establishment of transformed cells. Characterization of the effects of Cr(VI)-induced MAP kinase activity on downstream events (i.e., substrate phosphorylation, transcription factor activation, and gene expression) should increase our understanding of the specific cellular processes altered in response to Cr(VI) and their relationship to the development of neoplasms.

The relevance of our observations to established mechanisms of chromium-induced carcinogenesis is not known. Based on the ability of this metal to induce various types of DNA damage, current models of chromate exposure-related carcinogenesis characterize Cr(VI) as a mutagen (19, 20). In this context, cellular GSH is thought to increase the genotoxic and carcinogenic potential of Cr(VI) since reduction of this metal by GSH promotes some types of DNA damage (i.e., DNA strand breaks; Refs. 49 and 50). Conversely, our studies indicate that elevated cellular GSH opposes Cr(VI)-induced MAP kinase activation and would, therefore, preclude the involvement of resulting aberrant signaling in processes of transformation. Studies designed to determine the relative contribution of both actions of Cr(VI) (i.e., genetic/DNA damage and epigenetic/alterations in signal transduction) to carcinogenic processes should lead to a more comprehensive understanding of the manner by which this agent induces tumors in humans.

The present study demonstrates that Cr(VI) induces the persistent activation of ERK1 and ERK2, key regulatory kinases in various signal transduction pathways. Although the mutagenic and genotoxic effects of Cr(VI) is associated with its toxicity and carcinogenicity, our observed effect of this element on MAP kinases suggests epigenetic mechanisms of chromium action.

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