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Chromium (VI) Inhibits Heme Oxygenase-1 Expression In Vivo and in Arsenic-Exposed Human Airway Epithelial Cells

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Abstract

Inhaled hexavalent chromium (Cr(VI)) promotes lung injury and pulmonary diseases through poorly defined mechanisms. One hypothesis for this lung pathogenesis is that Cr(VI) silences induction of cytoprotective genes, such as heme oxygenase-1 (HO-1), whose total lung mRNA levels were reduced 21 days after nasal instillation of potassium dichromate in C57BL/6 mice. To investigate the mechanisms for this inhibition, Cr(VI) effects on basal and arsenic (As(III))-induced HO-1 expression were examined in cultured human bronchial epithelial (BEAS-2B) cells. An effect of Cr(VI) on the low basal HO-1 mRNA and protein levels in BEAS-2B cells was not detectable. In contrast, Cr(VI) added to the cells before As(III), but not simultaneously with As(III), attenuated As(III)-induced HO-1 expression. Transient transfection with luciferase reporter gene constructs controlled by the full length *ho-1* promoter or deletion mutants demonstrated that this inhibition occurred in the E1 enhancer region containing critical antioxidant response elements (ARE). Cr(VI) pretreatment inhibited As(III)-induced activity of a transiently expressed reporter construct regulated by three ARE tandem repeats. The mechanism for this Cr(VI)-attenuated transactivation appeared to be Cr(VI) reduction of the nuclear levels of the transcription factor Nrf2 and As(III)-stimulated Nrf2 transcriptional complex binding to the ARE *cis* element. Finally, exposing cells to Cr(VI) prior to co-exposure with As(III) synergized for apoptosis and loss of membrane integrity. These data suggest that Cr(VI) silences induction of ARE-driven genes required for protection from secondary insults. The data also have important implications for understanding the toxic mechanisms of low level, mixed metal exposures in the lung.

Hexavalent chromium (Cr(VI)) and arsenic (As(III)) are well known environmental and occupational hazards and are often found as mixed contaminants and wastes. Human epidemiological and animal studies associate inhalation or ingestion of these metals with

increased incidence of pulmonary diseases including asthma, chronic obstructive pulmonary disease, interstitial lung disease, and lung cancer (Enterline et al., 1995; Ding et al., 2000; Ferreccio et al., 2000; Rice et al., 2001; Leikauf, 2002; Barchowsky and O'Hara, 2003; Park et al., 2004; von Ehrenstein et al., 2005). It is likely that these metals interact to yield even greater risks of lung diseases or cancers. However, knowledge of the cellular and molecular events linking an individual or combined metal exposure to changes in airway epithelial defense or repair processes that trigger lung disease etiology is limited.

Inducible heme oxygenase-1 (HO-1) is an important cytoprotective enzyme that catalyses the rate limiting step in heme degradation into carbon monoxide (CO), biliverdin, and iron (Morse and Choi, 2002; Carter et al., 2004). In the lung, as in many other tissues, these metabolites generated by HO-1 play an important role in protecting against oxidant and inflammatory injuries stimulated by a range of environmental insults that include inhaled metals, bacterial sepsis, prolonged low or high oxygen tensions, and allergens (Choi et al., 1995, 1996; Almolki et al., 2004; Carter et al., 2004). It is thought that both CO and bilirubin, a metabolite of biliverdin, are the primary protective products in the reaction (Otterbein et al., 1999; Morse and Choi, 2002; Carter et al., 2004). In addition, CO reduces allergen-induced airway inflammation and hyperreactivity by stimulating guanylate cyclase in pulmonary smooth muscle cells (Almolki et al., 2004; Ryter et al., 2004). Loss or reduced expression of HO-1 contributes to several pulmonary diseases including adult respiratory distress syndrome, interstitial fibrosis, chronic obstructive pulmonary disease, and asthma (Carter et al., 2004). Many of these diseases are promoted by inhalation of metals, such as Cr(VI) or metal mixtures (Novey et al., 1983; Sorahan et al., 1987; Leikauf, 2002). However, metals differentially affect *ho-1* induction by eliciting actions in different regions of the *ho-1* promoter (Alam, 1994, 2000; Gong et al., 2001, 2002; Stewart et al., 2003). In addition to having direct effects on signaling molecules, many metals can cause persistent generation of reactive oxygen species, which is postulated to be the ultimate basis for their disease promoting and carcinogenic activity in the lung (Ding et al., 2000). Prolonged increase in inflammatory oxidants is a strong stimulus for *ho-1* induction to protect the lung (Morse and Choi, 2002; Carter et al., 2004).

As(III) is well known to induce *ho-1* through a combination of stress signaling pathways. The induction of HO-1 expression in response to arsenic is a protective response since inhibiting the activity of the induced HO-1 enhances As(III) toxicity (Lee et al., 2005). As(III) is a prime example of a complex environmental toxicant that activates or inhibits signaling pathways in cells through direct actions on protein thiols and through generation of reactive oxygen species (Barchowsky et al., 1996, 1999a,b; Kapahi et al., 2000; Roussel and Barchowsky, 2000; Pi et al., 2003). Consequently, As(III) activates the *ho-1* promoter through multiple *cis*-elements with a dominant requirement for nuclear factor E2 related factor 2 (Nrf2) and CREB proteins binding to the E1 enhancer region (Yu et al., 2000; Gong et al., 2002; Kietzmann et al., 2003). Gong et al. demonstrated that the antioxidant response element (ARE) (a.k.a: stress response element (StRE)) elements in the E1 enhancer are necessary for full As(III)-stimulated transactivation of the *ho-1* promoter (Gong et al., 2002). In certain cells, As(III) stimulates ARE transactivation through stress activated pathways involving upstream activators of both JNK1 and p38 γ (Yu et al., 2000; Kietzmann

et al., 2003). However, other p38 isoforms can inhibit As(III) and oxidant-induced ARE activity (Yu et al., 2000; Kietzmann et al., 2003). This complex signaling causes an array of changes in AP-1 and ATF-2 transcription factor proteins binding to the ARE sites along with Nrf2 to yield differential gene activation states (Gong et al., 2002).

Cr(VI) also increases cJun-N-terminal kinase (JNK) activity and AP-1 proteins binding to DNA (Ding et al., 2000; Barchowsky and O'Hara, 2003; O'Hara et al., 2003). However, Cr(VI) does not increase HO-1 expression in lung cells unless it is added in high toxic concentrations (Dubrovskaya and Wetterhahn, 1998). Concentrations of Cr(VI) needed to activate JNK in human airway cells slightly increase reactive oxygen species levels, but these may be below the threshold for generating significant signaling events (O'Hara et al., 2003). Instead, Cr(VI) stimulates selective members of the Src family kinase (SFK) (O'Hara et al., 2003; Vasant et al., 2003), which are upstream of selective JNK activation (O'Hara et al., 2003), but not activation of ERK or p38 (O'Hara et al., 2003; Ceryak et al., 2004). Despite selective JNK activation and increased AP-1 activity in Cr(VI)-treated cells, it is not clear why HO-1 is not induced by Cr(VI). However, a plausible explanation may involve the known differential effects of Cr(VI) on transcription factor complexes and transactivation in inducible genes.

The experiments presented below were initiated by the observation that in vivo Cr(VI) exposures decreased HO-1 mRNA levels in mouse lungs. Mechanistic experiments in human bronchial epithelial cells tested the hypothesis that low, non-cytotoxic Cr(VI) exposures inhibit inducible HO-1, which may potentiate injury to airway epithelial cells following exposure to As(III). These studies identified transcriptional regulation as the primary site of Cr(VI) action and demonstrate that Cr(VI) prevents ARE-dependent induction of *ho-1*. These novel findings support the hypothesis by confirming that exposure to Cr(VI) sensitizes the lung epithelium to injury from a secondary stressor.

MATERIALS AND METHODS

Cell culture and treatment

Human bronchial epithelial cells (BEAS-2B; ATCC, Rockville, MD) were cultured on a matrix of 0.01 mg/ml fibronectin (Invitrogen, Carlsbad, CA), 0.029 mg/ml Vitrogen 100 (COHESION, Inc., Palo Alto, CA), and 0.01mg/ml bovine serum albumin (BSA) in LHC-9 medium (Invitrogen). The cultures were maintained in LHC-9 medium at 37°C and 5% CO₂, and the medium was changed every 2 days. Unless stated otherwise, 1 day post-confluent cells were exposed to either potassium dichromate (Aldrich Chemical Co., Milwaukee, WI) or sodium arsenite (Fisher Scientific, Pittsburgh, PA) for the indicated times. Concentrations given in the results for exposures are the concentration of the respective metal ions not the formulas of the compounds (e.g., 2.5 μ M potassium dichromate = 5 μ M Cr(VI)).

Inhalation exposures

All experimental exposures were performed in accordance with institutional guidelines for animal safety and welfare. C57BL/6 mice (5–8 weeks of age) (Charles River Laboratories,

Wilmington, MA) were exposed to phosphate buffered saline (PBS) or PBS containing either 0.25 or 0.75 mg/kg Cr(VI) prepared from potassium dichromate via a single daily intranasal instillation for three consecutive days. These doses were based on prior experiments examining cell signaling protein induction in rats (D'Agostini et al., 2002; Izzotti et al., 2002) and are 2–3 orders of magnitude below the LD₅₀ for lung toxicity due to chromium in rodents. The intranasal route of exposure avoids interfering systemic inflammatory responses caused by acid irritation to the skin or mucous membranes cause by chromic acid in inhalation exposures. There is no difference in delivery of the soluble metal since mice are obligate nose breathers. Administration through the nose avoids interfering inflammatory responses caused by the surgery involved in intratracheal instillations. The mice were sedated with Avertin prior to instillation, and the maximum volume of each instillation was 25 μ l. Groups of mice (n = 5–10 per group) were euthanized with 90 mg/kg sodium pentobarbital 21 day following the last instillation. The chest cavity was opened, a polyurethane catheter was inserted into the trachea, and the lungs were instilled with PBS at a pressure of 25 cm water. The right and left lobes were separated by suturing. For histochemistry and histopathological analysis, the right lung lobes from both sham (saline) and Cr(VI)-exposed animals were placed in a tissue cassette overnight in 4% paraformaldehyde before embedding in paraffin blocks. Portions of the left lobe were placed into RNeasyTM (Ambion, Inc., Austin, TX), snap frozen, and stored at –80°C until RNA isolation.

RNA isolation and Real-time RT-PCR

Total RNA was harvested from mouse lung tissue or BEAS-2B cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA (0.5 μ g) was reverse transcribed with random primers and the resulting cDNA was amplified by real-time PCR using the MJ Research Opticon 2 (BioRad, Hercules, CA) with the following primer sets: human HO-1 (F) 5'-CCAGCAACAAAGTGCAAGATTC-3', (R) 5'-CTGCAGGAAGTGGATGCTG-3'; β -actin (F) 5'-GGGACCTGACCGACTACCTC, (R) 5'-GGGCGATGATCTTGATCTTC; mouse HO-1 (F) 5'-CAACAAGCAGAACCCAGTCTA-3', (R) 5'-TCTCCAGAGTGTTCATTTCGAG-3'; HPRT (F) 5'-GCTGGTGAAAAGGACCTCT-3', (R) 5'-CACAGGACTAGAACACCTGC-3'. Each PCR reaction contained 1 $\times$ SYBR[®] Green PCR Master Mix (Applied Biosystems, Foster City, CA), and 0.3 pmol each of forward and reverse primer. Following an initial 10 min incubation 95°C, thermal cycling was performed using 45 cycles of 94°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. Gene expression was quantified using standard curves for the respective cDNA products. All changes in HO-1 cDNA levels were normalized to changes in either β -actin (human) or HPRT (mouse) cDNA. Data are presented as the pg of normalized product per ml of reaction.

Transient transfections

The HO15Luc, E1Luc, E2Luc, (E1 $\pm$ E2)-Luc, p33Luc, and 3XSTRE-Luc plasmids have been previously described (Alam et al., 2000; Gong et al., 2002). BEAS-2B cells were co-transfected with the plasmid of interest and 0.2 μ g of enhanced green fluorescence protein plasmid (eGFP) (Clontech, Palo Alto, CA) using LipofectAMINE Plus (Invitrogen) in LHC-9 medium. After recovering overnight, cells were exposed for the desired amount of

time to Cr(VI) and/or As(III) and harvested for luciferase assays. Transfection efficiency was approximately 20%–30% as determined by GFP fluorescence measured using a fluorescent plate reader (ex 485 nm, em 508 nm).

Luciferase assay

At the end of experimental periods, cells were rinsed three times in PBS and then lysed in luciferase lysis buffer (25 mM glycylglycine, 4 mM EGTA, 15 mM MgSO₄, 1% Triton X-100, 1 mM DTT, 1 mg/ml BSA). Following centrifugation at 13,000g at 4°C for 5 min, lysate was mixed with luciferase assay buffer (25 mM glycylglycine, 15 mM potassium phosphate, 150 mM MgSO₄, 4 mM EGTA, 2 mM ATP, 1 mM DTT). Luciferin (400 μM) was added to each sample and relative light units (RLU) were determined for 20 sec in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). RLU were normalized to GFP readings followed by comparison to control.

Total and nuclear protein isolation

Cells were rinsed twice and then scraped from plates in stop buffer (10 mM Tris, pH 7.4, 10 mM EDTA, 5 mM EGTA, 0.1 M NaF, 0.2M sucrose, 100 μM sodium orthovanadate, 5 mM sodium pyrophosphate, and supplemented with protease inhibitors) and centrifuged at 400g for 10 min at 4°C. Total cell protein was isolated by scraping into lysis buffer (20 mM Tris, pH 7.5, 1% SDS, and supplemented with protease inhibitors). The lysates were boiled for 5 min followed by centrifugation at 13,000g for 5 min. In separate experiments, nuclear proteins were isolated using a modified Dignam's procedure, as previously described (Barchowsky et al., 1996; Shumilla et al., 1999). Sample protein concentrations were determined by the absorbance at 595 nm after addition of Coomassie blue dye (Pierce Biotechnology, Rockford, IL) using BSA as a reference standard.

Western blotting analysis

Western analysis was performed on isolated proteins separated by SDS–PAGE, as previously described (O'Hara et al., 2003). Primary antibodies included antibodies to HO-1 (Stressgen Biotechnologies, San Diego, CA), Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA), and β-Actin (SIGMA, Saint-Louis, MO). Reacted bands were detected by horseradish-peroxidase conjugated secondary antibodies and enhanced chemiluminescence substrates (Perkin Elmer, Boston, MA).

Electrophoretic mobility shift assay

At the end of experimental exposures, cells were rinsed 2× with and then scraped in PBS containing protease inhibitors. Cells were pelleted at 400g for 10 min, the supernatants were removed, and the cell pellets were frozen in liquid nitrogen. Whole cell protein extracts (WCE) were then prepared, essentially as described (Murata et al., 1999; Gong et al., 2002). Cell pellets were resuspended in 25 μl of extraction buffer 1 (20 mM HEPES (pH 7.9), 0.4M KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 25% glycerol, and protease inhibitors) and then lysed by three cycles of freeze thaw on dry ice. The lysates were centrifuged at 100,000g for 5 min and the supernatants were diluted with an equal volume of extraction buffer 2 (20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 25% glycerol, and protease

inhibitors). Reaction mixtures containing 3.5 µg of WCE, 40,000–60,000 CPM of ³²[P]-end labeled double stranded *ho-1* StRE probe (plus: 5'-TTTTCTGCTGAGTCAAGGTCCG-3') and binding buffer (18 mM HEPES (pH 7.9), 80 mM KCl, 2 mM MgCl₂, 10 mM dithiothreitol, 10% glycerol, 0.2 mg/ml BSA, 160 µg/ml poly (dI-dC)) were incubated at 25°C for 20 min. Products were resolved on a 5%, non-denaturing, polyacrylamide gel (PAGE), and detected by autoradiography. Supershift analyses were performed by incubating protein/DNA reaction mixtures as above, and then adding 2 µg of either anti-Nrf2 (H-300) or anti-c-jun/Ap-1 antibody (Santa Cruz) for an additional 2 h incubation at room temperature.

Cell viability and apoptosis assays

The viability of cells following Cr(VI) and As(III) exposures was determined by measuring retention of calcein dye (Molecular Probes, Inc., Eugene, OR). Briefly, cells grown in 12-well plates were untreated or exposed to individual metals for 8 h or to 5 µM Cr(VI) for 2 h followed by co-exposure with 1–5 µM As(III) for an additional 8 h. The cells were then washed twice with PBS, and incubated for 15 min at room temperature in PBS containing 1 µM Calcein AM. After rinsing the cultures, the relative fluorescence of retained dye was measured (ex 495, em 530) in a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA). The effect of co-exposures on the apoptotic endpoint of poly (ADP ribose) polymerase (PARP) cleavage by caspase was used to demonstrate that the combined effects of the metals was on the degree of cell death rather than the rate at which cells die. Cells were exposed to either As(III) or Cr(VI) alone or co-exposed to both metals for 24 h (Cr(VI) added 2 h before As(III)). Total protein was isolated as above and immunoblotting was performed with an antibody that recognizes both total and cleaved PARP (Cell Signaling Technologies, Inc., Danvers, MA).

Statistics

One-way analysis of variance (ANOVA) was used to determine significant differences between treatment groups and controls. If significant differences were proven, then the means of groups were then compared using Newman–Keuls post hoc test or Bonferroni's Multiple Comparison Test. All statistics were performed using GraphPad Prism, version 4.0 (GraphPad Software, San Diego, CA). Data are presented as means ± SD or as a percentage of control values.

RESULTS

Cr(VI) decreased HO-1 mRNA expression in vivo

HO-1 mRNA levels were used as a molecular marker of oxidative stress in the lungs of mice exposed to three daily instillations of either 0.25 mg/kg or 0.75 mg/kg Cr(VI), and allowed to recover for 1 or 21 days. The exposures were designed to mimic occupational exposures to levels of Cr(VI) that were associated with chronic disease, but not acute lethality. The recovery time points were chosen to capture the effects of both early inflammatory reactions and chronic changes in gene expression after Cr(VI) exposures. The images in Figure 1A indicate that after 21 day of recovery lungs from Cr(VI)-exposed mice exhibited no obvious changes in alveolar morphology, relative to sham (saline)-treated controls. A veterinary

pathologist, blinded to the exposures, found some mildly reactive and hyper-plastic bronchiolar epithelium in the mice treated with 0.75 mg/kg Cr(VI). Instead of a normal cuboidal morphology (Fig. 1A sham), some distal airways exhibited tall columnar to “hobnail”-shaped epithelial cells indicative of metaplasia or hyperplasia (Fig. 1A 0.25 or 0.75 mg/kg Cr(VI)). There were no overt signs of inflammation, necrotic zones, or cell death. There were also no differences between the body weights of the shams and the CrVI-exposed animals, indicating lack of general CrVI toxicity (data not shown). At the molecular level, there were no changes in HO-1 expression in 1 day recovery mice, relative to sham mice (data not shown). In contrast, HO-1 mRNA levels were significantly decreased in the 21 recovery day animals exposed to 0.75 mg/kg Cr(VI) (Fig. 1B). These data suggested that Cr(VI) produced prolonged silencing of pulmonary HO-1 expression.

Cr(VI) inhibits As(III)-induced HO-1 expression in BEAS-2B cells

To examine Cr(VI) effects on *ho-1* inducibility in human lung cells, HO-1 mRNA levels were measured in BEAS-2B cells exposed to 5 μ M Cr(VI) over a 10 h period. This exposure period was chosen since there is minimal toxicity associated with this level of Cr(VI) during this time period (Fig. 6 and (Shumilla and Barchowsky, 1999)) and the period allowed for examination of gene inducibility. The basal levels of HO-1 mRNA and protein in the BEAS-2B cells were barely detectable in either untreated or Cr(VI) exposed cells. Due to this low level of expression, a trend towards a decreased basal expression of HO-1 mRNA in the Cr(VI)-exposed cells did not reach significance. In contrast, As(III), a stressor known to induce *ho-1* (Alam et al., 1999; Gong et al., 2002), caused a dose-dependent increase in HO-1 expression over an 8 h period. Pre-incubation of the cells for 2 h with Cr(VI) and then co-exposing them to As(III) for 8 h attenuated, but did not abolish, As(III) induction of HO-1 mRNA and protein levels (Fig. 2A,C). There was no effect of Cr(VI) on As(III)-induced HO-1 mRNA levels when the two compounds were added simultaneously (Fig. 2B). These data are consistent with previous demonstrations of Cr(VI) effects on gene induction in response to cytokines or toxicants (Shumilla et al., 1999; Wei et al., 2004) and suggest that Cr(VI) requires time to modify the inducibility of transcriptional complexes.

Cr(VI) inhibits As(III)-induced HO-1 at the level of promoter transactivation

As(III) induces HO-1 expression at the transcriptional level through well defined enhancer regions in the *ho-1* promoter (Gong et al., 2002). To identify the region of the promoter that was responsible for Cr(VI) silencing of As(III) inducibility, luciferase reporter gene constructs under the control of the full length *ho-1* promoter or promoter deletion mutants were transiently transfected into BEAS-2B cells. Luciferase activity was compared in untreated cells, in cells exposed to Cr(VI) or As(III), and in cells exposed to Cr(VI) prior to co-exposure to As(III) (Fig. 3). The time period for As(III) exposures was extended to 12 h when luciferase expression reached an optimal level for comparisons. The data confirmed that As(III) required elements in the E1 and E2 enhancer regions for full transactivation of the *ho-1* promoter (Fig. 3). Cr(VI) had no effect on basal construct activity over a 12–14 h period. Pre-exposing the cells to Cr(VI) for 2 h and co-exposing them with As(III) for 12 h attenuated As(III) inducibility of both the full length and E2 mutant promoter constructs (Fig. 3). In contrast, there was no effect of Cr(VI) on the As(III) inducibility of the E1 mutant construct (Fig. 3). Eliminating both the E1 and E2 regions reduced As(III)

inducibility in the absence of Cr(VI), which confirms previous reports of the dominance of these enhancers in mediating the As(III) response (Gong et al., 2002).

Cr(VI) inhibits As(III)-stimulated ARE transactivation

The ability of Cr(VI) to inhibit As(III)-induced transactivation by ARE elements was investigated by transiently transfecting the cells with a reporter construct containing three tandem copies of the ARE (p3XStRE) consensus sequences. Consistent with the data in Figures 2 and 3, Cr(VI) had no effect on basal expression of the reporter construct (Fig. 4). In contrast, prior exposure and co-exposure with Cr(VI) significantly inhibited As(III)-stimulated transactivation of ARE reporter. Thus, transcriptional complexes at the ARE elements in the E1 enhancer appear to be the targets of Cr(VI) inhibition.

Cr(VI) attenuated As(III)-stimulated DNA binding of protein to the *ho-1* enhancer ARE

The DNA binding of transcriptional complexes to the *ho-1* ARE *cis* element and the nuclear levels of the ARE-binding transcription factor Nrf2 were examined to determine the mechanism for Cr(VI) silencing of ARE transactivation. First, the time course for As(III)-stimulated nuclear protein binding to a double stranded oligonucleotide probe containing an *ho-1* specific ARE sequence was determined. As seen in Figure 5A, As(III) stimulates DNA binding within 4 h, which is sustained beyond 12–24 h. This time course is consistent with both the endogenous *ho-1* gene expression data in Figure 2 and induction of the luciferase constructs. There was no effect of Cr(VI) on protein binding to the ARE over a 12 h period (Fig. 5B). However, exposing the cells with Cr(VI) for 2 h and then co-exposing them with As(III) for 12 h attenuated As(III)-stimulated ARE binding (Fig. 5B). Supershift analysis demonstrated that the As(III)-stimulated transcriptional complexes contained both Nrf2 and cJun, which is consistent with previous reports of As(III) activation of the ARE/StRE elements in *ho-1* (Gong et al., 2002). Immunoblotting of nuclear protein extracts with antibody specific for Nrf2 demonstrated that there is little basal retention of Nrf2 in the nucleus of control or Cr(VI)-treated cells. Pre-incubation of the cells with Cr(VI) partially inhibited As(III)-stimulated movement to or retention in the nucleus (Fig. 5C), which is consistent with the effect on DNA binding and silencing of gene induction.

Cr(VI) enhances the cytotoxicity of As(III)

To test the hypothesis that Cr(VI) reduces the ability of the BEAS-2B cells to induce a cytoprotective response to As(III), changes in cell viability were measured by the cellular retention of the fluorescent dye calcein AM. Calcein is trapped inside the cell after esterase cleavage to the acetoxymethyl ester side chain and loss of dye retention indicates loss of membrane integrity. The viability of cells exposed to varying doses of either As(III) or Cr(VI) was decreased in a dose-dependent manner after 8 h of exposure (Fig. 6). In combined exposure to the two metals, membrane integrity was synergistically reduced relative to viability in response to either Cr(VI) ($P < 0.05$) or As(III) ($P < 0.01$) alone. It is possible that this analysis measured differences in the rate of cell death rather than the degree of cell death. To address this point, immunoblotting for caspase-cleaved PARP was used to assay for synergy caused by the combined metal treatments. The data in Figure 6B confirmed that either 5 μ M Cr(VI) or 1 μ M As(III) added alone caused minimal PARP

cleavage over a 24 h period, relative to the amount of cleavage caused by 10 μ M As(III). In contrast, pre-incubation of the cells with Cr(VI) and then combined incubation with As(III) for 24 h synergistically increased PARP cleavage.

DISCUSSION

The mechanisms by which Cr(VI) causes pulmonary disease and lung cancer are unclear. One probable mechanism for promotion of lung cell injury by Cr(VI) is suppressed induction of genes, such as *ho-1*, that are essential for cytoprotection and injury repair. The data in the present study support a mechanism whereby Cr(VI) alters transcriptional complexes to suppress *ho-1* expression in vivo, and prevents As(III)-induced *ho-1* transactivation in human bronchiolar epithelial cells. Our studies implicate a novel pathway for the combined toxicity of these two important lung toxicants. These findings contribute to the expanding evidence that Cr(VI) limits protective responses and sensitizes cells to secondary insults by altering the activity of transcription factors or their ability to interact with transcriptional cofactors (Shumilla et al., 1999; Majumder et al., 2003; Wei et al., 2004). In the case of the current study Cr(VI) could be said to increase As(III) toxicity by silencing the induction of HO-1 needed to suppress the toxic effects of As(III) (Lee et al., 2005).

Inhibition of inducible, but not constitutive gene expression is a long-held tenet of Cr(VI) toxicity (Wetterhahn et al., 1989; Shumilla et al., 1998; Majumder et al., 2003; Wei et al., 2004). There is a significant body of evidence that suggests that this effect on inducibility results from formation of Cr-DNA adducts which interfere with efficient formation of transcriptional complexes or polymerase elongation of transcripts (O'Brien et al., 2003; Reynolds et al., 2004). However, in repair proficient cells, it can be argued that these lesions or potential oxidized bases resulting from intracellular Cr(VI) reduction would be rapidly removed and not have a significant effect on gene induction (Hailer-Morrison et al., 2003; Reynolds et al., 2004). In contrast, there is accumulating support for epigenetic mechanisms through which Cr(VI) enhances toxicity or reduces the capacity of cells to respond to environmental hazards. For example, Shumilla *et al.* demonstrated that cytokine-induced IL-8 expression in airway cells was inhibited by Cr(VI) disrupting NF- κ B transactivation (Shumilla et al., 1998). Cr(VI) prevented cadmium induction of MTF-1 driven genes, including metallothionein-1 and 2, as well as a zinc transporter (Majumder et al., 2003). In contrast to the effects of Cr(VI) on As(III) induction of *ho-1* in the current study (Fig. 2), Cr(VI) did not inhibit cadmium-induced HO-1 expression (Majumder et al., 2003). This further confirms the unique signaling caused by exposure to different metals. This signaling may involve differential effects at multiple signaling steps in *ho-1* induction or effects on proteins binding to different regions of the *ho-1* promoter (Alam, 1994; Alam et al., 1999; Gong et al., 2002; Suzuki et al., 2003). Cr(VI) is particularly unique since it has been reported to increase the DNA binding of many of the transcription factors responsible for *ho-1* induction, such as AP-1, Sp1, CREB, NF- κ B (Ye et al., 1995; Chen et al., 1997; Kaltreider et al., 1999; Shi et al., 1999), but does not induce *ho-1* in some lung cells (Dubrovskaya and Wetterhahn, 1998).

In keeping with the large body of literature on preferential silencing of inducible genes by Cr(VI), the data generated in the current study also demonstrated that Cr(VI) had little or no effect on basal HO-1 mRNA levels in cultured cells. However, Cr(VI) did have an effect on the basal expression of HO-1 in the intact lungs (Fig. 1B). The lung is a dynamic organ with multiple interacting cell types and the data may suggest that Cr(VI) silences background *ho-1* induction by either paracrine or autocrine factors. It is possible that culturing cells produces an artifactually lower amount of *ho-1* expression that makes Cr(VI) effects on basal expression difficult to observe. An alternative explanation might be that local high concentrations of Cr(VI) in the intact lung might have resulted in a high level of stress. Exact dosimetric comparison between the in vivo and cell culture exposures is extremely difficult, since Cr(VI) accumulates in the lung within the first cell layers it contacts and concentrates at bifurcation points (De Flora, 2000; O'Brien et al., 2003). High, stressful levels of Cr(VI) have been demonstrated to induce HO-1 mRNA levels in cell lines with a measurable basal production (Dubrovskaya and Wetterhahn, 1998). However, if local stressful levels were achieved in the mouse exposures, evidence of localized injury or cell death should have been apparent. This was not the case. Thus, since the current study is the first to examine Cr(VI) on *ho-1* expression in vivo, much more in depth analysis is warranted to resolve the mechanisms for Cr(VI) effects on this expression and its impact on susceptibility in the intact lung.

The data in Figure 2 indicate that Cr(VI) requires pre-exposure to attenuate *ho-1* inducibility. This is consistent with earlier results demonstrating that Cr(VI) inhibition of TNF-induced IL-8 expression (Shumilla et al., 1999) or benzo[a]pyrene induction of an array of metabolic genes (Wei et al., 2004) required that Cr(VI) be present before the second exposure. Wei *et al.* proposed that Cr(VI) silenced benzo[a]pyrene induction through a mechanism that involved locking histone deacetylase 1 into proximal promoters of induced genes and impairing interaction of the aromatic hydrocarbon receptor with these proximal promoters (Wei et al., 2004). It is possible that responses to As(III) or receptor mediated events (Shumilla et al., 1999; Wei et al., 2004) are more rapid than the effects of Cr(VI) and allow chromatin rearrangement or transcriptional factor complexing to occur before Cr(VI) can affect histone deacetylase activity or lock in the silenced configuration of the promoters. However, this mechanism would not explain how Cr(VI) induces gene expression through other elements (Tully et al., 2000). Further, the data in Figures 4 and 5 confirm that Cr(VI) has additional inhibitory effects at the level of transcription factors, which would complement effects on chromatin remodeling (Wei et al., 2004) or formation of transcriptional complexes (Shumilla et al., 1999; Majumder et al., 2003).

Cr(VI) inhibition of As(III)-stimulated protein binding to ARE elements (Figs. 4 and 5) may contribute to the inhibition of induction through the ARE elements and the E1 enhancer (Figs. 3 and 4). The E1 enhancer site is 268-bp long and contains consensus-binding sequences for ARE, Sp1, CAAT/enhancer binding protein (C/EBP), activator protein-4 (AP-4), and GATA (Alam, 1994; Alam et al., 1995). The ARE sites appear to be required for As(III)-induction of the *ho-1* gene (Gong et al., 2002). However, the protein complexes binding to these sites change composition with time and activation state (Gong et al., 2002). The predominant proteins regulating inducibility by As(III) are CREB and Nrf2 (Gong et al.,

2002). Gong *et al.* used EMSA supershift assays to demonstrate that the proteins binding to the ARE include Nrf2, ATF/CREB, AP-1, and Maf family members and that these family members change in the presence of As(III) to increase promoter activity (Gong et al., 2002). Heterodimer formation with the small MafG protein is essential for Nrf2 to transactivate *ho-1* (Gong et al., 2002). In contrast, heterodimers with the AP-1 family members JunD and JunB may limit promoter activity (Gong et al., 2002). It is plausible that different heterodimers are formed that either induce or silence the *ho-1* promoter and that Cr(VI) contributes to silencing the promoter by preventing Nrf2 translocation to or retention in the nucleus. Since Nrf2 stimulates transactivation of its own promoter (Alam et al., 1999; Kwak et al., 2002), the continued presence of Cr(VI) may have implications not only for prolonged decreases in *ho-1* expression (Fig. 1), but also for the large number of protective genes regulated by Nrf2 (Kwak et al., 2003). Additional studies are needed to determine whether Cr(VI) can affect the composition of heterodimers binding to the E1 enhancer, the duration of these changes in binding partners, and the broad implication of these changes on gene clusters.

In summary, our data support a unique mechanism through which Cr(VI) sensitizes lung cells to secondary insults. Exposure to Cr(VI) enhances the cytotoxic effects of secondary stresses, such as exposure to arsenic. The level of Cr(VI) inhibition appears to be interference with protein partnering that is required for activation of transcriptional complexes or possibly through inhibitory pathways that influence chromatin structure (Wei et al., 2004). These inhibitory effects on transcriptional proteins appear to a common mechanism for Cr(VI) for silencing gene induction (Shumilla et al., 1999; Majumder et al., 2003; Wei et al., 2004). The data presented here are significant in that they are the first to demonstrate that Cr(VI) can specifically inhibit the induction by another metal or metalloid of a cytoprotective gene, such as *ho-1*, in lung. In conclusion, these data suggest that instead of directly promoting injury, a more important mechanism in the pathogenesis of Cr(VI)-induced lung diseases may be the silencing of cytoprotective *ho-1* and other genes that allow the cells to survive additional toxic insults in mixed environmental exposures.

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Abbreviations

HO-1	heme oxygenase-1
Cr(VI)	hexavalent chromium
As(III)	arsenite
CO	carbon monoxide
SFK	Src Family Kinase

JNK	cJun-N-terminal kinase
ARE	antioxidant response element
StRE	stress response element
Nrf2	nuclear factor E2 related factor 2

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Fig. 1.

Cr(VI) decreased HO-1 mRNA expression in vivo. C57BL/6 mice were exposed to Cr(VI) by intranasal instillation with the indicated doses on three successive days. After a 21 day recovery, the animals were euthanized and lung lobes were either perfusion fixed for histological analysis (**A**) or extracted for total RNA (**B**). **A**: Thin, 5 μ m sections of lung tissues were stained with hematoxylin and eosin and imaged by standard light microscopy (original magnifications were 40 $\times$). Images show alveolar ductal regions (ad = alveolar duct; L = lumen of distal bronchiole). **B**: HO-1 and HPRT mRNA levels were measured by quantitative real-time RT-PCR as described in Materials and Methods. Data are reported as mean $\pm$ SD of the pg of HO-1 DNA product per ml of reaction volume corrected for constitutive HPRT mRNA levels (n = 5 to 6 mice/group; ** designates significant difference with $P < 0.01$).

Fig. 2.

Prior, but not simultaneous, exposure to Cr(VI) inhibits As(III)-induced HO-1 expression. BEAS-2B cells were incubated in the presence or absence of Cr(VI), As(III), or combined Cr(VI) and As(III). Following exposures, either RNA (**A**, **B**) or protein (**C**) was isolated for real-time RT-PCR analysis of mRNA levels or Western analysis of protein expression, as described in Materials and Methods. Treatment paradigms were: A, C no treatment (C), 5 μM Cr(VI) for 10 h, As(III) (1–5 μM) for 8 h (open bar), or 5 μM Cr(VI) for 2 h followed by As(III) for 8 h (striped bar). B no treatment (C), 5 μM Cr(VI) for 8 h, As(III) (1–5 μM) for 8 h (open bar), or 8 h exposure to Cr(VI) and As(III) added simultaneously (striped bar).

Values in A and B are mean $\pm$ SD pg of HO-1 PCR product corrected for β -actin expression. Values in C are mean $\pm$ SD arbitrary density units (ADU) from densitometric analysis of immunoblot images. The levels of significance are designated by *, **, and *** for increases above control values at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. ^, ^^, and ^^ designate significant inhibition of indicated As(III) induced HO-1 mRNA or protein levels at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Each treatment was repeated at least three times.

Fig. 3.

Cr(VI) inhibits As(III)-induced HO-1 at the level of transactivation. BEAS-2B cells were transiently transfected with luciferase reporter plasmids under the control of the full length, 1.5 kbp *ho-1* promoter (pHO15luc) and deletion mutants missing the indicated enhancer regions. Twenty four hours after transfection, the cells were left unexposed or exposed to 5 μ M Cr(VI) for 14 h (solid bar), 5 μ M As(III) for 12 h (open bar), or 5 μ M Cr(VI) for 2 h before adding 5 μ M As(III) for an additional 12 h (striped bar). The 12 h exposure period allowed for maximal As(III) induction of luciferase activity. The cells were lysed and luciferase assays were performed as described in Materials and Methods. The data are derived from two separate experiments with triplicate cultures of cells in each experiment. The results are reported as mean $\pm$ SD fold change in relative light units (RLU) of luciferase activity in exposed cells relative to unexposed cells. All As(III) treatments resulted in significant increases in luciferase activity (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). ^^ designates significant inhibition of As(III) induced HO-1 mRNA at the level of $P < 0.01$.

Fig. 4.

Cr(VI) inhibits As(III)-induced ARE transactivation. BEAS-2B cells were transiently transfected with a luciferase reporter plasmid under the control of three tandem ARE sites (p3XStRELuc). Twenty-four hours after transfection, the cells were left unexposed or were exposed to Cr(VI) or As(III) as in Figure 3. The cells were lysed and luciferase assays were performed as described in Materials and Methods. The data are derived from two separate experiments with triplicate cultures of cells in each experiment. The results are reported as mean $\pm$ SD fold change in RLU of luciferase activity in exposed cells relative to unexposed cells. Significant induction of luciferase above control cells is designated by * for $P < 0.05$ and ** for $P < 0.01$. Significant inhibition of As(III)-induced activity is designated by ^ for $P < 0.05$ or ^^ for $P < 0.01$.

Fig. 5.

Cr(VI) inhibits As(III)-stimulated increases in nuclear Nrf2 protein levels. **A:** BEAS-2B cell cultures were either not treated (n.t.) for 24 h or exposed to 5 μ M As(III) for the indicated times before extraction of proteins to determine the amount of transcriptional complex binding to a [32 P]-end labeled double stranded oligonucleotide containing an human *ho-1* ARE element. As a positive control for stress-induced ARE activation, cultures in the last two lanes were exposed to 50 μ M As(III) for 8 h. Protein/DNA complexes were resolved on 5% by non-denaturing PAGE and detected by autoradiography. Each lane represents a separate cell culture. **B:** In a separate experiment, cells were not treated, treated for 12 h with the positive control of 50 μ M As(III) (*), or incubated with indicated combinations of 5 μ M

Cr(VI) and 5 μ M As(III). For the co-exposure of Cr(VI) and As(III), Cr(VI) was added 2 h before adding As(III) for 12 h. In the last two lanes, proteins extracted from cultures in lanes 5 and 6 were incubated with the ARE probe and then with antibody to either Nrf2 or cJun. The bands for transcriptional complexes containing Nrf2 are indicated by the arrows. **C:** Nuclear proteins were extracted from unexposed control cells, cells exposed for 24 h to 5 μ M Cr(VI), cells exposed to 5 μ M As(III) for 4 h, or Cr(VI) for 24 h and then co-exposed with As(III) for 4 h. Nrf2 levels were measured by Western analysis and the results of densitometric quantitation of resulting protein bands are presented. Each bar presents the mean $\pm$ SD of the band density from three separate experiments. Significant difference from control at the level of $P < 0.001$ is designated by ***. The significant decrease of As(III)-stimulated Nrf2 protein is designated by ^^ ($P < 0.01$).

Fig. 6.

Co-exposure to Cr(VI) and As(III) causes synergistic decreases in cell integrity and increases in apoptosis. BEAS-2B cells were exposed to the indicated doses of Cr(VI) or As(III) for 8 h or pre-exposed to 5 μ M Cr(VI) for 2 h before adding As(III) for an additional 8 h. Retention of calcein dye was then used to measure viability, as described in Materials and Methods. These data were performed in triplicate in two independent experiments. Values given are means $\pm$ SD of fluorescence measured at ex 495, em 530. Statistical decreases in dye retention in cells exposed to individual metals are designated by ** for $P < 0.01$ or *** for $P < 0.001$. Statistical differences of the combined treatment from the effect of As(III) alone are designated by ^^^ for $P < 0.001$. **B:** To measure apoptosis, total cell protein was isolated from cell cultures that were untreated or exposed to the indicating amounts of metals for 24 h. For co-exposures, Cr(VI) was added 2 h before As(III). Whole and caspase-cleaved PARP levels were determined by Western analysis using an antibody that recognizes both protein forms.