MOLECULAR CARCINOGENESIS 28:225–235 (2000)

Disruption of Dioxin-Inducible Phase I and Phase II Gene Expression Patterns by Cadmium, Chromium, and Arsenic

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Recent work suggesting that cellular oxidative stress exerts an inhibitory effect on aromatic hydrocarbon receptor (AHR)–dependent gene expression led us to test the hypothesis that pro-oxidant environmental pollutants might alter the induction of detoxification genes by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an AHR ligand. We found that, in mouse hepatoma Hepa-1 cells, TCDD-inducible cytochrome P450, *Cyp1a1*, and nicotinamide adenine dinucleotide phosphate–quinone oxidoreductase (*Nqo1*) mRNA accumulation were differentially affected by cadmium (Cd^{2+}), chromium (Cr^{6+}), and arsenic (As^{3+}). Cadmium or arsenic did not change *Cyp1a1* mRNA levels but did enhance TCDD-inducible levels of *Nqo1* mRNA, an effect that paralleled the ability of these metals to activate a β-galactosidase gene reporter system regulated by an electrophile response promoter element. Chromium inhibited mRNA accumulation for both *Cyp1a1* and *Nqo1*. Manipulation of cellular thiol status did not modify the response to combined chromium-TCDD exposure, suggesting that the response was not caused by oxidative stress. Chromium did not block DNA-binding competence of the AHR and did not have an effect on mRNA stability, but it inhibited *Cyp1a1* gene transcription and the expression of an AHR-dependent luciferase reporter. These data indicate that coexposure to pro-oxidant metals and AHR ligands, which is common in the environment, can disrupt the regulation of phase I and phase II detoxification genes, leading to imbalances in gene expression that may have important consequences for the toxicity of complex mixtures. *Mol. Carcinog. 28:225–235, 2000.* © 2000 Wiley-Liss, Inc.

Key words: aromatic hydrocarbon receptor; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; cadmium; chromium; arsenic; mixtures; oxidative stress; electrophile response element; DNA crosslinks; transcription

INTRODUCTION

The aromatic hydrocarbon receptor (AHR) plays a central role in the toxicity of numerous aromatic compounds by controlling the expression of a battery of detoxification enzymes responsible for their metabolism [1]. The coordinated regulation of these detoxification genes suggests that, to avoid a serious imbalance in detoxification homeostasis, increases in production of reactive metabolites by phase I enzymes need to be accompanied by comparable increases in conjugation of hydrophilic groups by phase II enzymes. Hence, coordinated expression of the genes encoding phase I enzymes, such as the cytochromes P450 (CYP1A1, CYP1A2, and CYP1B1), and phase II enzymes, such as glutathione-*S*-transferase A1 (GSTA1), nicotinamide adenine dinucleotide phosphate–quinone oxidoreductase (NQO1), and UDP-dependent glucuronosyltransferease (UGT1A6), is an important determinant of toxicant fate.

The importance of the AHR in the toxic responses induced by many of its ligands, such as benzo(a)pyrene (BaP) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), has been well demonstrated in mouse strains harboring *Ahr* alleles that encode AHRs of different ligand affinities [2,3] and in *Ahr* knockout mice [4,5], but the role of nonligands as modifiers of these responses has been less well studied. Recent reports have demonstrated that modification of AHR-dependent gene expression can result from oxidative stress, suggesting that coexposure to AHR ligands and pro-oxidant environmental pollutants may disrupt the coordinated regulation of detoxification genes. The precise step in the AHR signaling pathway that is acted on by

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Received 29 December 1999; Revised 17 May 2000; Accepted 7 June 2000

Abbreviations: AHR, aromatic hydrocarbon receptor; CYP, cytochrome P450; Gsta1, glutathione-*S*-transferase A1; Nqo1, nicotinamide adenine dinucleotide phosphate–quinone oxidoreductase; UGT1A6, UDP-glucuronosyltransferase; BaP, benzo(a)pyrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; BSO, L-buthionine (S,R)-sulfoximine; NAC, *N*-acetyl cysteine; EpRE, electrophile response element; LDH, lactate dehydrogenase; SSC, sodium chloride/sodium citrate; SDS, sodium dodecyl sulfate; Ho, heme oxygenase; Sod1, Cu,Zn superoxide dismutase; Mt-I, metallothionein I; Gclr, glutamate cysteine ligase regulatory subunit; AhRE, aromatic hydrocarbon receptor response element; DMSO, dimethylsulfoxide.

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oxidative stress is not clear. Sulfhydryl-modifying agents have been shown to block binding of TCDD to the AHR [6–8] and AHR DNA binding [9,10], suggesting that AHR activation may be directly redox regulated. In addition, H_2O_2 has been shown to inhibit *Cyp1a1* inducibility [11] and disrupt reporter gene expression driven by AHR-responsive promoter sequences [12]. It remains unclear, however, whether these effects are mediated through the AHR or through other transcription factors, such as NF-1, which may be an important determinant of the oxidative regulation of the *Cyp1a1* gene [13,14].

The ability of cadmium, chromium, and arsenic to generate oxidative stress has been well documented [15,16]. Both cadmium and arsenic are able to bind sulfhydryl groups avidly, and changes in the levels of cellular thiols such as glutathione, either directly or through formation of reactive oxygen species, may be an important avenue for the biological activity of these metals [17–21]. In addition, increases in production of reactive oxygen species and lipid peroxidation products have been shown in both cadmium- [19,21–23] and arsenic- [18,20,24,25] treated cells and tissues. Increased formation of reactive oxygen species and reactive chromium intermediates has also been reported after chromium treatment and is thought to result from the intracellular reduction of hexavalent chromium by cellular reductants, such as glutathione or ascorbic acid [26–30]. Based on these diverse mechanisms of oxidative stress generation, we chose to evaluate markers for cellular oxidative stress encompassing multiple pathways, including glutathione depletion and induction of oxidative stress–responsive genes.

Many sources of environmental exposure to AHR ligands involve metal coexposure. For example, carcinogenic metals and AHR ligands are common contaminants of hazardous waste sites and are coreleased from sources such as fossil fuel combustion and municipal waste incineration and as components of cigarette smoke. Estimated human tissue concentrations in the low micromolar range for cadmium, chromium [31,32], and arsenic [reviewed in 33] have been reported, even in individuals without known exposure, thus confirming the high prevalence of background exposure. Based on the potential for exposure and serious nature of their toxic effects, cadmium, chromium, arsenic, BaP, and polychlorinated biphenyls, including the AHR-activating mixture arochlor 1254, continue to be high priorities for environmental remediation [34].

To investigate further the potential interactions between these environmental pollutants, we tested the effect of cadmium, chromium, and arsenic on AHR-dependent gene expression and on oxidative stress responses in mouse hepatoma Hepa-1 cells. We found that metal coexposure led to changes in the expression patterns of detoxification genes induced by TCDD.

METHODS

Cell Lines, Growth Conditions, and Chemical Treatments

Mouse hepatoma Hepa-1 cells were seeded to approximately 25–35% confluence and grown overnight in α-minimal essential medium (GibcoBRL Life Technologies, Grand Island, NY) containing 5% fetal bovine serum and 1% antibiotics, as described previously [35]. Cadmium chloride, potassium dichromate, and sodium arsenite (hereinafter referred to simply as cadmium, chromium, and arsenic; Sigma Co., St. Louis, MO) were dissolved fresh in sterile deionized water. L-buthionine (S,R)-sulfoximine (BSO) and *N*-acetyl cysteine (NAC) were dissolved in serum-free medium and stored at −20°C until use. Specific treatment regimens are described in the captions for each figure.

To assess AHR-dependent responses, we used a Hepa-1–derived cell line stably transformed with the luciferase reporter gene regulated by the AHR-binding domain in the promoter of the mouse *Cyp1a1* gene, pAhRDTKLuc3. The pAhRDTKLuc3 plasmid was constructed by subcloning three AHR-binding elements from the C57BL6 mouse *Cyp1a1* gene promoter (base pairs −1100 to −869) and the thymidine kinase minimal promoter into the reporter plasmid pGL3 basic (Promega, Madison, WI) [35]. Similarly, to determine electrophile-dependent responses, we used a Hepa-1 cell line stably transformed with a β-galactosidase–neomycin-resistance reporter plasmid, pEpREβgeo. The pEpREβgeo plasmid was generated by subcloning a single electrophile response element (EpRE) from the mouse *Gsta1* enhancer (base pairs −754 to −714) [36] in front of the metallothionein gene minimal promoter to drive expression of a β-galactosidase- and neomycin-resistance gene. These stably transfected cell lines were grown under conditions similar to those of Hepa-1 cells, with the addition of geneticin for cell selection.

Cytotoxicity Analysis

The effect of metal and TCDD treatments on cell viability was determined by measuring lactate dehydrogenase (LDH) release into the medium. Cells were plated and grown for 24 h in tissue culture medium as described above, the medium was subsequently removed, and the cells were rinsed twice with phosphate-buffered saline. Fetal bovine serum has significant LDH activity, and phenol red absorbs strongly at 420 nm even in the absence of cells. As a result, for the metal treatments, the medium was replaced with D-minimal essential medium without phenol red and supplemented with 0.25% fetal bovine serum, 2.5 mg/mL bovine serum albumin, and 1% antibiotics. Positive con-

trols included cells incubated in sterile water for 30 min before the addition of medium. Treatments were added to the medium, and 100 μL of medium was removed from each culture at 12 and 24 h after treatment. LDH activity in the medium was determined by using a TOX-7 assay kit as recommended by the manufacturer (Sigma Co.).

RNA Isolation and Northern Blot Analysis

RNA was isolated with Tri-reagent (MRC, Inc., Cincinnati, OH). Northern blotting and hybridizations were performed as described elsewhere [37]. Briefly, aliquots of 5–10 μg of total RNA were separated in a 1% agarose gel and transferred to NYTRAN Plus membranes (Schleicher & Schuell, Keene, NH) in 20 × sodium chloride/sodium citrate (SSC; 3 M NaCl and 0.3 M sodium citrate, pH 7.0). Blots were incubated for 1 h at 65°C in prehybridization buffer (0.45 M NaCl; 90 mM Tris-HC; pH 8; 6 mM EDTA; 0.1% sodium dodecyl sulfate (SDS); 2 mg/mL each Ficoll, polyvinylpyrrolidone; and bovine serum albumin; and 250 μg/mL yeast tRNA) and hybridized in the same buffer for at least 12 h with 1×10^6 dpm/mL of the appropriate probes. For a *Cyp1a1* probe, we used the StuI fragment derived from the 3′ untranslated region of the mouse cDNA. For a *Nqo1* probe, we used the full-length mouse cDNA, as described elsewhere [38]. Probes for heme oxygenase (*Ho*) and Cu,Zn superoxide dismutase (*Sod1*) were prepared from full-length rat cDNA clones, and the probe for metallothionein I (*Mt-I*) was prepared from a mouse cDNA clone, as described previously [39]. The primers GATTTGGTCAGGGAGTTTCC (sense) and AATGCAGTCAAATCTGGTGG (antisense) were used to amplify cDNA sequences of the regulatory subunit of glutamate cysteine ligase (*Gclr*) to generate a probe corresponding to nucleotides 180–607 relative to the translation initiation site. After hybridization, blots were washed in 0.3 × SSC and 0.1% SDS until the background was negligible and visualized by exposure to X-omat-AR film (Kodak, Rochester, NY). Intensity of the resulting signal was quantified on a Storm 860 phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Electrophoretic Mobility Shift Assay

Nuclear extract preparation and binding conditions were performed as described previously [40]. Briefly, 15 μg of nuclear protein extract was preincubated in binding buffer (10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 240 mM KCl, 1 μg of poly[dI-dC], and 20 mM HEPES, pH 7.8) for 15 min at room temperature. An AHR response element (AhRE) double-stranded probe end-labeled with [γ-^{32}P]ATP (Amersham Life Science, Arlington Heights, IL) was added (10 000 dpm/reaction), and the reaction was incubated for an additional 15 min at room temperature. The reaction products were separated by electrophoresis in 4.5% nondenaturing acrylamide gels. Gels were dried at 80°C for 1 h and exposed overnight to X-omat-AR film.

Nuclear Run-On Assays

Nuclei were prepared as described elsewhere [41]. Briefly, cells were harvested in ice-cold phosphate-buffered saline, spun down at 500 × *g*, and resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4; 3 mM $CaCl_2$; and 2 mM $MgCl_2$). The cells were recentrifuged as previously described and resuspended in 2 mL of lysis buffer and 2 mL of the same lysis buffer containing 1% NP-40. The cells were disrupted by 10 strokes in a dounce homogenizer, and the nuclei were pelleted by centrifugation as before. The supernatant was removed, and the nuclei were resuspended in storage buffer (50 mM Tris-HCl, pH 8.3; 40% glycerol; 5 mM $MgCl_2$; and 0.1 mM EDTA) and stored at −70°C. The hybridization targets were the full-length *Cyp1a1* cDNA [42] and, for actin, was derived from rat cDNA [43]. The hybridization targets were denatured in 100 mM NaOH for 30 min at room temperature, and 5 μg of each DNA was applied to a NYTRAN Plus membrane by filtration. Blots were neutralized with 6 × SSC, and the DNA was crosslinked to the support by ultraviolet irradiation. Run-on reactions were conducted as described previously [37], with minor modifications. For labeling of newly transcribed RNA, approximately 5×10^7 nuclei in 100 μL were added to 100 μL of 2 × reaction buffer (10 mM Tris-HCl, pH 8; 5 mM $MgCl_2$; 0.16 M KCl; 500 μM each ATP, GTP, and CTP; 5mM dithiothreitol; and 150 μCi/μL [α-^{32}P]UTP). The reaction was incubated at 30°C for 30 min and was stopped by the addition of 30 U of RQ1 DNase (Promega). After incubation for 10 min at 37°C, 600 μL of stop solution (2% SDS, 7 M urea, 0.35 M LiCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 8) was added. The reaction was incubated for 1 h at 56°C, and the labeled RNA was isolated by subsequent trichloroacetic acid and ethanol precipitations. Hybridization was performed in the same buffers as for Northern blot analysis, with 7.5×10^6 dpm/mL for 30 h at 65°C.

Statistical Analysis

Group comparisons were made by one-way analysis of variance. Differences were considered significant at $P < 0.05$.

RESULTS

Dose- and Time-Dependent Decreases in Cell Viability Generated by Metal Treatments

Studies of the biologic mechanisms of toxicity of cadmium, chromium, and arsenic in the existing literature have employed a wide dose range, perhaps reflecting different species and tissue sensitivity. To determine the appropriate concentrations for use in

our cell model (Hepa-1 cells), we evaluated the effect of metal treatments on release of LDH activity in the cell-culture medium relative to that of positive controls of cells lysed by hypotonic disruption. The cytotoxicity of the metals was dose and time dependent. After a 12-h incubation, only 50 μM chromium increased LDH activity. In contrast, treatment for 24 h resulted in significantly increased LDH release at 50 μM cadmium, 50 μM chromium, and 25 μM arsenic (Figure 1). In general, the cytotoxicity assay results corresponded well with direct observation of the cells because no changes in cell morphology were noted in any of the gene expression or reporter gene experiments after cadmium or chromium treatments at these doses for up to 8 h. In some cases, concentrations of 10 μM or greater of arsenic for 8 h increased cell rounding and the number of detached cells, although no effect of arsenic was noted for shorter treatment durations. These results indicate that, under the conditions of the treatments reported in this study, the effects of metals were not likely due to decreases in cell viability, although exposure of Hepa-1 cells at the higher doses could result in overt cytotoxicity for longer exposures.

Altered TCDD-Inducible mRNA Levels with Metal Cotreatment

To test the effect of metals on TCDD-inducible gene expression, *Cyp1a1* and *Nqo1* mRNA accumulation were measured in cells treated with 1 nM TCDD plus different concentrations of cadmium, chromium, or arsenic. To preclude effects of treatment due to cytotoxicity, cells were exposed to pro-oxidants for a maximum of 4 h. TCDD treatment induced both *Cyp1a1* and *Nqo1*, an effect that was

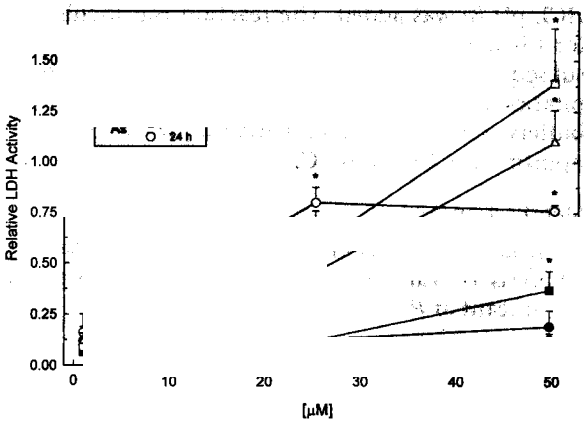

Figure 1. LDH release from metal-treated Hepa-1 cells. Cells were treated with cadmium, chromium, or arsenic at the indicated concentrations for 12 h or 24 h, and LDH activity was determined relative to cells incubated for 30 min under hypotonic conditions. Each point represents the mean ± SD of duplicate samples from two independent experiments. The relative LDH activity was compared with the low dose for each treatment, and significant differences ($P < 0.05$) are denoted with asterisks.

significantly decreased by 50 μM chromium to 23% and 36% of TCDD-inducible levels for these two genes, respectively (Figures 2 and 3). Cadmium or arsenic treatment had no effect on *Cyp1a1* induction by TCDD. Cadmium at 40 μM and arsenic at 3 μM and 10 μM appeared to increase the TCDD-inducible levels of *Nqo1* mRNA, but the magnitude of the enhancing effect of the metals was variable.

We also determined the ability of these metals to induce characteristic oxidative stress and metal responses by measuring mRNA levels of *Gclr*, *Ho*, and *Mt-I* (Figures 2 and 3). The effects of cadmium plus TCDD treatment on *Gclr* were similar to those observed for *Nqo1*, with cadmium generating only a weak induction (2.1-fold) at 40 μM that was not significantly different from TCDD treatment alone. Arsenic was a potent inducer of *Gclr*, with treatment at 10 μM plus TCDD resulting in a 28-fold increase in mRNA levels versus TCDD treatment alone. Chromium treatment had no effect on *Gclr* mRNA. The levels of *Ho* mRNA were greatly increased, 39-fold, over TCDD treatment by 10 μM arsenic plus TCDD and were not affected by the other metals. *Mt-I* mRNA levels were significantly increased by either 20 μM cadmium or 30 μM arsenic, whereas chromium treatment had no effect. It is unlikely that TCCD had any effect on the observed induction of oxidative stress or metal-responsive genes by cadmium (*Mt-I*) or arsenic (*Gclr*, *Ho*, and *Mt-I*) because TCDD treatment alone did not affect expression of these genes and increased mRNA levels were also observed for the metal-only treatments. In all cases, levels of *Sod1* mRNA remained unchanged, indicating that the observed mRNA changes for other genes were not the result of nonspecific changes in mRNA expression or differences in gel loading.

To determine whether pro-oxidant treatments induced electrophile-driven responses, Hepa-1 cells stably transformed with an EpRE-responsive β-galactosidase reporter were treated with different metal concentrations (Figure 4). β-Galactosidase activity was increased 4.1-fold by 25 μM cadmium and 5.6-fold by 5 μM arsenic. No response occurred after chromium treatment at any of the concentrations used. These results, which paralleled the pattern of mRNA accumulation for EpRE-regulated genes (*Nqo1* and *Gclr*), suggested that cadmium and arsenic, but not chromium, effectively induced oxidative stress, as indicated by activation of the electrophile response pathway.

Decreased Activation of an AHR-Responsive Luciferase Reporter with Chromium and Arsenic Cotreatment

Induction of *Cyp1a1* has frequently been used as a marker for AHR activation. Because chromium was able to inhibit *Cyp1a1* induction partially and the AHR has been reported to be regulated by redox changes, we tested the effects of metals on induc-

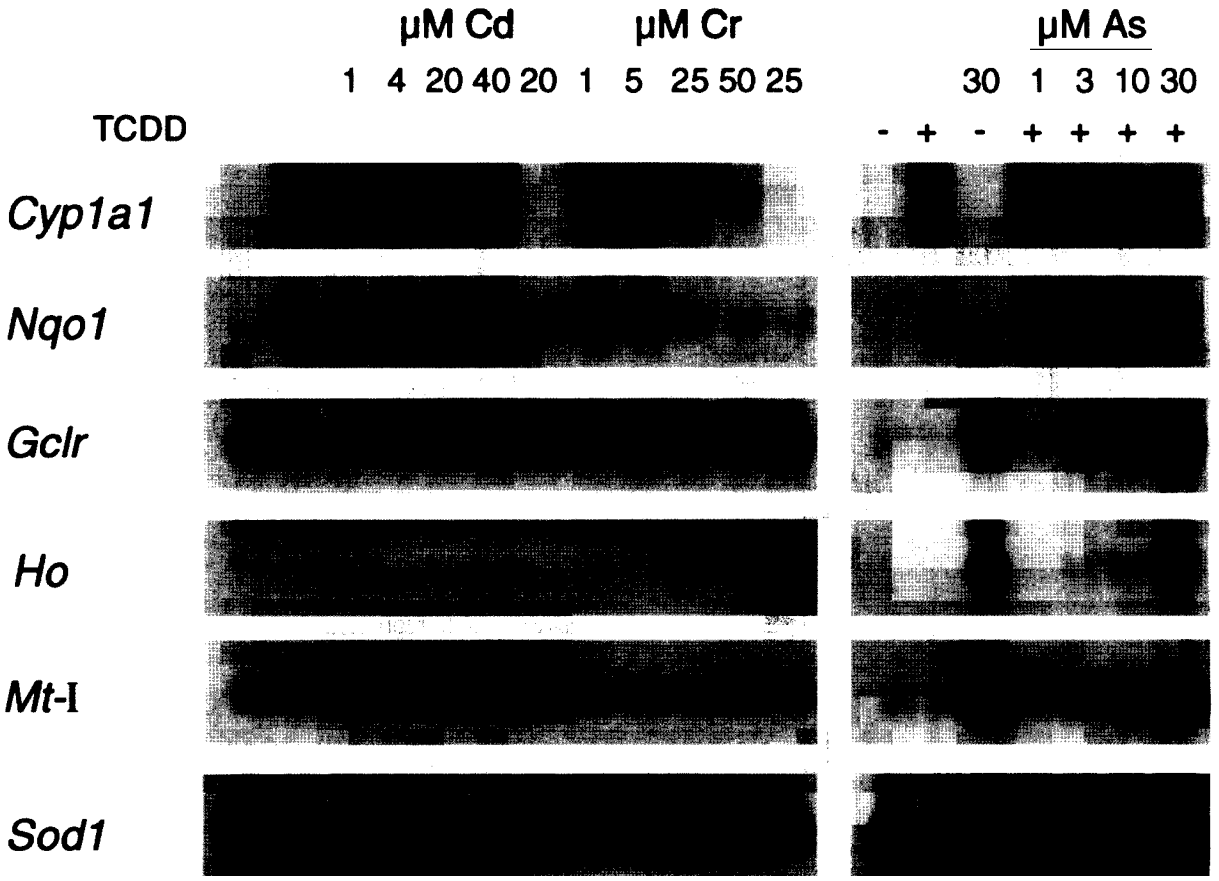

Figure 2. Northern blot analysis of mRNA from TCDD- and metal-treated Hepa-1 cells. Cells were treated with cadmium, chromium, or arsenic at the indicated concentrations for 30 min before cotreatment with 1 nM TCDD for 4 h. Northern blots were performed at least twice with RNA prepared from independently treated cell cultures. One representative result is shown.

tion of an AhRE-responsive luciferase reporter stably transformed in Hepa-1 cells. Treatment of these cells with 100 pM TCDD generated a significant 8.8-fold induction in luciferase activity (Figure 5). This concentration of TCDD causes submaximal gene induction effects and was used to determine whether any of the metal treatments would increase or decrease TCDD-inducible activation of the AHR. Addition of chromium or arsenic 30 min before TCDD decreased TCDD-inducible luciferase activity in a concentration-dependent fashion. Consistent with the northern blot analysis, cadmium did not generate any effect on TCDD-inducible luciferase activity.

We found that chromium inhibited AHR-mediated responses when administered 30 min before TCDD. Chromium generates oxidative stress rapidly [44–46], yet it inhibits BaP-induced mutagenesis when administered simultaneously with BaP but not when added before or after BaP [47]. Hence, analysis of the timing of chromium addition was of particular interest. To test the effect of the timing of chromium treatment on AHR-inducible responses, we measured the expression of AHR-dependent luciferase activity as a function of the time of chromium addition relative to TCDD addition. The greatest decrease in TCDD inducibility, to 14% of TCDD-inducible levels, occurred with the addition of chromium 2 h before TCDD (Figure 6). The effect became less severe when the two reagents were added simultaneously, and only a decrease to 50% of control levels was observed when chromium was added 2 h after TCDD. These results suggest that chromium interferes with an early step of inducible gene transcription.

Redox-Independent Changes in AHR-Responsive Gene Expression

To determine whether changes in AHR-dependent luciferase activity were due to metal-induced oxidative stress, we tested the effect of BSO and NAC addition on AHR-dependent luciferase activity. BSO depletes cellular glutathione levels in Hepa-1 cells under the present dosing regimen [48] and NAC protects against sulfhydryl-binding molecules by direct inactivation of electrophilic species. Therefore, if the effects of these metals on AHR-dependent gene expression had an oxidative stress basis, BSO should enhance the effect and NAC should decrease it. Chromium, at a concentration of 25 μM, inhibited TCDD inducibility to 36% of the level

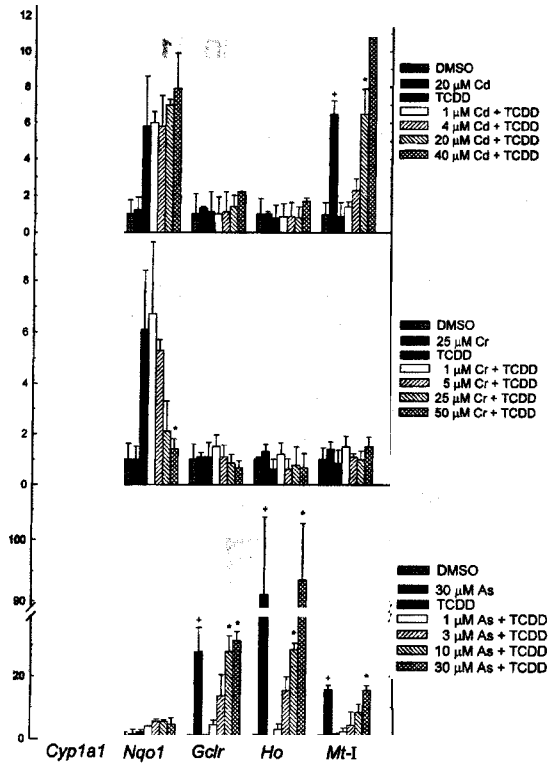

Figure 3. Quantitation of northern blot analysis. Northern blots were performed as described in Figure 1 and were quantitated on a Storm phosphorimager. The resulting band intensities were standardized to *Sod1* to control for gel loading and are presented relative to DMSO controls. Each bar represents the mean ± SD from at least two independent experiments. Metal-only treatments were compared with DMSO controls, and significant differences ($P < 0.05$) are denoted with a plus sign. Metal-TCDD cotreatments were compared with TCDD-treated cells, and significant differences ($P < 0.05$) are denoted with asterisks.

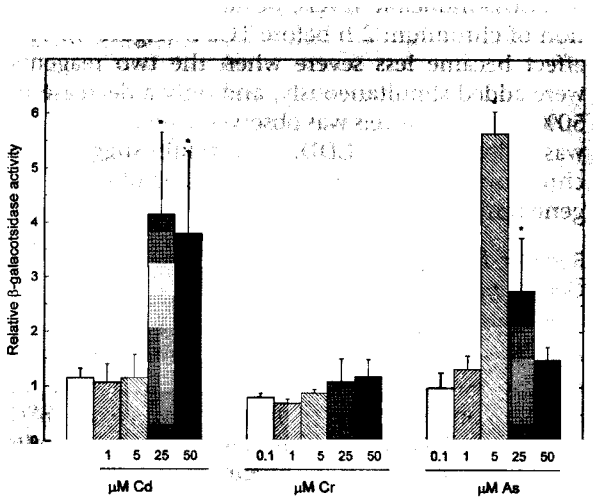

Figure 4. β-Galactosidase activity in Hepa-1 cells stably transformed with pEpREβgeo. Cells were incubated for 6 h in the presence of increasing concentrations of metals as indicated. β-Galactosidase activity was standardized to total protein mass and is presented as the fold increase in activity over untreated cells. Each bar represents the mean ± SD from three independent experiments. Significant differences ($P < 0.05$) from untreated cells are denoted with asterisks.

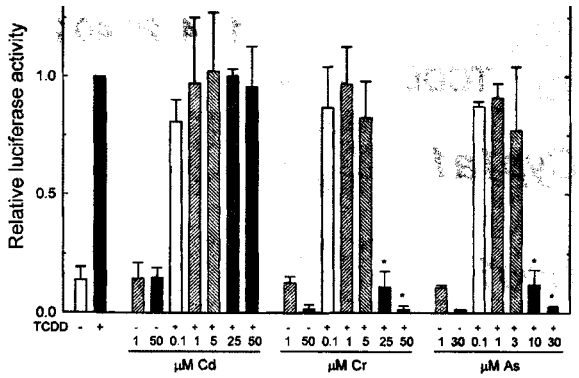

Figure 5. Luciferase activity in Hepa-1 cells stably transformed with pAhRDTKLuc3. Cells were treated with metals for 30 min at the indicated concentration before cotreatment with 100 pM TCDD for 8 h. Luciferase activity was standardized to total protein mass, and the results are reported relative to TCDD alone. Each bar represents the mean ± SD from three independent experiments. Metal-only treatments were compared with DMSO controls, and differences were not significant ($P > 0.05$). Metal-TCDD cotreatments were compared with TCDD-treated cells, and significant differences ($P < 0.05$) are denoted with an asterisk.

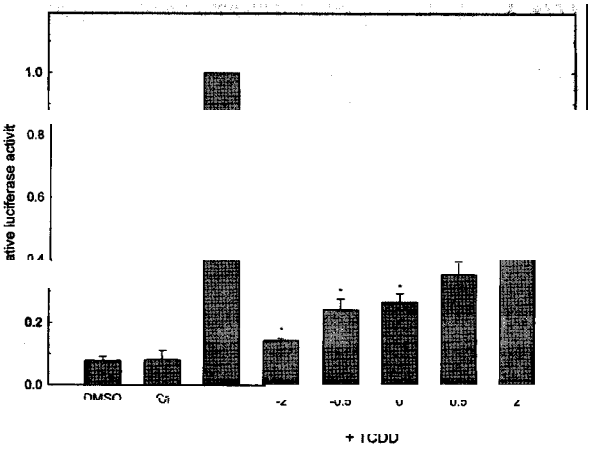

Figure 6. Effect of time of chromium addition on luciferase activity in Hepa-1 cells stably transformed with pAhRDTKLuc3. Cells were treated with 25 μM chromium at different times ranging from 2 h before to 2 h after addition of TCDD to 1 nM. The cells were incubated in the presence of TCDD for 8 h in all cases. Luciferase activity was standardized to total protein mass and is reported in relation to cells treated with TCDD alone. Each bar represents the mean ± SD from two independent experiments. Metal-only treatments were compared with DMSO controls and were not significantly different ($P > 0.05$). Metal-TCDD cotreatments were compared with TCDD-treated cells, and significant differences ($P < 0.05$) are denoted with an asterisk.

that occurred without chromium, and this effect was not altered by either BSO or NAC (Figure 7). In contrast, BSO enhanced the effect of arsenic on the TCDD response, decreasing luciferase activity from 90% to 30% of the levels generated by TCDD alone, whereas NAC had no significant effect. Neither BSO nor NAC treatment changed the lack of effect of cadmium. These findings support the conclusion that arsenic, but not chromium, generated an

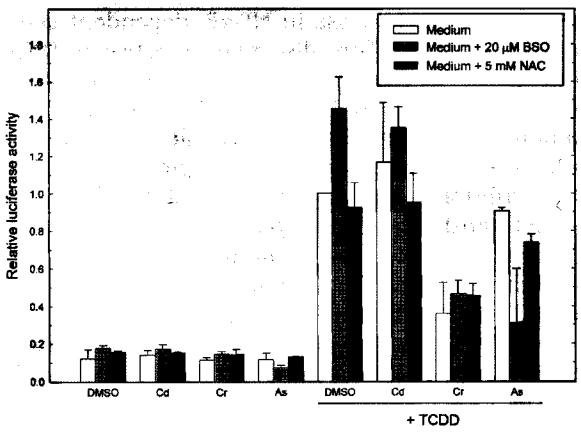

Figure 7. Effect of BSO and NAC on luciferase activity in Hepa-1 cells stably transformed with pAhRDTKLuc3. Cells were treated with 20 μM BSO for 12 h or with 5 mM NAC 2 h before the addition of TCDD to 1 nM. Arsenic (5 μM), cadmium (25 μM), or chromium (25 μM) was added 30 min before TCDD treatment. The cells were incubated in the presence of TCDD for 8 h. Luciferase activity was standardized to total protein mass and is presented in relation to cells treated with TCDD alone. Each bar represents the mean ± SD from two independent experiments. Metal-only treatments were compared with DMSO controls, and differences were not significant ($P > 0.05$). Metal-TCDD cotreatments were compared with TCDD-treated cells, and differences were not significant ($P > 0.05$).

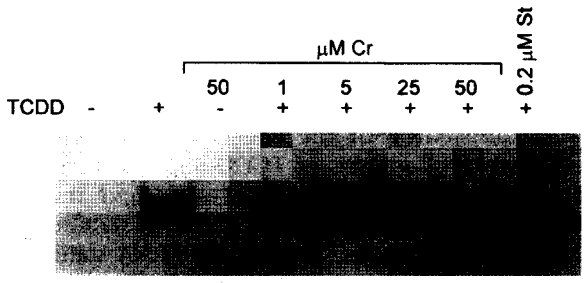

Figure 8. Determination of AHR activation by TCDD in the presence of chromium. Nuclear extracts were prepared from Hepa-1 cells treated with chromium at the indicated concentrations for 30 min or with 0.2 μM staurosporine for 1 h before the addition of TCDD to 1 nM. Cells were incubated for 2 h in the presence of TCDD in all cases. Nuclear extract proteins from cells treated with TCDD alone, TCDD plus increasing concentrations of chromium, or TCDD plus staurosporine were incubated with a ^{32}P-labeled AhRE probe for the electrophoretic mobility shift assays.

oxidative stress response responsible for alterations of AHR-dependent gene expression.

Transcriptional Inhibition of *Cyp1a1* and *Nqo1* by Chromium

The observation that chromium can inhibit inducible gene expression by directly interacting with DNA [reviewed in 26] prompted us to test the effect of chromium on transcriptional regulation of the genes under study. To test the effect of chromium on the DNA-binding competence of the AHR, we used electrophoretic mobility shift assays of nuclear extracts from Hepa-1 cells. TCDD treatment resulted in the formation of DNA-binding competent complexes of the AHR with its dimerization partner the AHR nuclear translocator as demonstrated by the shift of a ^{32}P-labeled AhRE probe. This effect was not altered by cotreatment with different concentrations of chromium (Figure 8). In contrast, a positive control with the protein kinase C inhibitor staurosporine blocked formation of DNA-protein complexes, consistent with pre-

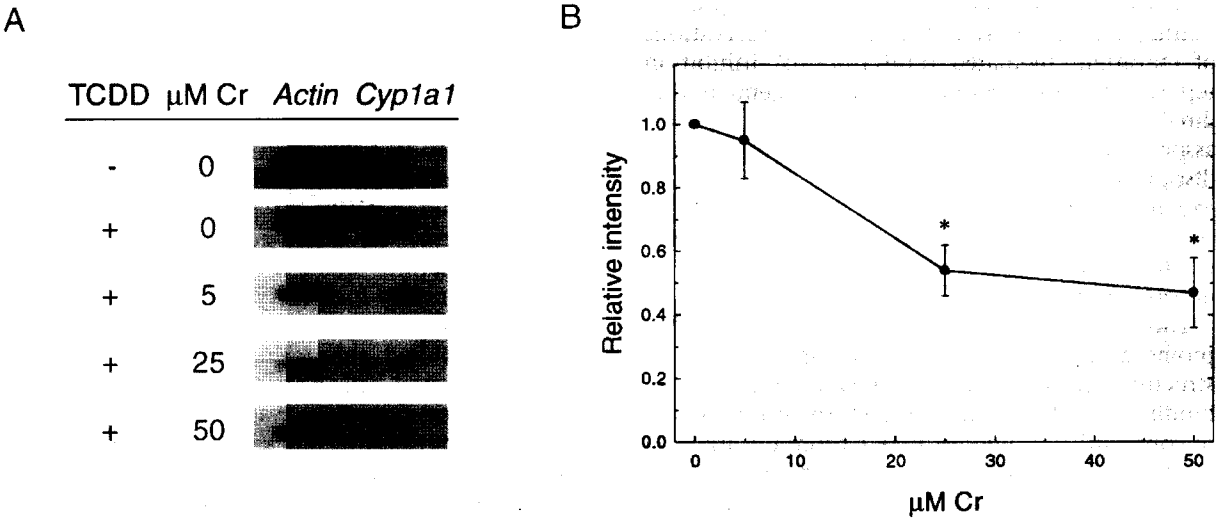

Figure 9. Nuclear run-on analysis of chromium and TCDD-treated Hepa-1 cells. Cells were treated with 0, 5, 25, or 50 μM chromium for 30 min and then cotreated with 1 nM TCDD for 2 h before harvesting for nuclei preparation. (A) Run-on analysis was performed four times; one representative result is shown. (B) Hybridization intensities for *Cyp1a1* were quantified and are shown relative to the intensity of the actin mRNA hybridization for cells receiving the same treatments. Each bar represents the mean ± SD from four independent experiments. Metal-TCDD cotreatments were compared with TCDD-treated cells, and significant differences ($P < 0.05$) are denoted with asterisks.

vious observations [40]. These data suggest that chromium does not impair AHR activation.

The ability of chromium to interact with DNA and selectively inhibit the expression of inducible genes was consistent with the effects observed in the Northern blot analysis of TCDD-responsive genes. Hence, the decrease in *Cyp1a1* and *Nqo1* mRNA levels could be due to changes in mRNA stability or in transcription rates. To determine RNA stability, we measured decay rates of *Cyp1a1*, *Nqo1*, and *Sod1* mRNA in TCDD-treated cells after blocking further transcription initiation with actinomycin D. The *Cyp1a1* mRNA half-life was not significantly affected by treatment with 25 μM chromium and neither were the half-lives of *Nqo1* and *Sod1* (data not shown). However, transcriptional run-on analyses showed that *Cyp1a1* transcription was induced by 1 nM TCDD and that induction was significantly decreased approximately to 50% by 25 or 50 μM chromium (Figure 9A and B). These data suggest that chromium acts by blocking gene transcription and not through direct inactivation of the AHR.

DISCUSSION

In this study we report that treatment with cadmium, chromium, or arsenic can differentially alter the TCDD-inducible expression of two genes, *Cyp1a1* and *Nqo1*, involved in phase I and phase II detoxification, respectively. Chromium decreased TCDD-inducible expression of both genes. The effects of chromium were not modulated by treatment with BSO or NAC, and chromium did not induce expression of *Gclr*, *Ho*, or an EpRE β-galactosidase reporter. Thus, the mechanism of action of chromium on AHR-dependent inducible gene expression does not appear to be related to oxidative stress or to electrophile-driven responses. Rather, our results are consistent with observations of chromium-mediated transcriptional inhibition reported for other inducible genes engendered by direct interactions of chromium with DNA or DNA-associated proteins [49–51]. It seems plausible that disruption of normal chromatin architecture would explain the inhibitory results of chromium in our studies because DNA conformation changes in the *Cyp1a1* gene promoter may in part drive its AHR-dependent induction [52].

The selective action of chromium on inducible promoters may result from a more open chromatin structure that offers a better target for chromium binding than the more closed chromatin structure of constitutive promoters [53]. Chromium-induced DNA-protein crosslinks are found preferentially in association with the nuclear matrix [54]. Because many transcriptional proteins associate with the nuclear matrix, crosslinks between DNA and these proteins may block their function. For example, intracellular reduction of hexavalent chromium, although stimulating DNA binding of NF-κB, did not lead to an increase in NF-κB–dependent gene expression [55]. This discordance between DNA binding of the transcription factor and gene induction was due to the ability of chromium to block the binding of the p65 NF-κB subunit to the cyclic AMP–responsive element-binding protein (p300), a transcriptional coactivator with intrinsic histone acetyl transferase activity [55].

In contrast to the inhibition observed with chromium, both cadmium and arsenic moderately enhanced the effect of TCDD on *Nqo1* mRNA levels. The fact that they did not influence TCDD inducibility of *Cyp1a1* suggests that they act through a mechanism distinct to phase II gene promoters. This observation is consistent with the ability of cadmium and arsenic to induce electrophile-driven responses in our experiments (Figure 4) and as demonstrated by others for the *Nqo1* [56,57] and *Gsta1* genes [36,58,59]. We observed only a limited enhancement of TCDD inducibility in our experiments, possibly because electrophile responses driven by these metals were masked by the high potency of TCDD as a Nqo1 inducer through the AhRE in the promoter of this gene [1] at the concentrations used in our experiments. If this were true, then the effect of these metals on phase I to phase II balance may be more dramatic at lower concentrations of TCDD than might be expected from environmental exposures.

Our results suggest that combined exposure to metals may alter phase I metabolism and phase II conjugation of polycyclic aromatic hydrocarbons. The potential of cadmium, chromium, and arsenic to modify downstream biologic effects, including xenobiotic metabolism resulting from phase I or phase II gene expression, however, has not been well defined and is an area of major interest. Cadmium has been shown to both decrease and increase P450-dependent metabolism [60–62], with responses differing in a dose- and sex-dependent manner. Treatment of cells in culture with BaP and cadmium resulted in additive mutagenic effects [63]. Cadmium has been recently proposed to enhance mutagenicity of Bap-diol epoxide by favoring formation of N2-guanine adducts rather than N7-guanine adducts [64]. Chromium has shown potentially inhibitory effects on AHR ligand inducible responses [65,66] and, in one recent report, inhibited the formation of BaP-induced mutations when cells were treated simultaneously but not when chromium was added before or after BaP [47]. Antioxidants blocked the inhibitory effect of chromium, suggesting that chromium-mediated oxidative stress was responsible for decreased yield of BaP-induced mutations. In vitro, arsenic inhibits ligand-inducible CYP1A1 and CYP1A2 activation [67], but in vivo mixed results have been reported, perhaps because of tissue- and species-specific differences [68,69]. In addition, arsenic and BaP

appear to have a positive interaction on lung tumorigenesis in animals [70,71].

The widely different biologic responses to metal and polycyclic aromatic hydrocarbon coexposures described in the existing literature highlight the need for additional studies to understand the parameters driving these responses. We have shown that metals can modulate biologic responses to AHR ligands at the gene expression level. The results suggest that metal and halogenated aromatic hydrocarbon coexposures represent significantly different health risks than would be anticipated based on evaluation of the toxic mechanisms of each class of compound evaluated singly. By extension, it is likely that the same argument applies to mixtures of metals and polycyclic aromatic hydrocarbon ligands of the AHR.

ACKNOWLEDGMENTS

We thank M. Z. Dieter for the Hepa-1 cell line stably transformed with pEpREβgeo. We also thank P. Gartside and M. Medvedovic for assistance with statistical analysis. This work was supported by NIEHS grants ES06273 and P30 ES06096 and by a US EPA STAR predoctoral fellowship to AM.

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