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# Mercury modulates the CYP1A1 at transcriptional and posttranslational levels in human hepatoma HepG2 cells

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ABSTRACT

Aryl hydrocarbon receptor (AhR) ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and metals, such as mercury ($Hg^{2+}$), are environmental co-contaminants and their molecular interaction may disrupt the coordinated regulation of the carcinogen-activating enzyme cytochrome P450 1A1 (CYP1A1). Therefore, we examined the effect of co-exposure to $Hg^{2+}$ and TCDD on the expression of the CYP1A1 in HepG2 cells. Our results showed that $Hg^{2+}$ significantly inhibited the TCDD-mediated induction of CYP1A1 at the mRNA, protein, and catalytic activity levels. At the transcriptional level, co-exposure to $Hg^{2+}$ and TCDD significantly decreased the TCDD-mediated induction of AhR-dependent luciferase reporter gene expression. Moreover, $Hg^{2+}$ did not affect CYP1A1 mRNA stability, while decreasing its protein half-life, suggesting the involvement of a posttranslational mechanism. Importantly, $Hg^{2+}$ increased the expression of heme oxygenase-1 (HO-1), a rate limiting enzyme in heme degradation, which coincided with further decrease in the CYP1A1 catalytic activity levels. Upon using a competitive HO-1 inhibitor, tin mesoporphyrin, heme precursor, hemin, or transfecting the HepG2 cells with siRNA for HO-1 there was a partial restoration of the inhibition of TCDD-mediated induction of CYP1A1 catalytic activity. In conclusion, we demonstrate that $Hg^{2+}$ down-regulates the expression of CYP1A1 at the transcriptional and posttranslational levels in HepG2 cells. In addition, HO-1 is involved in the modulation of CYP1A1 at the posttranslational level.



## 1. Introduction

Co-contamination of heavy metals, such as mercury ($Hg^{2+}$), with halogenated aromatic hydrocarbons (HAHs) such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), is a common environmental problem with multiple biological consequences. $Hg^{2+}$ is mostly considered highly toxic agent that is introduced into the environment through natural and/or industrial sources (Snow et al., 1989). Both aryl hydrocarbon receptor (AhR) ligands and heavy metals are ranked high on the list of the most hazardous xenobiotics in the environment, as reported by the Agency for Toxic Substances and Diseases Registry and the Canadian Environmental Protection Act. (ATSDR, 2005; CEPA, 2006). Studies on the carcinogenicity and mutagenicity of HAHs have demonstrated the role of cytochrome P450 1A1 (CYP1A1), a phase I xenobiotics-metabolizing enzyme, in bioactivating poly aromatic hydrocarbons (PAHs) to epoxide and diol-epoxide intermediates, which will subsequently lead to DNA and protein adducts formation (Shimada and Fujii-Kuriyama, 2004).

The AhR is a ligand-activated cytoplasmic transcription factor that belongs to the basic-helix-loop-helix protein family. The AhR plays a key role in the regulation of CYP1A1. This cytosolic inactive receptor exists attached to a complex of two heat shock proteins 90 (HSP90), hepatitis B virus X-associated protein (XAP2), and the chaperone protein p23 (Hankinson, 1995; Meyer et al., 1998). Upon ligand binding, the activated AhR dissociates from the cytoplasmic complex, and translocates to the nucleus where it dimerizes with the aryl hydrocarbon nuclear translocator (Arnt) (Whitelaw et al., 1994). Thereafter, the ligand/AhR/Arnt complex acts as a transcription factor that binds to a specific DNA recognition sequence, GCGTG, within the xenobiotic responsive element (XRE), located in the promoter region of a battery of genes termed AhR-regulated genes such as CYP1A1 (Denison et al., 1989; Nebert et al., 2004). The toxicological effects of HAHs, typified by TCDD, are mainly mediated through the activation of AhR and consequently CYP1A1. In fact, a strong correlation between the induction of CYP1A1 and cancer has been previously reported (McLemore et al., 1990).

The majority of published studies on AhR ligands toxicities have been conducted individually, yet human exposures are usually to

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mixtures of these ligands and metals such as $Hg^{2+}$. $Hg^{2+}$ is a metal that is widely used in the foundry, mining, and manufacturing industries and is a component in a number of electrical instruments and medical products such as thermometers, thermostats, dental amalgams, switches, and batteries (Gochfeld, 2003). Among metals, $Hg^{2+}$ is unique in that it is found in the environment in several physical and chemical forms. At room temperature, elemental (or metallic) $Hg^{2+}$ exists as a liquid (Zalups, 2000). As a result of its high vapor pressure, this form of $Hg^{2+}$ is released into the environment as $Hg^{2+}$ vapor. Hg also exists in three different oxidation states such as $Hg^0$, which exists in metallic form as vapor, and $Hg^+$ or $Hg^{2+}$, which can form stable organic compound such as methylmercury. Generally, the route and efficiency of exposure depends mainly on the oxidation state of Hg (Ercal et al., 2001; Tezel et al., 2001).

Previous reports from our laboratory and others have demonstrated that $Hg^{2+}$ alters the expression of the carcinogen-activating enzyme CYP1A1, at different signaling pathway levels (Korashy and El-Kadi, 2004; Vakharia et al., 2001). Therefore, the objective of this study was to determine the possible effects of $Hg^{2+}$ on the TCDD-mediated induction of CYP1A1, and to investigate the underlying molecular mechanisms involved in this pathway.

We provide here the evidence that $Hg^{2+}$ modulates the expression of CYP1A1 through affecting its transcriptional and posttranslational levels. The inhibitory effect of $Hg^{2+}$ on the TCDD-mediated induction of CYP1A1 catalytic activity might be in part due to its effect on HO-1, which will subsequently lead to the formation of a hollow functionless CYP1A1 protein. Furthermore, $Hg^{2+}$ significantly alters the expression of CYP1A1 protein stability, suggesting posttranslational down-regulation of CYP1A1 by $Hg^{2+}$.

## 2. Materials and methods

### 2.1. Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cycloheximide (CHX), 7-ethoxyresorufin, fluorescamine, anti-goat IgG peroxidase secondary antibody, hemin, protease inhibitor cocktail, and mercuric chloride ($HgCl_2$) were purchased from Sigma Chemical Co. (St. Louis, MO). Tin mesoporphyrin (SnMP), was purchased from Frontier Scientific Inc. (Logan, UT). TCDD, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). TRIzol reagent and Lipofectamine 2000 reagents were purchased from Invitrogen (San Diego, CA). High-Capacity cDNA Reverse Transcription Kit, SYBR® Green PCR Master Mix, human Hmox1 (HO-1) validated siRNA was purchased from Applied Biosystems (Foster City, CA). INTERFERin siRNA transfecting reagent was purchased from Polyplus transfection (Illkirch, France). Actinomycin-D (Act-D) was purchased from Calbiochem (San Diego, CA). Chemiluminescence Western blotting detection reagents were from GE Healthcare Life Sciences (Piscataway, NJ). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA). CYP1A1 mouse polyclonal primary antibody, GAPDH rabbit polyclonal antibody, and anti-rabbit IgG peroxidase secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-mouse IgG peroxidase secondary antibody was purchased from R&D Systems, Inc. (Minneapolis, MN). Luciferase assay reagents were obtained from Promega (Madison, WI). All other chemicals were purchased from Fisher Scientific (Toronto, ON).

### 2.2. Cell culture

HepG2 cell line, ATCC number HB-8065 (Manassas, VA), were maintained in Dulbecco's modified Eagle's medium (DMEM) with phenol red, supplemented with 10% heat-inactivated fetal bovine serum, 20 μM l-glutamine, 50 μg/ml amikacin, 100 IU/ml penicillin, 10 μg/ml streptomycin, 25 ng/ml amphotericin B, 0.1 mM non-essential amino acids, and vitamin supplement solution. Cells were grown in 75-$cm^2$ cell culture flasks at 37 °C in a 5% $CO_2$ humidified incubator.

### 2.3. Chemical treatments

Cells were treated in serum free medium with various concentrations of $Hg^{2+}$ (2.5–10 μM) in the absence and presence of 1 nM TCDD, and/or 5 μM SnMP and 80 μM hemin as described in figure legends. TCDD and SnMP were dissolved in dimethylsulfoxide (DMSO) and maintained in DMSO at −20 °C until use. $Hg^{2+}$ and hemin (10 mM stocks) were prepared freshly in double de-ionized water. In all treatments, the DMSO concentration did not exceed 0.05% (v/v).

### 2.4. Effect of $Hg^{2+}$ on cell viability

The effect of $Hg^{2+}$ on cell viability was determined using the MTT assay as described previously (Anwar-Mohamed and El-Kadi, 2009). MTT assay measures the conversion of MTT to formazan in living cells via mitochondrial enzymes of viable cells. In brief, HepG2 cells were seeded onto 96-well microtiter cell culture plates and incubated for 24 h at 37 °C in a 5% $CO_2$ humidified incubator. Cells were treated with various concentrations of $Hg^{2+}$ (2.5–50 μM) in the absence and presence of 1 nM TCDD. After 24 h incubation, the medium was removed and replaced with cell culture medium containing 1.2 mM MTT dissolved in phosphate buffered saline (PBS) (pH 7.4). After 2 h of incubation, the formed crystals were dissolved in isopropanol. The intensity of the color in each well was measured at a wavelength of 550 nm using the Bio-Tek EL 312e microplate reader (Bio-Tek Instruments, Winooski, VT).

### 2.5. RNA extraction and quantitative real-time PCR

After incubation with the test compounds for the specified time periods, total cellular RNA was isolated using TRIzol reagent, according to manufacturer's instructions (Invitrogen), and quantified by measuring the absorbance at 260 nm. For reverse transcription-polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized from 1.0 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit with random primers. Real-time PCR reactions were performed on an ABI 7500 real-time PCR system (Applied Biosystems), using SYBR® Green PCR Master Mix (Applied Biosystems). The amplification reactions were performed as follows: 10 min at 95 °C, and 40 cycles of 94 °C for 15 s and 60 °C for 1 min. Primers and probes for human CYP1A1 were: Forward primer 5′-CTA TCT GGG CTG TGG GCA A-3′, reverse primer 5′-CTG GCT CAA GCA CAA CTT GG-3′. Heme oxygenase-1 (HO-1): forward primer 5′-ATG GCC TCC CTG TAC CAC ATC-3′, reverse primer 5′-TGT TGC GCT CAA TCT CCT CCT-3′ and for β-actin: forward primer 5′-CTG GCA CCC AGG ACA ATG-3′, reverse primer 5′-GCC GAT CCA CAC GGA GTA-3′ were purchased from Integrated DNA technologies (IDT, Coralville, IA). The fold change in the level of *CYP1A1* or *HO-1* (target genes) between treated and untreated cells, corrected by the level of β-actin, was determined using the following equation: fold change = $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{(target)} - Ct_{(\beta\text{-actin})}$ and $\Delta(\Delta Ct) = \Delta Ct_{(treated)} - \Delta Ct_{(untreated)}$.

### 2.6. Protein extraction and Western blot analysis

Twenty-four hours after incubation with the test compounds, cells were collected in lysis buffer containing 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 cell homogenates 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. Proteins (50 μg) were resolved by denaturing gel electrophoresis, as described previously (Elbekai and El-Kadi, 2004). Briefly, the cell homogenates were dissolved in 1X sample buffer, boiled for 5 min, separated by 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. Protein blots were blocked for 24 h at 4 °C in blocking buffer containing 5% skim milk powder, 2% bovine serum albumin and 0.05% (v/v) Tween-20 in tris-buffered saline solution (TBS; 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris–base). After blocking, the blots were incubated with a primary polyclonal mouse anti-rat CYP1A1 antibody for 2 h at room temperature, and primary polyclonal rabbit anti-human GAPDH antibody for overnight at 4 °C in TBS containing 0.05% (v/v) Tween-20 and 0.02% sodium azide. Incubation with a peroxidase-conjugated anti-mouse IgG secondary antibody for CYP1A1 and anti-rabbit for GAPDH was carried out in blocking buffer for 1 h at room temperature. The bands were visualized with the enhanced chemiluminescence method according to manufacturer's instructions (GE Healthcare Life Sciences, Piscataway, NJ). The intensity of CYP1A1 protein bands was quantified, relative to the signals obtained for GAPDH protein, using ImageJ software.

### 2.7. Determination of CYP1A1 enzymatic activity

CYP1A1-dependent 7-ethoxyresorufin O-deethylase (EROD) was performed on intact living cells using 7-ethoxyresorufin as previously described (Anwar-Mohamed et al., 2008). Enzymatic activity was normalized for cellular protein content, which was determined using a modified fluorescent assay (Lorenzen and Kennedy, 1993).

### 2.8. Transient transfection and luciferase assay

HepG2 cells were plated onto 12-well cell culture plates. Each well of cells was transfected with 1.6 μg of XRE-driven luciferase reporter plasmid pGudLuc 6.1, generously provided by Dr. M.S. Denison (University of California, Davies), using lipofectamine 2000 reagent according to manufacturer's instructions (Invitrogen). Luciferase assay was performed according to manufacturer's instructions (Promega) as described previously (Elbekai and El-Kadi, 2007). Briefly, after incubation with test compounds for 24 h, cells were washed with PBS and a 200 μl of 1× lysis buffer was added into each well with continuous shaking for at least 20 min, then the content of each well was collected separately in 1.5 ml microcentrifuge tubes. Luciferase activities were analyzed in 100-μl cell extracts with the Luciferase Assay