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Mercury modulates the cytochrome P450 1a1, 1a2 and 1b1 in C57BL/6J mice: *in vivo* and *in vitro* studies

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ABSTRACT

In the current study C57BL/6J mice were injected intraperitoneally with Hg^{2+} in the absence and presence of TCDD. After 6 and 24 h the liver was harvested and the expression of Cyps was determined. *In vitro*, isolated hepatocytes were incubated with TCDD in the presence and absence of Hg^{2+}. At the *in vivo* level, Hg^{2+} significantly decreased the TCDD-mediated induction of Cyps at 6 h while potentiating their levels at 24 h. *In vitro*, Hg^{2+} significantly inhibited the TCDD-mediated induction of Cyp1a1 in a concentration- and time-dependent manner. Interestingly, Hg^{2+} increased the serum hemoglobin (Hb) levels in mice treated for 24 h. Upon treatment of isolated hepatocytes with Hb alone, there was an increase in the AhR-dependent luciferase activity with a subsequent increase in Cyp1a1 protein and catalytic activity levels. Importantly, when hepatocytes were treated for 2 h with Hg^{2+} in the presence of TCDD, then the medium was replaced with new medium containing Hb, there was potentiation of the TCDD-mediated effect. In addition, Hg^{2+} increased heme oxygenase-1 (HO-1) mRNA, which coincided with a decrease in the Cyp1a1 activity level. When the competitive HO-1 inhibitor, tin mesoporphyrin was applied to the hepatocytes there was a partial restoration of Hg^{2+}-mediated inhibition of Cyp1a1 activity. In conclusion, we demonstrate for the first time that there is a differential modulation of the TCDD-mediated induction of Cyp1a1 by Hg^{2+} in C57BL/6J mice livers and isolated hepatocytes. Moreover, this study implicates Hb as an *in vivo* specific modulator of Cyp1 family.

Introduction

Cyotchrome P450 enzymes (CYPs) play an important role in catalyzing oxidative metabolism of different xenobiotics, carcinogens, and protoxicants (Ortiz de Montellano, 1995; Porter and Coon, 1991). Much of the initial concerns regarding CYP1 enzymes activities were arisen from the field of chemical carcinogenesis and particularly studies utilizing polycyclic aromatic hydrocarbons (PAHs), *e.g.*, benzo[*a*]pyrene (Dipple et al., 1985; Heidelberger, 1975). Many experimental studies have been conducted to examine the inducibility of these enzymes involved in the metabolism of these procarcinogens (Conney, 1982). In addition, previous studies on the carcinogenicity and mutagenicity of PAHs have demonstrated the role of CYP1 family members particularly cytochrome P4501a1 (Cyp1a1) and 1a2 (Cyp1a2) in bioactivating these toxicants to epoxide and diol-epoxide intermediates, which will subsequently lead to DNA and protein adduct formation (Shimada and Fujii-Kuriyama, 2004). Cyp1a1, Cyp1a2 and Cyp1b1 are mainly regulated by the aryl hydrocabon receptor (AhR) with the exception of Cyp1b1 which also is regulated by 17 β-estradiol (Bhattacharyya et al., 1995; Hayes et al., 1996; Shimada et al., 1996, 1998).

The AhR is a cytosolic transcription factor that mediates many toxic and carcinogenic effects in animals and humans. The AhR is constitutively present in the cytosol as an inactive complex attached to two molecules of heat shock protein-90 (HSP90), the 23-kDa heat shock protein (p23), and a 43-kDa protein known as the AhR inhibitory protein (AIP) or hepatitis B virus X-associated protein 2 (XAP2) (Nebert and Duffy, 1997). The hydrophobic AhR inducers enter the cell by diffusion and bind to the AhR ligand binding domain. Upon ligand binding, the AhR-ligand complex dissociates from the cytoplasmic complex and translocates to the nucleus where it associates with aryl hydrocarbon nuclear translocator (ARNT) (Nebert and Duffy, 1997). The whole complex then acts as a transcription factor that binds to a specific DNA recognition sequence, termed the xenobiotic responsive element (XRE), located in the promoter region of a number of AhR-regulated genes.

Co-exposure to heavy metals typified by Hg^{2+}, and PAHs typified by TCDD is a very likely event with multiple biological consequences. Both Hg^{2+} and TCDD 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, 2011; CEPA, 2012).

Abbreviations: AhR, aryl hydrocarbon receptor; Hg^{2+}, mercury chloride; Cyp450s, cytochrome P450s; HO-1, heme oxygenase-1; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); XRE, xenobiotic responsive element.

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We have previously demonstrated that Hg^{2+} by itself or in the presence of AhR ligands such as TCDD, is capable of differentially altering Cyp1a1 gene and protein expression in addition to catalytic activity in both *in vivo* and *in vitro* situations (Amara et al., 2010, 2012; Korashy and El-Kadi, 2005). Most of the *in vitro* studies were conducted using the murine hepatoma Hepa 1c1c7 cells, human hepatoma HepG2 cells, and human isolated hepatocytes (Amara et al., 2010; Korashy and El-Kadi, 2005; Vakharia et al., 2001). The applicability of the results of such studies to *in vivo* situation has only recently been addressed (Amara et al., 2012). Despite these efforts, these studies were conducted on the extrahepatic organs such as kidney, lung and heart with regard to the effect of Hg^{2+} on AhR-regulated genes including cytochrome P450s family 1 in the absence and presence of ligands. Therefore, it was important to investigate the effect of Hg^{2+} on family 1 P450s in the liver and isolated hepatocytes of C57BL/6J mice. We hypothesize that Hg^{2+} differentially alters the expression of Cyp1a1 *in vivo* and *in vitro* in C57BL/6J mice. Thus, the objective of the current study was to investigate the effect of Hg^{2+} on Cyp1a1 *in vivo* using C57BL/6J mice livers, and *in vitro* using isolated mouse hepatocytes in the absence and presence of TCDD. Also, to explore the molecular mechanisms involved in this alterations.

Materials and methods

Materials

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), β-glucuronidase, arylsulfatase, chlorpromazine HCL, collagenase, collagen from rat tail, 7-ethoxyresorufin, 7-methoxyresorufin, fluorescamine, hemoglobin (Hb), anti-goat IgG peroxidase secondary antibody, protease inhibitor cocktail, mercury chloride (Hg^{2+}), Resveratrol and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Sigma Chemical Co. (St. Louis, MO). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA). High-Capacity cDNA Reverse Transcription Kit, SYBR Green SuperMix, and 96-well optical reaction plates with optical adhesive films were purchased from Applied Biosystems (Foster City, CA). Real-time PCR primers were synthesized by Integrated DNA Technologies Inc. Tin mesoporphyrin (SnMP), was purchased from Frontier Scientific Inc. (Logan, UT).TCDD, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). Chemiluminescence Western blotting detection reagents were from GE Healthcare Life Sciences (Piscataway, NJ). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA). Cyp1a1/1a2 mouse polyclonal primary antibody, glyceraldehyde-3-phosphate dehydrogenase (Gapdh) rabbit polyclonal antibody, heme oxygenase-1 (HO-1) goat polyclonal primary antibody, and anti-rabbit IgG peroxidase secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Cyp1b1 polyclonal primary antibody was purchased from BD Biosciences (Mississauga, ON). Anti-mouse IgG peroxidase secondary antibody was purchased from R&D Systems, Inc. (Minneapolis, MN). All other chemicals were purchased from Fisher Scientific (Toronto, ON).

Animals and ethics

Male C57BL/6J mice, aged 10–12 weeks (Charles River Laboratories) were used in this study. Mice were group-housed under standard conditions, three to five per cage with food and water available *ad libitum* and were maintained on a 12-h light/dark cycle. Mice were treated in compliance with the University of Alberta Health Sciences Animal Policy and Welfare Committee guidelines. All experiments included matched numbers of male mice.

Biohazard precaution

TCDD is toxic and a likely human carcinogen. All personnel were instructed as to safe handling procedures. Lab coats, gloves, and masks were worn at all times, and contaminated materials were collected separately for disposal by the Office of Environmental Health and Safety at the University of Alberta.

Animal treatment

Male C57BL/6J (22–30 g) mice were obtained from Charles River, Canada (Montreal, QC, Canada). Animals were injected intraperitoneally (i.p.) with Hg^{2+} (dissolved in saline) at 2.5 mg/kg, in the absence and presence of 15 μg/kg TCDD (dissolved in corn oil) injected i.p. The mice were divided into 4 groups. The first group ($n=12$) control mice received saline (0.4 mL) plus corn oil (0.4 mL). The second group ($n=12$) was Hg^{2+} treated mice which received Hg^{2+} dissolved in saline (0.4 mL) plus corn oil (0.4 mL) The third group ($n=12$) was TCDD treated mice which received TCDD dissolved in corn oil (0.4 mL) plus saline (0.4 mL). The fourth group ($n=12$) was Hg^{2+} plus TCDD treated mice which received Hg^{2+} dissolved in saline (0.4 mL) plus TCDD dissolved in corn oil (0.4 mL). Thereafter, the animals were euthanized after a single injection at 6 h ($n=6$) and 24 h ($n=6$) *via* cervical dislocation. Liver tissue was excised, immediately frozen in liquid nitrogen, and stored at −80 °C until further analysis. All experimental procedures involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. All animals were allowed free access to food and water throughout the treatment period.

Isolation of mouse hepatocytes and cell culture

Three solutions were utilized for the isolation of mouse hepatocytes as previously described (El-Kadi et al., 1997). Mouse hepatocytes were isolated by a two-step collagenase perfusion method as described previously (Seglen, 1976).

Chemical treatments

Cells were treated in serum free medium with Hg^{2+} (2.5, 5, and 10 μM) in the absence and presence of TCDD (1 nM) and/or Hb (1 μM) (Burgstahler and Nathanson, 1995). Hg^{2+} (10 mM stock) were prepared freshly in double de-ionized water. TCDD was dissolved in dimethylsulfoxide (DMSO) and maintained in DMSO at −20 °C until use. The concentrations of Hg^{2+} and TCDD used for the treatment of isolated mouse hepatocytes were chosen based on cell viability results in which Hg^{2+} at concentrations of 2.5–10 μM in the presence and absence of 1 nM TCDD did not affect cell viability. Therefore, all subsequent studies were conducted using the concentrations of 2.5–10 μM. In addition to MTT assay, these low μM concentrations of Hg^{2+} used in the present work are within the estimated human plasma and tissue levels in individuals without known exposure (Gerhardsson et al., 1988; Tezel et al., 2001). Taken together, chronic human exposure to this toxic metal, its longer half-life, and the high possibility of accumulations in the body tissues, particularly the liver (Barbier et al., 2005), make the concentrations used in the present study of high relevance to the corresponding human plasma and tissue levels and related to calculated exposure levels in the environment (ATSDR, 2011; CEPA, 2012). Hb was dissolved in DMEM and maintained in DMEM at −20 °C until use. In all treatments, the DMSO concentration did not exceed 0.01% (v/v).

RNA extraction and cDNA synthesis

Total RNA from the frozen tissues or treated cells for the specific time periods was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm. RNA quality was determined by measuring the 260/280 ratio. Thereafter, first strand cDNA synthesis was performed

by using the High-Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions.

Briefly, 1.5 μg of total RNA from each sample was added to a mix of 2.0 μL of 10× reverse transcriptase buffer, 0.8 μL of 25× dNTP mix (100 mM), 2.0 μL of 10× reverse transcriptase random primers, 1.0 μL of MultiScribe reverse transcriptase, and 3.2 μL of nuclease-free water. The final reaction mix was kept at 25 °C for 10 min, heated to 37 °C for 120 min, heated for 85 °C for 5 s, and finally cooled to 4 °C.

Quantification by real-time PCR

Quantitative analysis of specific mRNA expression was performed using real-time PCR by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems). The 25-μL reaction mix contained 0.1 μL of 10 μM forward primer and 0.1 μL of 10 μM reverse primer (40 nM final concentration of each primer), 12.5 μL of SYBR Green Universal Mastermix, 11.05 μL of nuclease-free water, and 1.25 μL of cDNA sample. Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. After sealing the plate with an optical adhesive cover, the thermocycling conditions were initiated at 95 °C for 10 min, followed by 40 PCR cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. The primers used in the current study were chosen from previously published studies and are listed in Table 1. Melting curve (dissociation stage) was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product.

Real-time PCR data analysis

The real-time PCR data were analyzed using the relative gene expression *i.e.* ($\Delta\Delta C_T$) method as described in Applied Biosystems User Bulletin No.2 and explained further by Livak and Schmittgen (2001). Briefly, the ΔC_T values were calculated in every sample for each gene of interest as follows: $C_{T\ \text{gene of interest}} - C_{T\ \text{reporter gene}}$, with β-actin as the reporter gene. Calculation of relative changes in the expression level of one specific gene ($\Delta\Delta C_T$) was performed by subtraction of ΔC_T of control (vehicle treated animals at 6 or 24 h time points) from the ΔC_T of the corresponding treatment groups. The values and ranges given in different figures were determined as follows: $2^{-\Delta(\Delta CT)}$ with $\Delta\Delta C_T + \text{S.E.}$ and $\Delta\Delta C_T - \text{S.E.}$, where S.E. is the standard error of the mean of the $\Delta(\Delta C_T)$ value.

Preparation of microsomal protein fractions

Liver microsomes were prepared by differential centrifugation of homogenized tissues as previously described (Barakat et al., 2001; Lin et al., 1991). Briefly, individual liver tissue was rapidly removed and washed in ice-cold potassium chloride [1.15% (w/v)]. Consequently, the liver was cut into pieces, and homogenized separately in cold sucrose solution (1 g of tissue in 5 mL of 0.25 M sucrose). After homogenizing, the tissues were separated by different ultracentrifugation steps. The final microsomal pellets were reconstituted in cold sucrose and stored at −80 °C. Thereafter, microsomal protein concentrations were determined by the Lowry method using bovine serum albumin as a standard (Lowry et al., 1951).

Table 1
Primers sequences for real-time PCR reactions.

Gene	Forward primer	Reverse primer
B-actin	5′-TAT TGG CAA CGA GCG GTT CC-3′	5′-GGC ATA GAG GTC TTT ACG GAT GTC-3′
Cyp1a1	5′-GGT TAA CCA TGA CCG GGA ACT-3′	5′-TGC CCA AAC CAA AGA GAG TGA-3′
Cyp1a2	5′-TGG AGC TGG CTT TGA CAC AG-3′	5′-CGT TAG GCC ATG TCA CAA GTA GC-3′
Cyp1b1	5′-AAT GAG GAG TTC GGG CGC ACA-3′	5′-GGC GTG TGG AAT GGT GAC AGG-3′
HO-1	5′-GTG ATG GAG CGT CCA CAG C-3′	5′-TGG TGG CCT CCT TCA AGG-3′

Protein extraction from hepatocytes

Twenty-four hours after incubation with the test compounds, cells were collected in lysis buffer containing 50 mM HEPES, 0.5 M NaCl, 1.5 mM MgCl, 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. The supernatant total cellular lysate was collected and stored at −80 °C.

Western blot analysis

Western blot analysis was performed using a previously described method (Amara and El-Kadi, 2011). Briefly, 20 μg of liver microsomal proteins or total cell lysates was separated by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), 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, 2% bovine serum albumin, and 0.5% Tween-20. After blocking, the blots were incubated with the following primaries: primary monoclonal mouse anti-rat Cyp1a1/1a2, primary polyclonal rabbit anti-rat Cyp1b1, and primary polyclonal rabbit anti-mouse actin for 2 h at room temperature. Incubation with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody for Cyp1b1 or actin or goat anti-mouse IgG secondary antibody for Cyp1a1/1a2 was carried out for another 2 h at room temperature. The bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare Life Sciences, Piscataway, NJ). The intensity of the protein bands were quantified, relative to the signals obtained for actin, using ImageJ software [National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij.].

Determination of Cyp1a1 enzymatic activity in hepatocytes

Cyp1a1-dependent 7-ethoxyresorufin O-deethylase (EROD), activity was performed on intact, monolayer living cells in 96-well plate with modifications. Cells were incubated with 100 μL/well of culture medium containing 8 μM 7-ethoxyresorufin, and a NAD(P)H-oxidoreductase inhibitor (dicoumarol 10 μM). After 45 min incubation at 37 °C in a CO_2 incubator, 75 μL of culture media from each well was transferred to white, opaque 96-well plates. 15 μL of 1:100 β-Glucuronidase/sulfatase diluted in phosphate-buffered saline, pH 7.2 was added to each well. Thereafter, the plate was incubated for 2 h at 37 °C to hydrolyze any hydroxyl resorufin conjugates. The fluorescence of the content in each well was measured using the Baxter 96-well fluorescence plate reader with excitation and emission wavelengths of 545 and 575 nm, respectively (Amara et al., 2012; Vakharia et al., 2001).

Microsomal incubation and measuring EROD and MROD catalytic activities

Microsomes from liver of different treatments (1 mg protein/mL) were incubated in the incubation buffer (5 mM magnesium chloride hexahydrate dissolved in 0.5 M potassium phosphate buffer pH = 7.4) at 37 °C in a shaking water bath (50 rpm). A pre-equilibration period of 5 min was performed. The reaction was initiated by the addition of 1 mM NADPH. The concentrations of substrate were 2 μM for both, EROD for Cyp1a1 and 7-methoxyresorufin (MROD) for Cyp1a2 and

Cyp1b1. After incubation at 37 °C (5 min for EROD, and 10 min for MROD assays), the reaction was stopped by adding 0.5 mL of cold methanol. The amount of resorufin formed in the resulting supernatant was measured using the Baxter 96-well fluorescence plate reader using excitation and emission wavelengths of 545 and 575 nm, respectively. Formation of resorufin was linear with incubation time and protein amount. Enzymatic activities were expressed as picomole of resorufin formed per minute and per milligram of microsomal proteins.

Transient transfection of hepatocytes and luciferase assay

Hepatocytes were plated onto 12-well cell culture plates. Each well of cells was transfected with 1.6 μg of XRE-driven luciferase reporter plasmid pGudLuc1.1, generously provided by Dr. M. S. Denison (University of California, Davies), and 0.1 μg of the renilla luciferase pRL-CMV vector, used for normalization, 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). In brief, after incubation with test compounds for 24 h, cells were washed with PBS, 100 μL of 1× passive lysis buffer was added into each well with continuous shaking for at least 20 min, and then the content of each well was collected separately in 1.5 mL microcentrifuge tubes. Enzyme activities were determined using a Dual-Luciferase reporter assay system (Promega). Quantification was performed using a TD-20/20 luminometer (Turner BioSystems, Sunnyvale CA).

Measuring serum Hb levels

Measurement of serum Hb levels was carried out as previously described (Vazquez et al., 1991). In brief, stock solution of Hb was prepared (1 mg/mL) and the exact Hb content was determined by the Hartree method (Hartree, 1972). The reaction mixture was prepared by the addition of the following solutions to a test tube, in the following order: 1.5 mL of 1% NaCl, 1 mL of glacial acetic acid, 0.01 mL of 0.25 M EDTA solution, 0.2 mL of 0.5 M chlorpromazine HCL solution, and 0.3 mL of 6% H_2O_2. After careful mixing, the solution was preincubated for 5 min at room temperature. Thereafter, 10 μL of serum or Hb standard solution were added and the reaction was allowed to continue for about 1 min. Absorbance was read at 1 min intervals against water at 525 nm.

Statistical analysis

The comparative statistical analysis of results was performed using SigmaStat for Windows (Systat Software, CA, USA). A one-way analysis of variance (ANOVA) followed by Duncan's multiple range *post hoc* test were used to assess the significance of differences between groups. The differences were considered significant when $P<0.05$. Results were calculated as mean ± S.E.M., and presented as mean + SEM for clarity.

Results

Effect of co-exposure to Hg^{2+} and TCDD on Cyp1a1, Cyp1a2, and Cyp1b1 mRNA levels in the liver of C57Bl/6J mice

At 6 and 24 h, Hg^{2+} alone failed to significantly affect Cyp1a1, Cyp1a2, or Cyp1b1 mRNA levels in the liver (Figs. 1A, B, and C). On the other hand, TCDD alone significantly induced Cyp1a1, Cyp1a2 and Cyp1b1 mRNA levels in the liver at 6 h by 12,400-, 11.7- and 6-fold, respectively, compared to the control (Figs. 1A, B, and C). At 24 h TCDD alone was able to significantly induce Cyp1a1, Cyp1a2, and Cyp1b1 mRNA levels in the liver by 5600-, 15-, and 220-fold, respectively, compared to the control (Figs. 1A, B, and C). When animals were co-exposed to Hg^{2+} and TCDD, Hg^{2+} at 6 h significantly inhibited the TCDD-mediated induction of Cyp1a1 and Cyp1a2 mRNA levels in the liver by −17.7- and −6.6-fold, respectively, compared to TCDD alone, while there was no effect of this co-exposure on Cyp1b1 mRNA levels (Figs. 1A, B, and C). On the contrary, Hg^{2+} at 24 h significantly potentiated the TCDD-mediated induction of Cyp1a1, Cyp1a2 and Cyp1b1 mRNA levels by 1.4-, 1.4- and 2.7-fold, compared to TCDD alone (Figs. 1A, B, and C).

Effect of co-exposure to Hg^{2+} and TCDD on Cyp1a and Cyp1b1 protein expression and catalytic activity levels in the liver of C57Bl/6 J mice

Our results showed that Hg^{2+} alone significantly increased Cyp1b1 protein expression levels in the liver by 1.8-fold, while it did not affect the Cyp1a protein levels (Fig. 2A). As expected, TCDD alone significantly induced Cyp1a and Cyp1b1 protein expression levels in the liver by 31- and 6-fold, respectively, compared to the

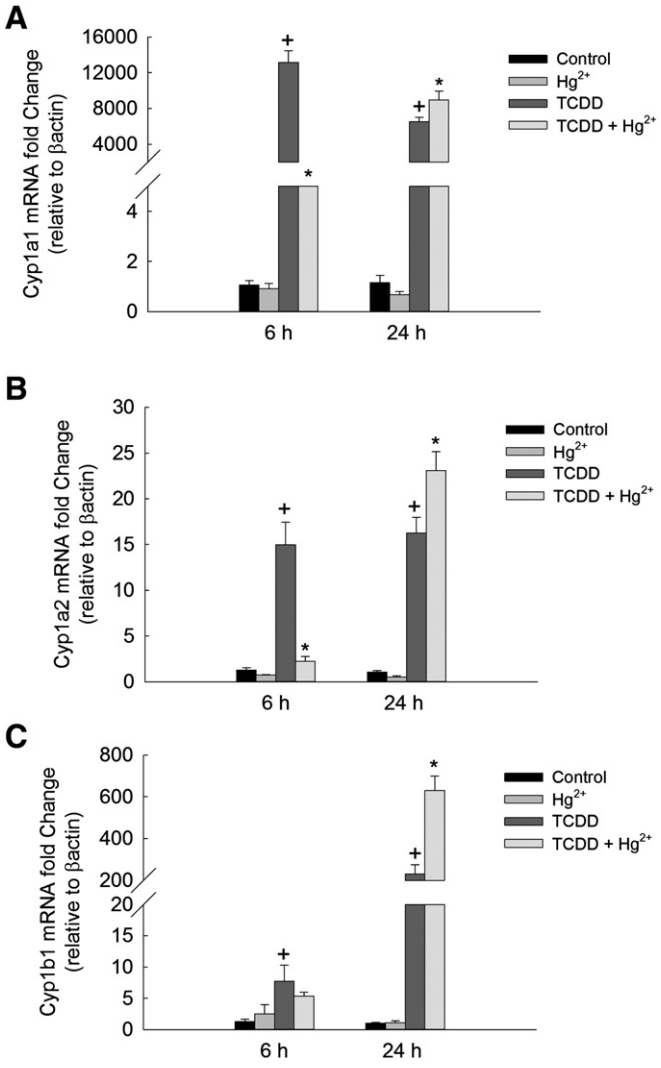

Fig. 1. Effect of co-exposure to Hg^{2+} and TCDD on liver Cyp1a1, Cyp1a2, and Cyp1b1 mRNA in C57BL/6J mice. Animals were injected i.p. with 2.5 mg/kg Hg^{2+} in the absence and presence of 15 μg/kg TCDD for 6 h and 24 h. First-strand cDNA was synthesized from total RNA (1.5 μg) extracted from liver and the expression of Cyp1a1, Cyp1a2, and Cyp1b1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. (+) $P<0.05$, compared to control (untreated animals); (*) $P<0.05$, compared to respective TCDD treatment.

control (Fig. 2A). When animals were co-exposed to Hg^{2+} and TCDD, Hg^{2+} significantly potentiated the TCDD-mediated induction of Cyp1a protein expression levels in the liver by 2.5- and 2.3-fold, respectively, compared to TCDD alone (Fig. 2A).

At the catalytic activity levels, Hg^{2+} alone did not significantly affect EROD or MROD activity in the liver (Figs. 2B and C). TCDD alone significantly induced EROD and MROD activities in the liver by 8- and 6-fold, respectively, compared to the control (Figs. 2B and C). However, when animals were co-exposed to Hg^{2+} and TCDD, Hg^{2+} significantly potentiated the TCDD-mediated induction of EROD and MROD activities in the liver by 2- and 2.4-fold respectively, compared to TCDD alone (Figs. 2B and C).

Effect of co-exposure to Hg^{2+} and TCDD on HO-1, mRNA level in the liver of C57Bl/6J mice

At 6 h Hg^{2+} and TCDD were able to significantly induce HO-1 mRNA levels in the liver by 13-and 4.6-fold respectively, compared to the control, while at 24 h they did not affect its mRNA levels (Fig. 3A). When animals were co-exposed to Hg^{2+} and TCDD, TCDD at 6 h significantly potentiated the Hg^{2+}-mediated induction of HO-1 mRNA levels in the liver by 7-fold, compared to Hg^{2+} alone (Fig. 3A). While, at 24 h it did not significantly affect the Hg^{2+}-mediated induction of HO-1 mRNA levels in the liver compared to Hg^{2+} alone (Fig. 3A).

Concentration- and time-dependent effect of co-exposure to Hg^{2+} and TCDD on Cyp1a1 mRNA levels in isolated mouse hepatocytes

To examine the effect of co-exposure to Hg^{2+} and TCDD on Cyp1a1 mRNA, isolated mouse hepatocytes were treated with various concentrations of Hg^{2+} (2.5–10 μM) in the presence of 1 nM TCDD (Fig. 4A). Thereafter, Cyp1a1 mRNA was assessed using real-time PCR. TCDD alone caused 308-fold increase in Cyp1a1 mRNA levels that was inhibited in a dose-dependent manner by Hg^{2+}. Initially, Hg^{2+} at the concentration of 2.5 μM caused a significant decrease in TCDD-mediated induction of Cyp1a1 mRNA levels by −1.3-fold. The maximum inhibition took place at the highest concentration tested (10 μM), which caused a decrease in the TCDD-mediated induction of Cyp1a1 mRNA levels by −3.7-fold (Fig. 4A).

To better understand the kinetics of Cyp1a1 mRNA in response to the co-exposure to Hg^{2+} and TCDD, the time-dependent effect was determined at various time points up to 24 h after treatment of isolated mouse hepatocytes with 1 nM TCDD in the absence and presence of 10 μM Hg^{2+}. Fig. 4B shows that TCDD-induced Cyp1a1 mRNA in a time-dependent manner. TCDD treatment caused a maximal induction of the Cyp1a1 mRNA by 412-fold at 12 h, compared to 0 h. However, a 22.6-fold of induction occurred as early as 3 h (Fig. 4B). In contrast, when isolated mouse hepatocytes were co-exposed to Hg^{2+} and TCDD, there was a significant decrease in the Cyp1a1 mRNA levels that occurred at 6 h by −1.7-fold, compared to TCDD alone. Similarly, Hg^{2+} significantly decreased the TCDD-mediated induction of Cyp1a1 mRNA levels at 12 h, and 24 h, by −1.6- and −2.7-fold, respectively, compared to TCDD alone (Fig. 4B).

Concentration-dependent effect of co-exposure to Hg^{2+} and TCDD on Cyp1a protein and catalytic activity in isolated mouse hepatocytes

To examine whether the observed inhibition of the TCDD-mediated induction of Cyp1a mRNA by Hg^{2+} is further translated to the protein and activity levels, isolated mouse hepatocytes were treated for 24 h with increasing concentrations of Hg^{2+} (2.5–10 μM) in the presence of 1 nM TCDD. Figs. 5A and B show that TCDD alone caused 3.8- and 15-fold increase in Cyp1a protein and catalytic activity, respectively. Of interest, Hg^{2+} decreased the TCDD-mediated induction of Cyp1a protein and catalytic activity levels in a dose-dependent manner. This inhibitory effect of Hg^{2+} on the Cyp1a protein and catalytic activity levels is in concordance with the observed effect at the mRNA levels, in which the initial significant inhibition took place at 2.5 μM Hg^{2+}, and reached the maximal inhibition at 10 μM (Figs. 5A and B).

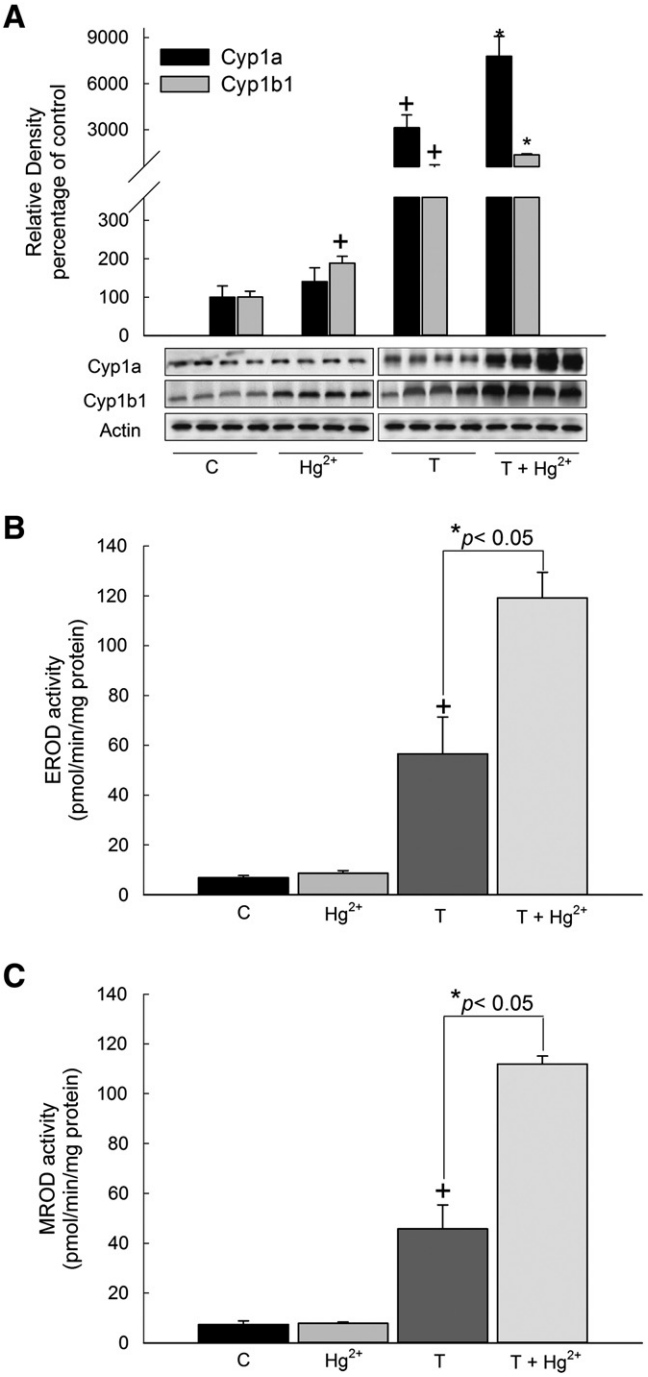

Fig. 2. Effect of co-exposure to Hg^{2+} and TCDD on liver Cyp1a and Cyp1b1 protein expression levels, and EROD and MROD activities in C57BL/6J mice. **(A)** Liver microsomal proteins were isolated after 24 h of treatment. 30 μg of microsomal proteins were separated on a 10% SDS-PAGE. Proteins were detected using the enhanced chemiluminescence method. The graph represents the relative amount of protein normalized to actin signals (mean ± SEM, $n = 6$), and the results are expressed as percentage of the control values taken as 100%. (B) and (C) EROD and MROD activities were measured using 7-ethoxyresorufin and 7-methoxyresorufin as substrates, respectively. The reaction was started by the addition of 1 mM NADPH and lasted for 5 min for EROD and 10 min for MROD. The reaction was terminated by the addition of ice cold acetonitrile. Values are presented as mean ± SEM ($n = 6$). (+) $P<0.05$, compared to control; (*) $P<0.05$, compared to respective TCDD treatment.

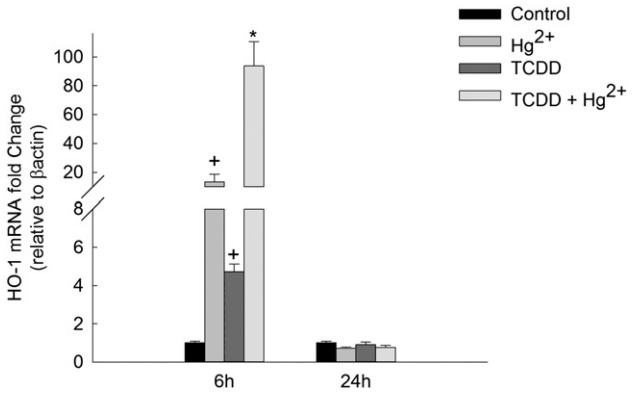

Fig. 3. Effect of co-exposure to Hg^{2+} and TCDD on liver HO-1 mRNA in C57BL/6J mice. Animals were injected i.p. with 2.5 mg/kg Hg^{2+} in the absence and presence of 15 μg/kg TCDD for 6 h and 24 h. First-strand cDNA was synthesized from total RNA (1.5 μg) extracted from liver and the expression of HO-1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. (+) $P<0.05$, compared to control (untreated animals); (*) $P<0.05$, compared to respective TCDD treatment.

Transcriptional and post-translational inhibition of Cyp1a by Hg^{2+}

In order to study the effect of Hg^{2+} on the AhR-dependent transcriptional activation, isolated mouse hepatocytes were transiently

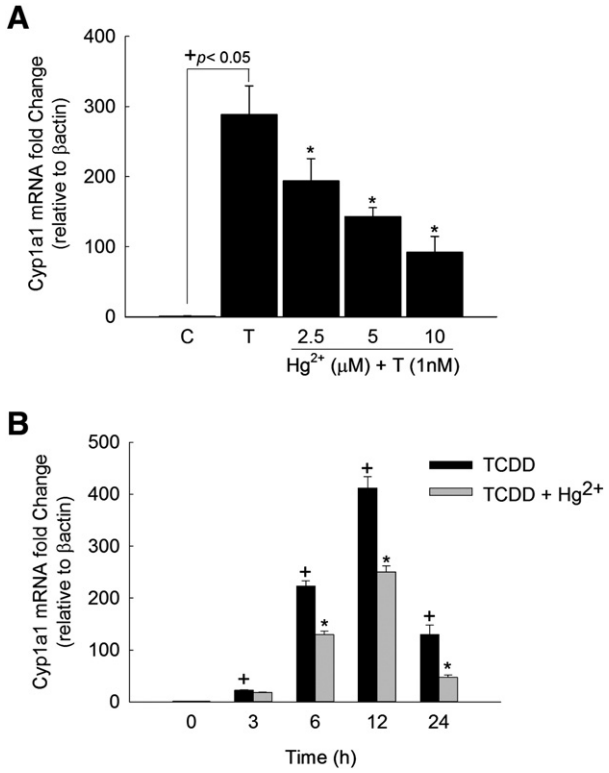

Fig. 4. Concentration- and time-dependent effect of Hg^{2+} on Cyp1a1 mRNA in isolated mouse hepatocytes. Hepatocytes were treated with increasing concentrations of Hg^{2+} in the presence of 1 nM TCDD for 6 h for concentration dependent (A) or for different time points for time-dependent (B). First-strand cDNA was synthesized from total RNA (1 μg) extracted from isolated mouse hepatocytes. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. (+) $P<0.05$, compared to control (C) (concentration = 0 μM or time = 0 h); (*) $P<0.05$, compared to respective TCDD (T) treatment.

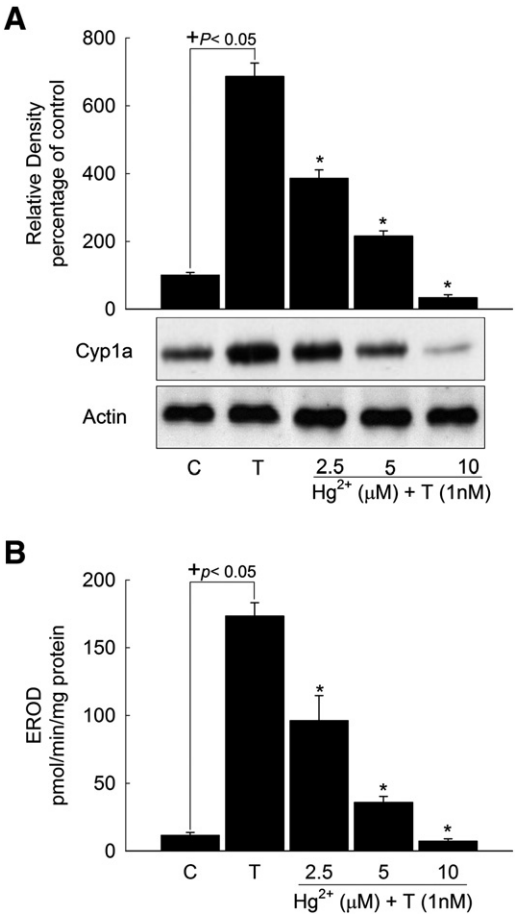

Fig. 5. Effect of Hg^{2+} on Cyp1a protein and catalytic activity in isolated mouse hepatocytes. Hepatocytes were treated with increasing concentrations of Hg^{2+} in the presence of 1 nM TCDD for 24 h for protein and catalytic activity. (A) Protein (20 μg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4 °C and then incubated with a primary Cyp1a antibody for 24 h at 4 °C, followed by 1 h incubation with secondary antibody at room temperature. Cyp1a protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to actin signals, which was used as loading control. One of three representative experiments is shown. (B) Cyp1a1 activity was measured in intact living cells treated with increasing concentrations of Hg^{2+}, in the absence and presence of 1 nM TCDD for 24 h. Cyp1a1 activity was measured using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SE ($n=6$). (+) $P<0.05$, compared to control (C); (*) $P<0.05$, compared to respective TCDD (T) treatment.

transfected with the XRE-driven luciferase reporter gene. Luciferase activity results showed that 10 μM Hg^{2+} alone did not affect the constitutive expression of the luciferase activity (Fig. 6). On the other hand, 1nM TCDD alone caused a significant increase of luciferase activity by 12.75-fold as compared to the control (Fig. 6). Interestingly, co-treatment with Hg^{2+} and TCDD significantly decreased the TCDD-mediated induction of luciferase activity by −1.8-fold (Fig. 6).

The effect of Hg^{2+} on HO-1 mRNA and the effect of SnMP as a competitive inhibitor of HO-1 on the posttranslational modification of Cyp1a1 catalytic activity by Hg^{2+}

The fact that Hg^{2+} inhibited the TCDD-mediated induction of Cyp1a1 at the catalytic activity level more than inhibiting its mRNA or protein levels prompted us to investigate the possible effect of Hg^{2+} on HO-1 mRNA levels. For this purpose isolated mouse hepatocytes were co-exposed to 2.5, 5.0 and 10 μM Hg^{2+} and 1 nM TCDD. Our results showed that, TCDD alone did not significantly affect HO-1 mRNA levels. In contrast, Hg^{2+} in the presence of 1nM TCDD was able to increase HO-1 mRNA in a dose-dependent manner by

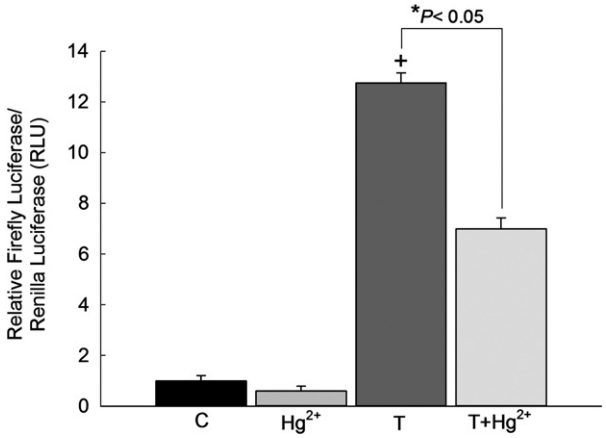

Fig. 6. Effect of Hg^{2+} on luciferase activity in isolated mouse hepatocytes. Hepatocytes were transiently transfected with the XRE-luciferase transporter plasmid pGudLuc1.1 and renilla luciferase plasmid pRL-CMV plasmid. Cells were treated with vehicle, Hg^{2+} (10 μM), TCDD (1 nM), TCDD (1 nM) + Hg^{2+} (10 μM) for 24 h. Cells were lysed and luciferase activity was measured according to manufacturer's instruction. Luciferase activity is reported relative to renilla activity. Values are presented as mean ± SE ($n=6$). (+) $P<0.05$, compared to control (C); (*) $P<0.05$, compared to respective TCDD (T) treatment.

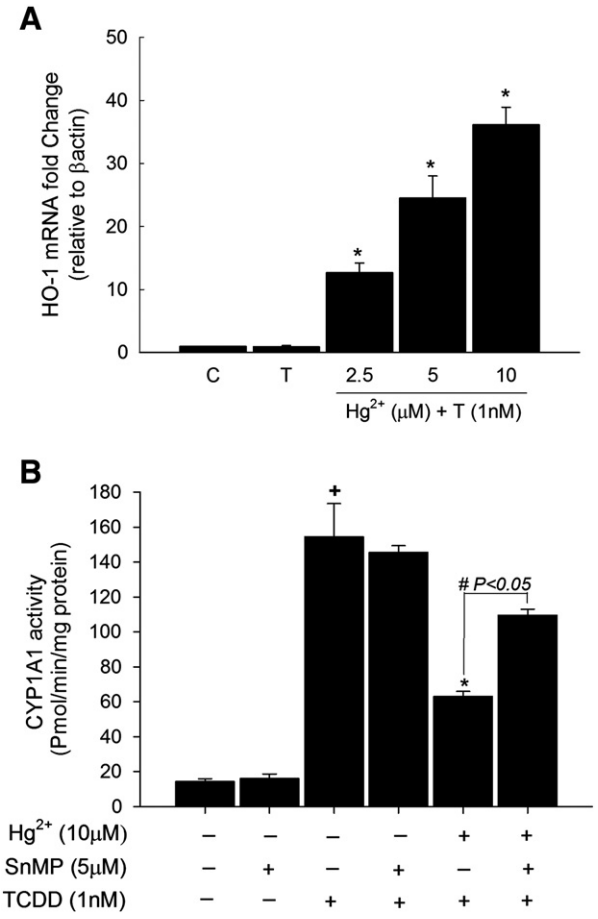

Fig. 7. The role of HO-1 in the inhibition of Cyp1a1 activity by Hg^{2+}. (A) Effect of Hg^{2+} on HO-1 mRNA. Hepatocytes were treated with increasing concentrations of Hg^{2+} in the presence of 1 nM TCDD for 6 h. First-strand cDNA was synthesized from total RNA (1 μg) extracted from hepatocytes. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. (+) $P<0.05$, compared to control (C) (concentration = 0 μM); (*) $P<0.05$, compared to respective TCDD (T) treatment. (B) Effect of SnMP as a competitive inhibitor of HO-1 on Hg^{2+}-mediated inhibition of Cyp1a1 activity. Hepatocytes were treated with 10 μM of Hg^{2+} and 1 nM TCDD in the presence and absence of 5 μM SnMP for 24 h for Cyp1a1 catalytic activity. Cyp1a1 activity was measured using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SE ($n=8$). (+) $P<0.05$, compared to control; (*) $P<0.05$, compared to respective TCDD treatment; (#) $P<0.05$, compared to respective Hg^{2+} + TCDD treatment.

12-, 24-, and 36-fold, respectively, compared to TCDD respective treatment (Fig. 7A).

To confirm the role of Hg^{2+}-induced HO-1 in decreasing the TCDD-mediated induction of Cyp1a1 catalytic activity, we examined the effect of HO-1 inhibitor, SnMP, on the Cyp1a1 catalytic activity that was inhibited by Hg^{2+}. For this purpose isolated mouse hepatocytes were co-exposed to 10 μM Hg^{2+} and 1 nM TCDD in the presence and absence of 5 μM SnMP. SnMP alone or in the presence of TCDD did not alter the Cyp1a1 catalytic activity. TCDD alone increased the Cyp1a1 catalytic activity by 14-fold. On the other hand, Hg^{2+} at the concentration of 10 μM decreased the TCDD-mediated induction of Cyp1a1 catalytic activity by −9-fold, compared to TCDD treatment (Fig. 7B). Intriguingly, SnMP partially reversed the Hg^{2+}-mediated decrease of Cyp1a1 activity to reach 8-fold compared to control. In spite of being successful in partially reversing the Hg^{2+}-mediated decrease of Cyp1a1 activity through inhibiting HO-1, SnMP was unable to completely restore the Cyp1a1 activity.

The effect of Hg^{2+} on serum Hb levels in vivo and the effect of Hb on XRE-luciferase activity in vitro

The discrepancy between the effects of Hg^{2+} on the TCDD-mediated induction of Cyp1a1 mRNA, protein, and catalytic activity levels *in vivo* and those observed at the *in vitro* level prompted us to examine the role of an endogenous mediator that could have influenced the effect of Hg^{2+} on the TCDD-mediated induction of Cyp1a1 *in vivo* but not *in vitro*. For this purpose, we measured serum Hb levels in C57BL/6J mice treated with Hg^{2+} in the absence and presence of TCDD. Our results demonstrated that Hg^{2+} increased serum Hb levels by 2-fold, compared to the control (Fig. 8A). In contrast, TCDD alone did not significantly affect serum Hb levels. Interestingly, when animals were co-exposed to Hg^{2+} and TCDD, Hg^{2+} increased serum Hb levels by 2.5-fold, compared to TCDD alone (Fig. 8A).

The results of Hg^{2+} on serum Hb levels further motivated us to examine its effect alone on the XRE-driven luciferase activity. For this purpose, isolated mouse hepatocytes were treated with 1 μM Hb in the absence and presence of 1nM TCDD. Our results demonstrated that Hb alone was able to significantly induce luciferase activity by 3.7-fold, compared to the control (Fig. 8B). TCDD alone significantly induce luciferase activity by 46.6-fold, compared to control. Interestingly, when isolated mouse hepatocytes were co-exposed to Hb and TCDD, Hb significantly potentiated the TCDD-mediated induction of luciferase activity by 1.27-fold, compared to TCDD alone (Fig. 8B).

In order to correlate the *in vivo* effects with those observed at the *in vitro* levels we tried to mimic the *in vivo* situation using an *in vitro* model. For this purpose, isolated mouse hepatocytes were treated with TCDD for 2 h in the absence and presence of Hg^{2+}. Thereafter, the treatment medium for cells receiving Hg^{2+} was replaced with another medium containing 1 μM Hb with TCDD. The reason behind replacing the medium with new treatment medium is that we were unable to detect any Hg^{2+} in the serum of animals who received Hg^{2+} treatment for 24 h using slow poke reactor (data not shown). In addition, we needed to remove Hg^{2+} from the medium to rule out any direct effect for Hg^{2+}. Our results showed that when cells were treated with Hg^{2+} and then treated with Hb there was no significant effect of Hb on the XRE-driven luciferase activity. Importantly, when cells were treated with Hg^{2+} and then co-exposed to TCDD and Hb, there was a significant potentiation to the XRE-driven luciferase activity by 1.98-fold, compared to Hg^{2+} in the presence of TCDD treatment (Fig. 8C).

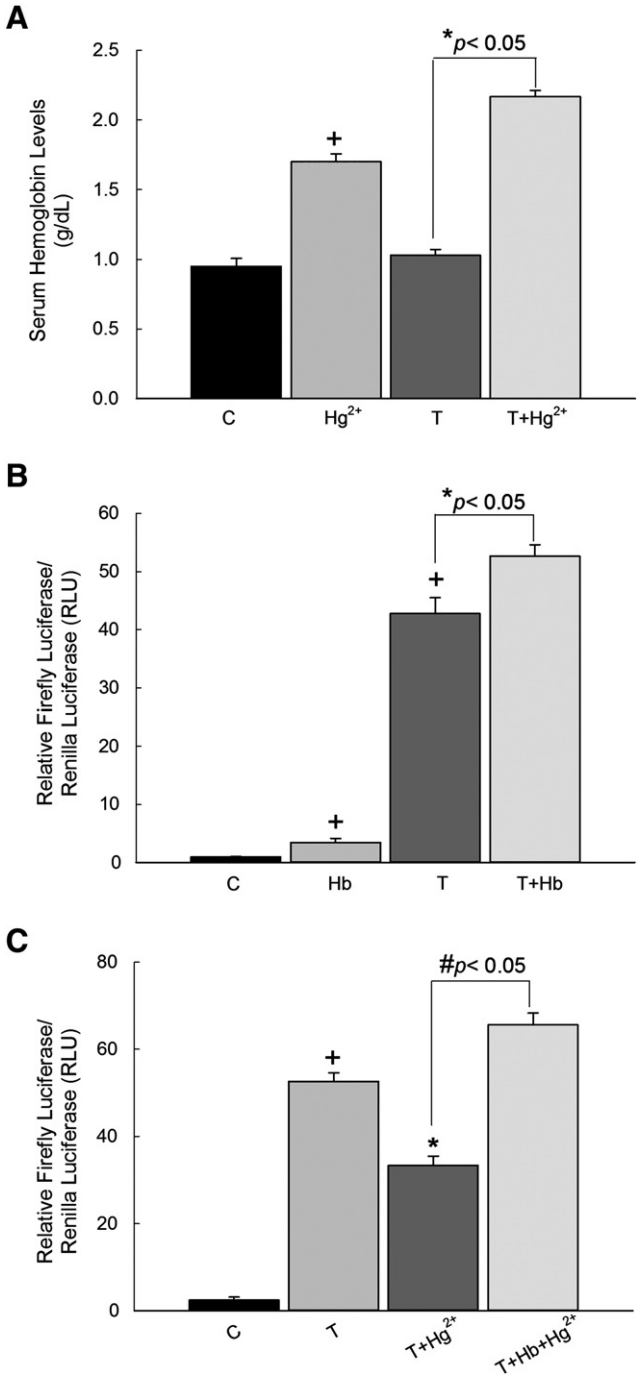

Fig. 8. Effect of Hg^{2+} on serum Hb levels *in vivo* and the effect of Hb on luciferase activity in isolated mouse hepatocytes. (A) Animals were injected i.p. with 2.5 mg/kg Hg^{2+} in the absence and presence of 15 μg/kg TCDD for 24 h. Total blood was collected and centrifuged to isolate serum. Serum Hb levels were measured as previously described under Materials and methods section. (B) Hepatocytes were transiently transfected with the XRE-luciferase transporter plasmid pGudLuc1.1 and renilla luciferase plasmid pRL-CMV plasmid. Cells were treated with vehicle, Hb (1 μM), TCDD (1 nM), TCDD (1 nM) + Hb (1 μM) for 24 h. Cells were lysed and luciferase activity was measured according to manufacturer's instruction. Luciferase activity is reported relative to renilla activity. Values are presented as mean ± SE ($n = 6$). (+) $P<0.05$, compared to control (C); (*) $P<0.05$, compared to respective TCDD (T) treatment. (C) Hepatocytes were transiently transfected with the XRE-luciferase transporter plasmid pGudLuc1.1 and renilla luciferase plasmid pRL-CMV plasmid. Cells were treated with vehicle, Hg^{2+} (10 μM), TCDD (1 nM), TCDD (1 nM) + Hg^{2+} (10 μM) for 2 h. Cells were then washed and cells that were treated with Hg^{2+} or TCDD + Hg^{2+} were further incubated with Hb for additional 24 h. Cells were lysed and luciferase activity was measured according to manufacturer's instruction. Luciferase activity is reported relative to renilla activity. Values are presented as mean ± SE ($n = 6$). (+) $P<0.05$, compared to control (C); (*) $P<0.05$, compared to respective TCDD (T) treatment.

Effect of Hb on the Cyp1a1 protein and catalytic activity levels

Western blot analysis was carried out to examine whether the obtained transcriptional activation of XRE by Hb is further translated to the protein level. Fig. 9A shows that Hb caused a significant concentration-dependent induction of the Cyp1a1 protein by 2.5-, 3-, and 4.5-fold when incubated for 24 h with Hb concentrations of 0.5, 1, and 2 μM, respectively. Expectedly, TCDD significantly induced the Cyp1a1 protein by 9-fold (Fig. 9A). To determine whether the effect of Hb on Cyp1a1 protein is further translated to Cyp1a1 catalytic activity, isolated mouse hepatocytes were incubated with increasing concentrations of Hb (0.5, 1 and 2 μM) for 24 h. Thereafter, Cyp1a1 catalytic activity was determined using EROD assay. Our results showed that Hb significantly induced Cyp1a1 catalytic activity in a concentration-dependent manner by 1.7-, 2.3-, and 3-fold with Hb concentrations of 0.5, 1, and 2 μM, respectively (Fig. 9B). Moreover, the positive control, TCDD, significantly induced Cyp1a1 catalytic activity by 14-fold (Fig. 9B).

To examine the role of AhR in the induction of Cyp1a1 catalytic activity by Hb, isolated mouse hepatocytes were preincubated with AhR

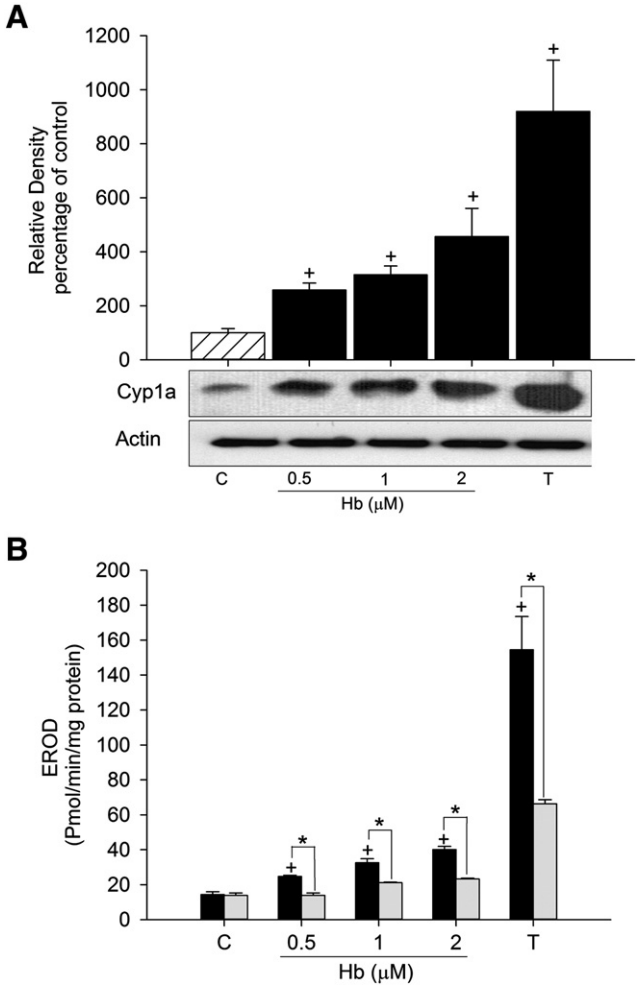

Fig. 9. Effect of Hb on Cyp1a1 protein and catalytic activity in isolated mouse hepatocytes. Cells were treated with increasing concentrations of Hb (0.5–2 μM) or TCDD (1 nM) as a positive control for 24 h. (A) Cells were then harvested and Cyp1a1 protein was determined using Western blot analysis. The graph represents the average optical density (±S.E.M.) of bands from three different experiments as a percent of control. (B) Cyp1a1 activity was determined using Cyp1a1-dependent EROD assay. To investigate the role of AhR in the induction of Cyp1a1 by Hb, cells were incubated with resveratrol (20 μM) for 2 h prior to the treatment with Hb for an additional 24 h. Values represent mean activity ± S.E.M. ($n = 8$). (+) $P<0.05$ compared to control, (C); (*) $P<0.05$ compared to the treatment.

antagonist, resveratrol, (20 μM) for 2 h before the treatment with Hb or TCDD for an additional 24 h. Our results showed that resveratrol significantly reduced the induction of Cyp1a1 at catalytic activity mediated either by Hb or TCDD (Fig. 9B).

Discussion

The current study provides the first evidence that Hg^{2+} differentially modulates the constitutive and TCDD-inducible Cyp1a and Cyp1b1expression and activity in C57BL/6J mouse liver and isolated mouse hepatocytes. Furthermore, the concentrations of Hg^{2+} and TCDD utilized in the current study were selected based on previous *in vivo* studies that used the same and different mouse strains (Hu et al., 1999; Mehra and Kanwar, 1980; Tanaka-Kagawa et al., 1998; Uno et al., 2008; Wong et al., 2010). The reasons of choosing these single doses is that because administration of repeated doses of Hg^{2+} leads to a considerable fall in total protein content in different tissues as previously studied, where the maximum decrease (39.6 %) being observed in the liver (Mehra and Kanwar, 1980). In addition, the LD50 values of mercury for various rodent species including rats, mice and guinea pigs range from 29.9 mg/kg to 57.6 mg/kg, and 21 mg/kg, respectively (RTECS, 1985–1986).

Our results showed that Hg^{2+} alone did not affect liver Cyp1a1, Cyp1a2, or Cyp1b1 at the mRNA, protein or catalytic activity levels, with the exception of Cyp1b1 protein expression levels. Importantly, Hg^{2+} inhibited the TCDD-mediated induction of liver Cyp1a1 and Cyp1a2 mRNA levels at 6 h while significantly potentiating the TCDD-mediated induction of liver Cyp1a1, Cyp1a2 and Cyp1b1 mRNA levels at 24 h. Interestingly, Hg^{2+} potentiated the TCDD-mediated induction of liver Cyp1a1, Cyp1a2, and Cyp1b1 protein expression levels with a concomitant potentiation in the EROD and MROD catalytic activity levels.

To the best of our knowledge, this is the first study to examine the effect of co-exposure to Hg^{2+} and TCDD on the AhR-regulated P450s in the liver of C57Bl/6J mice. The discrepancy between the effect of Hg^{2+} on the TCDD-mediated induction of Cyp1a1 and Cyp1a2 mRNA obtained at 6 h and 24 h time points might be due to multiple factors. We hypothesized that the effects observed at 6 h are due to the direct effect of Hg^{2+}. However, the effect after 24 h is an indirect effect that could be attributed to other endogenous factors. For example, Hg^{2+} might have affected other physiological processes or even triggered the release of other physiological modulators such as bilirubin or billiverdin which are known AhR-ligands (Denison and Nagy, 2003).

In the current study we have demonstrated that Hg^{2+} alone or in the presence of TCDD was able to induce liver HO-1 mRNA levels as early as 6 h. However, this induction was completely abolished after 24 h of treatment. Thus, this finding implies that HO-1 was almost completely eliminated after 6 h. These results are in agreement with previous studies which reported that HO-1 mRNA and protein half-life are 1.6 and 8 h, respectively (Lam et al., 2005; Panchenko et al., 2000).

When we treated isolated mouse hepatocytes with increasing concentration of Hg^{2+} in the presence of TCDD, Hg^{2+} decreased Cyp1a1 mRNA, protein, and catalytic activity levels in a dose-dependent manner. To the best of our knowledge, we are the first to report these effects in isolated mouse hepatocytes. In contrast, previous reports from our laboratory have demonstrated that Hg^{2+} potentiated the TCDD-mediated induction of Cyp1a1 mRNA and protein expression levels in the mouse liver derived cell line Hepa 1c1c7 cells after 6 h of treatment (Korashy and El-Kadi, 2005). The controversy between the effect of Hg^{2+} on Hepa 1c1c7 cells on one side and isolated mouse hepatocytes on the other side might be due to mechanistic differences in the regulation of Cyp1a1 gene expression upon treatment by TCDD which has been previously reported between Hepa 1c1c7 cells and the human HepG2 cells (Ramadoss and Perdew, 2005; Ramadoss et al., 2004). Furthermore, Hepa 1c1c7 cells are immortalized cells and thus might not represent the freshly isolated mouse hepatocytes due to genetic manipulations in Hepa 1c1c7 cells which could have affected the AhR regulatory pathway.

The transcriptional regulation of *Cyp1a1* gene expression by Hg^{2+} was also investigated. In this context, we have demonstrated that Hg^{2+} in the presence of TCDD was able to significantly decrease the AhR-dependent luciferase reporter gene expression (Fig. 7). Thus, the down-regulation of *Cyp1a1* gene expression by Hg^{2+} was mediated through an AhR-dependent mechanism. The ability of Hg^{2+} to inhibit the Cyp1a1 at the activity level more than that observed effect on the mRNA or protein expression levels in isolated mouse hepatocytes raised the question whether there is a post-translational modification that might have occurred to the Cyp1a1 protein. Evidence from our laboratory and others suggests a role of HO-1 in the Hg^{2+}-mediated decrease in Cyp1a1 catalytic activity levels in Hepa 1c1c7 cells, and HepG2 cells (Amara et al., 2010; Korashy and El-Kadi, 2005).

In this study, we have shown that Hg^{2+} significantly increased the HO-1 mRNA level at all concentrations tested, implying the possibility that Hg^{2+} might have decreased Cyp1a1 activity through degrading its heme content *via* HO-1. HO-1, an enzyme of 32 kDa, catalyzes the oxidative conversion of heme into billiverdin, which serves an important role in protecting cells from oxidative damage, such as free radicals (Marilena, 1997). HO-1 anchors to the endoplasmic reticulum membrane *via* a stretch of hydrophobic residues at the C-terminus (Schuller et al., 1998). Thus, it is expected to interact with CYP450s which are also endoplasmic reticulum-bound enzymes. The results of the current study are in agreement with our previous published data in which Hg^{2+} was shown to be able to significantly induce HO-1 mRNA expression in Hepa 1c1c7 and HepG2 cells (Amara et al., 2010; Korashy and El-Kadi, 2005). The fact that Hg^{2+} induces HO-1 with a consequent decrease in the heme pool could result in the failure to form a functioning Cyp1a1 protein. Moreover, the apoprotein would be more susceptible to proteasomal degradation (Anwar-Mohamed et al., 2009). However, this might have not occurred at the *in vivo* level due to the fact that HO-1 was not persistently elevated, and thus its effect could not be seen at the activity level.

The role of HO-1 in the down-regulation of Cyp1a1 at the catalytic activity level was supported by using SnMP as a competitive inhibitor of HO-1. In this regard, SnMP was able to partially prevent the decrease in TCDD-mediated induction of Cyp1a1 activity by Hg^{2+}. The observed effect of SnMP on the Hg^{2+}-mediated decrease of Cyp1a1 catalytic activity was solely through competitively inhibiting HO-1 protein and not through altering Cyp1a1 mRNA (data not shown). In addition, it was previously demonstrated that SnMP alone is incapable of producing any effect on HO-1 mRNA and Cyp1a1 mRNA and catalytic activity (Amara et al., 2010; Anwar-Mohamed and El-Kadi, 2010).

An important difference between our current *in vivo* and *in vitro* studies with regard to the effect of Hg^{2+} on the TCDD-mediated induction of Cyp1a1 mRNA, protein, and catalytic activity is the distribution of Hg^{2+} which might have contributed to the differential expression between *in vitro* which expressed the direct effect of the metal and *in vivo* which applied an indirect effect (Sundberg et al., 1998). As such, it has been demonstrated previously that metallic mercury is preferentially deposited in the kidneys and lungs than other vital organs in mice (Kim et al., 1995). However, at the *in vitro* level, Hg^{2+} was persistently present with the cells throughout the treatment time-course. Thus, the effect we observe *in vitro* is the direct effect of Hg^{2+} which would be the opposite case at *in vivo* situation.

It is well established that Hg^{2+} has higher binding capacity with thiol group such as in glutathione (GSH) which plays an important role in Hg^{2+} metabolism (Clarkson, 1997; Custodio et al., 2005). Whereas, human hemoglobin has six free thiol groups — one in

each of the α-chains and two in each of the β-chains, hemoglobin of other vertebrates may have several numbers of thiol functions (Lawn et al., 1980; Wilson et al., 1980). Rats for example, have ten reactive thiol functions per hemoglobin, three in each α-chain and two in the β-chain (Chua et al., 1975; Radosavljevic and Crkvenjakov, 1989). Previous study showed that about 80% of mercury can be recovered in red blood cells (RBC) (Trumpler et al., 2009). Since hemoglobin is the most highly abundant constituent of RBC besides water, a reaction between mercury and hemoglobin is expected. In this context, binding between mercury and hemoglobin has been proven (Janzen et al., 2011). Furthermore, Hg^{2+} even at low concentrations, was able to make an adduct formation with hemoglobin which in turn leads to increased plasma hemoglobin levels (Janzen et al., 2011).

The fact that Hg^{2+} possess higher capacity of binding to thiol group such as in hemoglobin, which in turn leads to increased Hb levels in the plasma, prompted us to measure serum Hb levels from animals treated with Hg^{2+} for 24 h (Clarkson, 1997; Custodio et al., 2005; Janzen et al., 2011). Our results demonstrated that Hg^{2+} in the absence and presence of TCDD increases serum Hb levels. Previous data from our laboratory have demonstrated that Hb is capable of increasing CYP1A1 activity in human HepG2 cells (Anwar-Mohamed and El-Kadi, 2010). Therefore, we examined its effect on XRE-driven luciferase activity in isolated mouse hepatocytes. Our results demonstrated that Hb in the absence and presence of TCDD was able to increase XRE-driven luciferase activity. These results motivated us to test Hb effect in the presence of Hg^{2+} in an attempt to mimic its *in vivo* effect. Our results demonstrated that Hb treated isolated mouse hepatocytes in the presence of Hg^{2+} and TCDD further potentiated the TCDD-mediated increase in the XRE-driven luciferase activity. Thus, the *in vivo* effect of Hg^{2+} on the TCDD-mediated induction of Cyp1a1 mRNA, protein, and catalytic activity levels at 24 h might be attributed to the direct effect of Hb on AhR signaling or due to an indirect effect through heme release, and heme degradation by-products (billiverdin and bilirubin).

In this context, mechanistic experiments have been carried out to explore whether the effect of Hb on Cyp1a1 luciferase activity is occurring through the AhR signaling pathway. Firstly, we tested the effect of Hb on Cyp1a1 protein expression using isolated mouse hepatocytes. Our results showed that Hb significantly induces the Cyp1a1 protein in a dose-dependent manner. Secondly we investigated its effect on Cyp1a1 catalytic activity levels in the absence and presence of a known AhR antagonist; resveratrol in isolated mouse hepatocytes. Our results showed that Hb was able to induce Cyp1a1 catalytic activity levels in a concentration-dependent manner. On the other hand, resveratrol significantly inhibited the Hb-mediated induction of Cyp1a1 activity, suggesting the involvement of an AhR-dependent mechanism. We postulate that this effect could be due to the direct effect of Hb or indirectly through its metabolic products heme, bilirubin, and biliverdin which are previously known AhR ligands (Phelan et al., 1998; Sinal and Bend, 1997).

In conclusion, the present study demonstrated for the first time that Hg^{2+} differentially modulates constitutive and TCDD-induced Cyp1a1 mRNA, protein, and activity between C57BL/6J mouse liver and isolated hepatocytes. Furthermore, the effect of Hg^{2+} indicate more complex regulation of Cyp1a1 at the *in vivo* level as there are several factors that can confound the *in vivo* results which are not present *in vitro*. In addition, caution should be taken when extrapolating *in vitro* data to *in vivo* situation that warrants further investigation.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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