Regulatory Mechanisms Modulating the Expression of Cytochrome P450 1A1 Gene by Heavy Metals

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We recently demonstrated that heavy metals, Hg^{2+}, Pb^{2+}, and Cu^{2+} induced Cyp1a1 gene expression, yet the mechanisms involved remain unknown. To explore the molecular mechanisms involved in the modulation of Cyp1a1 by heavy metals, Hepa 1c17 cells were treated with the metals in the presence and absence of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a potent Cyp1a1 inducer. Time-dependent effect study showed that all metals significantly induced the basal Cyp1a1 mRNA. This was apparent 3 h after treatment, and levels remained elevated for at least 24 h. At the inducible level, Hg^{2+} and Pb^{2+} further increased, while Cu^{2+} decreased, the TCDD-mediated induction of Cyp1a1 mRNA. The RNA synthesis inhibitor, actinomycin D, completely blocked the Cyp1a1 induction by heavy metals. The protein synthesis inhibitor, cycloheximide, and 26S proteasome inhibitor, carbobenzoxy-L-leucyl-L-leucyl-leucinal (MG-132), superinduced the metal-mediated induction of Cyp1a1 mRNA. In addition, all three metals induced aryl hydrocarbon receptor/xenobiotic-responsive element (AhR/XRE) binding, suggesting an AhR-dependent mechanism. Cyp1a1 mRNA and protein decay experiments showed that the three metals did not significantly affect the half-life of mRNA; however, they significantly decreased the degradation rate of its protein, implying a posttranslational regulation of the Cyp1a1 by the heavy metals. A significant decrease in TCDD-mediated induction of Cyp1a1 activity associated with an increase in HO-1 mRNA and a decrease in cellular heme content was observed after all metals treatment. This suggests that heme degradation plays a role in reducing Cyp1a1 activity. This is the first demonstration that heavy metals can directly induce Cyp1a1 gene expression in an AhR-dependent manner through transcriptional and posttranslational mechanisms.

Key Words: aryl hydrocarbon receptor; Cyp1a1; heavy metals; transcriptional; posttranscriptional.

Cytochrome P450 constitutes a major family of xenobiotic metabolizing enzymes which transform xenobiotics to nontoxic or carcinogenic metabolites (Guengerich, 2004). The cytochrome P450 1a1 (Cyp1a1) is of major interest because of its role in bioactivating procarcinogens and environmental pollutants such as halogenated aromatic hydrocarbons (HAHs) into carcinogenic and mutagenic intermediates (Guengerich, 2004; Shimada and Fujii-Kuriyama, 2004). Current knowledge of the mechanism of CYP1A1 induction by HAHs, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), an ubiquitous environmental contaminant and the most potent Cyp1a1 inducer tested to date, clearly suggests a transcriptional regulation. Thus binding of TCDD to the aryl hydrocarbon receptor (AhR), a cytosolic receptor, is the first step in a series of events leading to HAH-mediated carcinogenicity and mutagenicity (Guengerich, 2004; Pollenz, 2002; Whitlock, 1999).

AhR is a ligand-activated basic helix-loop-helix transcription factor located in the cytoplasm and is bound to two 90-kDa heat-shock proteins (HSP90) and an AhR interacting protein (AIP) (Heid et al., 2000; Ma and Whitlock, 1997; Pollenz, 2002). Activation of the AhR upon binding with a ligand, such as TCDD, causes dissociation of HSP90 and AIP. Subsequently, the ligand–receptor complex translocates to the nucleus, where it heterodimerizes with the transcriptional factor protein, aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR/ARNT complex then binds to a specific DNA sequence, called the xenobiotic-responsive element (XRE) located in the promoter region of the Cyp1a1 gene. This results in the initiation of the mRNA transcription process. Newly synthesized mRNA is then transported out of the nucleus where it is translated into protein (Pollenz, 2002; Whitlock, 1999).

The frequent persistent occurrence and accumulation of heavy metals in the environment, even in traces, and the potential to human exposure from numerous sources, including contaminated air, water, soil, and food, makes heavy metals some of the most hazardous and toxic substances in the...
cupper sulfate, resorufin, and 100× vitamin supplements were purchased from ICN Biomedicals Canada (Montreal, QC). Fetal bovine serum, gentamicin sulfate, L-glutamine, MEM nonessential amino acids solution, penicillin-streptomycin, random primers DNA labeling system, and TRIZol reagent were purchased from Invitrogen Co. (Grand Island, NY). Hybond-N-nylon membranes, Poly(dI-dC), and chemiluminescence Western blotting detection reagents were purchased from Amersham Canada (Oakville, ON). α-32P-DCTP and γ-32P-ATP (3000 Ci/mmol) were supplied by DNA Core Services Laboratory at the University of Alberta (Edmonton, AB). Acrylamide, N',N'-bis-methylene-acrylamide, ammonium persulfate, bromophenol blue, β-mercaptoethanol, glycine, nitrocellulose membrane (0.45 μm), and TEMED were purchased from Bio-Rad Laboratories (Hercules, CA). Cyp1a1 and actin goat polyclonal primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Skim milk was obtained from DIFCO Laboratories (Detroit, MI). Actinomycin D and carbobenzoxy-L-leucyl-L-leucyl-leucinal (MG-132) were purchased from Calbiochem (San Diego, CA). All other chemicals were purchased from Fisher Scientific Co. (Toronto, ON).

**Cell culture and treatments.** Murine hepatoma Hepa 1c1c7 cells (generously provided by Dr. O Hankinson, University of California, Los Angeles) were maintained in DMEM, without phenol red, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin sulfate, 4 mM L-glutamine, 10 mM HEPES, 100 μM nonessential amino acids, and vitamin supplement solution. Cells were grown at 37°C in 75-cm² tissue culture flasks under a 5% CO₂ humidified environment.

For enzyme activity assay, Hepa 1c1c7 cells were seeded in 96-well cell culture plates at a cell density of 7.5 × 10⁴ cells/well in DMEM culture media, and for RNA and protein assays at a cell density of 1–1.5 × 10⁶ cells/well in six-well cell culture plates. The cells were treated in serum-free media with TCDD dissolved in dimethylsulfoxide (DMSO) and/or metals dissolved in double deionized water, in the presence and absence of the chemical inhibitors actinomycin D (Act-D) dissolved in 75% ethanol, CHX dissolved in double deionized water, MG-132 dissolved in DMSO, or the combination of CHX and MG-132. The various metals were added 30 min prior to TCDD treatment or after the addition of chemical inhibitors, whenever applicable.

**RNA extraction and Northern blot analysis.** After incubation with the test compounds for the indicated time periods, total RNA was isolated from the cells using TRIZol reagent (Invitrogen) according to manufacturer’s instructions. Northern blot analysis was performed as described previously (Korashy and El-Kadi, 2004; Sambrook et al., 1989). Briefly, total RNA (20 μg) was electrophoresed on a 1.1% formaldehyde-agarose denaturing gel, transferred to Hybond-N-nylon membranes, and hybridized with a 32P-labeled cDNA probe specific for mouse Cyp1a1 and HO-1 (generously provided by Dr. J. R. Bend, University of Western Ontario, London). The nylon membrane blots were subsequently stripped and rehybridized with glyceraldehyde-3-phosphate dehydrogenase (Gapdh) cDNA probe, which was used as a loading control. The intensity of Cyp1a1 mRNA was quantified, relative to the densitometric signals obtained for Gapdh mRNA (TA Desnitometer, model TBX, Tobias Associates, Inc., Ivyland, PA).

**Protein extraction and Western blot analysis.** After incubation with the test compounds for the indicated time periods, Hepa 1c1c7 cells were collected in lysis buffer (50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 μl/ml of protease inhibitor cocktail). The total cellular proteins were obtained by incubating the cell lysates on ice for 1 h, with intermittent vortexing every 10 min, followed by centrifugation at 12,000 × g for 10 min at 4°C. The supernatant fractions were collected for determination of protein concentration using bovine serum albumin as a standard by the Lowry method (Lowry et al., 1951). Western blot analysis was performed using a previously described method (Korashy and El-Kadi, 2004; Sambrook et al., 1989). Briefly, 25 μg of protein from each treatment group was separated by 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and then electrophoretically transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C in...
blocking solution containing 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris-base (TBS), 5% skim milk powder, 2% bovine serum albumin, and 0.5% Tween-20. After blocking, the blots were incubated with a primary polyclonal goat anti-mouse Cyp1a1 antibody for 2 h at room temperature in TBS solution containing 0.05% (v/v) Tween-20 and 0.02% sodium azide. Incubation with a peroxidase-conjugated rabbit anti-goat IgG secondary antibody was carried out in blocking solution for 1 h at room temperature. The bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham Biosciences Inc., Piscataway, NJ). The blots were subsequently stripped in a solution containing 62.5 mM Tris-HCl pH 6.7, 100 mM 2-mercaptoethanol, and 2% SDS for 30 min at 50°C and thereafter reprobed with actin goat anti-human primary antibody, which was used as loading control, as described above.

**Determination of Cyp1a1 mRNA and protein half-lives.** Two independent experiments, Act-D- and CHX-chase experiments, were performed in order to determine the half-lives of the Cyp1a1 mRNA and protein, respectively. Hepa 1c1c7 cells were grown to 70% confluence in six-well cell culture plates and then preincubated with 1 mM TCDD for 12 or 24 h, to induce mRNA or protein, respectively. After the induction period, cells were then washed three times with fresh media to remove TCDD.

For the Act-D-chase experiment, the cells were incubated in fresh serum-free media containing 5 µg/ml Act-D, a transcription inhibitor, to block any further synthesis of RNA. Thirty min later, 5 µM Hg²⁺, 25 µM Pb²⁺, or 10 µM Cu²⁺ was added. Total RNA was isolated at 0, 1, 3, 6, 12, and 24 h after the addition of Act-D, followed by Northern blot quantification analysis.

For the CHX-chase experiment, the cells were incubated in fresh serum-free media containing 10 µg/ml CHX, a translation inhibitor, to block any further synthesis of protein. Thirty min later, 5 µM Hg²⁺, 25 µM Pb²⁺, or 10 µM Cu²⁺ was added. The cell protein was then extracted at 0, 1, 3, 6, 12, and 24 h after the addition of CHX, followed by Western blot quantification analysis.

In both experiments, Northern and Western blot analyses were quantified by densitometry. Cyp1a1 mRNA and protein half-lives were calculated from the slope of the semi-logarithmically transformed best fit line. The decay curves were analyzed individually using linear regression of mRNA or protein amount, expressed as percentage of mRNA or protein remaining versus time. The half-lives obtained from three separate experiments were then used to calculate the mean half-life (mean ± SEM, n = 3).

**Nuclear and cytosolic protein preparation.** Hepa 1c1c7 cells, plated at 1 × 10⁶ cells/100 mm dish, were grown to 90% confluence and harvested after treatment with 5 µM Hg²⁺, 25 µM Pb²⁺, 10 µM Cu²⁺, or 10 nM TCDD in serum-free media for 3 h. The nuclear proteins were then extracted and prepared as described previously (Rogers and Denison, 2002). Hepatic cytosol of untreated guinea pig (generously provided by Dr. M. S. Denison, University of California, Davis) was prepared as described previously (Brown et al., 2004) and then was incubated in vitro with 5 µM Hg²⁺, 25 µM Pb²⁺, 10 µM Cu²⁺, or 20 nM TCDD for 3 h.

**Gel electrophoretic mobility shift assay (EMSA).** XRE complementary oligonucleotides, 5'-GGAGTTGCGTGAGAAGAGCC-3' and 5'-GGCTCTTC-3', were synthesized, then annealed by heating to 70°C for 7 min, then allowed to cool to room temperature. The double-stranded XRE was then labeled with 3²P-ATP at the 5-end using T4 polynucleotide kinase (Invitrogen), according to the manufacturer’s instructions, and used as a probe for EMSA reactions. EMSA was performed as described previously (Gharavi and El-Kadi, 2005; Rogers and Denison, 2002). Briefly, aliquots of the nuclear extract (10 µg) or cytosolic protein (80 µg) were incubated for 30 min at room temperature in a reaction mixture (30 µl) containing 25 mM HEPES, pH 7.9, 80 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol (v/v), 1 µg salmon sperm DNA, and 5 µg poly(dI,dC). Thereafter, -1 ng (100,000 cpm) 3²P-labeled XRE was incubated with the mixture for another 30 min before being separated through a 4% nondenaturing polyacrylamide gel. For the competition assay, nuclear or cytosolic proteins were preincubated at room temperature for 30 min with a 100-fold molar excess of unlabeled XRE before the addition of the 3²P-labeled XRE. The gel was dried at 80°C for 1 h, and then visualized by autoradiography.

**Determination of Cyp1a1 enzymatic activity.** Cyp1a1-dependent 7-ethoxyresorufin O-deethylation (EROD) activity was performed on intact, living Hepa 1c1c7 cells, using 7-ethoxyresorufin as a substrate, as previously described (Kennedy et al., 1993; Korashy and El-Kadi, 2004). Enzymatic activity was normalized for cellular protein content, which was determined using a modified fluorescent assay (Korashy and El-Kadi, 2004; Lorenzen and Kennedy, 1993).

**Measurement of cellular heme content.** Cellular heme content was determined fluorimetrically as described previously (Ward et al., 1984). Briefly, Hepa 1c1c7 cells were plated in 100-mm Petri dishes for 48 h. The cells were then incubated in fresh serum-free media with 1 nM TCDD in the presence and absence of 5 µM Hg²⁺, 25 µM Pb²⁺, or 10 µM Cu²⁺ for 24 h. Pelleted cells were boiled in 2 M oxalic acid (100 µl) for 30 min and then rapidly resuspended in cold PBS (0.9 ml) followed by centrifugation at 14,000 × g for 15 min. The supernatant was assayed for protoporphyrin IX using Eclipse fluorescence spectrophotometer (Varian Australia PTY LTD., Australia) with excitation and emission wavelengths of 405 and 600 nm, respectively. Cellular heme content was normalized for cellular protein which was determined using Lowry method (Lowry et al., 1951).

**Statistical analysis.** All results are presented as mean ± SEM. The comparison of the results from the various experimental groups with their corresponding controls was carried out by a one-way analysis of variance (ANOVA) followed by Student–Newman–Keul’s test to assess significant differences from the control group. The differences were considered significant when p < 0.05.

**RESULTS**

**Time-Dependent Effects of Heavy Metals on the Constitutive and Inducible Expression of Cyp1a1 mRNA**

To better understand the kinetics of Cyp1a1 mRNA in response to metals, in comparison with TCDD, constitutive and TCDD-inducible Cyp1a1 mRNA contents were measured at various time points (0, 1, 3, 6, 12, and 24 h) following the incubation of Hepa 1c1c7 cells with vehicle, 5 µM Hg²⁺, 25 µM Pb²⁺, or 10 µM Cu²⁺ in the presence and absence of 1 nM TCDD. Figures 1A, 1B, and 1C show that the Cyp1a1 mRNA at various time points tested (data not shown).

Interestingly, the kinetics of Cyp1a1 mRNA in the presence of heavy metals was consistent with those obtained with TCDD, in which the onset of Cyp1a1 mRNA induction mediated by TCDD occurred at 3 h and reached the maximal level (110-fold) at 6 h, followed by a 30% decline of its maximal level (Figs. 1A, 1B, and 1C). Vehicle alone did not significantly alter the Cyp1a1 mRNA at various time points tested (data not shown).

At the inducible level, cotreatment of the cells with TCDD and either Hg²⁺ or Pb²⁺ (Figs. 1A and 1B) further increased the induction of Cyp1a1 mRNA, while TCDD and Cu²⁺ (Fig. 1C) diminished Cyp1a1 induction. A densitometric scan of the autoradiogram indicates that Hg²⁺ or Pb²⁺, in the presence of
TCDD, significantly increased the steady-state Cyp1a1 mRNA level by 30% and 22%, respectively \((p<0.05)\), whereas Cu\(^{2+}\) decreased the steady-state level by about 27% \((p<0.05)\). Taken together, the changes in the steady-state Cyp1a1 mRNA level by heavy metals reflect an alteration in the rate of synthesis and/or degradation of Cyp1a1 mRNA.

**Effect of Heavy Metals on the TCDD Concentration-Dependent Induction of Cyp1a1 mRNA**

To further examine the effect of heavy metals on the kinetics of Cyp1a1 mRNA induced by different concentrations of TCDD, Hepa 1c1c7 cells were treated with 5 \(\mu\)M Hg\(^{2+}\), 25 \(\mu\)M Pb\(^{2+}\), or 10 \(\mu\)M Cu\(^{2+}\) in the presence or absence of 1 nM TCDD for the time point indicated. Total RNA (20 \(\mu\)g) was separated on a 1.1% formaldehyde denaturing gel, transferred to nylon membranes, and hybridized with a \(^{32}\)P-labeled cDNA probe specific for mouse Cyp1a1. The blots were subsequently stripped and rehybridized with a cDNA probe specific for Gapdh, which was used as a loading control. The graph represents the relative amount of Cyp1a1 mRNA (mean \(\pm\) SEM, \(n=3\), and the Cyp1a1 band intensity was quantified by densitometry and normalized to Gapdh levels. One of three representative experiments is shown. *\(p<0.05\) compared with control, and *\(p<0.05\) compared with TCDD.

**FIG. 1.** Time-dependent effects of heavy metals on the constitutive and inducible expression of Cyp1a1 mRNA. Hepa 1c1c7 cells were treated with (A) 5 \(\mu\)M Hg\(^{2+}\), (B) 25 \(\mu\)M Pb\(^{2+}\), or (C) 10 \(\mu\)M Cu\(^{2+}\) in the presence or absence of 1 nM TCDD for the time point indicated. Total RNA (20 \(\mu\)g) was separated on a 1.1% formaldehyde denaturing gel, transferred to nylon membranes, and hybridized with a \(^{32}\)P-labeled cDNA probe specific for mouse Cyp1a1. The blots were subsequently stripped and rehybridized with a cDNA probe specific for Gapdh, which was used as a loading control. The graph represents the relative amount of Cyp1a1 mRNA (mean \(\pm\) SEM, \(n=3\)), and the Cyp1a1 band intensity was quantified by densitometry and normalized to Gapdh levels. One of three representative experiments is shown. *\(p<0.05\) compared with control, and *\(p<0.05\) compared with TCDD.

Transcriptional Regulation of Cyp1a1 Gene by Heavy Metals

Initially, we questioned whether the induction of Cyp1a1 mRNA by heavy metals (Figs. 1A, 1B, and 1C) is regulated at the transcriptional level. Therefore, a series of independent experiments were conducted.

Inhibition of metal-mediated Cyp1a1 mRNA induction by a RNA synthesis inhibitor. To investigate whether heavy metals are able to increase the \textit{de novo} Cyp1a1 RNA synthesis, Hepa 1c1c7 cells were treated with 5 \(\mu\)M Hg\(^{2+}\), 25 \(\mu\)M Pb\(^{2+}\), 10 \(\mu\)M Cu\(^{2+}\), or 1 nM TCDD in the presence or absence of 5 \(\mu\)g/ml Act-D, a RNA synthesis inhibitor, for 6 h. Total RNA was then isolated and quantified by Northern blot analysis. If metals increase the amount of Cyp1a1 mRNA through increasing its \textit{de novo} RNA synthesis, we would expect to observe a decrease in the content of Cyp1a1 mRNA after the inhibition of its RNA synthesis.

Figure 3 shows that in untreated cells, as expected, the amount of Cyp1a1 mRNA was barely detectable (lane 1). However, pretreatment of the cells with Act-D for 6 h completely inhibited the constitutive expression of Cyp1a1 mRNA (lane 2). In metal-treated cells, metals alone significantly increased the Cyp1a1 mRNA level (lanes 3, 5, and 7); however, pretreatment of the cells with Act-D completely
inhibited the induction of Cyp1a1 mRNA in response to metals (lanes 4, 6, and 8). Furthermore, the induction of Cyp1a1 mRNA by TCDD (lane 9), which is known to be a transcriptional event, was completely prevented by Act-D (lane 10). These results suggest that heavy metals increase the Cyp1a1 mRNA level by increasing its de novo RNA synthesis, in a manner similar to that observed with TCDD.

Superinduction of metal-mediated Cyp1a1 mRNA induction by a protein translation inhibitor. To further confirm the transcriptional regulation of the Cyp1a1 by heavy metals, we examined the effect of a protein translation inhibitor, CHX. For this purpose, Hepa 1c1c7 cells were treated for 6 h with 5 μM Hg2+, 25 μM Pb2+, or 10 μM Cu2+ in the presence or absence of 0.1, 1, or 10 nM TCDD, and total RNA was isolated and quantified by Northern blot analysis. If metals increase the transcription rate of Cyp1a1 under these conditions, we would expect to observe an increase in the accumulation of Cyp1a1 mRNA after the inhibition of its protein translation.

As shown in Figure 4, CHX alone caused a significant accumulation of Cyp1a1 mRNA 6-fold greater than DMSO-treated cells (lane 2). However, cotreatment of the cells with CHX and metals further increased the accumulation of Cyp1a1 mRNA by CHX (lanes 4, 6, and 8), a phenomenon known as “superinduction” (Israel et al., 1985; Joakim et al., 2004; Ma et al., 2000). Densitometric scan of the autoradiogram indicates that the rate of Cyp1a1 mRNA synthesis induced by Hg2+, Pb2+, or Cu2+, plus CHX were 2-, 3-, or 4-fold greater than those induced by each metal alone, respectively, and Cyp1a1 mRNA synthesis was about 25%, 37%, or 38% greater than those induced by CHX alone (p < 0.05). Furthermore, the cotreatment of the cells with CHX and TCDD superinduced the increase in Cyp1a1 mRNA level by CHX (lane 10). It should be noted here that, in superinduction studies, the weak
the transcriptional regulation of Cyp1a1. To test the possibility that inhibition of proteasomal-dependent degradation of AhR protein would increase Cyp1a1 transcription in response to metals, Hepa 1c1c7 cells were pretreated with 25 μM MG-132, a potent 26S proteasome inhibitor (Ma and Baldwin, 2000; Ma et al., 2000), with or without 10 μg/ml CHX, both in the presence and absence of Hg²⁺, Pb²⁺, Cu²⁺, or TCDD.

Treatment of Hepa 1c1c7 cells with either CHX or MG-132 alone for 6 h (Fig. 5), caused a 5- and 3-fold increase in the Cyp1a1 mRNA levels (lanes 2 and 3), respectively. Cotreatment of CHX and MG-132, however, did not further increase the accumulation of Cyp1a1 mRNA beyond what was observed with each inhibitor alone (lane 4). On the other hand, treatment of the cells with CHX and metals or MG-132 and metals, superinduced Cyp1a1 mRNA expression. Contrastingly, when the cells were pretreated with CHX and MG-132 in the presence of metals, the superinduction of the Cyp1a1 mRNA was greater than the sum of the increases of Cyp1a1 mRNA that was observed with exposure to each inhibitor alone (lanes 6–8, 10–12, and 14–16). Similar results were observed when the cells were pretreated with CHX and/or MG-132 in the presence of TCDD (lanes 18–20).

Taken together, these results suggest that the increased accumulation of Cyp1a1 mRNA in response to metals is an AhR-dependent pathway.

Activation of AhR/XRE binding by heavy metals. The effect of metals on AhR translocation to the nucleus and the binding to the XRE of the Cyp1a1 was measured by EMSA. Hepa 1c1c7 cells were treated with metals for 3 h, and their nuclear extracts were subjected to EMSA. Extracts from TCDD-treated cells were used as a positive control for AhR transformation. Figure 6 shows that Hg²⁺ or Pb²⁺, but not Cu²⁺, increased the DNA-binding capacity of the nuclear AhR, as shown by the intensity of the bands. The specificity of metal-induced AhR/ARNT heterodimer binding to XRE was confirmed by competition assay in the presence of 100-fold molar excess of unlabeled XRE.

To further assess the ability of heavy metals to directly activate AhR, EMSA was performed on untreated guinea pig hepatic cytosol previously incubated, in vitro, with either metals or TCDD for 3 h. Figure 7 shows that in vitro treatment of cytosol with heavy metals, at the indicated concentrations, induced the transformation of the AhR/ARNT/XRE complex, as determined by the shifted bands, as compared to TCDD. This implies that metals are capable of directly activating the AhR. Taken together, the EMSA results provide strong evidence that the induction of Cyp1a1 mRNA by heavy metals is both a transcriptional and an AhR-mediated event, in a manner similar to what was observed with TCDD.

Posttranscriptional Regulation of Cyp1a1 by Heavy Metals

To further investigate if the observed increase in Cyp1a1 mRNA by heavy metals could be attributed to a posttranscriptional stabilization of the mRNA, Act-D-chase experiments
assessing the half-life of Cyp1a1 mRNA in the presence and absence of heavy metals were performed. If metals do in fact stabilize the Cyp1a1 mRNA, an increase in mRNA half-life should be observed. As shown in Figure 8, Cyp1a1 mRNA decayed rapidly with an apparent half-life of 3.8 ± 0.33 h. In addition, all three metals did not significantly alter the half-life of Cyp1a1 mRNA, indicating that the increase of Cyp1a1 mRNA transcripts in response to heavy metals were not due to a posttranscriptional stabilization of the mRNA.

Posttranslational Regulation of Cyp1a1 by Heavy Metals

The sustained increase in Cyp1a1 mRNA in response to heavy metals (Figs. 1A, 1B, and 1C) prompted a further investigation into whether heavy metals could modify the stability of Cyp1a1 protein. Therefore, the effect of heavy metals on the Cyp1a1 protein half-life was determined using CHX-chase experiments. Figure 9 shows that Cyp1a1 protein is a short-lived protein with an estimated half-life of approximately 2.71 ± 0.02 h (Fig. 9). Interestingly, the three metals significantly decreased the rate of Cyp1a1 protein degradation, implying a posttranslational regulation of the Cyp1a1 by heavy metals.

Inhibition of TCDD-Inducible Cyp1a1 Catalytic Activity by Heavy Metals Is a Heme-Mediated Mechanism

To further examine whether the increases in Cyp1a1 mRNA and protein levels after heavy metal treatments are reflected at the activity level, we investigated the effect of heavy metals on the kinetics of the basal and TCDD-inducible Cyp1a1 activity. Therefore, Hepa 1c1c7 cells were treated with 5 μM Hg2+, 25 μM Pb2+, or 10 μM Cu2+, or 1 nM TCDD for 6 h. Total RNA (20 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to nylon membranes, and hybridized with a 32P-labeled cDNA probe specific for mouse Cyp1a1. The blots were subsequently stripped andrehybridized with a cDNA probe specific for Gapdh, which was used as a loading control. The graph represents the relative amount of Cyp1a1 mRNA (mean ± SEM, n = 3), and the Cyp1a1 band intensity was quantified by densitometry and normalized to Gapdh levels. One of three representative experiments is shown. *p < 0.05 compared with DMSO, †p < 0.05 compared with CHX, ‡p < 0.05 compared with MG-132, and §p < 0.05 compared with CHX plus MG-132 treatment.

FIG. 5. Effect of 26S proteasome inhibitor on heavy metal-mediated increase in Cyp1a1 mRNA. Hepa 1c1c7 cells were treated with 10 μg/ml CHX, a protein synthesis inhibitor, 25 μM MG-132, 26S proteasome inhibitor, or CHX plus MG-132, 30 min before exposure to 5 μM Hg2+, 25 μM Pb2+, 10 μM Cu2+, or 1 nM TCDD for 6 h. Total RNA (20 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to nylon membranes, and hybridized with a 32P-labeled cDNA probe specific for mouse Cyp1a1. The blots were subsequently stripped and rehybridized with a cDNA probe specific for Gapdh, which was used as a loading control. The graph represents the relative amount of Cyp1a1 mRNA (mean ± SEM, n = 3), and the Cyp1a1 band intensity was quantified by densitometry and normalized to Gapdh levels. One of three representative experiments is shown. *p < 0.05 compared with DMSO, †p < 0.05 compared with CHX, ‡p < 0.05 compared with MG-132, and §p < 0.05 compared with CHX plus MG-132 treatment.

The ability of heavy metals to induce the Cyp1a1 mRNA, but not the catalytic activity, prompted further investigation. Therefore, we hypothesized that the decrease in TCDD-inducible Cyp1a1 activity is attributed to decreased cellular heme content. To test this hypothesis, Hepa 1c1c7 cells were treated for 6 h with 5 μM Hg2+, 25 μM Pb2+, or 10 μM Cu2+ in the presence and absence of increasing concentrations of TCDD (0.1, 1, and 10 nM) and Northern blot analysis for HO-1 mRNA, a rate limiting enzyme of heme degradation, was
carried out. Our results clearly showed that all metals significantly increased HO-1 mRNA levels, which coincided with the inhibition of Cyp1a1 activity (Figs. 10A and 10B), indicating that all three metals increased heme degradation. To further confirm the role of heme degradation in reducing Cyp1a1 activity, we have measured the cellular heme content. Figure 10C shows that all three metals significantly decreased the cellular heme contents of TCDD-treated Hepa 1c1c7 cells. Taken together, these results suggest that the decreased cellular heme content, at least in part, plays a role in reduced Cyp1a1 catalytic activity observed in heavy metals treated cells.

DISCUSSION

We provide here the first evidence that heavy metals modulate Cyp1a1 gene expression at transcriptional and post-transcriptional levels. We show that all metals induce Cyp1a1 mRNA in a time-dependent manner. The RNA synthesis inhibitor, Act-D, completely blocked Cyp1a1 induction by metals, indicating a requirement of de novo RNA synthesis. The protein synthesis inhibitor, CHX and proteasome inhibitor, MG-132 superinduced the metal-mediated induction of Cyp1a1 mRNA. EMSA showed that all metals cause activation or transformation of the AhR, indicating that AhR-dependent mechanisms contributed to the Cyp1a1 induction. Cyp1a1 mRNA and protein decay experiments demonstrated that the three metals did not significantly affect the half-life of mRNA; however, significantly they decreased the degradation rate of its protein, implying a posttranslational regulation of the Cyp1a1 by heavy metals. Following metals treatment, a significant reduction in AhR ligand-mediated induction of Cyp1a1 activity was observed along with an increase in HO-1 and a decrease in cellular heme content.

Knowledge of the regulation of Cyp1a1 gene expression shows that the activation of a cytosolic transcriptional factor, AhR, is the first step in a series of molecular events promoting Cyp1a1 transcription and translation processes. To examine whether the regulation of Cyp1a1 gene expression by heavy metals is a transcriptional and/or posttranscriptional event, a series of experiments were carried out. Initially, we have found that all tested metals significantly increased the basal Cyp1a1 mRNA levels in a time-dependent manner. Interestingly, the induction pattern of Cyp1a1 mRNA by heavy metals was similar to those obtained with TCDD, in which heavy metals caused a rapid increase in Cyp1a1 mRNA, which was
evident after 3 h of treatment, reaching steady-state after 12 h. Although heavy metal-mediated induction of Cyp1a1 mRNA was 10-fold lower than those induced by TCDD, the level of induction was 15-fold higher than control. On the other hand, the TCDD-mediated induction of Cyp1a1 mRNA was further increased by Hg\(^{2+}\) and Pb\(^{2+}\), but was decreased by Cu\(^{2+}\) cotreatment. The alteration in Cyp1a1 mRNA steady-state levels in response to metals can therefore be attributed to transcriptional and/or posttranscriptional mechanisms.

In the current study, the transcriptional regulation of Cyp1a1 gene expression by heavy metals was demonstrated through different approaches. First, the inhibition of the RNA transcription, using Act-D, completely abolished the induction of Cyp1a1 mRNA in response to heavy metals, implying that metals increase the \textit{de novo} Cyp1a1 RNA synthesis, in a manner similar to that obtained with TCDD. Second, the coadministration of metals with CHX, a protein translation inhibitor, and/or MG-132, a 26S proteasome inhibitor, superinduced the Cyp1a1 mRNA in response to metals. Superinduction of Cyp1a1 mRNA by CHX or MG-132 has been previously reported in Hepa 1c1c7 (Gharavi and El-Kadi, 2005; Ma \textit{et al.}, 2000; Sindhu and Kikkawa, 1999) and in other cell lines (Daujat \textit{et al.}, 1991; Jojakim \textit{et al.}, 2004; Lamb and Franklin, 2002). Recent studies have demonstrated that the superinduction of Cyp1a1 gene by CHX or MG-132 is a transcriptional mechanism and reflects a change in the
synthesis, rather than stabilization, of Cyp1a1 mRNA (Joiakim et al., 2004; Ma and Baldwin, 2000, 2002).

Interestingly, it has been previously reported that superinduction of the transcription of AhR-regulated genes by CHX or MG-132 is a gene-specific effect. For example, it has been shown that Cyp1a1 and TCDD-inducible poly(ADP-ribose) polymerase (TiPARP), which are regulated by AhR, are superinduced by CHX or MG-132 (Ma, 2002), whereas the induction of phase II AhR-regulated genes, NAD(P)H: quinone oxidoreductase (Nqo1) and glutathione S-transferase Ya subunit (Gst ya), by TCDD requires both AhR and nuclear factor erythroid 2-related factor 2 (Nrf2) (Ma et al., 2004). This is because Nrf2 is a labile protein, and therefore, the induction is susceptible to CHX but not MG-132. Although the exact mechanism of superinduction is not clear, several proposed mechanisms have been reported. The most acceptable mechanism suggests the presence of a labile transcriptional protein that negatively regulates the expression of Cyp1a1. This is supported by the

FIG. 10. Effect of heavy metals on the TCDD-concentration-dependent effect on (A) Cyp1a1 activity, (B) HO-1 mRNA, and (C) cellular heme content. Hepa 1c1c7 cells were treated with 5 μM Hg2+, 25 μM Pb2+, or 10 μM Cu2+ in the presence and absence of 0.1, 1, or 10 nM TCDD for indicated time. (A) Cyp1a1 activity was measured in intact living cells using a 96-well cell culture plates using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SEM (n = 8). (B) Total RNA (20 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to nylon membranes, and hybridized with a 32P-labeled cDNA probe specific for mouse HO-1. The blots were subsequently stripped and rehybridized with a cDNA probe specific for Gapdh, which was used as a loading control. The graph represents the relative amount of HO-1 mRNA (mean ± SEM, n = 3), expressed as % of the control, and the HO-1 band intensity was quantified by densitometry and normalized to Gapdh levels. One of three representative experiments is shown. (C) Cellular heme content was measured by fluorimetric assay using excitation/emission wavelengths of 405/600 nm. Values are presented as mean ± SEM (n = 4). *p < 0.05 compared to DMSO, and **p < 0.05 compared to TCDD.
observations that inhibition of protein synthesis, using CHX, decreases the concentration of repressor proteins, such as IkBa (Ma et al., 2000), resulting in an increase in the rate of Cyp1a1 transcription (Israel et al., 1985; Lamb and Franklin, 2002; Lusska et al., 1992). Moreover, it has been hypothesized that inhibition of the turnover of AhR protein by a protein synthesis inhibitor (Ma and Baldwin, 2002; Ma et al., 2000) or proteasome inhibitor (Ma and Baldwin, 2000; Pollenz, 2002) accounts for the superinduction of Cyp1a1, based on the fact that AhR activation is an integral step for the induction of Cyp1a1 gene expression (Ma et al., 1995). Taken together, these results suggest that the superinduction of Cyp1a1 in response to metals in the presence of CHX or MG-132 is both a transcriptional and an AhR-dependent pathway; in a manner similar to that obtained with TCDD (Ma and Baldwin, 2002; Sindhu and Kikkawa, 1999).

The direct evidence for the involvement of AhR in the transcriptional regulation of Cyp1a1 by heavy metals is supported by our previous observation that heavy metals dose-dependently induced Cyp1a1 mRNA in wild-type, but not in mutant AhR-deficient Hepa 1c1c7 cells (Korashy and El-Kadi, 2004). Further evidence comes from the result of the EMSA. The presence of a nuclear AhR complex is dependent on ligand binding to the cytosolic receptor, nuclear translocation of the liganded AhR, its heterodimerization with ARNT, and subsequent specific and high-affinity DNA binding (Phelan et al., 1998). Our results not only suggest that metals can induce AhR transformation, nuclear accumulation, and DNA binding, similar to that which is observed with classical AhR ligands such as TCDD, but also support a role for the AhR in the induction of Cyp1a1 by heavy metals. Surprisingly, unlike Hg$^{2+}$ and Pb$^{2+}$, Cu$^{2+}$ fails to induce the nuclear AhR/ARNT/XRE complex formation. At present, we have no mechanistic explanation for the differential effects of Cu$^{2+}$ in vitro and in intact cells. However these results indicate the involvement of different molecular processes.

The mechanism by which heavy metals directly activate the in vitro AhR translocation is still unknown. However, several mechanisms of activation of the AhR by nonclassical ligands were proposed. In this regard, it has been recently demonstrated that the divalent metal arsenite induced AhR nuclear translocation and binding to the Cyp1a1 gene promoter in Hepa 1c1c7 cells, possibly by disrupting the molecular interaction between AhR and its associated protein, HSP90 (Kann et al., 2005). Based on these observations, we postulate that heavy metals may bind to the AhR-associated proteins, or to a non-TCDD binding site on the receptor, resulting in AhR conformational changes which lead to activation of the receptor and its translocation to the nucleus (Werlinder et al., 2001).

To test the hypothesis that heavy metals may modulate Cyp1a1 mRNA at the posttranscriptional level, we assessed the turnover rate of Cyp1a1 mRNA using an Act-D chase experiment. Our results showed that the Cyp1a1 mRNA induced by TCDD is short-lived, with an estimated half-life of 3.8 h. Our results are in agreement with previous reports that the half-life of Cyp1a1 mRNA induced by TCDD in Hepa 1c1c7 cells ranges from 3–4.5 h (Chen et al., 1995; Miller et al., 1983). Previous studies have reported that the half-life for Cyp1a1 mRNA induced by a different AhR ligand such as dimethylnaphthalene in MCF7 cells was about 7 h (Ciolino and Yeh, 1999), whereas in human hepatoma HepG2 cells the half-life was about 2.5 h (Lekas et al., 2000). The differences in the half-lives could be attributed to different cell lines, Cyp1a1 inducers, and/or the type of RNA synthesis inhibitor used. In our study, the Cyp1a1 mRNA half-life in Hepa 1c1c7 cells treated with metals was not statistically different from those of TCDD-induced Cyp1a1 mRNA, indicating that metals did not alter the stability of Cyp1a1 mRNA. In agreement with our results, but with a different metal, it has been recently reported that arsenite did not affect the Cyp1a1 mRNA stability in HepG2 cells (Bessette et al., 2005).

The inhibitory effects of heavy metals on the induction of Cyp1a1 activity by TCDD suggest a posttranslational mechanism, though the posttranslational regulation of Cyp1a1 is not well studied. This is the first report, to our knowledge, that showed that Hepa 1c1c7 Cyp1a1 protein is short-lived, with a half-life of about 2.7 h. Similarly, in V79 cells, the half-life of Cyp1a1 protein was 2.8 h (Werlinder et al., 2001). In the present study, all three metals significantly decreased the degradation rate of Cyp1a1 protein, providing the first evidence that metals are capable of regulating Cyp1a1 gene expression through a posttranslational mechanism.

Interestingly, the stabilization of Cyp1a1 protein by heavy metals was in contrast with the inhibitory effects of metals on the induction of Cyp1a1 activity-mediated by TCDD. Although it is unclear how heavy metals decreased the induction of Cyp1a1 activity, it has been previously demonstrated that the decrease in heme availability, the prosthetic group of CYP450, causes a reduction in the Cyp1a1 activity (Meyer et al., 2002). This is supported by our current observations that heavy metals induced HO-1 mRNA expression and decreased cellular heme content. Another potential mediator that may be involved in the inhibition of TCDD-mediated induction of Cyp1a1 activity by heavy metals is reactive oxygen species (ROS). We have recently demonstrated that increased ROS production by the AhR ligands, benzo[a]pyrene, 3-methylcholanthrene, and β-naphthoflavone, at high concentrations were accompanied by a decrease in the Cyp1a1 catalytic activity, but not the mRNA or protein expression levels, which were significantly induced in a concentration-dependent manner (Elbekai et al., 2004). The AhR-mediated decrease in Cyp1a1 activity was reversed by the antioxidant N-acetylcycteine, suggesting a direct involvement of ROS in the inhibition of Cyp1a1 activity. Furthermore, recent data from our laboratory showed that all metals used in the current study are able to generate ROS (data not shown).
and posttranslational levels. Furthermore, the inhibitory effects of heavy metals on the TCDD-inducible Cyp1a1 activity are attributed, at least in part, to decreased heme availability.

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