Free Radical Biology and Medicine 58 (2013) 52–63

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

# Differential modulation of cytochrome P450 1a1 by arsenite in vivo and in vitro in C57BL/6 mice

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ARTICLE INFO

*Article history:*
Received 12 September 2012
Received in revised form
14 January 2013
Accepted 22 January 2013
Available online 29 January 2013

*Keywords:*
AhR
Arsenite
Cyp1a1
Cyp1a2
Cyp1b1
HO-1
Free radicals

ABSTRACT

Heavy metals, typified by arsenite (As(III)), have been implicated in altering the carcinogenicity of aryl hydrocarbon receptor (AhR) ligands, typified by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), by modulating the induction of the Cyp1a1 enzyme, but the mechanism remains unresolved. In this study, the effects of As(III) on Cyp1a1 expression and activity were investigated in C57BL/6 mouse livers and isolated hepatocytes. For this purpose, C57BL/6 mice were injected intraperitoneally with As(III) (12.5 mg/kg) in the absence and presence of TCDD (15 μg/kg) for 6 and 24 h. Furthermore, isolated hepatocytes from C57BL/6 mice were treated with As(III) (1, 5, and 10 μM) in the absence and presence of TCDD (1 nM) for 3, 6, 12, and 24 h. At the in vivo level, As(III) decreased the TCDD-mediated induction of Cyp1a1 mRNA at 6 h while potentiating its mRNA, protein, and catalytic activity levels at 24 h. At the in vitro level, As(III) decreased the TCDD-mediated induction of Cyp1a1 mRNA in a concentration- and time-dependent manner. Moreover, As(III) decreased the TCDD-mediated induction of Cyp1a1 protein and catalytic activity levels at 24 h. Interestingly, As(III) increased the serum hemoglobin (Hb) levels in animals treated for 24 h. Upon treatment of isolated hepatocytes with Hb alone, there was an increase in the nuclear accumulation of AhR and AhR-dependent luciferase activity. Furthermore, Hb potentiated the TCDD-induced AhR-dependent luciferase activity. Importantly, when isolated hepatocytes were treated for 5 h with As(III) in the presence of TCDD and the medium was then replaced with new medium containing Hb, there was potentiation of the TCDD-mediated effect. Taken together, these results demonstrate for the first time that there is a differential modulation of the TCDD-mediated induction of Cyp1a1 by As(III) in C57BL/6 mouse livers and isolated hepatocytes. Thus, this study implicates Hb as an in vivo-specific modulator.



The aryl hydrocarbon receptor (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, the 23-kDa heat shock protein, and a 43-kDa protein known as the AhR-inhibitory protein or hepatitis B virus X-associated protein 2 [1]. 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 the aryl hydrocarbon receptor nuclear translocator (Arnt) [1]. 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. Among these genes are those encoding a number of xenobiotic-metabolizing enzymes, including four phase I enzymes (cytochrome P450 1A1 (Cyp1a1), Cyp1a2, Cyp1b1, and Cyp2s1) and four phase II enzymes (NAD(P)H:quinone oxidoreductase-1, glutathione *S*-transferase A1, cytosolic aldehyde dehydrogenase 3, and UDP-glucuronosyltransferase 1A6) [1,2].

Several AhR agonists, with the exception of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are substrates for the inducible AhR-regulated phase I enzymes. The conversion of these AhR ligands into diol epoxide compounds specifically by Cyp1a1 and Cyp1a2 results in the formation of covalent adducts when these genotoxic metabolites react with guanines in critical genes, potentially initiating tumorigenesis and other toxic responses [3]. The coexposure to heavy metals, typified by sodium arsenite (As(III)), and dioxins, typified by TCDD, is a very likely event, as As(III) and TCDD are ranked first and fifth, respectively, 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 [4,5]. There are several approaches that can be used to measure AhR activity. Examples of

*Abbreviations:* AhR, aryl hydrocarbon receptor; As(III), arsenite; Cyp, cytochrome P450; HO-1, heme oxygenase-1; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic-responsive element

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http://dx.doi.org/10.1016/j.freeradbiomed.2013.01.012

these assays would include *CYP1A1* gene and protein expression, Cyp1a activity, and finally XRE-driven luciferase reporter activity.

Previous studies examining the effect of As(III) on AhR activity have shown that As(III) 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 different in vivo and in vitro systems [6]. Most of the in vitro studies were conducted using the murine hepatoma Hepa 1c1c7 cells, human hepatoma HepG2 cells, and isolated hepatocytes from rats and humans [6]. On the other hand, a few studies examined the effect of As(III) on AhR in vivo using guinea pig, rat, and mouse models. Despite these efforts, these studies were very contradictory with regard to the effect of As(III) on Cyp1a1 in the absence and presence of ligands. These contradictory results were mostly to be seen between the in vivo and the in vitro studies [6].

Therefore, we hypothesize that As(III) differentially alters the expression of Cyp1a1 in vivo and in vitro in C57BL/6 mice. Thus, the objective of this study was to investigate the effects of As(III) in the absence and presence of TCDD on Cyp1a1 in vivo using C57BL/6 mouse livers and in vitro using isolated hepatocytes.

## 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, As(III), and Dulbecco's modified Eagle medium (DMEM) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TRIzol reagent, Alexa Fluor 488 donkey anti-goat secondary antibody, and Prolong Gold mounting medium were purchased from Invitrogen (Carlsbad, CA, USA). The 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, USA). Real-time PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Tin mesoporphyrin (SnMP) was purchased from Frontier Scientific (Logan, UT, USA). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, $>99\%$ pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). Chemiluminescence Western blotting detection reagents were from GE Healthcare Life Sciences (Piscataway, NJ, USA). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Cyp1a1/1a2 mouse polyclonal primary antibody, AhR goat polyclonal primary antibody, Arnt goat polyclonal antibody, GAPDH rabbit polyclonal antibody, and anti-rabbit IgG peroxidase secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cyp1b1 rabbit polyclonal primary antibody was purchased from BD Biosciences (Mississauga, ON, Canada). Anti-mouse IgG peroxidase secondary antibody was purchased from R&D Systems (Minneapolis, MN, USA). Glass coverslips (22-mm diameter; Cat. No. 72224-01) were purchased from Electron Microscopy Sciences (Hatfield, PA, USA). All other chemicals were purchased from Fisher Scientific (Toronto, ON, Canada).

### *Animals and ethics*

Male C57BL/6 mice, ages 10–12 weeks (Charles River Laboratories, Montreal, QC, Canada) 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 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 probable 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/6 (22–30 g) mice were obtained from Charles River, Canada. The animals were injected intraperitoneally (ip) with As(III) (as sodium arsenite dissolved in saline) at 12.5 mg/kg in the absence and presence of 15 μg/kg TCDD (dissolved in corn oil) injected ip. The mice were divided into four groups. The first group ($n=12$), control mice, received saline (0.4 ml) plus corn oil (0.4 ml). The second group ($n=12$), As(III)-treated mice, received As(III) dissolved in saline (0.4 ml) plus corn oil (0.4 ml). The third group ($n=12$), TCDD-treated mice, received TCDD dissolved in corn oil (0.4 ml) plus saline (0.4 ml). The fourth group ($n=12$), As(III) plus TCDD-treated mice, received As(III) dissolved in saline (0.4 ml) plus TCDD dissolved in corn oil (0.4 ml). The animals were euthanized at 6 ($n=6$) or 24 h ($n=6$) after a single injection via cervical dislocation. Liver tissues were excised, immediately frozen in liquid nitrogen, and stored at $-80\,^{\circ}C$ until further analysis. All experimental procedures involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee.

### *Isolation of mouse hepatocytes and cell culture*

Three solutions were utilized for the isolation of mouse hepatocytes as previously described [7]. Solution A contained (in mM) NaCl 115, KCl 5, $KH_2PO_4$ 1, Hepes 25, EGTA 0.5, and glucose 5.5 and heparin 56.8 μg/ml in deionized water. Solution B was solution A with $CaCl_2$ 1 mM, trypsin inhibitor 0.25 μM, and collagenase 0.025%. Solution C contained 100 ml of solution B supplemented with $MgSO_4$ 1.2 mM and 1 ml of DMEM. Solutions A and B were adjusted to pH 7.4 by adding HCl 1 N and filtered through a 22-μm membrane before use.

Mouse hepatocytes were isolated by a two-step collagenase perfusion method as described previously [8]. Mice were anesthetized, a midline laparotomy was performed, and the portal vein was cannulated, while the suprahepatic and the inferior cava veins were cut. All tubing and solutions were maintained at 37 °C and saturated with 95% $O_2$:5% $CO_2$. The liver was perfused via the portal vein with 25 ml of solution A, by use of a peristaltic pump at a flow rate of 5 ml/min for 5 min, and then with 35 ml of solution B at a flow rate of 5 ml/min for 7 min, until the liver appeared completely blanched and softened. The liver was maintained wet with saline during the entire period of perfusion.

After in situ perfusion, the liver was removed and placed in a petri dish containing 30 ml solution C. The capsule was stripped away from one side of the liver, and the cells were detached by brushing the liver with a plastic comb and filtered through cotton gauze. The suspension of cells was incubated in a shaker water bath at 37 °C for 5 min with 95% $O_2$:5% $CO_2$, filtered through a (70-μm) cell strainer, and divided into two aliquots, which were placed on ice. Once the temperature of 4 °C was reached, the cells were centrifuged at 100 g for 2 min, the supernatant was aspirated, and the sediment was resuspended in DMEM and recentrifuged at 100 g for 2 min, an operation that was repeated twice.

**Table 1**
Primer sequences used for real-time PCR.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| β-Actin | 5′-TATTGGCAACGAGCGGTTCC-3′ | 5′-GGCATAGAGGTCTTTACGGATGTC-3′ |
| Cyp1a1 | 5′-GGTTAACCATGACCGGGAACT-3′ | 5′-TGCCCAAACCAAAGAGAGTGA-3′ |
| Cyp1a2 | 5′-TGGAGCTGGCTTTGACACAG-3′ | 5′-CGTTAGGCCATGTCACAAGTAGC-3′ |
| Cyp1b1 | 5′-AATGAGGAGTTCGGGCGCACA-3′ | 5′-GGCGTGTGGAATGGTGACAGG-3′ |
| HO-1 | 5′-GTGATGGAGCGTCCACAGC-3′ | 5′-TGGTGGCCTCCTTCAAGG-3′ |

The supernatant was discarded and the sediment was resuspended in DMEM to obtain $0.5 \times 10^6$ cells $ml^{-1}$.

Each well of the plastic culture plates (24-well; Falcon, Becton–Dickinson Labware, Franklin Lakes, NJ, USA) was coated with 500 μl of type I rat-tail collagen (50 μg/ml acetic acid 0.02 N) overnight, and the wells were rinsed with deionized water and washed once with serum-free DMEM before the hepatocytes ($0.25 \times 10^6 \times 500$ $\mu l^{-1}$ per well) were added in DMEM supplemented with 10% fetal bovine serum, 1 μM insulin, 50 ng/ml dexamethasone phosphate, 100 IU/ml penicillin G, 10 μg/ml streptomycin, and 25 ng/ml amphotericin B. The plastic culture plates were incubated at 37 °C in a cell culture incubator with 95% $O_2$:5% $CO_2$. Viability was assessed before and after the incubation period by the trypan blue (0.2%) exclusion method, and in both instances the viability was over 90%.

*Chemical treatments for cells*

Cells were treated in serum-free medium with As(III) (1, 5, and 10 μM) in the absence and presence of TCDD (1 nM) and/or Hb (0.02 g/ml) as described in the figure legends. As(III) (10 mM stock) was prepared fresh in double deionized water. TCDD was dissolved in dimethyl sulfoxide (DMSO) and maintained in DMSO at −20 °C until use. 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).

*Effect on cell viability*

The effect of As(III) on cell viability was determined using the MTT assay as described previously [9]. The MTT assay measures the conversion of MTT to formazan in living cells via mitochondrial enzymes of viable cells. In brief, hepatocytes were seeded onto 96-well microtiter cell culture plates and incubated for 24 h at 37 °C in a 5% $CO_2$ humidified incubator. Cells were treated with various concentrations of As(III) (1–50 μM) in the absence and presence of 1 nM TCDD. After 24 h incubation, the medium was removed and replaced with cell culture medium containing 1.2 mM MTT dissolved in phosphate-buffered saline (PBS; pH 7.4). After 2 h of incubation, the crystals that formed 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, USA).

*RNA extraction and cDNA synthesis*

Total RNA from the frozen tissues or cells treated 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 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; Table 1), 12.5 μl of SYBR Green Universal Master Mix, 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 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 annealing/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.

*Real-time PCR data analysis*

The real-time PCR data were analyzed using the relative gene expression, i.e., the $\Delta\Delta C_T$ method as described in Applied Biosystems User Bulletin No. 2 and explained further by Livak and Schmittgen [10]. Briefly, the $\Delta C_T$ 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 $\Delta C_T$ of the control (vehicle-treated animals at 6- or 24-h time point) from the $\Delta C_T$ of the corresponding treatment group. The values and ranges given in the figures were determined as follows: $2^{-\Delta(\Delta CT)}$ with $\Delta\Delta C_T + SE$ and $\Delta\Delta C_T - SE$, where SE 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 [11,12]. Briefly, individual lung and kidney tissues were rapidly removed and washed in ice-cold potassium chloride (1.15% (w/v)). Consequently, they were cut into pieces and homogenized separately in cold sucrose solution (1 g of tissue in 5 ml of 0.25 M sucrose). After homogenization, the tissues were separated by ultracentrifugation. 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 [13].

*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 sodium chloride, 1.5 mM magnesium chloride, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 μl/ml 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 total cellular lysate supernatant was collected and stored at −80 °C.

*Western blot analysis*

Western blot analysis was performed using a previously described method [14]. Briefly, 20 μg of liver microsomal proteins or total cell lysates was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (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, 5% skim milk, 2% bovine serum albumin, and 0.5% Tween 20. After being blocked, the blots were incubated for 2 h at room temperature with the following primaries: primary polyclonal mouse anti-rat Cyp1a1/1a2, primary polyclonal rabbit anti-rat Cyp1b1, primary polyclonal rabbit anti-mouse actin, or primary polyclonal goat anti-mouse GAPDH. 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, or rabbit anti-goat IgG secondary antibody for GAPDH 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). The intensity of the protein bands was quantified, relative to the signals obtained for actin, using ImageJ software (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij).

*Determination of Cyp1a1 and Cyp1a2 enzymatic activities in hepatocytes*

Cyp1a1-dependent 7-ethoxyresorufin *O*-deethylase (EROD) and Cyp1a2-dependent 7-methoxyresorufin *O*-deethylase (MROD) activities were assessed on intact, monolayer living cells using 7-ethoxyresorufin or 7-methoxyresorufin as substrate, respectively, as previously described [15,16]. Enzymatic activity was normalized for cellular protein content, which was determined using a modified fluorescence assay [17].

*Microsomal incubation and measuring Cyp1a1 and Cyp1a2 catalytic activities*

Microsomes from liver of various 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 preequilibration period of 5 min was performed. The reaction was initiated by the addition of 1 mM NADPH. The concentration of substrate was 2 μM for 7-ethoxyresorufin (EROD) for Cyp1a1 and 7-methoxyresorufin (MROD) for Cyp1a2. After incubation at 37 °C (5 min for EROD and 10 min for MROD assay), 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 picomoles 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 at Davis, USA), and 0.1 μg of the *Renilla* luciferase pRL-CMV vector, used for normalization, using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). Luciferase assay was performed according to the manufacturer's instructions (Promega, Madison, WI, USA) as described previously [18]. 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 a 1.5-ml microcentrifuge tube. 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, USA).

*Measuring serum Hb levels*

Measurement of serum Hb levels was carried out as previously described [19]. In brief, a stock solution of Hb was prepared (1 mg/ml) and the exact Hb content was determined by the Hartree method [20]. 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 was added and the reaction was allowed to continue for about 1 min. Absorbance was read at 1-min intervals against water at 525 nm.

*Nuclear extract preparation*

Nuclear extracts were prepared from primary hepatocytes treated for 2 h with vehicle or 1 μM Hb. All nuclear protein extractions were performed on ice with ice-cold reagents. Protease inhibitors were added to reagents before use, and the NE-PER nuclear extraction kit (Pierce, Rockford, IL, USA) was utilized to obtain nuclear fractions. The fractions were then stored at −80 °C until further analyses. Protein concentrations for the extracts were determined using the method of Lowry et al. [13].

*Immunoprecipitation of AhR protein*

To examine the effects of Hb on the translocation of AhR, primary hepatocytes were treated for 3 h with vehicle or 1 μM Hb. Thereafter, AhR protein was immunoprecipitated from nuclear fractions (1000 μg protein) using anti-AhR goat polyclonal antibody bound to protein A/G–Sepharose overnight at 4 °C. The AhR and Arnt were visualized by Western blot analysis using anti-AhR goat polyclonal antibody and anti-Arnt goat polyclonal antibody. Primary antibodies were detected with peroxidase-conjugated rabbit anti-goat IgG secondary antibody for AhR and Arnt. The bands were visualized with the enhanced

chemiluminescence method according to the manufacturer's instructions (Amersham, Arlington Heights, IL, USA).

*Immunocytochemical analysis of AhR localization*

Plated cells on collagen-coated glass coverslips were treated for 1 h with either vehicle or 1 μM Hb. Treated cells were fixed using 3.7% (w/v) paraformaldehyde in CSK buffer (10 mM Pipes, pH 6.8; 10 mM NaCl; 300 mM sucrose; 3 mM $MgCl_2$; 2 mM EDTA) at room temperature. Cells were then washed twice with PBS, 10 min each, and then permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature. The cells were then washed twice with PBS, 10 min each, before being incubated with blocking buffer (1 × PBS, 1% donkey serum, 0.1% Tween 20) for 10 min at room temperature. Before proceeding with further steps, controls were determined to be no antibodies control, no primary antibody + secondary antibody control, and no secondary antibody + primary antibody control. Thereafter, blocked cells were incubated with anti-AhR goat polyclonal antibody diluted in blocking buffer (1:2000) for 1 h at 4 °C. The cells were then washed three times with PBS, 10 min each, before being incubated with Alexa Fluor 488 (green) donkey anti-goat secondary antibody diluted in blocking buffer (1:2000). The cells were then washed three times with PBS, 10 min each, and thereafter the DNA was stained with DAPI (blue) diluted in PBS (1:15000). The stained coverslips were rinsed with double-deionized water to remove excess DAPI. Coverslips were then dried on a paper towel and 3–5 μl of Prolong Gold mounting medium was added to each coverslip before it was mounted on a microscope glass slip. Images were obtained using spinning disk confocal, integrated by Quorum Technologies (Guelph, ON, Canada) on an Olympus IX-81 stand with a Yokogawa CSU-X11 confocal scan unit, with a 100 × /1.4 NA lens obtaining an image pixel size of 109 nm.

*Statistical analysis*

Data are presented as the mean ± standard error of the mean. Control and treatment measurements were compared using a one-way analysis of variance followed by a Student–Newman–Keuls post hoc comparison. A result was considered statistically significant when $P < 0.05$.

## Results

*Effect of coexposure to As(III) and TCDD on Cyp1a1, Cyp1a2, and Cyp1b1 mRNA levels in the liver of C57BL/6 mice*

At 6 and 24 h, As(III) alone failed to significantly affect Cyp1a1, Cyp1a2, or Cyp1b1 mRNA levels in the liver (Figs. 1A, 1B, and 1C). On the other hand, TCDD alone significantly induced Cyp1a1, Cyp1a2, and Cyp1b1 mRNA levels in the liver at 6 h by 7633-, 16.8-, and 7.7-fold, respectively, compared to control (Figs. 1A, 1B, and 1C). At 24 h TCDD alone significantly induced Cyp1a1, Cyp1a2, and Cyp1b1 mRNA levels in the liver by 4851-, 17.4-, and 2583-fold, respectively, compared to control (Figs. 1A, 1B, and 1C). When animals were coexposed to As(III) and TCDD, As(III) at 6 h significantly inhibited the TCDD-mediated induction of Cyp1a1 and Cyp1a1 mRNA levels in the liver by −2.6- and −2.2-fold, respectively, compared to TCDD alone, whereas there was no effect of this coexposure on Cyp1b1 mRNA levels at 6 h (Figs. 1A, 1B, and 1C). On the other hand, As(III) at 24 h significantly potentiated the TCDD-mediated induction of Cyp1a1 mRNA levels by 1.2-fold, compared to TCDD alone, whereas it did not affect the TCDD-mediated induction of Cyp1a2 or Cyp1b1 mRNA levels in the liver (Figs. 1A, 1B, and 1C).

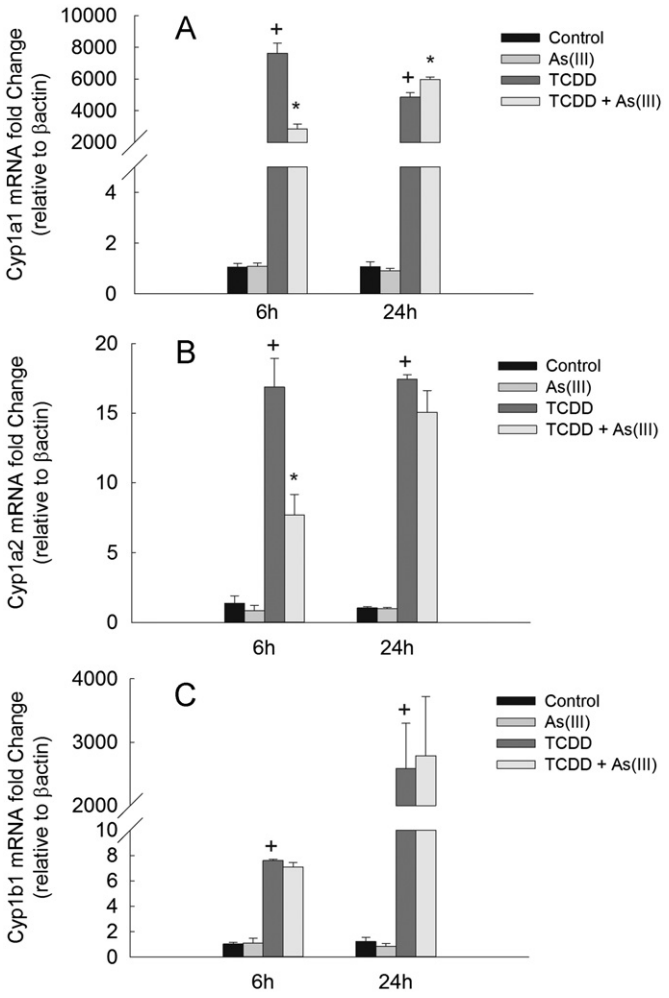


**Fig. 1.** Effect of coexposure to As(III) and TCDD on liver Cyp1a1, Cyp1a2, and Cyp1b1 mRNA in C57BL/6 mice. Animals were injected ip with 12.5 mg/kg As(III) in the absence and presence of 15 μg/kg TCDD for 6 and 24 h. First-strand cDNA was synthesized from total RNA (1.5 μg) extracted from liver and the expression of Cyp1a1, Cyp1a2, and Cyp1b1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using an 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.

*Effect of coexposure to As(III) and TCDD on Cyp1a and Cyp1b1 protein expression levels and EROD and MROD catalytic activity levels in the liver of C57BL/6 mice*

Our results showed that As(III) alone did not significantly affect Cyp1a or Cyp1b1 protein expression levels in the liver (Fig. 2A). TCDD alone significantly induced Cyp1a and Cyp1b1 protein expression levels in the liver by 6.2- and 4.6-fold, respectively, compared to control (Fig. 2A). On the other hand when animals were coexposed to As(III) and TCDD, As(III) significantly potentiated the TCDD-mediated induction of Cyp1a protein expression levels in the liver by 2.4- and 1.6-fold, respectively, compared to TCDD alone (Fig. 2A).

At the catalytic activity level, As(III) alone did not significantly affect EROD or MROD activity in the liver (Figs. 2B and 2C). TCDD alone significantly induced EROD and MROD activities in the liver by 5.8- and 6.4-fold, respectively, compared to control (Figs. 2B and 2C). When animals were coexposed to As(III) and TCDD, As(III) significantly potentiated the TCDD-mediated induction of EROD and MROD activities in the liver by 1.9- and 1.4-fold, respectively, compared to TCDD alone (Figs. 2B and 2C).

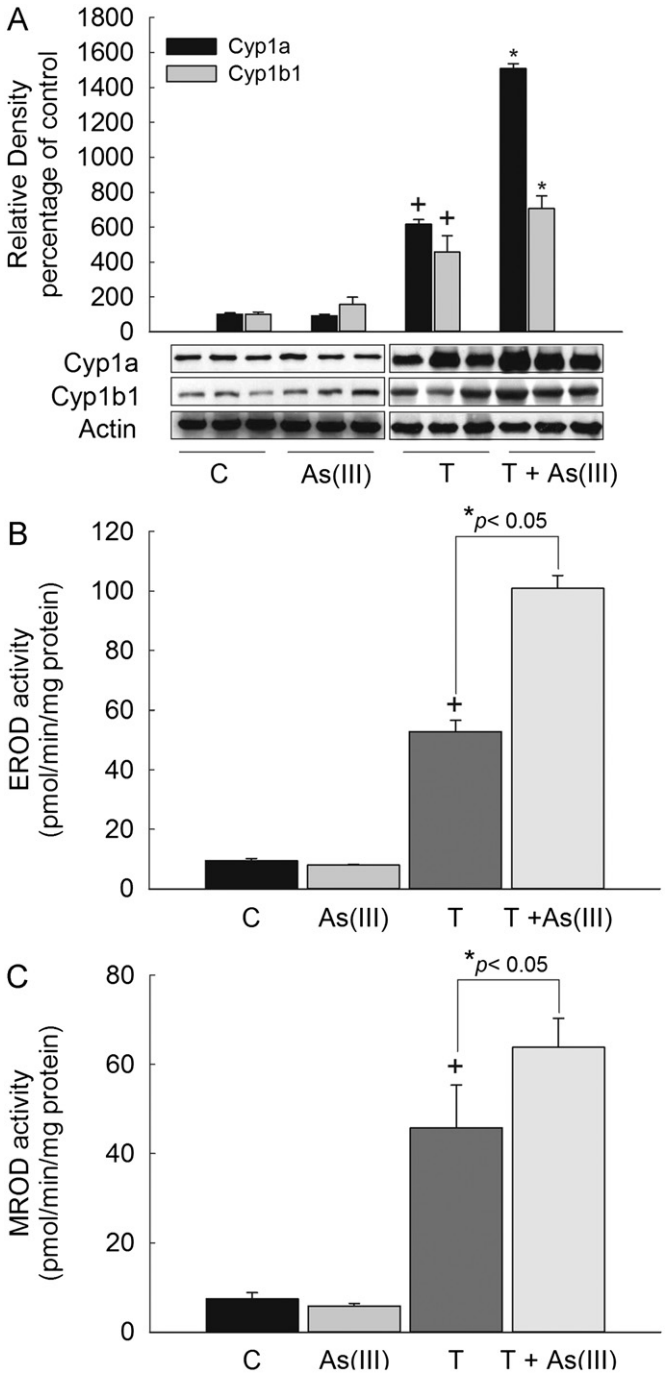


**Fig. 2.** Effect of coexposure to As(III) and TCDD on liver Cyp1a and Cyp1b1 protein expression levels and EROD and MROD activities in C57BL/6 mice. (A) Liver microsomal proteins were isolated after 24 h of treatment. Thirty micrograms of microsomal proteins was separated by 10% SDS–PAGE. Proteins were detected using the enhanced chemiluminescence method. The graph represents the relative amounts of protein normalized to actin signals (mean ± SEM, $n=6$), and the results are expressed as a percentage of the control values taken as 100%. (B and C) EROD and MROD activities were measured using 7-ethoxyresorufin and 7-methoxyresorufin as substrate, 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 the mean ± SEM ($n=6$). $+P<0.05$, compared to control; $^{*}P<0.05$, compared to respective TCDD treatment.

*Effect of coexposure to As(III) and TCDD on HO-1 mRNA levels in the liver of C57BL/6 mice*

As(III) alone at 6 h significantly induced HO-1 mRNA levels in the liver by 28.9-fold, compared to control, whereas at 24 h it did not affect its mRNA levels (Fig. 3). TCDD alone at 6 or 24 h did not significantly affect HO-1 mRNA levels in the liver compared to control (Fig. 3). When animals were coexposed to As(III) and TCDD, there was a significant potentiation to the As(III)-mediated induction of HO-1 mRNA levels in the liver by 2.6-fold, compared to As(III) alone (Fig. 3). On the other hand, TCDD at 24 h did not significantly affect the As(III)-mediated induction of HO-1 mRNA levels in the liver compared to As(III) alone (Fig. 3).

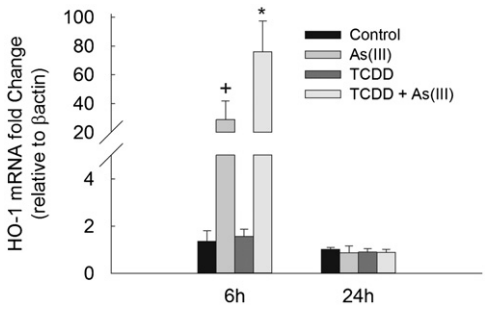


**Fig. 3.** Effect of coexposure to As(III) and TCDD on liver HO-1 mRNA in C57BL/6 mice. Animals were injected ip with 12.5 mg/kg As(III) in the absence and presence of 15 μg/kg TCDD for 6 and 24 h. First-strand cDNA was synthesized from total RNA (1.5 μg) extracted from liver and the expression of HO-1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using an 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.

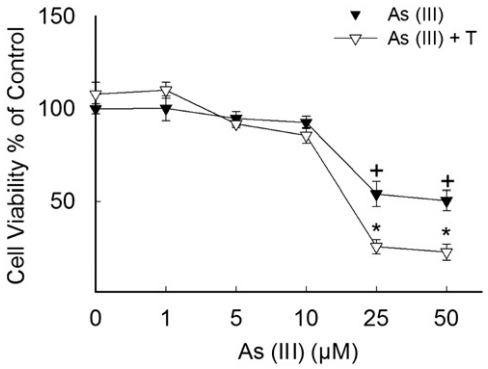


**Fig. 4.** Effect of As(III) on cell viability. Isolated hepatocytes were treated for 24 h with As(III) (0, 5, 10, 25, and 50 μM) in the absence and presence of 1 nM TCDD. Cell cytotoxicity was determined using the MTT assay. Data are expressed as the percentage of untreated control (which is set at 100%) ± SE ($n=8$). $+P<0.05$, compared to control (concentration 0 μM); $^{*}P<0.05$, compared to respective TCDD treatment.

*Effect of coexposure to As(III) and TCDD on cell viability*

To determine the nontoxic concentrations of As(III) to be utilized in this study, isolated hepatocytes from C57BL/6 mice were exposed for 24 h with increasing concentrations of As(III) (1–50 μM) in the absence and presence of 1 nM TCDD, and thereafter cytotoxicity was assessed using the MTT assay. Fig. 4 shows that As(III) at concentrations of 1–10 μM in the presence and absence of 1 nM TCDD did not affect cell viability (Fig. 4). Therefore, all subsequent studies were conducted using the concentrations of 1–10 μM.

*Time- and concentration-dependent effect of coexposure to As(III) and TCDD on Cyp1a1 mRNA levels in isolated hepatocytes*

To examine the effect of coexposure to As(III) and TCDD on Cyp1a1 mRNA, isolated hepatocytes were treated with various

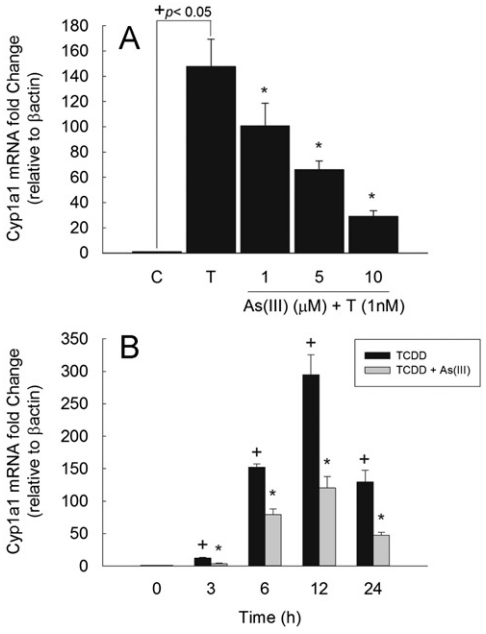


**Fig. 5.** Concentration- and time-dependent effects of As(III) on Cyp1a1 mRNA in isolated hepatocytes. Hepatocytes were treated (A) with increasing concentrations of As(III) in the presence of 1 nM TCDD for 6 h for concentration dependent or (B) for various times for time dependent. First-strand cDNA was synthesized from total RNA (1 μg) extracted from isolated hepatocytes. cDNA fragments were amplified and quantitated using an 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.

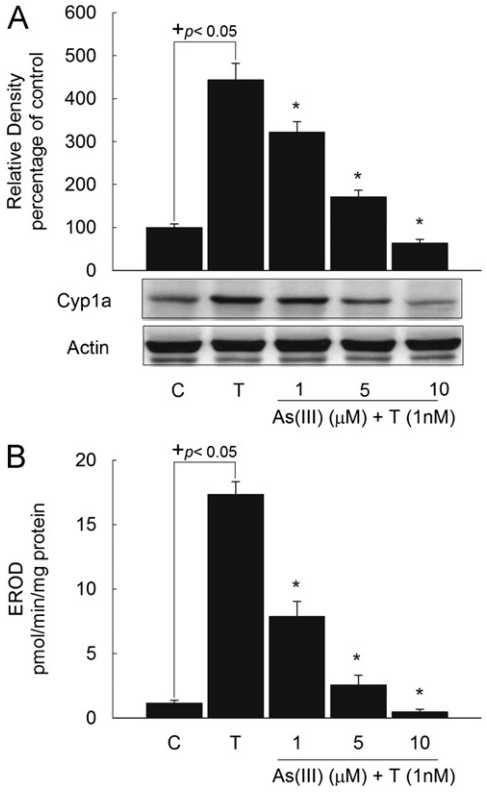


**Fig. 6.** Effects of As(III) on Cyp1a protein and catalytic activity in isolated hepatocytes. Hepatocytes were treated with increasing concentrations of As(III) in the presence of 1 nM TCDD for 24 h for protein and catalytic activity. (A) Protein (20 μg) was separated by 10% SDS–PAGE and transferred 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 the 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 As(III), 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 the mean ± SE ($n=6$). $+P<0.05$, compared to control (C); $^{*}P<0.05$, compared to respective TCDD (T) treatment.

concentrations of As(III) in the presence of 1 nM TCDD (Fig. 5A). Thereafter, Cyp1a1 mRNA was assessed using real-time PCR. TCDD alone caused a 147-fold increase in Cyp1a1 mRNA levels that was inhibited in a dose-dependent manner by As(III). Initially, As(III) at the concentration of 1 μM caused a significant decrease in TCDD-mediated induction of Cyp1a1 mRNA levels by −1.5-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 −5.1-fold (Fig. 5A).

To better understand the kinetics of Cyp1a1 mRNA in response to the coexposure to As(III) and TCDD, the time-dependent effect was determined at various time points up to 24 h after treatment of isolated hepatocytes with 1 nM TCDD in the absence and presence of 5 μM As(III). Fig. 5B shows that TCDD induced Cyp1a1 mRNA in a time-dependent manner. TCDD treatment caused a maximal induction of the Cyp1a1 mRNA by 294-fold at 12 h, compared to 0 h. However, a 12.3-fold induction occurred as early as 1 h (Fig. 5B). In contrast, when isolated hepatocytes were coexposed to As(III) and TCDD, there was a significant decrease in the Cyp1a1 mRNA levels, which occurred as early as 3 h, of 3.3-fold, compared to TCDD alone. Similarly, As(III) significantly decreased the TCDD-mediated induction of Cyp1a1 mRNA levels at 6, 12, and 24 h, by −1.9-, −2.5-, and −2.7-fold, respectively, compared to TCDD alone (Fig. 5B).

*Concentration-dependent effects of coexposure to As(III) and TCDD on Cyp1a protein and catalytic activity in isolated hepatocytes*

To examine whether the observed inhibition of the TCDD-mediated induction of Cyp1a mRNA by As(III) is further translated to the protein and activity levels, isolated hepatocytes were treated for 24 h with increasing concentrations of As(III) in the presence of 1 nM TCDD. Figs. 6A and 6B show that TCDD alone caused 4.4- and 17.3-fold increase in Cyp1a protein and catalytic activity, respectively. Of interest, As(III) decreased the TCDD-mediated induction of Cyp1a protein and catalytic activity levels in a dose-dependent manner. This inhibitory effect of As(III) 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 1 μM As(III) and reached the maximal inhibition at 10 μM (Figs. 6A and 6B).

*Transcriptional and posttranslational inhibition of Cyp1a by As(III)*

Isolated hepatocytes were transiently transfected with the XRE-driven luciferase reporter gene to study the effect of As(III) on the AhR-dependent transcriptional activation. Luciferase activity results showed that 5 μM As(III) alone did not affect the constitutive expression of the luciferase activity (Fig. 3). On the other hand, 1 nM TCDD alone caused a significant increase in luciferase activity by 12.8-fold compared to control (Fig. 7A). Interestingly, cotreatment with As(III) and TCDD significantly decreased the TCDD-mediated induction of luciferase activity by −2.4-fold (Fig. 7A).

The fact that As(III) inhibited the TCDD-mediated induction of Cyp1a1 at the catalytic activity level more than inhibiting its

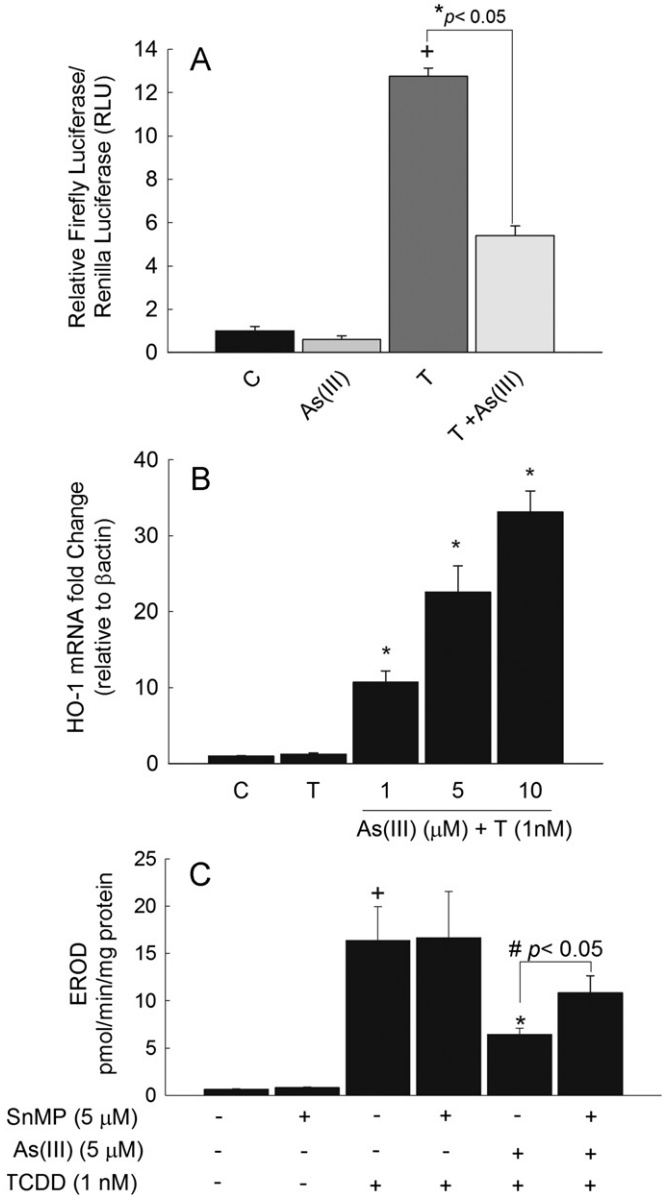


**Fig. 7.** Effects of As(III) on (A) luciferase activity and (B) HO-1 mRNA in isolated hepatocytes, and (C) the effect of SnMP as a competitive inhibitor of HO-1 on Cyp1a1 catalytic activity. (A) Hepatocytes were transiently transfected with the XRE–luciferase transporter plasmid pGudLuc1.1 and *Renilla* luciferase plasmid pRL-CMV. Cells were treated with vehicle, As(III) (5 μM), TCDD (1 nM), or TCDD (1 nM)+As(III) (5 μM) for 24 h. The cells were lysed and luciferase activity was measured according to the manufacturer's instructions. Luciferase activity is reported relative to *Renilla* activity. Values are presented as the mean ± SE ($n=6$). $+P<0.05$, compared to control (C); $^*P<0.05$, compared to respective TCDD (T) treatment. (B) Hepatocytes were treated with increasing concentrations of As(III) 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 an 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 respective TCDD (T) treatment. (C) Hepatocytes were treated with 5 μM As(III) and 1 nM TCDD in the presence and absence of 5 μM SnMP for 24 h. EROD activity was measured using 7-ethoxyresorufin as a substrate. $+P<0.05$, compared to control (concentration 0 μM); $^*P<0.05$, compared to respective TCDD treatment; $\#P<0.05$, compared to respective TCDD+As(III) treatment.

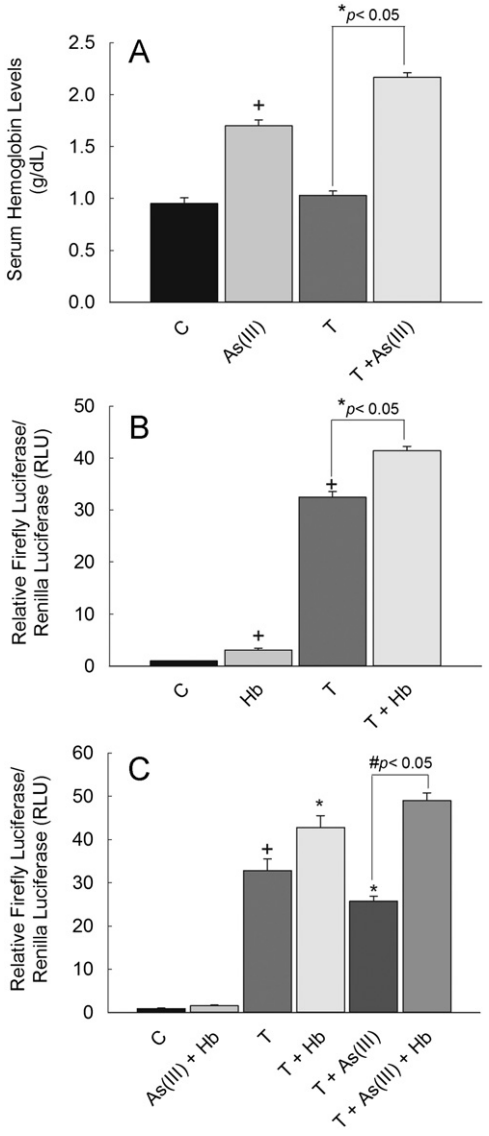


**Fig. 8.** Effect of As(III) on serum Hb levels in vivo and the effect of Hb on luciferase activity in isolated hepatocytes. (A) Animals were injected ip with 12.5 mg/kg As(III) in the absence and presence of 15 μg/kg TCDD for 24 h. Total blood was collected and centrifuged to isolate serum. Serum Hb levels were measured as described under Materials and methods. (B) Hepatocytes were transiently transfected with the XRE–luciferase transporter plasmid pGudLuc1.1 and *Renilla* luciferase plasmid pRL-CMV. Cells were treated with vehicle, Hb (1 μM), TCDD (1 nM), or TCDD (1 nM)+Hb (1 μM) for 24 h. Cells were lysed and luciferase activity was measured according to the manufacturer's instructions. Luciferase activity is reported relative to *Renilla* activity. Values are presented as the 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. Cells were treated with vehicle, As(III) (5 μM), TCDD (1 nM), or TCDD (1 nM)+As(III) (5 μM) for 5 h. The cells were then washed and further incubated with Hb for an additional 24 h. The cells were lysed and luciferase activity was measured according to the manufacturer's instructions. Luciferase activity is reported relative to *Renilla* activity. Values are presented as the mean ± SE ($n=6$). $+P<0.05$, compared to control (C); $^*P<0.05$, compared to respective TCDD (T) treatment; $\#P<0.05$, compared to respective TCDD+As(III) treatment.

mRNA or protein levels prompted us to investigate the possible effect of As(III) on HO-1 mRNA levels. For this purpose isolated hepatocytes were coexposed to 5 μM As(III) and 1 nM TCDD. Our results showed that TCDD alone did not significantly affect HO-1 mRNA levels. On the other hand, As(III) in the presence of 1 nM TCDD was able to increase HO-1 mRNA by 10-, 22-, and 33-fold, respectively, compared to control (Fig. 7B).

The possibility that As(III)-mediated induction of HO-1 might be contributing to lowering Cyp1a1 catalytic activity encouraged us to further investigate the possible role of HO-1 in this inhibitory effect. For this purpose isolated hepatocytes were coexposed to 5 μM

As(III) and 1 nM TCDD in the presence and absence of 5 μM SnMP. SnMP alone caused no effect on the Cyp1a1 catalytic activity. Similarly, the TCDD-mediated induction of Cyp1a1 catalytic activity in isolated hepatocytes was not affected by SnMP treatment. As(III) at the concentration of 5 μM, however, decreased the TCDD-mediated induction of Cyp1a1 catalytic activity. Importantly, SnMP partially restored Cyp1a1 activity that was decreased by As(III). As such, treating the cells with SnMP in the presence of both As(III) and TCDD gave a partial restoration of the As(III)-mediated downregulation of Cyp1a1 catalytic activity induced by TCDD (Fig. 7C). Despite being successful in partially reversing the As(III)-mediated decrease in Cyp1a1 activity by inhibiting HO-1, SnMP was unable to completely restore Cyp1a1 activity, implying the presence of a transcriptional mechanism in addition to the posttranslational modification.

*The effect of As(III) on serum Hb levels in vivo and the effect of Hb on XRE–luciferase activity in vitro*

The discrepancy between the effects of As(III) 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 As(III) on the TCDD-mediated induction of Cyp1a1 in vivo but not in vitro. For this purpose, and given the fact that As(III) is a potent hemolysis-inducing agent, we measured serum Hb levels in C57BL/6 mice treated with As(III) in the absence and presence of TCDD. Our results demonstrated that As(III) increased serum Hb levels by 1.8-fold, compared to control (Fig. 8A). On the other hand, TCDD alone did not significantly affect serum Hb levels. Interestingly, when animals were coexposed to As(III) and TCDD, As(III) increased serum Hb levels by 2.1-fold, compared to TCDD alone (Fig. 8A).

The results of As(III) on serum Hb levels further motivated us to examine its effect alone on the XRE-driven luciferase activity. For this purpose, isolated hepatocytes were treated with 1 μM Hb in the absence and presence of 1 nM TCDD. Our results demonstrated that Hb alone was able to significantly induce luciferase activity by 3.1-fold, compared to control (Fig. 8B). As expected, TCDD alone significantly induced luciferase activity by 32-fold, compared to control. Interestingly, when isolated hepatocytes were coexposed to Hb and TCDD, Hb significantly potentiated the TCDD-mediated induction of luciferase activity by 1.3-fold, compared to TCDD alone (Fig. 8B).

To correlate the in vivo effects with those observed at the in vitro level we tried to mimic the in vivo situation in vitro. For this purpose, isolated hepatocytes were treated with As(III) for 5 h in the absence and presence of TCDD, and thereafter, the treatment medium for cells receiving As(III) was replaced with 1 μM Hb in the absence and presence of TCDD. The reason behind replacing the medium with new treatment medium is that we were unable to detect As(III) or any of its metabolites in the serum of animals who received As(III) treatment for 24 h using a slowpoke reactor (data not shown). In addition, we needed to remove As(III) from the medium to rule out any direct effect of As(III).

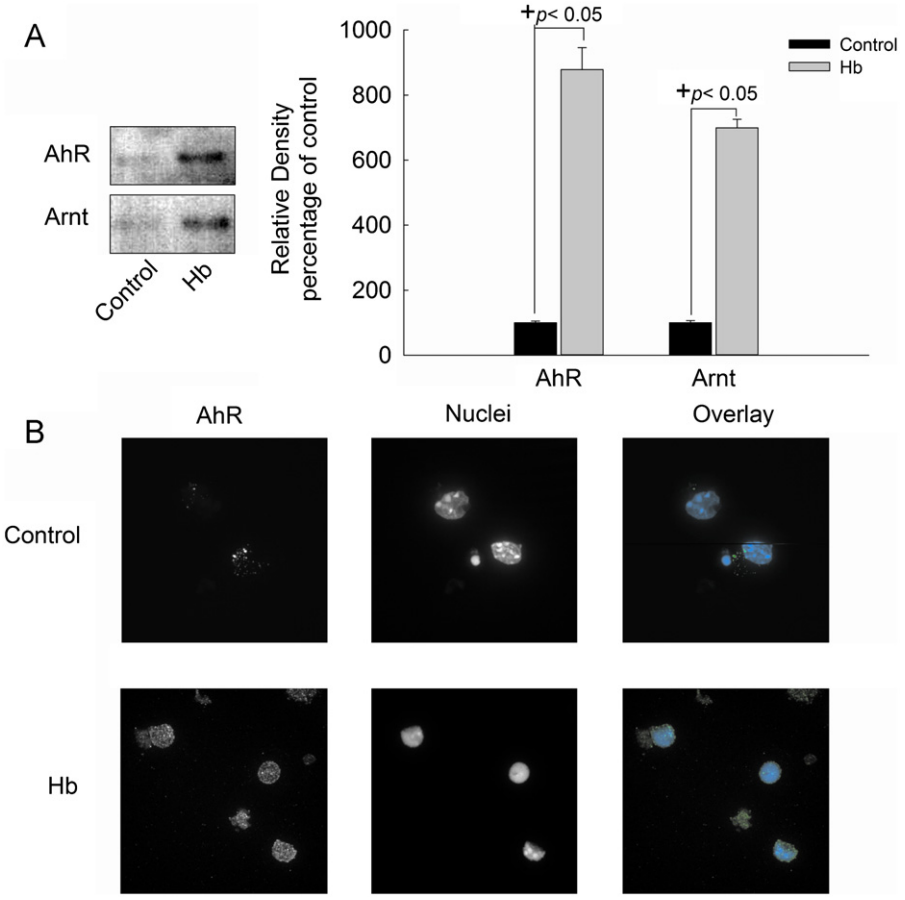


**Fig. 9.** Effect of Hb on AhR subcellular localization in isolated hepatocytes. (A) AhR protein was immunoprecipitated from nuclear fractions of treated isolated hepatocytes. Immunoprecipitates were then analyzed by Western blot analysis. AhR and Arnt proteins were detected using the enhanced chemiluminescence method. One of three representative experiments is shown. $+P<0.05$, compared to control (C). (B) Immunofluorescence microscopy of AhR protein subcellular localization in isolated hepatocytes. Cells were plated on collagen-coated glass coverslips, treated for 1 h with 1 μM Hb, and thereafter fixed and stained as described under Materials and methods. The magnification of each micrograph is identical. Cells were all stained at the same time and the results are representative of three independent experiments.

Our results showed that when cells were treated with As(III) and then treated with Hb there was no significant effect of Hb on the XRE-driven luciferase activity. On the other hand, when cells were treated with TCDD and Hb there was further potentiation to the TCDD-mediated induction of XRE-driven luciferase activity. Furthermore, when cells were exposed to As(III) and then TCDD was added after removing the As(III), there was a significant inhibition of the XRE-driven luciferase activity compared to TCDD alone; however, this inhibition was lower than that of coexposure to As(III) and TCDD (Fig. 7A). Importantly, when cells were treated with As(III) and then coexposed to TCDD and Hb, there was a significant potentiation of the XRE-driven luciferase activity by 1.5-fold, compared to TCDD alone (Fig. 8C).

*Effect of Hb on AhR protein localization*

The fact that Hb was able to increase the XRE-driven luciferase activity prompted us to investigate whether Hb was able to do so by activating AhR with a subsequent translocation to the nucleus. For this purpose, we examined the potential effect of Hb on AhR translocation to the nucleus using immunoprecipitation followed by Western blot analysis and immunocytochemical analysis. Isolated hepatocytes were treated with either vehicle or Hb for 2 h, followed by extraction of nuclear extracts and immunoprecipitation of AhR. Our results showed that Hb alone increased the nuclear accumulation of AhR by 8.8-fold compared to vehicle-treated cells (Fig. 9A). Importantly, Arnt protein coprecipitated with AhR from nuclear fractions of cells treated with Hb, indicating that Hb was able to activate AhR and change its conformation to the DNA-binding form (Fig. 9A). To confirm that Hb induced the nuclear accumulation of AhR, we examined the subcellular location of AhR upon treatment with Hb using immunocytochemical analysis. For this purpose isolated hepatocytes were treated for 1 h with Hb followed by fixing and staining as detailed under Materials and methods. Fig. 9B shows that AhR is a cytosolic protein, as vehicle-treated cells did not have significant localization of AhR to the nucleus. Interestingly, Hb-treated cells increased the nuclear localization of AhR, confirming the induction of the XRE-driven luciferase activity.

## Discussion

In this study we have demonstrated that As(III) differentially modulates the constitutive and TCDD-inducible Cyp1a expression and activity in C57BL/6 mouse liver and isolated hepatocytes. Furthermore, the concentrations of As(III) and TCDD utilized in this study were selected based on previous in vivo and in vitro studies that used the same concentrations of both As(III) and TCDD on C57BL/6 mice and Hepa 1c1c7 cells [21–25].

In this study As(III) by itself did not affect liver Cyp1a1, Cyp1a2, or Cyp1b1 at the mRNA, protein, or catalytic activity level. Importantly, As(III) 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 and not affecting Cyp1a2 or Cyp1b1 mRNA levels at 24 h. Furthermore, As(III) did potentiate 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.

Although there is no previous study that examined the effect of As(III) on the constitutive and TCDD-inducible liver Cyp1a1, Cyp1a2, or Cyp1b1 mRNA or protein expression levels, one previous study has shown that As(III) alone was capable of lowering EROD activity in CD57BL/6 mouse liver [26]. There is one difference between our study and the previously published study, in that As(III) was administered subcutaneously, in a dose closer to what we used in this study (11 mg/kg) [26]. Importantly, in the same study EROD activity was not affected at 48 and 72 h post-treatment with As(III), suggesting that the release of As(III) from a subcutaneous injection was delayed compared to the intraperitoneal injection used in the current study and thus when As(III) was eliminated after 48 and 72 h its effect was eliminated, too. In addition, previous reports have demonstrated that As(III) inhibited the β-naphthoflavone-mediated induction of the Cyp1a1 catalytic activity in the liver of the guinea pig [27]. Furthermore, in Wistar rats As(III) decreased total hepatic P450 content and mono-oxygenase activities of several P450s, including Cyp1a1 [28]. The discrepancy between these previously published studies and our current study could be attributed to species differences, route of administration, and the use of different AhR ligand.

The opposing effects of As(III) on the TCDD-mediated induction of Cyp1a1 and Cyp1a2 mRNA between the 6- and the 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 nonmetabolized As(III) in the form of sodium arsenite. However, the effect after 24 h is an indirect effect that could be attributed to other metabolic and kinetic factors summarized as follows: first, the distribution and elimination half-life of As(III) are different from those of TCDD, with TCDD possessing a longer half-life than As(III) and thus the persistent effect after 24 h of treatment [29,30]. Second, As(III) is metabolized in the liver to mono-, di-, and trimethyl arsenates and arsenites, which in turn will have differential effects on Cyp1a1 and Cyp1a2 mRNA, protein, and catalytic activity levels [31]. Third, there have been previous reports that have shown a biphasic effect of As(III) at different concentrations, and the observed changes in the expression of AhR-regulated enzymes could be attributed to these biphasic effects [32]. Fourth, As(III) might have affected other physiological processes or even triggered the release of other physiological modulators such as bilirubin and biliverdin, which are also AhR ligands [33]. Fifth, As(III) is a potent hemolytic agent that is known to bind to hemoglobin from red blood cells, causing hemolysis and increasing plasma hemoglobin levels [34].

To test our hypothesis that the effects of As(III) on the TCDD-mediated induction of Cyp1a1 and Cyp1a2 mRNA level at 6 h are in fact due to the direct effects of As(III), we measured As(III) levels in the serum of animals treated for 24 h. Interestingly, we could not detect As(III) in the serum of animals treated for 24 h (data not shown). In line with these findings, we have also shown that As(III) 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, these results imply that As(III) was almost completely eliminated after 24 h.

When we treated isolated hepatocytes with increasing concentrations of As(III) in the presence of TCDD, As(III) decreased Cyp1a1 mRNA, protein, and catalytic activity levels in a dose-dependent manner. In this regard, we are the first to report these effects in isolated hepatocytes. The transcriptional regulation of *Cyp1a1* gene expression by As(III) was also investigated. In this regard, we have shown that As(III) alone or in the presence of TCDD was able to significantly decrease the AhR-dependent XRE-driven luciferase reporter activity. In agreement with our results Maier et al. showed that As(III) decreased XRE-driven luciferase activity in Hepa 1 cells [35]. Similarly, studies on primary cultures of chick and rat hepatocytes showed that As(III) decreased total P450 and 3-methylcholanthrene-mediated induction of Cyp1a1 activity in chick hepatocytes, and Cyp1a1 mRNA, protein, and catalytic activity in rat hepatocytes [36–38]. The effect of As(III) was also tested in primary human hepatocytes in which As(III)

decreased polycyclic aromatic hydrocarbon-mediated induction of CYP1A2 at the mRNA level, whereas it decreased protein and catalytic activity levels of CYP1A1 and CYP1A2 [39]. In mouse Hepa 1c1c7 cells we have previously demonstrated that As(III), in the presence of several AhR ligands, significantly inhibited Cyp1a1 catalytic activity, possibly by increasing HO-1 [25].

The ability of As(III) to inhibit Cyp1a1 at the activity level more than was observed on the mRNA or protein expression level in isolated hepatocytes raised the question whether there is a posttranslational modification that might have occurred to the Cyp1a1 protein. Evidence from our laboratory and others suggests a role for HO-1 in the As(III)-mediated decrease in Cyp1a1 catalytic activity levels in Hepa 1c1c7 cells, HepG2 cells, and rat isolated hepatocytes [27,36,38,40,41]. In this study we have shown that As(III) increases HO-1 mRNA levels. Thus it is plausible that As(III), by inducing HO-1, will act to decrease the heme pool, which could result in failure to form functioning Cyp1a1 protein, which is at the same time more susceptible to proteasomal degradation [6]. However, this might not have occurred at the in vivo level because HO-1 was not persistently elevated, and thus its effect could not be seen at the activity level.

To dissect the role of HO-1 in the As(III)-mediated decrease in Cyp1a1 catalytic activity levels in isolated hepatocytes we treated the cells with SnMP, an inhibitor of HO-1, in an attempt to restore Cyp1a1 catalytic activity. If As(III) decreases the TCDD-mediated induction of Cyp1a1 catalytic activity by degrading its heme via HO-1, then inhibiting HO-1 will restore the TCDD-mediated induction of Cyp1a1 catalytic activity levels. Our results showed that inhibiting HO-1 using SnMP partially restores the As(III)-mediated decrease in Cyp1a1 catalytic activity. In agreement with this finding, we have previously shown that in the primary cultures of rat hepatocytes and human hepatoma HepG2 cells the effect of As(III) on CYP1A1 catalytic activity was reversed by SnMP, confirming the role of HO-1 in the As(III)-mediated inhibition of the TCDD-mediated induction of CYP1A1 catalytic activity [38,41].

An important difference between our in vivo and in vitro results with regard to the effect of As(III) on the TCDD-mediated induction of Cyp1a1 mRNA, protein, and catalytic activity is the kinetics factor. which in the in vivo case could have played a role in eliminating the effect of As(III) after 24 h of treatment especially at the gene expression level. In this regard, previous studies have shown that ip administered As(III) had a terminal half-life of 9 h with more than 90% of the dose being eliminated within 24 h [29]. Also, the distribution of As(III) might have contributed to this differential expression [29]. As such, it has been demonstrated previously that As(III) is deposited in the kidneys and lungs preferentially over other vital organs such as the liver in C57BL/6 mice after 24 h of treatment [29]. However, at the in vitro level, As(III) or its metabolites are persistently present with the cells throughout the treatment time course. Thus, the effect we see in vitro is either for As(III) or for a mixture of arsenicals after being metabolized.

The fact that As(III) causes hemolysis with a subsequent release of Hb to plasma prompted us to measure serum Hb levels from animals treated with As(III) for 24 h [34]. Our results demonstrated that As(III) in the absence and presence of TCDD increases serum Hb levels. In addition, we have previously demonstrated that Hb is capable of increasing CYP1A1 activity in human HepG2 cells [41]. Therefore, we examined its effect on XRE-driven luciferase activity in isolated 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 the Hb effect in the presence of As(III) in an attempt to mimic its in vivo effect. Our results demonstrated that Hb-treated hepatocytes in the presence of As(III) and TCDD further potentiated the TCDD-mediated increase in the XRE-driven luciferase activity. Thus, the in vivo effect of As(III) 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.

In this study we have demonstrated that Hb increases XRE-driven luciferase activity by increasing AhR nuclear accumulation as evident by nuclear extract immunoprecipitation of AhR and immunocytochemical analysis of AhR localization. However, the internalization of Hb by the isolated hepatocytes is a prerequisite for causing these effects. In this regard, it has been shown previously that Hb forms a very tight complex with haptoglobin, a plasma glycoprotein synthesized by hepatocytes [42]. The complex is then internalized by the hepatocytes through a receptor-mediated endocytosis [43]. The increase in AhR-dependent luciferase activity by Hb could be explained by the release of heme and heme degradation by-products such as biliverdin and bilirubin, which are at the same time endogenous AhR ligands [33]. Another heme degradation by-product through HO-1 is carbon monoxide (CO). CO by itself is known to bind and inhibit heme-containing proteins such as Cyp1a1 [44]. However, the presence of excessive amounts of Hb, which is a CO scavenger, might act to protect Cyp1a1 and other heme-containing proteins from CO. In agreement with our findings, previous studies have shown that Hb was able to induce CYP2E1 activity in primary human hepatocytes [45] and E47 cells without altering protein level [46].

In conclusion, this study demonstrated for the first time that As(III) differentially modulates constitutive and TCDD-induced Cyp1a1 mRNA, protein, and activity between C57BL/6 mouse liver and isolated hepatocytes. Furthermore, the effect of As(III) in vivo could not be simply extrapolated from in vitro studies as there are several factors that can confound the in vivo results that are not present in vitro.

## Acknowledgments

This work was supported by Natural Sciences and Engineering Research Council of Canada Discovery Grant RGPIN 250139 to A.O.S. A.A.-M. is the recipient of an Alberta Ingenuity Graduate Scholarship and an Izaak Walton Killam Memorial Graduate Scholarship. I.E.A. is the recipient of a Libyan Government Scholarship. The authors are grateful to Dr. Stephen Ogg, University of Alberta, for his help with confocal microscopy, and Dr. M.S. Denison (University of California, Davis, CA, USA) for providing us with XRE–luciferase reporter plasmid pGudLuc1.1.

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