Posttranslational mechanisms modulating the expression of the cytochrome P450 1A1 gene by methylmercury in HepG2 cells: A role of heme oxygenase-1

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HIGHLIGHTS

- MeHg does not alter CYP1A1 mRNA or protein levels.
- MeHg significantly inhibited CYP1A1 activity.
- MeHg exerts its effect on CYP1A1 activity via HO-1.
- Inhibition or knockdown of HO-1 revert the effects of MeHg on CYP1A1 activity.
- MeHg induces NQO1 and HO-1 possibly through ARE.

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ABSTRACT

Recently we demonstrated the ability of mercuric chloride (Hg2+) in human hepatoma HepG2 cells to significantly decrease the TCDD-mediated induction of Cytochrome P450 1A1 (CYP1A1) mRNA, protein, and catalytic activity levels. In this study we investigated the effect of methylmercury (MeHg) on CYP1A1 in HepG2 cells. For this purpose, cells were co-exposed to MeHg and TCDD and the expression of CYP1A1 mRNA, protein, and catalytic activity levels were determined. Our results showed that MeHg did not alter the TCDD-mediated induction of CYP1A1 mRNA, or protein levels; however it was able to significantly decrease CYP1A1 catalytic activity levels in a concentration-dependent manner. Importantly, this inhibition was specific to CYP1A1 and was not radiated to other aryl hydrocarbon receptor (AhR)-regulated genes, as MeHg induced NAD(P)H:quinone oxidoreductase 1 mRNA and protein levels. Mechanistically, the inhibitory effect of MeHg on the induction of CYP1A1 coincided with an increase in heme oxygenase-1 (HO-1) mRNA levels. Furthermore, the inhibition of HO-1 activity, by tin mesoporphyrin, caused a complete restoration of MeHg-mediated inhibition of CYP1A1 activity, induced by TCDD. In addition, transfection of HepG2 cells with siRNA targeting the human HO-1 gene reversed the MeHg-mediated inhibition of TCDD-induced CYP1A1. In conclusion, this study demonstrated that MeHg inhibited the TCDD-mediated induction of CYP1A1 through a posttranslational mechanism and confirms the role of HO-1 in a MeHg-mediated effect.

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1. Introduction

Cytochrome P450s (CYPs) are the major enzymes involved in xenobiotic metabolism accounting for around 75% of the total transformations of xenobiotics to either non-toxic or carcinogenic metabolites (Guengerich, 2008). Among these enzymes, CYP1A1 is of major interest because of its role in bioactivating procarcinogens and environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) into carcinogenic and mutagenic intermediates (Guengerich, 2004). Halogenated aromatic hydrocarbons (HAHs), such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), are metabolically and chemically stable and highly lipophilic in the environment, with binding affinities in the pM to nM range (Denison and Nagy, 2003). Therefore, due to their persistence, HAHs are compounds of environmental concern. In this regard, it is well documented that CYP1A1 bioactivates PAHs to epoxide and diol-epoxide intermediates that subsequently lead to DNA and protein adducts formation (Shimada and Fuji-Kuriyama, 2004). HAHs can further enhance their carcinogenic effects by
inducing the expression of the CYP1A1 gene, thereby increasing the levels of bioactivated PAHs intermediates (Nebert et al., 2004). The current knowledge of the mechanism of CYP1A1 induction by TCDD, the most potent CYP1A1 inducer tested to date (Mimura and Fujii-Kuriyama, 2003), suggests a transcriptional regulation, in which the binding of TCDD to a cytosolic transcription factor, the aryl hydrocarbon receptor (AhR), is the first step in a series of events leading to carcinogenicity and mutagenicity (Whitlock, 1999). The TCDD/AhR complex translocates to the nucleus where it heterodimerizes with the AhR nuclear translocator (ARNT) transcription factor, this whole complex of the TCDD/AhR/ARNT then binds to xenobiotic responsive elements (XRE) located in the promoter region of CYP1A1 resulting in the initiation of the mRNA transcription process (Pollenz, 2002; Whitlock, 1999).

One of the most common environmental problems that possesses multiple biological consequences, particularly to the xenobiotic metabolizing enzyme systems in the body is co-contamination with complex mixtures of HAHs and heavy metals (Amara et al., 2010). Heavy metals and HAHs are common contaminants of hazardous waste sites and are co-released from sources such as fossil fuel combustion, municipal waste incineration, and as components of tobacco smoke (Mclemore et al., 1990). Both HAHs 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 (ATSDR, 2011) and the Canadian Environmental Protection Act (CEPA, 2012).

Among the heavy metals, mercury is of potential interest since it is liberated from the earth’s crust to the biosphere through degassing from volcanic areas or evaporation from the oceans in the form of elemental vapor (Hg^0). The natural emissions are estimated to be between 2700 and 6000 tons per year (Lindberg et al., 1987). In addition, human activities have been estimated to add another 2000–3000 tons to the total release of mercury to the environment (Lindberg et al., 1987). Part of the emitted inorganic mercury becomes oxidized to Hg^2+ and then methylated or transformed into organomercurials. The methylation is believed to involve a nonenzymatic reaction between Hg^2+ and a methylcobalamin compound (analog of vitamin B_12) that is produced by bacteria (Wood and Wang, 1983). This reaction takes place primarily in aquatic systems. The intestinal bacterial flora of various animal species including fish is also, able to convert ionic mercury into methylmercuric (MeHg) compounds. MeHg is the most frequently encountered organic mercury compound in the environment (Wood and Wang, 1983). Moreover, increases in industrialization and changes in the environment during the twentieth century, has become one of the major sources that made humans and animals more exposed to numerous chemical forms of mercury, including MeHg (Fitzgerald and Clarkson, 1991). Inasmuch as mercury is ubiquitous in the environment, it is nearly impossible for most humans to avoid exposure to MeHg.

Previous reports, from our laboratory and others have demonstrated that inorganic mercury in the form of mercuric chloride (Hg^2+), alters the expression of the carcinogen-activating enzyme CYP1A1 at different stages along its signaling pathway and hence could affect the mutagenicity and carcinogenicity of HAHs (Amara et al., 2010; Korashy and El-Kadi, 2005). Therefore, the objective of the current study was to investigate whether the same effect on CYP1A1 expression gene would be observed upon exposure to MeHg in the human hepatoma HepG2 cells. To the best of our knowledge, this manuscript provides the first evidence for the ability of MeHg to reduce the TCDD-mediated induction of CYP1A1 by TCDD in HepG2 cells through a posttranslational mechanism.

2. Materials and methods

2.1. Material

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), fluororescine, anti-goat IgG peroxidase secondary antibody, protease inhibitor cocktail, and methyl mercury (II) chloride (MeHgCl) were purchased from Sigma Chemical Co. (St. Louis, MO). Tin mesoporphyrin (SmNP), 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 Hox11 (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, GADPH 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 μL·gl−1·glutamine, 50 μg/mL amikacin, 100 μL/mL penicillin, 25 μg/mL streptomycin, 2.0 μg/mL amphotericin B, 0.1 mM nonessential amino acids, and vitamin supplement solution. Cells were grown in 75-cm² cell culture flasks at 37 °C in a 5% CO₂ humidified incubator.

2.3. Chemical treatments

Cells were treated in serum free medium with various concentrations of MeHg (1.25–5 μM) in the absence and presence of 1 nM TCDD, and/or 5 μM SnMP as described in figure legends. TCDD and SnMP were dissolved in dimethylsulfoxide (DMSO) and maintained in DMSO at −20 °C until use. MeHg (10 nM stock) was prepared freshly in double de-ionized water. In all treatments, the DMSO concentration did not exceed 0.05% (v/v).

2.4. Effect of MeHg on cell viability

The effect of MeHg on cell viability was determined using the MTT assay as described previously (Amara et al., 2010). MTT assay measures the conversion of MTT to formazan in 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% CO₂ humidified incubator. Cells were treated with various concentrations of MeHg (1.25–20 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 cDNA synthesis

Six hours after incubation with the test compounds, cells were collected and total RNA was isolated using TRizol reagent (Invitrogen) according to the manufacturer’s instructions and quantified by measuring the absorbance at 260 nm. Thereafter, first-strand cDNA synthesis was performed 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 10−4 reverse transcription (RT) buffer, 0.8 μL 25 × dNTP mix (100 mM), 2.0 μL 10−4 RT random primers, 1.0 μL MultiScribe reverse transcriptase, and 3.2 μL 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.

2.6. Quantification by real-time PCR

Quantitative analysis of specific mRNA expression was performed by 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). Twenty-five microliter reactions 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. The primers used in this study were chosen from previously published studies (Rushworth et al., 2008; Westerink and Schoonen, 2007) and are listed in Table 1.
Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td>5'-CTATCTGGGCTTGTTGCCAA-3'</td>
<td>5'-CTGTCCTAAGCGCCAACCTTGG-3'</td>
</tr>
<tr>
<td>NQO1</td>
<td>5'-CGGACACCTTGATATCCAG-3'</td>
<td>5'-CGTTTCTCCATCCTCTCACGG-3'</td>
</tr>
<tr>
<td>HO-1</td>
<td>5'-ATGCCTCCTGTTGCAACATC-3'</td>
<td>5'-CTGTGGCCATCCTCCTCCCT-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-CTGGACCGACCAACATG-3'</td>
<td>5'-GGCCGTCCCACACGAGTCT-3'</td>
</tr>
</tbody>
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Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. After the plate was sealed 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 anneal/extension at 60 °C for 1 min. A 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.

2.7. Real-time-PCR data analysis

The real-time PCR data were analyzed using the relative gene expression, i.e., the ΔΔCT method, as described in Applied Biosystems User Bulletin No. 2 and explained further by Livak and Schmittgen (2001). In brief, the primers used in this study were tested to avoid primer dimers, self-primer formation, or nonspecific amplification. To ensure the quality of the measurements, each plate included, for each gene, a negative control and a positive control. For each sample, a threshold cycle (Ct) was calculated based on the time (measured by the number of PCR cycles) at which the reporter fluorescence emission increased beyond a threshold level (based on the background fluorescence of the system). The triplicate measurements for each sample were averaged to give an average Ct value for each group, after removing the outliers (Agarici et al., 2005). The samples were diluted in such a manner that the Ct value was observed between 15 and 30 cycles. Results were expressed using the comparative CΔCT method as described in User Bulletin 2 (Applied Biosystems). Briefly, the CΔCT values were calculated in every sample for each gene of interest as follows:

\[ C_{\Delta \Delta CT} = C_{\Delta CT} - C_{\Delta CT, \text{control}} \],

where CΔCT is the Ct value of the tested gene, CΔCT,reporter is the Ct value of the reporter gene, and CΔCT,control is the Ct value of the control group. The values and ranges given in different figures were determined as follows: with ΔΔCT > SE and ΔΔCT < SE, where SE is the standard error of the mean of the ΔΔCT value (User Bulletin 2; Applied Biosystems).

2.8. 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 NaCl, 1.5 mM magnesium chloride, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 μg/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 et al., 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.

2.9. Determination of CYP1A1 enzymatic activity

CYP1A1-dependent 7-ethoxyresorufin O-deethylase (EROD) was performed on intact living cells using 7-ethoxyresorufin as previously described (Amara et al., 2010). Enzymatic activity was normalized for cellular protein content, which was determined using a modified fluorescent assay (Lorenzen and Kennedy, 1993).

2.10. Transient transfection and luciferase assay

HepG2 cells were plated onto 12-well cell culture plates. Each well of cells was transfected with 1 μg of XRE-driven luciferase reporter plasmid DNA (Promega) generously provided by Dr. M.S. Denison (University of California, Davis), 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 System (Promega) on a TD-20/20 luminometer (Turner BioSystems, Sunnyvale, CA). Luciferase activity is reported as emitted light per well as a percent of control, vehicle treated cells.

2.11. Transflecting HepG2 with HO-1 siRNA

HepG2 cells were plated onto 24-well cell culture plates. Each well of cells was transfected with HO-1 siRNA at the concentration of 20 nM using INTERFERIN reagent according to manufacturer’s instructions (Polyplus). HO-1 siRNA sequences were sense: CAA AUG CAG UAU UUU UGU UTT, and antisense: AAG AAA AUU ACU GCA UUU Gag. Transfection efficiency was determined using real-time PCR to detect HO-1 mRNA posttransfection at 6, 12, and 24 h. Therefore, cells were treated 6 h posttransfection with TCDD in the absence and presence of MeHg (5 μM) for 6 h to determine HO-1 and CYP1A1 mRNA levels, or 24 h to determine CYP1A1 catalytic activity levels.

2.12. Statistical analysis

The comparative analysis of the results from various experimental groups with their corresponding controls was performed using SigmaStat for Windows (Systat Software, Inc., CA). A t-test was carried out to assess statistical significance between control and TCDD treatments. Thereafter, a one-way analysis of variance (ANOVA) followed by Dunnett’s test was carried out to assess statistical significance between treatment groups compared to TCDD. For mRNA and protein half-lives statistical significance was assessed using two-way ANOVA followed by Dunnett’s test. The differences were considered significant when P < 0.05.

3. Result

3.1. Effect of co-exposure to MeHg and TCDD on cell viability

To determine the non-toxic concentrations of MeHg to be utilized in the current study, HepG2 cells were exposed for 24 h with increasing concentrations of MeHg (1.25–20 μM) in the absence and presence of 1 nM TCDD, thereafter cytotoxicity was assessed using the MTT assay. Fig. 1 shows that MeHg at concentrations of 1.25–5 μM in the presence and absence of 1 nM TCDD did not affect cell viability. However, the highest concentration tested

![Fig. 1. Effect of MeHg on cell viability. HepG2 cells were treated for 24 h with MeHg (1.25–20 μM) in the absence and presence of 1 nM TCDD. Cell cytotoxicity was determined using MTT assay. Data are expressed as percentage of untreated control (which is set at 100%) ± SE (n = 8). *P < 0.05, compared to control (concentration = 0 μM); †P < 0.05, compared to respective TCDD treatment.](image)
(10–20 μM), significantly reduced the cell viability by 35% and 90%, respectively (Fig. 1). Therefore, all subsequent studies were conducted using the concentrations of 1.25–5 μM.

3.2. Concentration-dependent effect of co-exposure to MeHg and TCDD on CYP1A1 mRNA

To examine the effect of co-exposure to MeHg and TCDD on CYP1A1 mRNA, HepG2 cells were treated with various concentrations of MeHg (1.25–5 μM) in the presence of 1 nM TCDD (Fig. 2). Thereafter, CYP1A1 mRNA was assessed using real-time PCR. Our results show that TCDD alone significantly induced CYP1A1 mRNA by 45-fold. Furthermore, MeHg was unable to significantly affect the TCDD-mediated induction of CYP1A1 mRNA at all concentrations tested (Fig. 2).

3.3. Concentration-dependent effect of co-exposure to MeHg and TCDD on CYP1A1 protein and catalytic activity

HepG2 cells were treated for 24 h with increasing concentrations of MeHg (1.25–5 μM) in the presence of 1 nM TCDD. Fig. 3A shows that TCDD alone significantly induced CYP1A1 protein levels by 24-fold. In agreement with the CYP1A1 mRNA results, MeHg did not significantly affect the TCDD-mediated induction of CYP1A1 protein levels at all concentrations tested. On the other hand, TCDD alone significantly induced the CYP1A1 catalytic activity by 16.5-fold, which was significantly decreased by MeHg in a concentration-dependent manner by ~2.4–6.4– and ~17.3-fold with the concentrations of 1.25, 2.5, and 5 μM, respectively, compared to TCDD treatment (Fig. 3B).

3.4. Effect of MeHg and TCDD mixture on XRE-driven luciferase reporter gene

HepG2 cells were plated onto 12-well cell culture plates. The XRE-driven luciferase reporter gene and the Renilla luciferase pRL-CMV vector, used for normalization, were co-transfected into HepG2 cells. Luciferase activity results showed that 5 μM MeHg alone did not affect the constitutive expression of the luciferase activity compared to control (Fig. 4). On the other hand, 1 nM TCDD alone caused a significant increase in the luciferase activity by 62-fold compared to control. Importantly, co-treatment with MeHg and TCDD did not affect the TCDD-mediated induction of the XRE-driven luciferase activity compared to TCDD alone (Fig. 4).

3.5. Direct effect of MeHg on TCDD-mediated induction of CYP1A1 catalytic activity

To examine the possible direct inhibitory effect of MeHg on CYP1A1 catalytic activity, HepG2 cells were treated for 24 h with 1 nM TCDD. Thereafter, cells were incubated with increasing concentrations of MeHg for 2 h, and the CYP1A1 catalytic activity levels were determined in intact living cells using EROD assay. Our results showed that TCDD alone significantly increased CYP1A1 catalytic activity by 17-fold compared to control (Fig. 5A). In contrast, MeHg
did not cause any direct inhibitory effect on the TCDD-mediated induction of CYP1A1 catalytic activity levels (Fig. 5A).

3.6. The effect of supplementing NADPH on the MeHg-mediated decrease in CYP1A1 activity

To investigate whether the MeHg-mediated decrease in CYP1A1 activity is a result of decreased intracellular NADPH, HepG2 cells were treated for 24 h with 5 μM MeHg with or without 1 nM TCDD and the CYP1A1 activity was assessed in total cellular lysates in the presence of NADPH. Fig. 5B shows that TCDD alone caused an 18-fold increase in CYP1A1 catalytic activity. On the other hand, MeHg decreased the TCDD-mediated induction of CYP1A1 catalytic activity by −8.6-fold in total cell lysate supplemented with NADPH (Fig. 5B).

3.7. Effect of co-exposure to MeHg and TCDD on HO-1 mRNA

The inverse relationship between HO-1 expression and CYP1A1 activity directed us to probe the role of MeHg in inhibiting the TCDD-mediated induction of CYP1A1 at the catalytic activity level. Therefore, we examined the effect of MeHg on HO-1 mRNA, a rate limiting enzyme in heme degradation. For this purpose, HepG2 cells were treated with increasing concentrations of MeHg (1.25–5 μM) in the presence of 1 nM TCDD. Thereafter, HO-1 mRNA was measured using real-time PCR. Our findings show that TCDD alone did not alter HO-1 mRNA level. However, co-exposure to TCDD and MeHg significantly increased the HO-1 mRNA level by 1.2-, 1.6-, and 4-fold with concentrations of 1.25, 2.5, and 5 μM, respectively. Thus, HO-1 might be participating in the MeHg-mediated decrease of the TCDD-mediated induction of CYP1A1 at the catalytic activity levels (Fig. 6A).

3.8. Effect of SnMP on the posttranslational modification of CYP1A1 catalytic activity by MeHg

The fact that MeHg inhibited the TCDD-mediated induction of CYP1A1 at the catalytic activity level but not at the mRNA or protein levels prompted us to investigate the possible role of HO-1 in this inhibitory effect. For this purpose HepG2 cells were co-exposed to 5 μM MeHg and 1 nM TCDD in the presence and absence of 5 μM SnMP. Our results showed that, SnMP alone caused no effect on the CYP1A1 catalytic activity. Similarly, the TCDD-mediated induction of CYP1A1 catalytic activity was not affected by SnMP treatment. On the other hand, MeHg at the concentration of 5 μM decreased the TCDD-mediated induction of CYP1A1 catalytic activity. Intriguingly, SnMP completely reversed the MeHg-mediated decrease in CYP1A1 activity. Upon treatment of the cells with SnMP in the presence of both MeHg and TCDD, there was a complete restoration of the MeHg-mediated inhibition of CYP1A1 catalytic activity induced by TCDD (Fig. 6B).

3.9. Effect of HO-1 siRNA on MeHg-mediated inhibition of CYP1A1 catalytic activity

Despite using selective pharmacological inhibitor such as SnMP to inhibit HO-1 activity; it was of importance to confirm our hypothesis that the MeHg-mediated increase in HO-1 is responsible for the inhibition of CYP1A1 at the catalytic activity level. Therefore, we took a genetic approach to confirm whether or not HO-1 is involved in the MeHg-mediated decrease of the TCDD-mediated
induction of CYP1A1 at the catalytic activity. Therefore, HepG2 cells were transfected with the human HO-1 siRNA for 6 h, and then the cells were treated with 5 μM MeHg in the presence and absence of 1 nM TCDD. Our results showed that HO-1 siRNA significantly decreased HO-1 mRNA by −4.5-fold, compared to control (Fig. 7A). On the other hand, MeHg was able to increase HO-1 mRNA levels, in the absence and presence of TCDD, by 24-fold compared to control. When the cells were transfected with HO-1 siRNA, and then treated with MeHg alone or in the presence of TCDD there was a statistically significant decrease in HO-1 mRNA by −4.7- and −5.2-fold, respectively, compared to MeHg alone (Fig. 7A). Furthermore, the Silencer select negative control siRNA did not affect the inducible level of HO-1 mRNA by MeHg, eliminating the possibility that the inhibitory effects of HO-1 siRNA might have been due to any toxicity.

Looking at CYP1A1 catalytic activity, MeHg alone or in the presence of HO-1 siRNA did not affect CYP1A1 catalytic activity (Fig. 7B). TCDD alone increased the CYP1A1 catalytic activity by 21-fold, whereas MeHg significantly decreased the TCDD-mediated induction of CYP1A1 catalytic activity by −5.8-fold compared to TCDD alone. Interestingly, when HepG2 cells were transfected with HO-1 siRNA and then co-exposed to MeHg and TCDD, MeHg was unable to maintain the inhibitory effect on CYP1A1 catalytic activity when compared to non-transfected cells (Fig. 7B).

### 3.10. Concentration-dependent effect of MeHg on the constitutive and TCDD-inducible NQO1 mRNA and protein levels

To investigate whether the inhibitory effect of MeHg is specific for CYP1A1 or can affect other AhR-regulated genes, the effect of MeHg on the expression of NADPH:quinone oxidoreductase 1 (NQO1) was examined. For this purpose, HepG2 cells were treated with various concentrations of MeHg in the absence and presence of 1 nM TCDD. Initially, MeHg at 1.25, 2.5, and 5 μM caused a concentration-dependent increase in NQO1 mRNA levels by 1.6-, 2-, and 2.4-fold, respectively compared to control. Moreover, TCDD alone caused a 2.3-fold increase in NQO1 mRNA level that was further potentiated by MeHg in a concentration-dependent manner...
reaching 1.15-, 1.54-, and 1.87-fold, respectively, with 1.25 μM, 2.5 μM, and 5 μM, compared to TCDD alone (Fig. 8A). In agreement, with the NQO1 mRNA results, MeHg alone significantly increased the constitutive NQO1 protein in a dose-dependent manner by 1.5-, 2.7-, and 3.7-fold at 1.25, 2.5, and 5 μM, respectively (Fig. 8B). Furthermore, TCDD alone caused a 3.5-fold increase in NQO1 protein. Importantly, MeHg at the concentrations of 2.5 and 5 μM but not 1.25 μM was able to significantly potentiate the TCDD-mediated induction of NQO1 protein levels by 1.48- and 1.71-fold, respectively, compared to TCDD alone (Fig. 8B).

3.11. Effect of MeHg and TCDD mixture on ARE-driven luciferase reporter gene

In order to examine whether the induction of both HO-1 and NQO1 mRNA levels by MeHg is through an ARE-dependent pathway, HepG2 cells were transiently transfected with the ARE-driven luciferase reporter plasmid. Luciferase activity results showed that MeHg alone significantly induced the ARE-luciferase activity by 2-fold, compared to the control (Fig. 8C). Expectedly, TCDD alone significantly induced ARE-luciferase activity by 2.1-fold compared to the control (Fig. 8C). Furthermore, co-treatment with 5 μM of MeHg significantly potentiated the TCDD-mediated induction of ARE-luciferase activity by 1.6-fold compared to TCDD alone (Fig. 8C).

4. Discussion

MeHg is a highly toxic non-essential environmental element that is neither created nor biodegradable (Barbier et al., 2005). The concentrations of MeHg used in the present study were chosen after determining the ability of a wide range of concentrations to modulate the expression of CYP1A1 without significantly affecting HepG2 cell viability (Fig. 1). Moreover, the estimated human plasma and tissue concentrations of mercury in individuals without known exposure have been reported to be in the μM range (Gerhardsson et al., 1988; Tezel et al., 2001) and within the range that might be expected to be encountered in the environment (ATSDR, 2011; CEPA, 2012; Vakharia et al., 2001a,b). Taken together, chronic human exposure to this long half-life toxic metal in addition to the high risk of accumulation in the body tissues, particularly the liver (Barbier et al., 2005), make the concentrations used in the present study of high relevancy to the corresponding human plasma and tissue levels, and calculated exposure levels in the environment (ATSDR, 2011; CEPA, 2012).

The capacity of mercury to modulate the induction of CYP1A1 expression by TCDD has been reported previously from our laboratory and others in murine Hepa 1c1c7 cells, human hepatoma HepG2 and primary human hepatocytes, at the mRNA, protein, and catalytic activity levels (Amara et al., 2010; Korashy and El-Kadi, 2005; Vakharia et al., 2001a). However, our efforts along with others were mainly concerned with inorganic mercury, namely mercuric chloride (Hg²⁺). Therefore, it was of importance to investigate whether other forms of mercury, especially organic forms, such as MeHg will exhibit an effect on CYP1A1 mRNA, protein, and catalytic activity levels. Mechanistically it would be very hard to investigate the effect of MeHg on CYP1A1 in vivo. This is because MeHg is demethylated to inorganic mercury in rat liver slices and in vivo in CD-1 mice through a non-enzymatic reaction (Khan and Wang, 2010; Sundberg et al., 1998; Yasutake and Hirayama, 2001). In addition, the elimination of MeHg in mice feces is a very according to the manufacturer’s instructions. Luciferase activity is reported as relative light units (RLU). Values are presented as mean ± SE (n = 6). *P < 0.05, compared with control (C); †P < 0.05, compared with the respective TCDD (T) treatment.
induction of CYP1A1 activity despite supplementation of the enzymatic system with excessive NADPH. Thus, the effect of MeHg on the TCDD-mediated induction of CYP1A1 activity does not involve an effect on intracellular NADPH levels. Third, HO-1 gene expression, a rate-limiting enzyme in heme catabolism, has been shown to alter cellular heme, the prosthetic group of CYP, content and hence the enzymatic activity of heme containing enzymes (Kikuchi et al., 2005). Interestingly, our results showed that MeHg–mediated inhibition of the TCDD-mediated induction of CYP1A1 activity was accompanied with a proportional increase in HO-1 mRNA levels. These results imply that MeHg might have decreased CYP1A1 activity through degrading its heme content via the induction of HO-1. Although, total heme content was not measured in the current study, we have previously demonstrated in Hepa 1c1c7 cells that Hg2+ caused a significant decrease in heme availability, causing a subsequent reduction in Cyp1a1 activity, suggesting a direct correlation between HO-1 and Cyp1a1 (Korashy and El-Kadi, 2005). In the current study, the role of HO-1 in the inhibition of CYP1A1 at the catalytic activity level is supported by series of evidence. First, the inhibition of HO-1 activity by administration of SnMP, a specific inhibitor of HO-1, completely abolished the MeHg–mediated inhibition of the TCDD-mediated induction of CYP1A1 activity. In this regard, it has been previously shown that the effect of SnMP is solely occurring through competitive inhibition of HO-1 protein and not through altering CYP1A1 expression (Amara et al., 2010; Anwar-Mohamed and El-Kadi, 2010). Second, knockdown of HO-1 using siRNA against HO-1 reversed the MeHg–mediated inhibition of TCDD-mediated induction of CYP1A1 activity. In agreement with our results, data from our laboratory and others have previously demonstrated that in HepG2 cells, knockdown HO-1 partially reverses the effect of metals such as Hg2+ and As3+ on the TCDD-mediated induction of CYP1A1 catalytic activity (Amara et al., 2010; Anwar-Mohamed and El-Kadi, 2010; Bessette et al., 2009).

Lastly, we investigated whether the inhibitory effect of MeHg is specific for CYP1A1. Therefore, the effect of MeHg on the expression of NQO1 was examined. Our findings show that MeHg was able to significantly increase the constitutive and TCDD-inducible NQO1 mRNA and protein levels, which is consistent with our previous findings with Hg2+ (Amara and El-Kadi, 2011), confirming that the inhibitory effect of MeHg is specific for CYP1A1. With the fact that NQO1 along with HO-1 are up-regulated in response to oxidative stress stimuli, such as hydrogen peroxide through the activation of ARE in their promoter regions, we examined the effect of MeHg on the ARE-driven luciferase activity. Our results demonstrated that MeHg increased ARE-luciferase activity. In this context, several studies have reported the capability of heavy metals including Hg2+ (Kaliman et al., 2001; Stohs and Bagchi, 1995), to induce HO-1. Furthermore, it has been previously reported that MeHg increases the nuclear accumulation of the transcription factor nuclear factor–erythroid 2-like 2 (Nrf2) in isolated mouse hepatocytes (Lamsa et al., 2010) which is the transcription factor that binds to and activates the ARE initiating the transcription of NQO1 and HO-1. An explanation offered for this phenomenon is that metal-mediated increase in oxidative stress, including the production of reactive oxygen species (ROS) (Kaliman et al., 2001; Stohs and Bagchi, 1995) and lipid peroxidation (Ossola et al., 1997; Stohs and Bagchi, 1995) might be in part responsible for the nuclear accumulation of Nrf2 and the subsequent activation of the ARE (Inamdar et al., 1996). Taken together, we suggest that induction of ROS by Hg2+ (Korashy and El-Kadi, 2005) triggers ARE activation and induction of NQO1 in addition to HO-1 which subsequently decreases CYP1A1 activity.

In conclusion, data presented here clearly demonstrate for the first time that MeHg decreases the TCDD-mediated induction of CYP1A1 through a posttranslational mechanism and confirms the role of HO-1 in the MeHg-mediated effect.
Conflict of interest statement

The authors declare that there is no conflict of interest.

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