"If I have seen further it is by standing on the shoulders of giants"

Isaac Newton (1642-1727)

University of Alberta

MODULATION OF ARYL HYDROCARBON RECEPTOR (AHR)-REGULATED XENOBIOTIC METABOLIZING ENZYMES BY ARSENITE

by

Anwar Gamal Abd El-Naser Anwar-Mohamed

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This work is dedicated to:

My parents,

GAMAL ABD EL-NASER ANWAR MOHAMED

&

BADRIA NAGY SABRA HAMMAM

To my future wife, with all love and respect

MONA ELSAYED GABR

And last but not least, my Brother and Sister,

MOHAMED & AYA

Thank you

ABSTRACT

Aryl hydrocarbon receptor (AhR) ligands and heavy metals are environmental co-contaminants and their molecular interaction may disrupt the coordinated regulation of AhR-dependent phase I and II drug metabolizing enzymes. As such, the effect of the heavy metal, As(III), on the AhR-regulated genes cytochrome P450 1A1 (CYP1A1), CYP1A2, CYP1B1, NAD(P)H: quinone oxidoreductase (NQO1), and glutathione *S*-transferase a1 subunit (GSTA1) was investigated. In HepG2 cells, As(III) significantly inhibited CYP1A1 at the mRNA, protein, and catalytic activity levels in a concentration- and time-dependent manner. As(III) significantly increased heme oxygenase-1 (HO-1), which coincided with a decrease in the CYP1A1 catalytic activity levels. The use of an HO-1 inhibitor, tin mesoporphyrin, a heme precursor, hemin, or transfecting the HepG2 cells with siRNA against HO-1 partially reversed the As(III)-mediated inhibition of the TCDD-mediated induction of CYP1A1 catalytic activity. Similarly, in rat primary hepatocytes, As(III) decreased CYP1A1, CYP1A2, CYP3A23, and CYP3A2 expression, and inhibiting As(III)-mediated induction of HO-1 partially restored the enzymatic activity of these P450s. In C57Bl/6 mice, As(III) modulated the constitutive and TCDD-induced AhR-regulated genes in a time-, tissue-, and AhR-regulated enzyme-specific manner. Additionally, As(III) increased the serum hemoglobin (Hb) levels in animals treated for 24h, and upon treatment of mouse 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, implicating Hb as an in vivo-specific modulator. Investigating the possible role of As(III)

metabolites as an alternative modulator to CYP1A1, we found that methylated pentavalent arsenic metabolites are bifunctional inducers as they increase *CYP1A1* through activating the AhR/XRE signaling pathway and they increase *NQO1* through activating the Nrf2/ARE signaling pathway in addition to the AhR/XRE pathway. Future studies are required to determine the exact role of AhR-and Nrf-2-regulated genes in the initiation and progression of malignancies.

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-ve siRNA	Scrambled control siRNA (Silencer Negative Select)
3MC	3-Methylcholanthrene
7ER	7-Ethoxyresorufin
7MR	7-Methoxyresorufin
AA	Arachidonic acid
AhR	Aryl hydrocarbon receptor
AhRE	Ah-responsive element
ANOVA	Analysis of variance
AP-1	Activator protein-1
APL	Acute promyelocytic leukemia
ARE	antioxidant-response element
ARNT	Aryl hydrocarbon receptor nuclear translocator
As(III)	Arsenite
ATCC	American type culture collection
ATSDR	Agency for Toxic Substances and Diseases Registry
AUBPs	AU rich element binding proteins
B[a]P	Benzo[a]pyrene
bHLH	Basic-helix-loop-helix
β–NF	β-naphthoflavone
CBP	CREB-binding protein
cDNA	Complementary DNA
CoPP	Cobalt protoporphyrin
CYP	Cyptochrome P450
CYP1A1 or Cyp1a1	Cytochrome P450 1A1
CYP1A2 or Cyp1a2	Cytochrome P450 1A2
CYP1B1 or Cyp 1b1	Cytochrome P450 1B1
CYP3A23	Cytochrome P450 3A23
CYP3A2	Cytochrome P450 3A2
CYP2S1	Cytochrome P450 2S1
DCPIP	2,6-Dichlorophenolindophenol
DFB	[3-[(3,4-Difluorobenzyl)oxy]-5,5-dimethyl-4-[4-

	(methylsulfonyl)phenyl]furan-2(5*H*)-one]
DFH	[3-hydroxy-5,5-dimethyl-4-[4 (methylsulfonyl)phenyl]furan-2(5*H*)-one]
DMA(V)	Diemthylarsinic acid
DMEM	Dulbecco's modified Eagle's medium
DMEs	Drug metabolizing enzymes
DMSO	Dimethyl sulfoxide
DRE	Dioxin responsive element
DTNB	5,5′-Dithiobis-(2-nitrobenzoic acid)
EH	Epoxide hydrolasae
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
EROD	7-Ethoxyresorufin *O*-deethylase
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GR	Glucocorticoid receptor
GSH	Reduced glutathione
GSTA1	Glutathione-*S*-transferase A1
H_2O_2	Hydrogen peroxide
HAHs	Halogenated aromatic hydrocarbons
Hb	Hemoglobin
HDAC	Histone deacetylase
Hepa 1c1c7	Murine hepatoma Hepa 1c1c7
HepG2	Human hepatoma HepG2
HIF-1α	Hypoxia inducible factor α
HO	Heme oxygenase
HO-1	Heme oxygenase 1
HSP90	90 kDa heat-shock protein
i.p.	Intraperitoneal
JNK	c-JUN NH_2-terminal kinase
Keap1	Kelch-like ECH associating protein 1
MAPKs	Mitogen-activated protein kinases
MMA(III)	Monomethylarsonous acid

MMA(V)	Monomethylarsonic acid
mRNA	Messenger RNA
MROD	7-Methoxyresorufin *O*-demethylation
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear Factor-Kappa B
NLS	Nuclear localization signal
NQO1	NAD(P)H:quinone oxidoreductase 1
NR	Nuclear receptors
NRE	Negative regulatory elements
Nrf2	Nuclear factor erythroid 2-related factor-2
p23	23-kDa Heat shock protein
PAGE	Polyacrylamide gel electrophoresis
PAHs	Polycyclic aromatic hydrocarbons
PAS	Per-Arnt-Sim
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pGudLuc1.1	Mouse XRE-luciferase reporter plasmid
pGudLuc6.1	Human XRE-luciferase reporter plasmid
PI3K	Phosphotidylinositol 3-kinase
PKC	Protein kinase C
pRL-CMV	Renialle luciferase reporter plasmid
PXR	Pregnane X recpetor
RAR	Retinoic acid receptor
Rif	Rifampin or rifampicin
RLU	Relative light unit
ROS	Reactive oxygen species
RT	Reverse transcription
RXRs	Retinoid X receptors
SDS	Sodium dodecyl sulfate
siRNA	Short interference RNA
SnMP	tin-mesoprophyrene
SUL	Isothiocyanate sulforaphane

TBS	Tris-buffered saline
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TKs	Tyrosine kinases
TMA(V)	Trimethylarsine oxide
V(V)	Vanadium as vanadate
XAP2	Hepatitis B virus X-associated protein 2
XRE	Xenobiotic responsive element

CHAPTER 1

1. INTRODUCTION

Versions of this chapter have been previously published.

1.1. ARYL HYDROCARBON RECEPTOR (AHR)

1.1.1. AHR FROM A HISTORICAL PERSPECTIVE

Since its discovery in the 1970s, the aryl hydrocarbon receptor (AhR) has attained notoriety as a front-line site of action for highly toxic environmental contaminants such as the polycyclic aromatic hydrocarbons (PAHs) and the halogenated aromatic hydrocarbons (HAHs) (Nebert and Bausserman, 1970). Poland and Glover were the first to identify the receptor protein as they found that it was responsible for the reported changes in enzyme activities induced by PAHs and HAHs (Poland and Glover, 1973). Following this notion, they discovered that this receptor is found in the cytosolic fraction of the C57BL/6 mice, and furthermore it reversibly binds to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent ligand of AhR ever known (Goujon et al., 1972; Nebert and Gielen, 1972; Poland et al., 1976). Soon after this discovery, a hypothesis was put forward linking the binding of AhR to the HAHs and PAHs and observed biological changes (Poland and Glover, 1973; Landers and Bunce, 1991). It was thus proposed that binding of HAHs and PAHs to this receptor in the cytosol would stimulate a conformational change in this receptor, allowing it to shuttle into the nucleus, bind to DNA, and subsequently initiate the transcription process (Landers and Bunce, 1991).

1.1.2. AHR SIGNALING PATHWAY

Conglomerates of studies have identified the AhR as a member of the basic-helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) family of transcription proteins. Inactive AhR resides in the cytoplasm bound to two 90-kDa heat-shock proteins (HSP90), the 23-kDa heat shock protein (p23), and hepatitis B virus X-associated protein 2 (XAP2). Upon ligand binding, the AhR-ligand complex dissociates from the cytoplasmic complex and translocates to the nucleus where it dimerizes with the aryl hydrocarbon nuclear translocator (ARNT) (Nebert and Duffy, 1997). The whole complex then acts as a transcription factor that binds to a specific DNA recognition sequence, termed the xenobiotic responsive element (XRE), thereby enhancing the transcription of an array of genes. Among these genes are those encoding a number of drug metabolizing enzymes (DMEs), including four phase I

enzymes [cytochrome P450 1A1 (CYP1A1), CYP1A2, CYP1B1, and CYP2S1] and four phase II enzymes [NAD(P)H: quinone oxidoreductase-1 (NQO1), glutathione-*S*-transferase A1 (GSTA1), cytosolic aldehyde dehydrogenase-3 and UDP-glucuronosyltransferase 1A6 (UGT1A6) (Nebert and Duffy, 1997; Rivera et al., 2002) (Figure 1.1)].

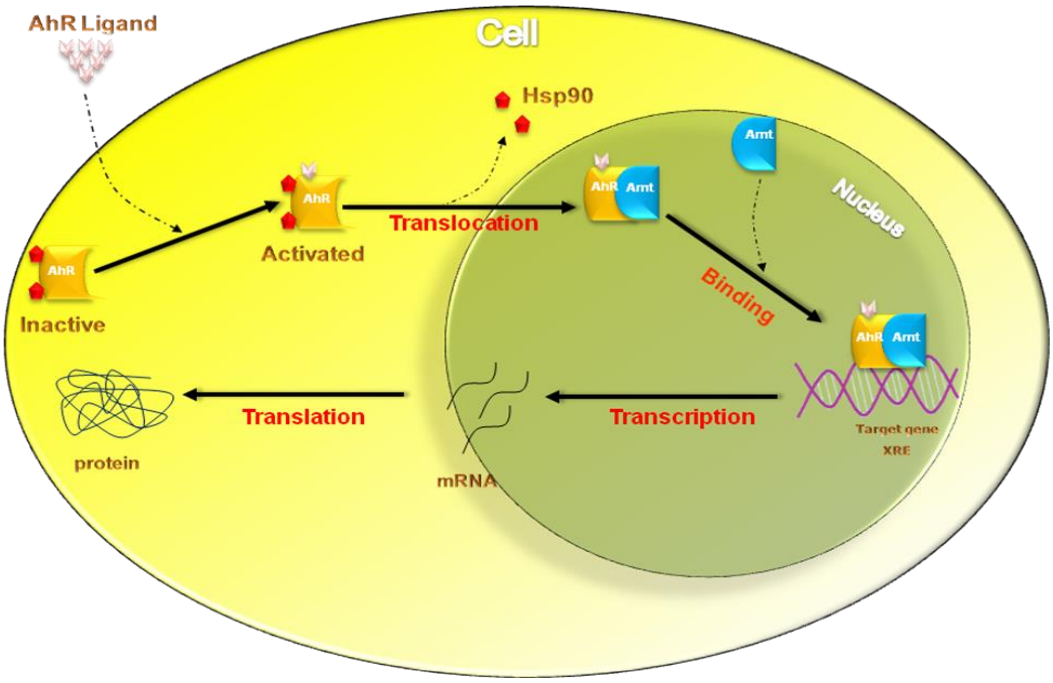

Fig. 1.1 A working model for the AhR signaling pathway.
(Adapted from (Hankinson, 1995)).

The AhR protein contains several regions which aid in protein and DNA binding. The bHLH motif located in the N-terminal of the protein contains two functionally distinctive and highly conserved domains (Fukunaga et al., 1995) (Figure 1.2). The first is the basic-region which is mainly involved in the binding of AhR to DNA. The second is a helix-loop-helix domain which is involved in protein-protein interactions. Members of the bHLH family also include Drosophila circadian rhythm protein period (Per), the ARNT, and the Drosophila neurogenic protein single minded (Sim) (Schmidt et al., 1993; Schmidt and Bradfield, 1996; Schmidt et al., 1996). The AhR protein contains also two Per-ARNT-Sim (PAS) domains, PAS-A and PAS-B domains. These PAS domains are

involved in secondary interactions with other PAS-containing proteins; an example of that would be the interaction of AhR with ARNT (Schmidt et al., 1993; Schmidt et al., 1996). Additionally, the PAS-B domain contains the AhR ligand binding site (Kazlauskas et al., 2001). Finally, a glutamine (Q) rich region allocated within the C-terminal was found to be responsible for the transcriptional activation upon AhR binding to DNA (Fukunaga et al., 1995).

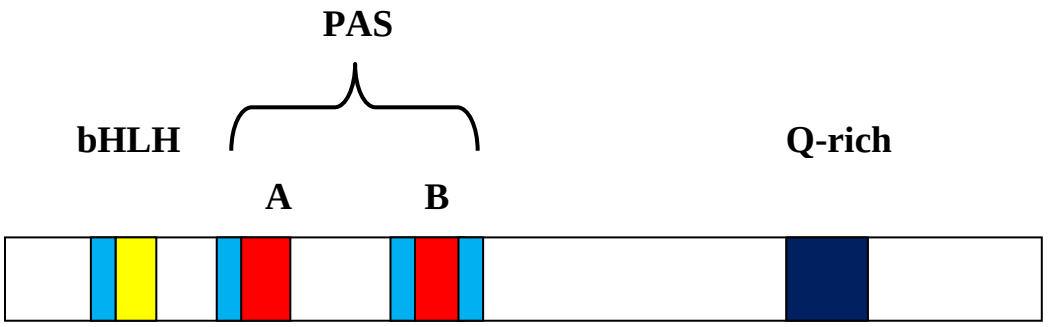

Fig. 1.2 Function domains of the AhR protein.
(Adapted from (Fukunaga et al., 1995)).

While the HSP90 interacts with the PAS-B and bHLH domains of the AhR, mounting evidence supports the role of HSP90 in maintaining the high affinity ligand binding conformation of the AhR, and repressing its intrinsic DNA-binding activity (Dolwick et al., 1993). The p23 is thought to stabilize the interaction between HSP90 and AhR (Kazlauskas et al., 1999), and further protects the receptor from being degraded through proteolysis, in addition to preventing the premature binding of AhR to ARNT. On the other hand, XAP2 interacts with the C-terminus of HSP90 and the nuclear localization sequence (NLS) of the AhR, preventing inappropriate trafficking of the receptor to the nucleus (Ikuta et al., 1998; Kazlauskas et al., 2000).

1.1.3. RECEPTOR ACTIVATION AND DNA BINDING

Activation of the AhR is a multi-stage process that starts with ligand binding, followed by the release of the AhR protein from the cytoplasmic complex, and its

translocation into the nucleus. The ligand binding domain of the AhR exists within the PAS B domain (Dolwick et al., 1993). Ligand binding induces a conformational change that leads to exposure of NLS that allows for the active transport of the AhR across the nuclear membrane (Ikuta et al., 1998; Kazlauskas et al., 2000). Hetero-dimerization with ARNT leads to DNA binding and subsequent activation of transcription. The recognition sequence of the AhR-ARNT dimer, the XRE, is also known as the dioxin- or Ah-responsive element (DRE or AhRE). The XRE is asymmetrical (5`-TNGCGTG-3`), suggesting that both the AhR and ARNT bind to different parts of the consensus sequence. It is believed that ARNT binds to the sequence at 5`-GTG-3` while the AhR binds 5` to these nucleotides (Hankinson, 1995). Binding of the heterodimer leads to chromatin remodeling through the recruitment of multiple co-activators, and the subsequent increase in gene transcription (Hankinson, 1995).

1.2. ACTIVATION OF THE AHR

1.2.1. LIGAND DEPENDENT ACTIVATION OF AHR

In vitro studies showed that the binding site for HSP90 within the AhR overlaps with the ligand-binding site (Whitelaw et al., 1993) and masks the AhR-NLS (Ikuta et al., 1998). Because the three-dimensional structure of the AhR is not determined yet, quantitative structure-activity relationship studies are commonly used to gain insight into the nature of the ligand-receptor interactions. Theoretically, there are two hypotheses for AhR interaction with its ligands (Mhin et al., 2002). First, electrostatic interaction, in which effective interaction of the ligand with the receptor depends on the molecular electrostatic potential around the ligand (Mhin et al., 2002). For example, all dioxin compounds that were able to activate the AhR share a unique molecular charge distribution pattern, which was dramatically changed with the chlorination pattern (Mhin et al., 2002). The second hypothesis is based on molecular polarizability and the distance between the receptor and the ligand (Mhin et al., 2002). In this regard, it has been shown that the AhR pocket can bind planar ligands with maximum dimensions of 14 Å ×

12 Å × 5 Å, that mainly depends upon the ligand's electronic and thermodynamic features (Denison and Nagy, 2003).

Although ARNT and the AhR of all species are about 20% identical in amino acid sequence, ARNT does not have any ligand binding capacity and therefore appears to be free from any repressive effect by HSP90 (Hankinson, 1995). Some evidence suggests that ARNT promotes dissociation of the AhR-HSP90 complex and targets the AhR to its nuclear site of action (Pollenz, 2002).

1.2.2. LIGAND INDEPENDENT ACTIVATION OF AHR

There are several lines of evidence to support the presence of a ligand-independent regulation of the AhR. Using an antagonist, 3′-methoxy-4'-aminoflavone (MNF), that can block the induction of CYP1A1 by TCDD, it was shown that omeprazole can induce CYP1A1 despite the presence of the antagonist (Ma and Whitlock, 1996). This specific mechanism has been shown to be tyrosine kinase dependent as it was blocked by herbimycin A, while it is not independent of the AhR, as the nuclear accumulation of the DNA binding of the AhR was observed (Ma and Whitlock, 1996). Interestingly, the induction of CYP1A1 by TCDD was not affected by herbimycin A, suggesting two distinct pathways for AhR activation (Kasai and Kikuchi, 2010). In light of the extensive evidence that pharmacological inhibitors mediate potential non-specific effects, these studies must be interpreted with caution. α-naphthoflavone is a partial agonist/antagonist depending on the concentration tested (Timme-Laragy et al., 2007). At low concentrations (<10 μM), α-naphthoflavone acts as an antagonist. However, at higher concentrations (>10 μM), α-Naphthoflavone acts as an agonist with a reduced affinity to AhR (Carver et al., 1994). This agonism was also prevented by herbimycin A (Kikuchi et al., 1998). More direct evidence for the presence of ligand-independent regulation of AhR can be demonstrated by the constitutive expression of CYP1A1 in a number of human lung cancer cell lines despite the absence of an exogenous AhR ligand (McLemore et al., 1989). Importantly, one must be cautious in supporting such hypothesis as there might be an indirect

generation of AhR ligands. To illustrate this line of thought, malassezin [2-(1H-indol-3-yl-methyl)-1H-indol-3-carbaldehyde] which is extracted from a strain of yeast is a non-classical AhR ligand-agonist by itself, but treatment with catalytic HCl liberates indolo[3,2,-b]carbazole (ICZ) which itself is a classic AhR ligand-agonist (Wille et al., 2001).

1.2.3. NON-CLASSICAL AHR LIGANDS

In addition to the classical AhR ligands typified by HAHs and PAHs, the AhR has been found to bind to synthetic compounds whose structure and physiochemical properties differ from these classical ligands (Denison and Nagy, 2003). The structural diversity of these "non-classical" AhR ligands is clearly evident by their molecular structures. For instance, carbaryl, a carbamate insecticide, primaquine, omeprazole, and the MAPK inhibitor SB203580 are all examples of novel AhR ligands whose structures deviate significantly from the classic planar, hydrophobic structure of the HAHs and PAHs (Denison et al., 1989; Werlinder et al., 2001; Backlund and Ingelman-Sundberg, 2004; Korashy et al., 2011). Not surprisingly, however, the majority of these ligands have been reported to be relatively weak inducers of the AhR. Nevertheless, the identification of these novel "non-classical" ligands paved the way for the identification of endogenous AhR ligands that can induce the AhR during physiological and/or pathological conditions (Denison and Nagy, 2003).

1.2.4. ENDOGENOUS AHR LIGANDS

Identification of an active AhR in cell culture and tissue slices in the absence of an exogenous AhR ligand prompted the search for possible endogenous AhR ligands (Singh et al., 1996). These observations lead to the identification of a large number of endogenous compounds capable of activating the AhR *in vitro*. Not surprisingly, these structurally distinct classes of compounds are relatively weak inducers of the AhR. Furthermore, their ability to activate the AhR *in vivo* is yet to be confirmed. Endogenous AhR ligands are grouped into several categories, including indoles, tetrapyrroles, and arachidonic acid metabolites (Denison and

Nagy, 2003). Indole-containing substances are primarily endogenous metabolites of tryptophan which is by itself in addition to several of its naturally occurring metabolites, including tryptamine, indoleacetic acid, indigo, and indirubin, have all been reported to activate the AhR in yeast and mammalian cells (Adachi et al., 2001). Products of heme degradation have also been found to activate the AhR signaling pathway. Heme degradation byproducts with the tetrapyrrole structure, including biliverdin and bilirubin, activate the AhR in cultured cells at physiologically relevant concentrations (Sinal and Bend, 1997; Phelan et al., 1998). Hydrophobic metabolites of arachidonic acid, most notably lipoxin A4 and prostaglandins (PGs) also activate the AhR (Seidel et al., 2000). In fact, several prostaglandins, including PGG2, PGD3, and PGH1 induce DNA binding of the AhR complex *in vitro* (Seidel et al., 2000). The physiological and/or pathological relevance of the ability of various endogenous metabolites to induce the AhR is currently under intensive investigation. However, a role for the AhR in normal development and physiological/biochemical processes is apparent due to the development of several physiological and developmental abnormalities in AhR knockout animals.

1.3. AHR REGULATED GENES

The toxicological relevance of AhR ligands stems from their ability to induce a host of genes in hepatic and extra-hepatic tissues. Several HAHs and PAHs not only serve as agonists of the AhR, but are also substrates for the induced phase I enzymes. The conversion of AhR ligands into diol epoxide compounds by CYP1A1, CYP1A2, or CYP1B1 results in the formation of covalent adducts when these genotoxic metabolites react with guanines in critical genes, potentially initiating tumorigenesis and other toxic responses (Spink et al., 2002). On the other hand, the induction of phase II enzymes is counterproductive to this process as NQO1 and GST a serve as a detoxification mechanism for many mutagenic and carcinogenic metabolites (Spink et al., 2002).

1.3.1. PHASE I AHR REGULATED CYPS

The CYPs are the terminal oxidases of an electron transfer system in the endoplasmic reticulum (Nebert and Russell, 2002). They are heme-containing proteins in which this heme-group is alligned to a cysteine molecule that is essential for thiol-ligand binding for the heme iron (Poulos, 2005). CYPs are made up of 400-500 amino acids with molecular weights ranging between 45 and 50 kda (Poulos, 2005). CYPs are expressed in almost every tissue (Spink et al., 2002).

CYPs are classified based on their amino acid sequence homology (Nelson, 2006; Sim and Ingelman-Sundberg, 2006). Members in a gene family are those who share more than 40% amino acid sequence identity. Similarly, members of the same subfamily have greater than 55% amino acid sequence identity and lie within the same cluster on a chromosome. Therefore, the family is designated by an Arabic number, whereas the subfamily is designated by a capital letter, followed by an Arabic number which represents a specific enzyme (Nelson, 2006; Sim and Ingelman-Sundberg, 2006). In addition, italic fonts are usually used to refer to the gene representing the enzyme (for example, *CYP1A1).* On the other hand, small letters are used to describe mouse enzymes (for example, Cyp1a1).

It is well documented that different families of CYPs participate in the oxidative metabolism of endogenous substrates such as steroids, fatty acids, and eicosanoids (Ramana and Kohli, 1998). In contrast, only the mammalian CYP1, 2, and 3 families are known to be involved in the metabolism of xenobiotics through different signaling pathways (Ramana and Kohli, 1998). Of these families, the CYP1 family member, CYP1A2, comprises 15 % of the total CYPs, which in turn comprise 70-80% of phase I drug metabolizing enzymes (Nebert and Dalton, 2006).

The CYP1A subfamily is comprised of only two members, CYP1A1 and CYP1A2. CYP1A1 is primarily an extra-hepatic enzyme since its constitutive

expression is low (Nebert et al., 2004). On the other hand, CYP1A2 is primarily a hepatic enzyme that is constitutively expressed. Nonetheless, the CYP1A1 may be induced 8-16 fold in the liver to a level that exceeds that of both constitutive and inducible expression of hepatic CYP1A2. In contrast to other members in the CYP1 family, CYP1B1 is inducible in numerous tissues, mainly the liver, lung, kidneys, and ovaries (Bhattacharyya et al., 1995). A difference between the enzymes also arises in their substrate specificity. While CYP1A2 preferably metabolizes hydrophilic amines, CYP1A1 and CYP1B1 metabolize the more hydrophobic PAHs and HAHs (Nebert and Dalton, 2006). Despite the difference in their tissue expression, the regulation of the three members in the CYP1 family is mainly transcriptional, regulated by the AhR.

1.3.1.1. CYP1A1

Among the CYPs, CYP1A1 has received considerable attention since it is highly induced by a broad range of xenobiotics such as PAHs and HAHs via the AhR-XRE transcription pathway (Denison and Nagy, 2003). CYP1A1 is capable of producing polar, toxic, or even carcinogenic, metabolites from various AhR ligands, including PAHs. Dissection of the mammalian *CYP1A1* gene revealed multiple regulatory elements which modulate its expression, the first being the XRE, several of which are localized approximately 1 kb upstream of the transcription start site in all mammalian *CYP1A1* genes (Hines et al., 1988). Secondly, there are three glucocorticoid responsive elements (GREs) in the human, rat, and mouse C*YP1A1* gene in the first intron, responsible for the modulation of PAH-induced CYP1A1 expression by glucocorticoids (Monostory et al., 2005). For example, it has been demonstrated that the inducibility of CYP1A1 by different PAHs and HAHs, which are known to be potent AhR ligands, is potentiated by the action of glucocorticoid receptors (Monostory et al., 2005). Of interest, exon 1 is a non-coding region and the initiation codon of *CYP1A1* gene expression is located within exon 2 (Monostory et al., 2005). Thus, binding of ligand-activated glucocorticoid receptor (GR) to the GRE sequences in the first intron will interact with the initiation complex on the CYP1A1 promoter

(XRE), and consequently enhance the level of induction of CYP1A1 enzyme by AhR-ligands transcriptionally (Monostory et al., 2005). On the other hand the GR will not be able to initiate the transcription process alone. Lastly, the presence of a negative regulatory element (NRE), located between -560 and -831 bp, inhibits the constitutive expression of CYP1A1 due to an interaction with the nuclear transcription factor Oct-1 (Hines et al., 1988).

The regulation of CYP1A1 has been extensively studied, but it is not yet completely understood. Changes in physiological conditions, including stressful conditions such as hyperoxia (Hazinski et al., 1995) and suspension of cells (Sadek and Allen-Hoffmann, 1994a; Sadek and Allen-Hoffmann, 1994b), or induction of differentiation (Crawford et al., 1997), increase CYP1A1 expression in the absence of an exogenous ligand. With the identification of an active AhR in cell culture and tissue slices in the absence of an exogenous AhR ligand (Singh et al., 1996), it was proposed that a ligand-independent mechanism may be responsible for AhR activation and subsequent CYP1A1 induction (Denison and Nagy, 2003). Further evidence for this theory arose from studies that showed that inhibition of nuclear export by leptomycin B or mutation of the AhR NES resulted in nuclear accumulation of AhR in the absence of exogenous ligand (Richter et al., 2001). These studies suggest that AhR shuttles between the nucleus and the cytosol in the absence of exogenous ligand. The exact mechanisms governing the ligand-independent activation of AhR are still not clear. However, it has been reported that activation of the cyclic adenosine monophosphate (cAMP) mediator (Oesch-Bartlomowicz et al., 2005) or mitogen-activated protein kinase (MAPK) signaling pathways (Ikuta et al., 2004) increase AhR nuclear translocation.

Theories regarding ligand-independent activation of the AhR have been shadowed with the identification of a large number of endogenous compounds with the ability to activate the AhR *in vitro*. These ligands have been grouped into several

categories, including indoles, tetrapyrroles, and arachidonic acid metabolites (Denison and Nagy, 2003). Indole-containing substances are primarily endogenous metabolites of tryptophan. Tryptophan and several of its naturally occurring metabolites, including tryptamine, indoleacetic acid, indigo, and indirubin, have been reported to activate the AhR in yeast and mammalian cell cultures (Heath-Pagliuso et al., 1998; Adachi et al., 2001). Products of the heme degradation pathway have also been found to activate the AhR signaling pathway. Bilirubin and biliverdin activate the AhR in cultured cells at physiologically relevant concentrations (Sinal and Bend, 1997; Phelan et al., 1998). Hydrophobic products of arachidonic acid metabolism, most notably lipoxin A4 and prostaglandins (PGs) also activate the AhR (Schaldach et al., 1999; Seidel et al., 2001). In fact, several prostaglandins, including PGG2, PGD3, and PGH1, induced DNA binding of the AhR complex *in vitro* (Seidel et al., 2001). Interestingly, these endogenous ligands seem to be relatively weak inducers as compared to the classic inducers (i.e. PAHs and HAHs).

The induction of Cyp1a1 by this structurally distinct class of compounds brings into light several identifiable factors that can influence the capacity of an AhR ligand to induce CYP1A1. AhR affinity plays a major role in determining the expression level of CYP1A1. This is illustrated in C57BL/6 mice which are sensitive to PAHs and HAHs (Nebert and Gelboin, 1969), and in DBA/2 mice which possess a lower affinity AhR and thus have lower induced Cyp1a1 levels (>15-fold difference) (Nebert and Gielen, 1972). Similarly, human beings also have >12-fold variation in AhR affinity that was positively correlated to cancer incidents, particularly in cigarette smokers (Nebert et al., 2004). In addition to receptor affinity, the recruitment of different co-activators of the transcription complex also influences the capacity of the AhR to induce CYP1A1 expression (Hankinson, 2005). It has been well documented that the chromatin core is comprised of a pair of histone proteins. Co-activators play an essential role in remodeling chromatin structure and relieving the transcription repressive effects of nucleosomes (Hankinson, 2005). The AhR signaling pathway is modulated by

several nuclear co-activators such as CBP/p300, SRC-1, SRC-2, RIP140, ERAP140, SMRT, and ATPase-dependent chromatin remodeling factors such as BRG-1 (Kobayashi et al., 1996; Kobayashi et al., 1997; Kumar and Perdew, 1999; Kumar et al., 1999; Nguyen et al., 1999; Beischlag et al., 2002; Rushing and Denison, 2002; Wang and Hankinson, 2002). Studies examining the role of these co-activators in the regulation of AhR-regulated genes by heavy metals are in their infancy. Some are available that can be included here.

1.3.1.2. CYP1A2

Besides CYP1A1, CYP1A2 is an important phase I enzyme that is involved in the metabolic activation of several procarcinogens including aryl amines and heterocyclic amine compounds which are present in cigarette smoke and char-coal broiled food. Therefore, elevated levels of CYP1A2 either through genetic polymorphism or through induction by an AhR ligand like TCDD might predispose to cancer (Kohle and Bock, 2007). CYP1A2 catalyzes the O-dealkylation of 7-ethoxyresorufin and 7-methoxyresorufin (Liu et al., 2001b; Uchida et al., 2002) and is involved in the metabolism of several pharmaceutical compounds such as acetaminophen, caffeine, imipramine, phenacetin, propranolol, and theophylline (Brosen, 1995; Tonge et al., 1998; Obase et al., 2003; Cheng et al., 2006). Furthermore, CYP1A2 has been correlated with tobacco-related cancers (Smith et al., 1996). In addition, the constitutive expression of Cyp1a2 in wild-type mice liver but not in AhR knockout mice strongly imply that Cyp1a2 is regulated by AhR (Quattrochi et al., 1998; Uchida et al., 2002). This was supported by the observations that TCDD markedly induces CYP1A2 in primary cultures of human hepatocytes through AhR-dependent mechanisms (Zhang et al., 2006). However, an AhR-independent regulation of CYP1A2 has also been reported previously. In a sequence analysis study of the human *CYP1A2* gene, it was revealed that there are two sequences homologus to the binding site of the AP-1, in addition to the XRE (Quattrochi et al., 1998).

1.3.1.3. CYP1B1

CYP1B1 is constitutively expressed in hepatic and extra-hepatic tissues and is highly induced by PAHs and TCDD through an AhR-dependent mechanism (McFadyen et al., 2001; Shimada and Fujii-Kuriyama, 2004). The enzyme catalyzes oxygenation of PAHs and aryl amines to reactive intermediates (Shimada and Fujii-Kuriyama, 2004). In this regard several lines of evidence demonstrated a correlation between CYP1B1 induction and breast cancer development (Spink et al., 1998; Ragin et al., 2010). As such, the ability of CYP1B1 to metabolize 17β-estradiol to 4-hydroxylated products is one of the postulated mechanisms by which CYP1B1 induces breast cancer development (Spink et al., 1998). These 4-hydroxylated products are recognized as potent carcinogens with high affinity for estrogen receptors that can cause breast cancer in women (Liehr and Ricci, 1996; Spink et al., 1998).

Two XREs have been identified at −834 and −853 in the 5′-flanking region of the human CYP1B1 gene to which liganded AhR/ARNT binds (Tsuchiya et al., 2005). Furthermore, it has been shown that the mutual interaction of these two XREs is important for the regulation of CYP1B1 (Tsuchiya et al., 2005). Additional studies demonstrated that the nuclear transcriptional factor Sp1 plays a crucial role in the constitutive and inducible transcriptional regulations of the human *CYP1B1* gene. In addition, a computer-assisted homology search identified a potential ERE on the human CYP1B1 promoter (between −63 and −49), and the treatment of ER-positive MCF-7 cells with estradiol induced CYP1B1 mRNA expression (Tsuchiya et al., 2005). Specific binding of ER to the putative ERE was demonstrated by chromatin immunoprecipitation assays and gel shift analyses. These findings clearly indicated that the human CYP1B1 is regulated by estrogen via ER.

1.3.2. PHASE II AHR REGULATED ENZYMES

Phase II drug metabolizing enzymes including GSTA1, and UGT1A6 but not the cytosolic ALDH3 or NQO1 catalyze drug-conjugation reactions (Swinney et al.,

2006; Anwar-Mohamed et al., 2013). Such an effect helps in detoxifying xenobiotics and carcinogenic metabolites formed by phase I drug metabolizing enzymes. Numerous studies have shown that these enzymes are regulated by the antioxidant responsive element (ARE) in addition to the XRE (Friling et al., 1990; Chen and Kunsch, 2004; Miao et al., 2005; Xu et al., 2005).

1.3.2.1. NQO1

NQO1 (also known as nicotinamide quinone oxidoreductase 1, DT-diaphorase, quinone reductase type 1, or menadione reductase), is a cytosolic flavoenzyme that catalyzes a two-electron one-step reduction of a broad range of substrates (Anwar-Mohamed et al., 2013). NQO1 plays a pivotal role in detoxifying quinones to their corresponding hydroquinone derivatives (Lind et al., 1982). Such an effect helps in maintaining endogenous antioxidants like ubiquinone and vitamin E in their reduced and active forms, thus protecting tissues from mutagens, carcinogens, and oxidative stress damage (Ross, 2004).

Being a reductase enzyme, it was first classified as a phase I drug metabolizing enzyme according to classical toxicology definitions as it serves to introduce functional groups to xenobiotics which is a typical behavior of this class of drug metabolizing enzymes (Nioi et al., 2003). In spite of being unable to catalyze conjugation reactions which is a unique feature of phase II drug metabolizing enzymes, NQO1 has been included in this class because it cannot introduce new functional groups into inert lipophilic xenobiotics (Prochaska et al., 1985). Moreover, it has been shown to be potentially induced by cancer chemopreventive agents along with other members of phase II drug-conjugating enzymes including glutathione *S*-transferase (GST) and UDP-glucuronosyl transferase (UGT) isoenzymes (Prochaska et al., 1985). However, on this specific occasion it might be ultimately incorrect to categorize NQO1 as a phase II drug metabolizing enzyme because NQO1 has also been shown to be induced by certain inducers of the phase I CYPs such as TCDD (Prochaska and Talalay, 1988). Perhaps the most noticeable feature of this enzyme that earned it phase II membership is its ability

to catalyze a two-electron reduction of several environmental xenobiotics and endogenous compounds (Ross, 2004; Korashy et al., 2007).

In addition to its wide distribution across different human tissues and organs, NQO1 has been found to be exceptionally elevated in a wide variety of human tumors and cell lines (Schlager and Powis, 1990; Winski et al., 1998). Among the three different forms of the NQOs identified to date, NQO1 is the most extensively studied enzyme. Particularly, NQO1 has been shown to play a critical role in protection against free radicals and mutagenicity, and hence is part of a cellular defense mechanism (Vasiliou et al., 2006). It is believed that NQO1 achieves that through three different mechanisms. The first is its direct catalytic action and this occurs if the chemical insult happens to be a quinone. The second mechanism would be its indirect antioxidant function. Thirdly, it stabilizes p53 protein which serves primarily as a transcriptional factor, and plays an important role in preserving genomic integrity, or the elimination of damaged or tumorigenic cells (Dinkova-Kostova and Talalay, 2000; Ross et al., 2000; Talalay and Dinkova-Kostova, 2004).

Constitutive NQO1 expression is tissue type-specific, in which maximum induction of the NQO1 mRNA was observed in liver and kidneys followed by lung and the heart (Jaiswal, 2000; Joseph et al., 2000). *NQO1* gene expression can be induced through two separate regulatory elements associated with its 5`-flanking region. The first pathway includes activation of a cytosolic transcription factor, the AhR as discussed previously. The second pathway involves activation of the ARE, which does not require functional AhR. In fact, the increased expression of *NQO1* gene expression in response to oxidative stress caused by agents such as isothiocyanate sulforaphane (SUL), *tert*-butylhydroquinone (t-BHQ) and H_2O_2 occurs primarily through the ARE signaling pathway (Venugopal and Jaiswal, 1996; Itoh et al., 1997). Perturbation in the redox status of the cell activates the nuclear factor erythroid 2-related factor-2 (Nrf2), a redox-sensitive member of the cap ‘n’ collar basic leucine zipper (CNC bZip) family of

transcription factors (Itoh et al., 1997). Subsequently, Nrf2 dissociates from its cytoplasmic tethering polypeptide, Kelch-like ECH associating protein 1 (Keap1), and then translocates into the nucleus, dimerizes with a musculoaponeurotic fibrosarcoma (MAF) protein, and thereafter binds to and activates ARE (Ma et al., 2004; Korashy et al., 2007) (Figure 1.3.).

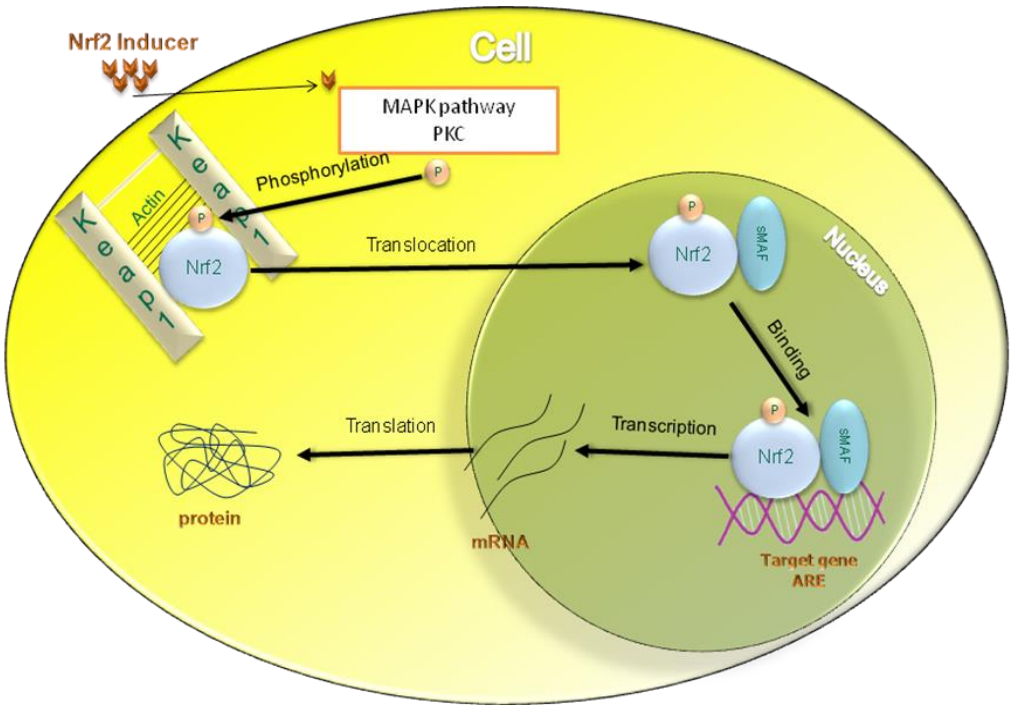

Fig. 1.3 A working model for the Nrf2 signaling pathway.
(Adapted from (Jaiswal, 2004; Anwar-Mohamed et al., 2013)).

1.3.2.2. GSTA1

GSTs are another group of detoxifying drug metabolizing enzymes with the ability to conjugate electrophiles including those represented by a carbon, sulfur, oxygen, or nitrogen atom. As such, the GSTs' substrate group is extremely large, the majority of which are either xenobiotics and their metabolites or products of oxidative stress. Arene oxides, quinones, epoxides, and hydroperoxides are some of the most toxicologically relevant functional groups susceptible to GSH conjugation (Hayes and Pulford, 1995). The conjugation reaction mediated by GSTs results in the formation of a thioether bond and hence a metabolite that is less reactive and more soluble, so it is easily excreted from the body. GSH

conjugation also tags the molecule for export from the cell through multidrug resistance-associated protein (MRP1 and MRP2) (Hayes and Pulford, 1995, Hayes et al., 2005).

GSTs comprise a subset of three superfamilies of enzymes which are in cytosolic, mitochondrial, and microsomal fractions. Mammalian cytosolic isoenzymes are dimers, categorized in 7 families designated as Alpha (A1-A5), Mu (M1-M5), Pi (P1), Sigma (S1), Theta (T1,T2), Omega (O1-O2) , and Zeta (Z1) (Hayes et al., 2005, Mannervik et al., 1985). In humans, a total of 16 Gst subunits have been identified that form 16 homodimers. In addition, members of the Alpha and Mu classes form a large number of heterodimers with members of the same class (Hayes and Pulford, 1995, Hayes et al., 2005).

Gst a1 and a2 are constitutively expressed and are induced by many xenobiotics and physiological stress. Fingerprinting of the promoter region has identified several cis acting elements in the 5′-flanking region of the rat *Gst a2* gene, including the ARE, the XRE, the glucocorticoid responsive element (GRE), and a Barbie box element responsive to phenobarbital (Hayes and Pulford, 1995). The promoter of the murine *Gst a1* gene, on the other hand, contains two tandomly arranged ARE and a Barbie box element. Surprisingly, although the intron and exon structures of the human and rodent *GSTA* genes are similar, there are striking differences in their mode of transcriptional regulation. Human *GST A* genes do not contain an ARE or XRE (Suzuki et al., 1994). Rather, transcriptional activation of the human *GSTA* genes occurs through an AP-1 and AP-2 consensus sequence and a GRE (Whalen and Boyer, 1998). Moreover, hepatic nuclear factor 1 has been located in the human *GST A* promoter region and functions as an enhancer (Clairmont et al., 1994).

1.4. CROSS-TALK OF THE AHR SIGNALING PATHWAY

A number of divergent points of cross-talk of the AhR signaling pathway with other signal transduction pathways will be discussed in this section. These

different cross-talk pathways can be classified into three major categories: 1) Co-activators and co-repressors involved in AhR regulation, 2) Phosphorylation cascades involved in AhR regulation, 3) Cross-talks of AhR with other nuclear receptors.

1.4.1. CO-ACTIVATORS AND CO-REPRESSORS INVOLVED IN AHR REGULATION

Data on AhR and other signal transduction pathways support a role for co-activators in cross-talk between signaling molecules. It has been well documented that the chromatin core is comprised of a pair of histone proteins. Co-activators play an essential role in remodeling chromatin structure and relieving the transcription repressive effects of nucleosomes (Hankinson, 2005). Co-activators which increase histone acetylation of the chromatin result in transcriptional activation, whereas co-repressors increase histone deacetylase activity, causing transcriptional repression. The AhR signaling pathway is modulated by several nuclear co-activators such as the CREB binding protein (CBP), p300, steroid receptor co-activator 1 and 2 (SRC 1 and 2), receptor interacting protein 140 (RIP140), estrogen receptor-associated protein of 140 kD (ERAP140), silencing mediator for retinoid acid and thyroid hormone receptors (SMRT) and ATPase-dependent chromatin remodeling factors such as Brahma-related gene 1 (BRG-1) (Anwar-Mohamed et al., 2009).

Functional interactions of AhR and estrogen receptor (ER) with ERAP140 and SMRT suggest a possible competition for limited pools of these co-regulators, or a mutual inhibitory cross-talk between AhR and ER (Nguyen et al., 1999). Two independent studies have shown that the co-repressor SMRT directly interacts with AhR. However, due to the conflicting findings between the two studies, which might be due to cell-specific effects, it has become difficult to generalize an effect of SMRT on the AhR-dependent gene expression. The co-activator RIP140 interacts directly with AhR in different cell types (Kumar et al., 1999). The recruitment of this co-activator by AhR or other transcription factors leads to

enhanced XRE-driven luciferase reporter activity (Kumar et al., 1999). Studies using AhR null embryonic fibroblasts showed that when these cells are transfected with AhR vector, the resultant AhR protein was bound to p300/CBP and was required for p300 DNA synthesis (Tohkin et al., 2000). In 293T cells AhR binds to different SRC-1 family proteins. Similarly, overexpression of different SRC-1 proteins increased the ligand-dependent XRE-driven luciferase reporter activity in Hepa 1c1c7 cells (Beischlag et al., 2002). Importantly, competition for CBP did not seem to mediate the AhR cross-talks with ER and NF-κB (Harnish et al., 2000). However, the cross-talk between AhR and NF-κB is thought to be through mutual interactions with SRC-1, p300, and CBP co-activators, leading to mutual inhibitory effect between the two transcription factors (Ke et al., 2001).

1.4.2. PHOSPHORYLATION CASCADES INVOLVED IN AHR REGULATION

Because AhR is a "phosphoprotein", its phosphorylation status plays an important role in modulating its activity (Chen and Tukey, 1996). Protein Kinases (PKs), Mitogen Activated Protein Kinases (MAPKs), and Tyrosine Kinases (TKs) have been implicated in AhR signaling (Long et al., 1998). Thus, studies investigating the phosphorylation of AhR may provide novel mechanism(s) for ligand-independent activation of AhR.

1.4.2.1. PROTEIN KINASE C (PKC)

The first evidence supporting a positive interaction between AhR and PKC was demonstrated by studies on mice in which the inhibition of PKC antagonized ligand-activated AhR/ARNT DNA binding and subsequently decreased *Cyp1* gene expression (Carrier et al., 1992). Similarly, several laboratories have independently demonstrated that PKC activation increased XRE-driven reporter activity and blocking PKC activity with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) decreased AhR-mediated induction of CYP1A1 and CYP1A2 mRNA, and CYP1A1 activity (Okino et al., 1992). Intriguingly, some early reports on PKC

indicated that phosphorylation of one of the AhR core complex proteins (AhR, ARNT, HSP90, p23 or XAP2) was necessary for AhR-mediated induction of CYP1A1 mRNA, and AhR/ARNT binding to DNA. Moreover, inhibition of PKC by TPA or staurosporine in mice Hepa-1 cells, human keratinocytes, and MCF-7 cells decreased the TCDD-mediated induction of CYP1A1 mRNA and catalytic activity via inhibiting the AhR/ARNT DNA binding (Okino et al., 1992).

AhR contains a Nuclear Localization Sequence (NLS), composed of amino acid residues 13-16 and 37-39, and a Nuclear Export Signal (NES) (Ikuta et al., 2004). In contrast to the previous studies, it was shown that phosphorylation of NLS at Ser12 and Ser36 by PKC inhibited the nuclear accumulation of AhR (Ikuta et al., 2004). In addition, when these Ser residues were substituted with Ala, AhR nuclear translocation was not affected by PKC-mediated phosphorylation, whereas replacement with Asp retained the mutant AhR in the cytoplasm (Ikuta et al., 2004). Thus, despite the conflicting reports about the role of PKC in AhR regulation, it is apparent that AhR activation is tightly regulated by PKC, which might be cell- and species-specific.

1.4.2.2. MITOGEN ACTIVATED PROTEIN KINASES (MAPKS)

MAPKs are serine threonine kinases, involved in inflammatory responses, apoptosis, cell growth and further mitogenic and developmental events. The three families of MAPKs are: extracellular signal regulated kinases (ERK1/2), c-Jun N-terminal/stress-activated protein kinases (JNK/SAPK), and the p38s; they are all important intracellular signal transduction mediators (Cobb and Goldsmith, 2000). MAPK activities are controlled by MAPK kinase kinase-MAPK kinase (MAPKKK-MAPKK) signaling cascades, in which MAPKs are activated by MAPKK-dependent phosphorylation, and MAPKKs are activated by MAPKKK-dependent phosphorylation (Cobb and Goldsmith, 2000). As a general rule ERK1 and 2 are involved in regulating mitogenic and developmental events, and the four p38 isoforms are involved in inflammatory responses, apoptosis, and cell cycle regulation (Weston and Davis, 2007). The three JNK isoforms are mainly

involved in cellular signaling, immune system, stress-induced apoptosis, carcinogenesis, and diabetes (Weston and Davis, 2007).

Three different AhR ligands, TCDD, benzo[*a*]pyrene (B[*a*]P), and benzo[*a*]pyrene-diolepoxide, activate JNK in mouse Hepa-1 cells, human lung carcinoma A549 cells, AhR-deficient CV-1 cells, and in AhR-positive and AhR-deficient mouse embryonic fibroblasts, suggesting that TCDD-mediated activation of MAPK is independent of AhR (Tan et al., 2002). Inversely, TCDD-stimulated MAPKs appear to be important for the induction of CYP1A1 (Tan et al., 2002). For example, TCDD-induced modulation of epithelial morphology causes the activation of JNK. These TCDD-mediated effects can be mimicked by constitutive expression of AhR (Diry et al., 2006). Furthermore, ablation of JNK2 and ERK decreases TCDD-mediated induction of CYP1A1 mRNA in mouse thymus and testis (Tan et al., 2002). It has been further noted that the induction of CYP1A1 and CYP1B1 mRNA and protein levels in response to UV radiation in human keratinocytes was partially due to JNK and p38 activation (Shimizu et al., 1999). The interaction between AhR and ERK appears to be critically linked as ERK inhibitors were shown to prolong TCDD-induced AhR degradation (Chen et al., 2005). Interestingly, in Hepa 1c1c7 cells it was shown that over-expression of ERK1 promoted AhR degradation, implying an important role of ERK in AhR proteolysis (Chen et al., 2005). Furthermore, phosphorylation of Ser68 in AhR NES by p38 activated AhR export from the nucleus, prior to its degradation (Fujii-Kuriyama and Mimura, 2005). Moreover, constitutively active MEK1, which is a MAPKK upstream of ERK1/2, increased TCDD-mediated induction of CYP1A1 mRNA via AhR (Andrieux et al., 2004). Based on the previous studies investigating the cross-talk between MAPKs and AhR, it can be concluded that AhR ligands contribute to the upregulation of several MAPKs which will consequently exert a positive effect on AhR nuclear accumulation and nuclear export.

1.4.2.3. TYROSINE KINASES (TKS)

Bombick and coworkers were the first to report an AhR-dependent modulation of tyrosine kinase. In addition, several reports suggested a requirement for tyrosine phosphorylation in AhR transactivation potential (Bombick and Matsumura, 1987). Direct interactions of the AhR complex with $pp60^{src}$, a tyrosine kinase, were observed in mouse hepatic cytosol using a cell-free system (Blankenship and Matsumura, 1997). Tyrosine phosphorylation has also been suggested as a requirement for AhR/ARNT complex DNA binding. For example, phosphorylation sites in two tyrosine domains of the C-terminus of the AhR are required for the formation of the functional AhR/ARNT heterodimer (Mahon and Gasiewicz, 1995). Furthermore, phosphorylation in a single tyrosine domain of the N-terminus is essential for proper recognition of the AhR for PKC-dependent phosphorylation, for binding of the AhR to its cognate DNA sequence, and for full transcriptional activity (Minsavage et al., 2004).

1.4.3. CROSS-TALK OF AHR WITH OTHER NUCLEAR RECEPTORS

For the last couple of decades extensive studies have been made to investigate the possible cross-talk between different nuclear receptors (NR) and the AhR. Of interest, there have been several attempts to explain this cross-talk: e.g. competitive binding of different NRs to a DNA-binding site, selective dimerization with other NRs prior to the DNA-binding step, and finally binding of different ligands that would probably affect the recruitment of a wide array of co-activators and/or co-repressors. Therefore, in the following section we will focus on seven NR cross-talks with AhR, namely nuclear factor erythroid 2-related factor-2 (Nrf2), ER, glucocorticoid receptor (GR), retinoid activated receptors and retinoid X receptors (RARs and RXRs), NF-κB, activator protein-1 (AP-1), and hypoxia inducible factor 1α (HIF-1α).

1.4.3.1. NUCLEAR FACTOR ERYTHROID 2-RELATED FACTOR-2 (NRF2)

The XRE was identified to be the DNA motif that up-regulates a battery of genes including phase I and phase II drug metabolizing enzymes. Similarly, the antioxidant responsive element (ARE), was identified to be the DNA motif that up-regulates specific phase II genes such as *NQO1, GSTA1, UGT1A6, ALDH3*, and heme oxygenase (*HO-1*) through the Nrf2/ARE signaling pathway. Nrf2 is a redox-sensitive member of the cap 'n' collar basic leucine zipper (CNC bZip) family of transcription factors (Itoh et al., 1997). In response to oxidative stress, Nrf2 dissociates from its cytoplasmic tethering polypeptide, Kelch-like ECH associating protein 1 (Keap1), translocates into the nucleus, and dimerizes with a musculoaponeurotic fibrosarcoma (MAF) protein, and thereafter binds to and activates the ARE (Ma et al., 2004). The proximity of the CYP1A1 promoter (XRE) and ARE suggested a cross-talk and functional overlap between the two signaling pathways (Miao et al., 2005; Kohle and Bock, 2006).

Conglomerates of studies have shown that bifunctional inducers, which activate both XRE and ARE signaling pathways, require a direct cross-talk between the XRE- and ARE-mediated pathways for the induction of several phase II genes (Kohle and Bock, 2006). Of interest, it has been reported that the induction of *NQO1* by selective ARE inducers requires the presence of the AhR, suggesting a more direct cross-talk between the XRE- and ARE-mediated pathways (Miao et al., 2005). Furthermore, it has been suggested that mouse Nrf2 is under the control of AhR as AhR ligands increased Nrf2 mRNA transcripts (Miao et al., 2005). Inversely, it was demonstrated that the expression of AhR, and subsequently CYP1A1, in addition to CYP2B1 is partially dependent on Nrf2 in Hepa 1c1c7 cells, implying that Nrf2 modulates AhR, constitutive androstane receptor (CAR) and their downstream targets (Shin et al., 2007). Reduction of AhR mRNA levels in Nrf2 knockout mice compared to wild type provides further support for this hypothesis (Shin et al., 2007). Moreover, AhR mRNA levels were

increased in Keap1 knockout mice, inferring a direct effect of Nrf2 in regulating AhR (Shin et al., 2007).

1.4.3.2. ESTROGEN RECEPTOR (ER)

Ohtake and co-workers have demonstrated that the estrogenic action of AhR agonists could be exerted through a direct interaction between the AhR/ARNT complex and the unliganded ER in the absence of 17β-estradiol (Ohtake et al., 2003). The use of AhR and ER knockout mice provided further support for this hypothesis. As such, 3-methylcholanthrene (3MC) was unable to activate the estrogen-responsive genes, namely *c-Fos* and vascular endothelial growth factor (*VEGF*), in both AhR and ER knockout mice (Ohtake et al., 2003). Incongruously, Hoivik et al. found no effect of estrogen on the CYP1A1 induction in both mouse hepatoma Hepa 1c1c7 and human breast cancer MCF-7 cells (Hoivik et al., 1997).

To date, no estrogen responsive elements (EREs) in the *CYP1A1* gene have been identified. However, a mutual inhibitory effect between the binding of ERα and AhR to their corresponding response elements has been previously reported and is a matter of debate (Kharat and Saatcioglu, 1996). For example, estradiol had no effect on the AhR/XRE binding, while on the other hand ERα-mediated suppression of induced CYP1A1 was successfully reversed by both an ER antagonist and by co-expression of nuclear factor-1 (NF-1), a transcription factor that interacts with both AhR and ERα. Therefore, these results suggested a direct cross-talk between AhR and ER-α through competing on a common transcription factor, NF-1 (Ricci et al., 1999). The competition between AhR and ER-α is not only limited to NF-1 as it was shown that both nuclear receptors also compete for several other co-activators such as RIP140, ERAP140, and SMRT (Kumar et al., 1999; Nguyen et al., 1999). Inversely, AhR-ligands were shown to down-regulate ER-dependent gene expression in human MCF-7 cells and in rodent estrogen-responsive tissues (Safe et al., 1998). Importantly, TCDD was shown to inhibit the interaction of ERα with its ligand and its response element (Gierthy et al.,

1996). Thus, these results suggest a potential competitive cross-talk between AhR and ERα for common co-activators (Nguyen et al., 1999).

The contradictory effects of estradiol on the AhR-regulated genes could be attributed to species-specific effects, the concentration of estradiol tested, and the tissue origin of the cell line utilized. These factors will determine the degree and the direction of response upon exposure to estradiols, while the cell line-specific effects will be in fact related to the changing levels of certain transcription factors or co-activators among different cell lines from the same species.

1.4.3.3. GLUCOCORTICOID RECEPTOR (GR)

Previous reports have demonstrated that the inducibility of CYP1A1 by different PAHs and HAHs, which are known to be potent AhR ligands, is potentiated by the action of GR (Monostory et al., 2005). The C*YP1A1* gene first intron contains three GREs (Monostory et al., 2005), while exon 1 is a non-coding region and the initiation codon of *CYP1A1* gene expression is located within exon 2 (Monostory et al., 2005). Thus, binding of ligand-activated GR to the GRE sequences in *CYP1A1* first intron will interact with the initiation complex (AhR/ARNT) on the XRE, and consequently enhance the level of induction of CYP1A1 mRNA by AhR-ligands (Monostory et al., 2005). Importantly, however, the GR will not be able to initiate the transcription process alone in the absence of the initiation complex.

In contrast to CYP1A1, *CYP1B1* gene expression was suppressed by dexamethasone in fibroblasts via a GR-dependent mechanism. An explanation offered for this awkward response is that the dexamethasone effect was mediated by a 256 bp DNA fragment carrying the XRE response element but not the GRE (Brake et al., 1998). Thus, one may speculate that dexamethasone might act differentially to down-regulate *CYP1B1* gene expression. In addition, the modulation of both *CYP1A1* and *CYP1B1* probably involves protein-protein

interactions between the GR and other transcription factors such as AhR, or competition for a common co-activator (Konig et al., 1992).

1.4.3.4. RETINOID ACTIVATED RECEPTORS AND RETINOID X RECEPTORS (RARS AND RXRS)

Retinoic acid (RA) has been identified as the most potent vitamin A metabolite that regulates a wide array of physiological processes including growth, differentiation, cell proliferation, and morphogenesis (Gambone et al., 2002). The physiological effects of RA are mediated by nuclear proteins RAR-α,β,γ, and RXR-α,β,γ (Chambon, 1996). It is believed that RXRs are the master regulators among other RA receptors because they dimerize with either themselves to form homo-dimers, or with most of the nuclear transcription factors, forming hetero-dimers (Vecchini et al., 1994). These receptor complexes then interact with the DNA cis-acting RA response element (RARE) to modulate the transcription of target genes (Mader et al., 1993).

The effect of RA on the regulation of *CYP1A1* is contradictory. For example, studies carried out on keratinocytes showed that RA was able to down-regulate or up-regulate CYP1A1 gene expression (Vecchini et al., 1994). Vecchini and co-workers demonstrated that in keratinocytes the *CYP1A1* gene promoter contains an unusual RARE element (Vecchini et al., 1994). In contrast, other studies on hepatocytes showed that RA had a minimal effect on CYP1A1 or CYP1A2 mRNAs, while selective ligands for RARs and RXRs caused a pronounced decrease in hepatic CYP1A2 expression *in vivo* (Howell et al., 1998).

1.4.3.5. NUCLEAR FACTOR-KB (NF-KB)

NF-κB is a family of transcription factors that plays a critical role in regulating gene expression (Shen et al., 2005). The NF-κB family is composed of six known proteins, NF-κB1, NF-κB2, RelA, RelB, c-Rel, and v-Rel, that can form homodimers as well as heterodimers with each other to bind to enhancer

sequences (Tian et al., 2002). Coimmunoprecipitation assays in Hepa 1c1c7 (Tian et al., 1999) and human breast cancer (Kim et al., 2000) cell extracts demonstrated physical and functional interactions between AhR and RelA subunit of NF-κB. These studies suggested that activation of one signaling pathway could significantly down-regulate the other. This has been demonstrated experimentally in Hepa 1c1c7 cells in which activation of NF-κB suppressed the expression of *Cyp1a1* at the transcription level (Ke et al., 2001). Although it is not clear whether such interaction occurs at the cytoplasmic or nuclear levels, several studies suggested that the interaction of NF-κB and AhR primarily occurs in the cytoplasm since ARNT was not found to dimerize with RelA in the absence of a ligand (Tian et al., 1999). It has been reported that unactivated AhR and NF-κB in the cytoplasm are kept away by being sequestered by their inhibitory proteins, HSP90 and inhibitory κB protein (IκB), respectively. However, once activated by TCDD and tumor necrosis factor-α (TNF-α), AhR and NF-κB, respectively, would then interact (Tian et al., 1999). Although the details of cytoplasmic interactions of RelA and AhR are still undetermined, transient transfection of Hepa 1c1c7 cells with AhR did not alter IκB levels, suggesting that the repressive effects are not mediated through the induction of IκB (Tian et al., 1999). On the other hand, the observation of a competition between RelA and AhR for binding to transcriptional co-activators and co-repressors strongly suggests a nuclear cross-talk between AhR and NF-κB. This was supported by Tian and coworkers who demonstrated that activation of NF-κB by TNF-α suppressed *Cyp1a1* gene expression through abolishing histone acetylation, which is an initial step for gene expression, resulting in inactivation of the *Cyp1a1* promoter (Ke et al., 2001). Furthermore, the suppressive effect of β-naphthoflavone, a potent AhR ligand, on NF-κB-enhancer driven luciferase reporter gene was reversed by the AhR antagonist, α-naphthoflavone (Tian et al., 1999).

Another postulated mechanism for the suppression of AhR by NF-κB activation is through activation of aryl hydrocarbon receptor repressor (AhRR). In this regard,

a NF-κB binding site (κB) was found in the promoter region of AhRR; therefore activation of NF-κB will result in induction of AhRR expression that heterodimerizes with ARNT and subsequently suppresses AhR activation and the expression of its regulated genes (Baba et al., 2001).

1.4.3.6. ACTIVATOR PROTEIN-1 (AP-1)

AP-1 is a heterodimeric complex of the leucine-zipper proteins c-Jun and c-Fos which are involved in a wide range of physiological and pathological conditions, such as cell proliferation, apoptosis, cell cycle control, tumor promotion, and carcinogenesis (Shen et al., 2005). Upon activation by a large number of stimuli, including proinflammatory cytokines, oxidative stress, and tumor promoters, AP-1 binds to TPA responsive elements (TRE) within the promoter regions of several target genes (Shen et al., 2005). AP-1 activity has been shown to be regulated by MAPK signaling pathways such as JNK, ERK1/2, and p38 (Shen et al., 2005). Once activated, JNK translocates to the nucleus where it phosphorylates c-Jun to potentiate its transcriptional activity which results in the induction of *c-Jun* and other AP-1 target gene transcription (Shen et al., 2005).

A well-established link between the AP-1 signaling pathway and the expression of AhR-regulated genes was demonstrated previously (Yao et al., 1997; Suh et al., 2002). The role of AP-1 in the modulation of *CYP1A1* and *CYP1A2* gene expression is controversial. Several previous studies have shown that TCDD inhibited LPS-induced DNA binding and transcriptional activity of AP-1 in murine lymphoma WT CH12.LX, but not in AhR-deficient BCL-1 cells (Suh et al., 2002). Furthermore, the observation that AhR antagonists attenuated TCDD-induced inhibition of AP-1 binding in CH12.LX cells (Suh et al., 2002) strongly suggests coordination between AhR and AP-1 signaling pathways. In contrast, treatment of Hepa 1c1c7 cells with TCDD or B[*a*]P caused an increase in c-Fos and c-Jun mRNA levels, which was associated with an increase in the DNA-binding activity of AP-1, suggesting that AP-1 activation requires a functional AhR-XRE complex (Hoffer et al., 1996). In addition, it has been shown that

induction of CYP1A2 activity in HepG2 cells in response to 3MC is mediated through activation of AP-1 DNA binding (Quattrochi et al., 1998).

1.4.3.7. HYPOXIA INDUCIBLE FACTOR 1A (HIF-1A)

The dimerization partner of AhR, ARNT is sometimes called HIF-1β (Eguchi et al., 1997). In addition to binding with AhR, ARNT dimerizes with HIF-1α to form an HIF-1 complex (Salceda et al., 1996). HIF-1α is also a member of the bHLH/PAS family of transcription factors. Upon the formation of HIF-1 and the subsequent binding to the hypoxia response element (HRE), the induction of transcription of hypoxia-related genes such as vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) is initiated (Laughner et al., 2001). Because of the potential competition between AhR and HIF-1α over ARNT, certain studies have supported the notion that the limiting cellular factor ARNT would influence the intensity of activation between the two pathways (Chan et al., 1999). In this sense, reciprocal cross-talk between hypoxia and TCDD signal transduction pathways has been demonstrated to occur *in vitro* and *in vivo* (Chan et al., 1999). Hypoxia on one hand would down-regulate the expression of AhR-regulated genes despite the presence of dioxin, while on the other hand increased oxygen supplementation would reverse these effects (Chan et al., 1999).

1.5. AHR AND PHYSIOLOGICAL DISEASES

Although the AhR expression levels are altered in different disease states, the cause-effect relationship between AhR on one side and the disease states on the other side remains a matter of debate. Therefore, in this section we are going to illustrate the recent findings with regard to the mutual interaction between AhR and different disease states, namely: carcinogenesis, reproduction and teratogenicity, cardiovascular diseases, immune diseases, and neural diseases.

1.5.1. CARCINOGENESIS

The carcinogenicity of AhR ligands including TCDD has been recently found to involve the AhR (Moennikes et al., 2004). Animal studies (multi-site carcinogenicity) and increased overall cancer mortality from industrial cohorts in several countries (Netherlands, Germany, and United States) lend support to this hypothesis (Safe, 2001). Epidemiologic data from the industrial cohorts with high exposure to AhR ligands showed that the pattern of increased types of cancer (lung, gastrointestinal, soft tissue sarcoma, non-Hodgkin lymphoma) varied considerably between these cohorts (Safe, 2001). For instance, the standard mortality ratios for non-Hodgkin lymphoma were 4.6 and 3.8 in German and Dutch studies (Kogevinas et al., 1995; Hooiveld et al., 1998), while the value in the United States was 0.9 (Fingerhut et al., 1991). Because most industrial cohorts were exposed to TCDD as a trace co-contaminant along with other contaminants such as chlorinated phenols, phenoxyherbicides, PCB, PAH, and metals, the increased incidence of cancer in these human cohorts could not be only TCDD-dependent but rather AhR-dependent. Importantly, association between AhR ligands including PAHs or HAHs, and breast cancer incidences was reported (Mukherjee et al., 2006), particularly in genetically susceptible subsets of women (Schlezinger et al., 2006). The risk of breast cancer recurrence was associated with adipose tissue PCB concentrations. However, several recent prospective and retrospective studies failed to confirm an association between HAH exposure and increased breast cancer risk (Schlezinger et al., 2006).

TCDD-induced tumors which were species-, age-, and sex-variable have been observed in different rodent models. With the fact that TCDD is not genotoxic by itself, it is likely that these carcinogenic effects are associated with the action of TCDD as a tumor promoter rather than tumor initiator (Tritscher et al., 1992). Major target organs for the TCDD-induced tumorigenesis are the liver, thyroid, oral cavity and lung in rats, and the liver, thymus, and skin in mice (Huff et al., 1994). In a 2-year feeding study, TCDD induced hepatocellular carcinogenesis in female but not male Sprague-Dawley rats that was estrogen-dependent (Kociba

and Schwetz, 1982). To the contrary and in the same study it was shown that the anti-tumorigenic effects of TCDD were due to blocking ER action (Kociba and Schwetz, 1982). This inhibitory effect of TCDD resulted in inhibition of multiple age-dependent spontaneous tumors including: pituitary adenomas, pituitary adenocarcinomas, interfollicular adenoma of the thyroid, benign uterine tumors, and benign neoplasma of the mammary gland (Mann, 1997).

The AhR appears to have constitutive expression and activity in established cell lines such as the adult T-cell leukemia (ATL) and in some primary ATL cell cultures, suggesting the existence of oncogenic activity associated with AhR expression in this tumor type (Barouki et al., 2007). In contrast, it was shown that acute lymphoblastic leukemia (ALL) REH and chronic myeloid leukemia (CML) K562 cell lines did not express AhR and had higher levels of AhR promoter methylation (Barouki et al., 2007). Interestingly, the AhR was methylated in 33% of 21 human primary ALL tumors (Barouki et al., 2007), suggesting that AhR silencing in this tumor type might be reflecting an AhR-mediated tumor suppression activity.

By far the most frequently studied set of genes induced by the AhR are those encoding phase I CYP enzymes, CYP1A1, CYP1A2, and CYP1B1, which metabolize at least some environmental AhR ligands into mutagenic intermediates (Castell et al., 2005). The role of CYP1 enzymes in mediating PAH toxicity can be demonstrated from epidemiological studies showing a correlation between the high CYP1 inducibility phenotype in cigarette smokers and cancers of the lung, larynx, and oral cavity (Senft et al., 2002). Xenobiotics have been shown to be activated by the CYP1 family into genotoxic and carcinogenic metabolites. Carcinogenic PAHs are activated by several drug metabolizing enzymes to highly mutagenic metabolites responsible for their toxicity (Shimada, 2006). The major activation pathway for PAHs, however, is through the formation of diol epoxides by members of the CYP and epoxide hydrolase enzymes (Shimada, 2006). B[*a*]P, the prototype carcinogenic PAH, is metabolized by CYP1A1 to B[*a*]P-7,8-oxide

which is subsequently hydrolyzed by epoxide hydrolase to (+)- and (-)-B[*a*]P-7,8-diol. CYP1A1 is then again responsible for the conversion of these metabolic intermediates into the toxic bay region metabolites (-)-B[*a*]P-7,8-diol-9,10-oxide-1, (+)-B[*a*]P-7,8-diol-9,10-oxide-2, (+)-B[*a*]P-7,8-diol-9,10-oxide-1, and (-)-B[*a*]P-7,8-diol-9,10-oxide-2 (Shimada, 2006). All four epoxides are highly mutagenic, yet (+)-B[*a*]P-7,8-diol-9,10-oxide-2 was identified as the most tumorgenic of the metabolites (Kapitulnik et al., 1978). The toxicity of other bay region epoxide metabolites has similarly been reported for other PAHs, including benzo[*a*]anthracene-3,4-diol, benzo[*b*]fluoranthene-9,10-diol, and benzo[*c*]phenanthrene-3,4-diol (Shimada, 2006).

Other members of the CYP1 family are also involved in xenobiotic activation. CYP1B1 metabolizes PAHs, aryl- and heterocyclic amines to carcinogenic metabolites (Shimada et al., 1996). In fact, *Cyp1b1*-null mice were not able to metabolize 7,12-DMBA and were resistant to the formation lymphomas and other malignancies caused by 7,12-DMBA (Buters et al., 1999). Furthermore, CYP1B1 is the principal precipitant of PAH-mediated immunotoxicity (Fujii-Kuriyama and Mimura, 2005). Interestingly, *in vitro* studies demonstrated a 10-fold higher activity for CYP1B1 than CYP1A1 in converting B[*a*]P to B[*a*]P-7,8-diol in a reconstituted human enzyme system (Shimada et al., 1999). However, similar rates of activation were reported for CYP1A1 and CYP1B1 in the activation of various PAH diols, including (-)-B[*a*]P-7,8-diols, 7,12-DMBA-3,4-diol, and B[*a*]A-3,4-diol (Conney, 1982; Amin et al., 1995). On the other hand, CYP1A2 demonstrated different enzyme kinetics as it had slower rates in the activation of these diols (Shimada, 2006).

Although there has been no clear link between exposure to heavy metals such as As(III) and vanadium (V(V)) and their mediated effects on AhR activity to carcinogenesis, there have been few attempts to explain some of these anticancer effects. As such As(III) (Trisenox^{TM}) has been successfully used for the treatment of acute promyelocytic leukemia and multiple myeloma (Hussein, 2001; Murgo,

2001). In addition, it is also being considered for treatment against solid tumors (Murgo, 2001). Similarly, V(V) compounds exert protective effects against chemical-induced carcinogenesis in animals by modifying various xenobiotic enzymes, possibly the AhR-regulated CYP1 family. Thus, V(V) inhibits carcinogen-derived reactive metabolites generation through this mechanism. Moreover, recent studies have suggested V(V) as an effective non-platinum metal antitumor agent (Kostova, 2009). As(III) and V(V) are believed to mediate their anticancer effect mainly through three different mechanisms: firstly by inactivation of the carcinogen-generating metabolizing enzymes such as CYP1A1, secondly through affecting cell proliferation, and lastly, through inducing cellular oxidative stress (Evangelou, 2002).

1.5.2. REPRODUCTION AND TERATOGENICITY

Previous studies have demonstrated that lack of AhR gene expression in AhR-deficient mice along with the unscheduled activation of AhR by HAHs result in adverse effects in female reproduction organs leading to impaired reproduction (Baba et al., 2005). For example, female AhR-deficient mice exhibit defects in multiple reproductive aspects, such as conception, litter size, and pup survival rate (McMillan and Bradfield, 2007b). These defects are further reflected in the decreased number of ovulated oocytes and a disordered estrous cycle (Baba et al., 2005). Both estrous cycle and oocyte development in the ovary are controlled by the complementary actions of the anterior pituitary hormones, luteinzing hormone (LH) and follicle stimulating hormone (FSH), and ovarian steroid hormones such as androgens, estrogens, and progestins (Gong et al., 1996). In a normal estrous cycle, these hormonal signals induce the maturation of granulosa cells surrounding the oocyte, triggering follicle rupture that would subsequently cause the release of the oocyte into the fallopian tube (Tornell et al., 1991). In this regard, the number of mature follicles and corpora lutea (CL) were significantly reduced in the super-ovulation process in AhR-deficient mice compared to wild-type (Barnett et al., 2007). Although the serum LH and FSH levels were not changed between AhR-deficient and wild-type mice, at all stages of the estrous

cycle, estradiol synthesis was lower in the AhR-deficient mice and was shown to be responsible for these reproduction defects (Barnett et al., 2007).

During the last couple of decades efforts have been made to better understand the relationship between estradiol synthesis-mediated reproduction defects and AhR deficiency. It is well documented that estrogen is synthesized from cholesterol in the ovary (Gruber et al., 2002), and it is therefore speculated that all the enzymatic machinery involved in estradiol synthesis is present in the ovary. Specifically, FSH secreted from the anterior pituitary binds to FSH receptor (FSHR) and stimulates expression of CYP aromatse (CYP19) gene (Gonzalez-Robayna et al., 2000), a rate-limiting enzyme in estrogen synthesis that converts testosterone to estradiol (Gonzalez-Robayna et al., 2000). In contrast, other steroidogenic enzymes such as CYP11A, CYP 17α-hydroxylase (CYP17), 3β-hydroxysteroid dehydrogenase (3β-SHD), and 17β-hydroxysteroid dehydrogenase (17β-SHD) are constitutively expressed (Hatano et al., 1994). In this regard, it has been demonstrated that the expression of these constitutive enzymes with the exception of CYP19 were not changed between AhR-deficient and wild-type mice (Hollingshead et al., 2006). Interestingly, in AhR-deficient mice CYP19 was not induced, compared to wild-type mice, in the proestrous stage leading to significantly lower ovarian estradiol concentrations (McMillan and Bradfield, 2007b). Furthermore, the reproductive defects in CYP19- or ER-deficient mice were similar to those observed in AhR-deficient mice, confirming its role in regulating CYP19 and subsequently controlling the levels of estradiol (Baba et al., 2005).

In addition to regulating the estrous cycle, AhR has been shown to play an important role during the pre-implantation period. As such, AhR is constitutively expressed in the uterine vasculature and developing tissues between the embryo and mother (Bremer et al., 2007). In addition to reduced fertility, AhR-deficient female mice are unable to raise their pups to the weaning age (Abbott et al., 1999), and these pups in return have lower survival rates than those raised by

wild-type female mice, an impairment that might be more related to mammary glands.

Activating the AhR beyond physiological limits by means of exposure to potent inducers such as TCDD affects developing embryos of several species. Thus, it has become apparent that TCDD-induced teratogenesis requires AhR, and the first step in this cascade is the ligand-activation of AhR. In developing mice, prenatal exposure to AhR ligands such as TCDD results in teratogenic effects at doses below those that can cause maternal or embryo/fetal toxicity (Li et al., 2006). Teratogenic effects mediated by AhR include thymic involution, cleft palate, hydronephrosis, aberrant cardiac development, and inhibition of ventral, dorsolateral, and anterior prostatic bud development (Wells et al., 2010). Thymic involution and hydronephrosis were also observed in mice harboring mutant AhR that is constitutively active in the absence of a ligand (Choi et al., 2006). These results highlight the importance of proper AhR regulation during development.

The AhR non-responsive mouse strain DBA/2 expresses an AhR encoded by the *Ahr*^{d} allele that binds TCDD with low affinity (Reichard et al., 2005). In contrast, the C57BL/6 strain expresses an AhR that is encoded by *AhR*^{b1} allele that binds TCDD with high affinity (Wells et al., 2010). Comparing the fetuses of both strains revealed that DBA/2 fetuses exposed to TCDD developed cleft palate and hydronephrosis to a much lower extent than C57BL/6 fetuses (Wells et al., 2010). Similarly, AhR-deficient fetuses displayed minimal incidences of cleft palate and hydronephrosis when compared to AhR wild-type fetuses (Wells et al., 2010). Therefore, the existence of AhR and the affinity of AhR to TCDD are undeniably important in determining the teratogenic outcome.

Despite the prominent role of AhR in mediating teratogenicity, the embryonic activity of most CYPs is low to negligible, and their role in xenobiotic bioactivation is still undetermined (Hines, 2008). However, it was previously reported that embryo cultures exposed to B[*a*]P and 2-acetylaminofluorene have

elevated levels of both CYP1A1 and CYP1A2 mediating the bioactivation of these xenobiotics (Juchau et al., 1992). In humans, CYP3A5 and CYP2C19 are the most abundant forms throughout pregnancy, and therefore they might play a role in the teratogen bioactivation (Hines, 2008). Human CYP-mediated bioactivation has been implicated, yet not proven, in fetal hydantoin syndrome caused by *in utero* exposure to phenytoin (Winn and Wells, 1995). The activities of some CYPs increase in the fetus later and could contribute to the teratogenic outcome. In contrast, there are some developmentally expressed CYPs such as CYP1B1/2 in rodents and CYP3A7 in humans which have high activity during embryonic development but decline rapidly at birth. These enzymes might play an important role in the bioactivation of teratogens such as B[*a*]P and aflatoxin B1 which are highly metabolized by CYP3A7 (Winn and Wells, 1995).

1.5.3. CARDIOVASCULAR DISEASES

AhR protein expression has been most commonly studied in the liver where its concentration ranges from 20 to 300 fmol/mg of cytosolic protein (Swanson and Bradfield, 1993; Hahn, 1998). In contrast, very little is known about the expression and function of the AhR in the heart. Investigational studies have demonstrated that defects in the resolution of fetal vascular structure were responsible for such phenotype (McMillan and Bradfield, 2007b). In the embryonic vasculature, the flow of blood partially bypasses the liver sinusoids via a shunt known as *ductus venosus* (DV) (Venkat-Raman et al., 2001), which directly connects the inferior vena cava and the portal vein. In normally developing embryos, the DV closes shortly after birth, forcing oxygen- and nutrient-rich blood to migrate through liver sinusoids (McMillan and Bradfield, 2007b). In the absence of AhR, the DV remains open throughout adulthood, and as a consequence this will lead to reduced postnatal liver growth due to deprivation of nutrients and oxygen (McMillan and Bradfield, 2007b). In addition to the DV phenotype, AhR-deficient mice have been shown to suffer from cardiac hypertrophy, hypertension, and elevated levels of the potent vasoconstrictors endothelin-1 and angiotensin-II (Lund et al., 2003). Interestingly, mounting

evidence indicates that vascular AhR signaling is mechanistically linked to shear stress which can be mimicked by blood flow through the vascular system (Korashy and El-Kadi, 2006a). As such, several independent reports have demonstrated that the AhR is highly activated by cellular exposure to fluid shear. Interestingly, recent evidence indicated that fluid shear stress activates the AhR through a direct effect on serum low density lipoprotein (LDL) function and structure, however, AhR activation through modified LDL does not seem to be solely through shear-induced modifications (McMillan and Bradfield, 2007a).

Early studies on the expression of AhR in different organ systems of healthy human subjects showed that AhR mRNA was expressed in several organs in the order (from higher to lower): placenta>lung>heart>kidney> liver (Dolwick et al., 1993). The intensity and localization of AhR was analyzed in the left ventricle of healthy persons as well as ischemic and dilative cardiomyopathy subjects (Mehrabi et al., 2002). In this study it was shown that AhR protein levels were two-fold higher in dilative and ischemic cardiomyopathy than in healthy subjects, suggesting a possible cause-effect relationship between AhR over-expression and cardiovascular diseases.

Studies conducted on embryonic chicken heart showed that both AhR and ARNT are constitutively expressed during the cardiogenesis process in the myocytes of aorta, atrium, ventricle, and atrioventricular canal, but not in the endothelial cells lining the heart valves and septa (Catron et al., 2001; Kanzawa et al., 2004). Surprisingly, although both chicken embryonic heart and liver express the same level of the AhR protein, TCDD was able to induce the hepatic but not cardiac *CYP1A1* gene (Kanzawa et al., 2004). This discrepancy suggests that the TCDD-mediated signaling cascades of cardiac AhR are different from those in the liver, and implies the presence of a tissue-specific transcriptional activation of the AhR (Kanzawa et al., 2004).

ARNT serves as a common dimer partner for both AhR and HIF-1α. A target gene downstream in the signaling cascade of HIF-1α is the VEGF (Semenza et al., 1999). Licht and coworkers showed that the expression of HIF-1α in the cardiovascular system is essential for embryonic cardiogenesis and blood vessel development, in which inhibition of HIF-1α activity resulted in a thin ventricular wall, disarranged endocardium, and impaired remodeling of the embryonic mouse vascular system (Licht et al., 2006). Of particular interest, C57BL/6J mouse embryo exhibited a high level of HIF-1α expression in the myocardial wall and atrioventricular canal of the developing heart (Jain et al., 1998).

Although little is known about the expression of CYPs in the cardiovascular system, the expression of cardiac AhR-regulated CYPs remains a matter of debate. Studies on AhR-deficient and wild-type mice demonstrated very low to negligible levels of cardiac constitutive expression of CYP1A1 and CYP1A2 mRNA and catalytic activities (Choudhary et al., 2005). In contrast, CYP1B1 mRNA is predominantly expressed at a significant level in the heart of both strains, representing a total of 13% of total cardiac CYPs (Choudhary et al., 2005). This was also observed in adult, but not fetal human heart (Choudhary et al., 2005). At the inducible level, both cardiac *CYP1A1* and *CYP1B1* genes were significantly induced by different AhR ligands such as B[*a*]P, PCB 126, β-naphthoflavone, and 3MC in different mammalian species (Granberg et al., 2000). Furthermore, cardiac CYP1A1-dependent EROD activity was induced by 4-fold in PCB 126-treated wild-type mice, while this induction was completely abolished in the AhR-deficient mice (Granberg et al., 2000). Similarly, Thackaberry and coworkers showed that TCDD was able to induce *Cyp1a1* and *Cyp1b1* gene expression by 13- and 5-fold, respectively, while it did not alter *Cyp1a2* gene expression in wild-type mouse fetuses (Thackaberry et al., 2005). In contrast to rodents, studies in human and chicken embryos demonstrated persistent expression of CYP1A1 in the cardiovascular tissue. For example, embryonic chicken heart has shown a strong constitutive expression of CYP1A1 protein (Walker et al., 1997).

The importance of these AhR-regulated CYPs lies behind their prominent role in the degradation, synthesis and/or metabolism of cardioactive endogenous substances such as arachidonic acid, prostaglandins, and thyroid hormones which have been shown to be effectively contributing to the pathogenesis of cardiovascular diseases (Rifkind et al., 1990). Although the exact mechanism of this effect is not fully understood, it has been experimentally demonstrated that the induction of CYP1A1 mediated the metabolism of arachidonic acid to hydroxyeicosatrienoic acid (HETE), epoxyeicosatrienoic acid (EET), and PGE2 metabolites in hearts of both rat and chicken embryos (Annas et al., 1998). Furthermore, CYP1B1 can metabolize arachidonic acid to both mid-chain HETEs and EETs, suggesting that these endogenous metabolites are involved in AhR-mediated cardiotoxicity (Choudhary et al., 2004). Bearing in mind that HETE is a potent vasoconstrictor, and EET is a vasodilator, the exposure to different AhR-ligands will be detrimental to cardiovascular pathophysiology. In this regard, we have recently demonstrated that 3MC- and B[*a*]P-induced cardiac hypertrophy in male Sprague-Dawley rats is partially due to increased HETE production by the AhR-regulated CYPs, CYP1A1 and CYP1B1 (Aboutabl et al., 2009).

The cardiotoxic effects of As(III) were reported earlier in patients exposed to pesticides, metallurgy, textiles, glass and paint (Butany et al., 2009). Due to the fact that As(III) in the form of As_2O_3 is now being used for the treatment of refractory acute promyelocytic leukaemia, its cardiotoxic effects have also been well documented (Butany et al., 2009). As(III) has known cardiographic abnormalities that include low flat T-wave, sinus tachycardia, prolonged QT intervals, atrioventricular blocks, multifocal ventricular tachycardia and ventricular fibrillation (Ohnishi et al., 2000). The most common change is prolongation of the QT interval, seen in 38% (n=99) of patients treated with As(III) for advanced malignancies (Barbey et al., 2003). As(III)-induced myocarditis has also been reported in the literature (Hall and Harruff, 1989). In contrast, Cu(II) supplementation has been shown to prevent or ameliorate

cardiomyopathy (Lynes et al., 2007). As such, it has been observed that dietary Cu(II) supplementation (20 mg Cu(II)/kg diet) can reverse an experimentally induced cardiac hypertrophy and improve heart contractile function in mice with established heart hypertrophy and dysfunction induced by ascending aortic constriction (Jiang et al., 2007). Inversely, it was also shown that ascending aortic constriction caused a decrease in cardiac Cu(II) levels, which can be corrected by dietary Cu(II) supplementation (Uriu-Adams et al., 2010). Altogether, despite the reported cardiac effects of both As(III) and Cu(II), there have been not attempts to correlate these effects to the alteration in AhR-regulated CYPs.

1.5.4. IMMUNE DISEASES/ INFLAMMATION

Immunotoxicology occurs via adverse interference by xenobiotics with the immune system, such as allergic reactions, drug-induced autoimmunity or immunosupression by TCDD (Johnson et al., 2000). In this regard, TCDD has been shown to be immunosuppressive in laboratory animals. This immunosuppressive effect has been shown to be not only limited to TCDD, but also to other AhR ligands such as PAHs and HAHs (Silkworth et al., 1995). In general, the immunosuppressive effects of these AhR ligands are: decreased host resistance to infectious disease, and suppressed humoral and cell-mediated immune responses (Silkworth et al., 1995). In addition to its immunosuppressive effect, TCDD promotes inflammatory responses via up-regulating the production of inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) (Fan et al., 1997).

In an effort to distinguish AhR-mediated TCDD toxicity from that resulting from alternative pathways, the AhR-deficient mice were compared with wild-type mice regarding the role of AhR in immunosupression (Kerkvliet et al., 2002). AhR-deficient mice are defective in T cell differentiation and are more susceptible to bacterial infection. Most of the cells that participate in immune responses either constitutively or inducibly express AhR at a certain level throughout the responsive process. Importantly, many of the genes involved in immune response

such as IL-1Rs, IL-6, IL-18, and all 9 Toll-Like Receptors (TLRs) have XRE sequences in their promoters, although it is not clear whether or not AhR is directly involved in controlling their expression (Fujii-Kuriyama and Mimura, 2005).

In contrast to the previously mentioned effect, studies carried out on AhR-deficient mice have shown that treatment with LPS rendered these mice more susceptible to septic shock (Sekine et al., 2009). In addition, these mice were also hypersensitive to LPS-induced lethal shock compared to their littermate wild-type mice (Sekine et al., 2009). Upon treatment with LPS, the serum levels of IL-6, TNF-α, and IL-1β were higher in AhR-deficient mice compared to wild-type (Sekine et al., 2009). In agreement with these *in vivo* studies, it was shown that isolated LPS-treated peritoneal macrophages from AhR-deficient mice had much higher IL-6 secretion than those from wild-type mice (Sekine et al., 2009). In an effort to confirm the role of AhR in the release of IL-6 from these macrophages, AhR-deficient mice macrophages transfected with AhR expression plasmid showed suppression of IL-6 expression upon LPS treatment (Kimura et al., 2009). Inversely, it was also demonstrated that LPS treatment enhances AhR expression in peritoneal macrophages (Kimura et al., 2009). An explanation offered to explain the AhR suppressive effect is that AhR by forming a complex with Stat1 and NF-κB leads to inhibition of the promoter activity of the *IL-6* gene (Kimura et al., 2009). With regard to IL-1β secretion, it was shown that AhR activated the expression of Pai-2, an inhibitor of caspase-1 activation that functions in the process of IL-1β secretion (Mimura and Fujii-Kuriyama, 2003). Treatment of wild-type macrophages with AhR ligands markedly enhanced Pai-2 expression, and similarly transduction of Pai-2 expression in AhR-deficient macrophages restored IL-1β secretion (Matsumura, 2009). Activated AhR together with NF-κB but not ARNT directly enhanced Pai-2 gene expression by binding to its promoter (Matsumura, 2009; Sekine et al., 2009). Using the human macrophage cell line U937, it was shown that TCDD induced the expression of IL-8, Bcl (B-cell chemoattractant), and Baff (B-cell activating factor) (Vogel et al., 2007; Vogel

and Matsumura, 2009). These increases in gene expression were shown to happen with a concomitant binding of the AhR-RelB complex to κB binding site in the promoters of these genes (Vogel and Matsumura, 2009). Interestingly, this process was ARNT-independent, suggesting the presence of a new transcriptional mechanism that involves TCDD-activated AhR.

It has been previously reported that AhR ligands stimulate naïve T cells (Th0) to differentiate in to helper T-cells that either suppress or accelerate the immune responses by modulating effector T cell proliferation and cytokine secretion (Fujii-Kuriyama and Mimura, 2005). Interestingly, it was also shown that the profound suppression of acute graft versus host (GVH) response in TCDD-treated mice was associated with the generation of donor-derived $CD4^+$ $CD25^+$ T_{reg} cells that was AhR-dependent (Funatake et al., 2005). In addition, the promoter of the *FoxP3* gene, a transcription factor that plays a master role in T_{reg} cell differentiation, contains functional XRE sequences that are responsible for inducing this gene in the presence of activated AhR (Sekine et al., 2009). Furthermore, a chromatin immunoprecipitation (ChIP) assay revealed that AhR is recruited to the *Foxp3* promoter (Sekine et al., 2009).

Previous studies have demonstrated that the AhR-regulated CYPs such as CYP1A1 and CYP1A2 were resistant to the down-regulation induced by interferon unlike other CYPs such as CYP3A2, CYP2C11, CYP2C12 and CYP2E1 which were significantly down-regulated in response to interferon (Cribb et al., 1994). In primary rat and human hepatocytes, the media obtained from activated Kupffer cells in addition to IL-1 decreased the total P450 content of these cells (Muntane-Relat et al., 1995). Subsequently, recombinant cytokines, including IL-1α, IL-1β, IL-6, TNF, and tumor growth factor were shown to suppress the expression of CYP1A1 and CYP1A2 in rodents *in vivo*, and in HepG2 cells *in vitro* (Vrzal et al., 2004). Importantly, the suppressive effects of TNF-α and IL-1β are at least in part due to a direct NF-κB action (Tian et al., 1999). In this regard, a few studies have suggested that in addition to the

transcriptional inhibition of CYP1A1 and CYP1A2 by these cytokines, these cytokines might also increase the turn-over rate of these transcripts (Delaporte and Renton, 1997).

It has been clearly demonstrated that TCDD causes chronic and sustained oxidative stress in animals, likely due to the production of reactive oxygenated metabolites by CYP1 enzymes (Dalton et al., 2002). The production of reactive oxygen species (ROS) occurs also during inflammation and/or infections (Coussens and Werb, 2002). Thus, ROS produced *in vivo* and *in vitro* have been implicated in the cascade of events leading to the loss of CYPs during inflammation (Symons and King, 2003). As such, it has been shown that hydrogen peroxide and oxidative stress cause loss of hepatic CYP1A1 and CYP1A2 mRNAs in isolated rat hepatocytes (Renton, 2001).

Metallothionein serves as one of the points of intersection for many of the immune and autoimmune disease states (Lynes et al., 2007). Although this small stress response protein is not a member of the heat-shock protein family, it serves many roles in both normal and stressed cells, acting as a reservoir of essential heavy metals such as Cu(II), as a scavenger for heavy metal toxicants such as Hg(II), Cd(II) and their associated free radicals, and as a regulator of transcription factor activity (Vasak, 2005). With the striking fact that AhR wild-type mice have relatively lower expression of metallothioneins compared to AhR-deficient mice (Tijet et al., 2006), it might be a possibility that in the presence of AhR and the consequent reduction of functional metallothionein, animals exposed to either Cd(II) or Hg(II) would have an elevation in immunotoxicity mediated by these heavy metals (Lynes et al., 2007).

1.5.5. NEURONAL DISEASES

During development, exposure to TCDD and related compounds affects a wide array of brain functions even at low doses (Mandal, 2005). An earlier report showed that exposure of rats to 10-100 nM TCDD stimulated Ca^{2+} uptake in

hippocampal neurons within 40 s (Xie et al., 2006). Moreover, Ca^{2+} uptake was antagonized by the calcium channel blocker nifedipine (Xie et al., 2006). In the nervous system of zebra fish, TCDD-induced effects were shown to be exerted through ligand-activated AhR together with ARNT (Mandal, 2005). In addition to zebra fish, AhR and its partner ARNT were identified in the olfactory bulb, cerebral and cerebellar cortices, and hippocampus of TCDD-exposed rats (Huang et al., 2003). Moreover, it has been suggested that TCDD could have significant endocrine-disruptive effects on the gonadal and thyroid hormone axes, as well as neural-disrupting action on neural transmission and neural network formation (Brouwer et al., 1999).

According to literature, Han-Wistar rats are among the most resistant of all mammals to TCDD toxicity (Mandal, 2005). The reason behind this resistance is due to a mutation in the AhR gene that alters and truncates the C-terminal transactivation domain of the AhR protein (Mandal, 2005). Of interest, Han-Wistar rats show normal acute induction of the CYP1 family genes. With the fact that TCDD-mediated toxicity in rats develops over weeks and sometimes over months of exposure time (Walker et al., 1998), it is reasonable to conclude that the TCDD-induced neural toxicity necessitates acute and chronic AhR activity to mediate these effects.

Exposure to environmental toxins such as PAHs and HAHs in genetically predisposed individuals might have an important role in aetiopathogenesis of neurodegenerative disorders (Bains and Shaw, 1997). For example, Parkinson's disease has been associated with mutations in the *CYP2D6* and *CYP1A1* genes (Tan et al., 2000). In addition, hepatic CYP1A2 has been shown to be involved in the metabolism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine to the neurotoxic metabolite 1-methyl-4-phenylpyridinium ion which produces a Parkinson's-like syndrome (Coleman et al., 1996). In addition to CYP1A1 and CYP1A2, CYP1B1 is highly expressed in the human blood-brain barrier, brain microvessels, brain glial cells, and dura mater (Dauchy et al., 2008; Dutheil et al., 2009). Therefore,

due to its high level of expression, CYP1B1 might be a contributor to neurodegenerative disorders.

Pb(II) is a ubiquitous environmental pollutant in the industrial environment that poses a serious threat to human health (Rahbari and Goharrizi, 2009). It has been demonstrated that heavy metals may play a role in the pathogenesis of diseases of nervous system such as multiple sclerosis, amylotrophic lateral sclerosis, and Alzheimer's disease (Mishra, 2009). For example, Waterman and co-workers have shown that Pb(II) exposure leads to the production of autoantibodies against neural proteins, including myelin basic protein and glial fibriallary acidic protein, concluding that Pb(II) can aggravate neurological disease by increasing the immunogenicity of nervous system proteins (Waterman et al., 1994). However, the role of AhR-regulated CYPs in this regulation is undeniably unknown.

1.6. HEAVY METALS

Although there are numerous studies examining the toxic effects of individual AhR ligand forms, there are relatively few reports of the combined toxic effects of AhR ligands and other environmental co-contaminants. Among these, environmental co-contaminants of most concern are heavy metals, typified by arsenite (As(III)). Heavy metals are found in air, water, soil, and food. As(III) comes at the first position in the list of most hazardous environmental contaminants listed on the Agency for Toxic Substances and Disease Registry (ATSDR) (Klaassen, 2001; ATSDR, 2011).

1.6.1. AS(III) EXPOSURE AND METABOLISM

Arsenic is widely distributed in the environment and can be found as organic and inorganic compounds in the trivalent (As(III)) or the pentavalent (As(V)) states (Klaassen, 2001). Inorganic As(III) is released into the environment from primary copper, zinc, and lead smelters and glass, pesticide, and herbicide manufacturing (Klaassen, 2001). It also found in cigarette smoke, arsenic-treated wood, and agricultural fertilizers (Patrick, 2003).

Exposure to arsenic species occurs through the oral and inhalation routes. Depending on the chemical form, 50-95% of ingested As(III) or As(V) is absorbed through the oral route, although absorption of less soluble species, such as lead As(V), tends to be much lower. Dermal absorption on the other hand is quite low, reaching only 4.5% after 24 h. Following absorption, arsenic is distributed evenly to all tissues, with little tendency to accumulate in organs although it has been shown to concentrate in nails and hair (Klaassen, 2001).

Inorganic As(III) is transported intracellularly via aquaporins 7 and 9 (Liu et al., 2004). Intracellulary, As(V) is reduced to As(III), which is subsequently methylated by an oxidative reaction to monomethylarsonic acid (MMA(V)). Subsequent reduction/oxidation and methylation reactions convert MMA(V) to monomethylarsonous acid (MMA(III)) and dimethylarsinic acid (DMA(V)) (Spiegelstein et al., 2003). Methylated As(III) may then flow out of the cell down its concentration gradient through aquaporin 9 (Liu et al., 2004), or may form glutathione conjugates which are pumped out of the cell by ATP-dependent transporters (Liu et al., 2001a). Urinary excretion of inorganic and methylated As(III) accounts for 75% of the absorbed As(III) dose. A small percentage of the absorbed dose is excreted in the feces, in sweat, and by desquamation of skin (Klaassen, 2001).

The toxicity of trivalent inorganic arsenicals has been known for thousands of years. Ingestion of 70-180 mg of inorganic As(III) results in an acute illness characterized by the presence of fever, anorexia, hepatomegaly, cardiac arrythmias and cardiovascular failure, peripheral neuropathy, various hematopoietic effects, and possibly death (Franzblau and Lilis, 1989; Klaassen, 2001). Chronic As(III) exposure has also been linked to the development of hematopoietic abnormalities, neurotoxicity, cirrhosis and hepatoportal sclerosis, endocrine disruption, cardiovascular disease, and immunotoxicity (Chen and Wang, 1990).

Not surprisingly, exposure to As(III) induces malignant transformation in multiple organ systems. As(III)-induced hyperpigmentation and hyperkeratosis may lead to Bowen's disease and basal cell and squamous cell carcinomas(Yu et al., 2006). As(III) exposure has also been linked to hepatocellulary carcinoma, angiosarcoma, bladder cancer, and epidermoid bronchogenic carcinoma (Chen and Wang, 1990; Marsh et al., 1998).

1.6.2. AS(III) TOXICITY AND OXIDATIVE STRESS

The exact cellular mechanisms of As(III)'s toxicity are not completely understood. It is known that As(III) induces deletion mutations, chromosomal aberrations, and DNA methylation, and alters signal transduction, cellular differentiation, and gene expression. All these mechanisms, however, are associated with the development of oxidative stress (Ercal et al., 2001).

As(III) has been shown to stimulate the production of superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) in various cellular systems including human vascular smooth muscle cells (Lynn et al., 2000), human keratinocyte cells (Pi et al., 2003), human–hamster hybrid cells (Liu et al., 2001a), and HEL30 cells (Corsini et al., 1999). Human studies confirm these findings with a reported increase in serum lipid peroxide levels in populations exposed to high As(III) concentrations in drinking water (Pi et al., 2003) and a decrease in plasma antioxidant capacity (Hsueh et al., 1998).

As(III) induces the production of ROS as a result of its intrinsic ability to accumulate in the mitochondria and alter cellular respiration. As(III) binds thiol groups of the dihydrolipoic acid cofactor of pyruvate dehydrogenase, inhibits succinic dehydrogenase in the electron transport chain, and competes with phosphate during oxidative phosphorylation (Klaassen, 2001; Patrick, 2003). The resultant inhibition of mitochondrial respiration stimulates ATPase, decreases

ATP production, and results in the production of ROS (Klaassen, 2001; Patrick, 2003).

The role of ROS in mediating the cellular mechanisms of As(III) toxicity has been extensively studied at the molecular and supramolecular levels. 8-Hydroxy-2'-deoxyguanosine, a major product of ROS-induced DNA base-modification, was significantly higher in arsenic-related skin neoplasms than in arsenic-unrelated Bowen's disease (Matsui et al., 1999). As(III)-induced oxidative stress is responsible for the apparent changes in cellular signaling pathways and aberrations in the cell cycle (Jung et al., 2003; Navarro et al., 2006). Moreover, superoxide dismutase, catalase, glutathione, and other antioxidants decrease $O_2^{\cdot -}$ production and preclude the development of chromosomal damage in fibroblasts. As(III) is also known to alter the function of various transcription factors, affect promoter function of various genes, and modify enzyme activities through oxidative mechanisms (Mei et al., 2002; Schuliga et al., 2002; Pi et al., 2003).

1.6.3. THE THERAPEUTIC BENEFITS OF AS(III)

Despite its toxicity, As(III) has been used as a therapeutic agent for more than 2400 years in Traditional Chinese Medicine and in ancient Greek and Roman times (Shen et al., 1997). Unexpectedly, As(III), as arsenic trioxide, is being used for the treatment of acute promyelocytic leukemia (APL) and multiple myeloma (MM) (Shen et al., 1997; Hussein, 2001; Murgo, 2001).

Evidence supporting the use of As(III) in APL is overwhelming. A daily dose of 10 mg (0.15 mg/kg) of arsenic trioxide was shown to produce a 72.7% clinical remission rate in newly diagnosed patients treated for 6-weeks, for up to 2 cycles (Niu et al., 1999). Furthermore, 85% of relapsed parients achieved complete remission (Niu et al., 1999; Soignet et al., 2001). The mechanism(s) by which As(III) exhibits its anti-cancer effects are still uncertain. One APL-specific mechanism involves degradation of the promyelocytic leukemia (PML)-retinoic

acid receptor α (RARα) fusion protein, a product of t(15:17) translocation, causing the induction of differentiation and apoptosis (Chen et al., 1997).

The anticancer effects of As(III) are not exclusive to APL. As(III) is also effective in the treatment of myelodysplastic syndromes (Vey et al., 2006) and primary liver and gallbladder cancers (Qian et al., 2001). Studies have also documented its effectiveness in inhibiting proliferation and inducing apoptosis of human colonic, breast, and pancreatic cancer cells (Li et al., 2002; Li et al., 2004). The mechanism of action of As(III) in cancers lacking the PML-RARα fusion protein may be multiple and various. Various reports have linked the generation of ROS to the efficacy of As(III) in cancer (Jing et al., 1999). The apoptotic effects of As(III) are related to its effect on mitochondrial function and the production of ROS and consequent release of cytochrome c and activation of caspase-9 and caspase-3 (Jing et al., 1999; Gazitt and Akay, 2005).

Paradoxically, however, ascorbic acid (AscA) enhances the anticancer effect of As(III) (Hussein, 2001). AscA enhanced the anti-lymphoma and antimyeloma effects of As(III) without additional toxicity to normal tissue (Bachleitner-Hofmann et al., 2002; Campbell et al., 2007). The anticancer effect of As(III) undeniably occurs by multiple mechanisms, and the mechanism by which AscA enhances these effects remains another question to be answered. These therapeutic benefits, however, highlight a gap in our knowledge on the pharmacology of heavy metals in the human body.

1.6.4. THE EFFECT OF AS(III) ON AHR ACTIVITY BIOMARKERS

Early reports have demonstrated that As(III) inhibited the β-naphthoflavone-mediated induction of CYP1A1-dependent 7-ethoxyresorufin-O-deethylation (EROD) activity in the liver and kidney, but not lung, of guinea pig (Falkner et al., 1993). In contrast, As(III) potentiated the β-naphthoflavone-mediated induction of CYP1A1 catalytic activity in the lungs while decreasing β-naphthoflavone-mediated induction of CYP1A1 activity in the kidneys and liver

(Falkner et al., 1993). In Wistar rats As(III) decreased total hepatic CYP content and monoxygenase activities of several CYPs including CYP1A1 (Siller et al., 1997). Similarly, studies on primary cultures of chick and rat hepatocytes showed that As(III) decreased total CYP and 3MC-mediated induction of CYP1A1 activity in chick hepatocytes, and CYP1A1 mRNA, protein and catalytic activity in rat hepatocytes (Jacobs et al., 1998; Jacobs et al., 1999). The effect of As(III) was also tested in primary human hepatocytes in which As(III) decreased PAH-mediated induction of CYP1A2 but not CYP1A1 at mRNA levels, while it decreased protein and catalytic activity levels of both isozymes in these cells (Vakharia et al., 2001a). In mouse Hepa 1c1c7 cells we have shown that As(III), in the presence of several AhR ligands, inhibited Cyp1a1 catalytic activity while potentiating its mRNA and protein levels (Elbekai and El-Kadi, 2004).

Although the effect of As(III) on CYP1A1 activity does not always parallel its effect on the expression on CYP1A1 mRNA which reflects AhR activity, almost all studies have reported a decrease in CYP1A1 catalytic activity in hepatic and extra-hepatic tissues and cells in response to As(III). Thus, multiple, but common, underlying pathways may be involved. As such, As(III)-dependent decrease in CYP1A1 catalytic activity was accompanied by either a decrease, increase, or no change in its mRNA levels. In addition to AhR-regulated CYPs, As(III) also decreases the catalytic activity levels of other CYPs that are not regulated by AhR (Albores et al., 1989). Thus, it is apparent that As(III) may have a direct effect on the function of the CYP protein, independent of its effect on transcriptional regulation. As such, it has been well documented that As(III) interacts with critical cysteine residues of many intercellular proteins, thus altering their functions (Del Razo et al., 2001). For example, As(III) has been shown to prevent the activation of NF-κB via interacting with cysteine 179 in the activation loop of the IκB kinase catalytic subunit, and subsequently inhibiting the dissociation of IκB from NF-κB which is a necessity for NF-κB activation (Kapahi et al., 2000). On the other hand, CYP activity critically depends on the binding of iron heme to sulfur atom of a conserved cysteine residue in the apoprotein (Gonzalez, 1988).

Therefore, As(III) might inhibit CYP1A1 catalytic activity by competing with heme for binding to the critical cysteine residue in the apoprotein (Vernhet et al., 2003). Another possibility is that metal-induced reactive oxygen species (ROS) may oxidize thiol groups in cysteine residues, directly or indirectly through the formation of reactive nitrogen species, of the CYP1A1 protein, causing loss of protein function (Bogdan, 2001). Furthermore, ROS may also interact with the heme Fe^{2+}, leading to heme destruction and enzyme inactivation (Lowe et al., 1998).

HO-1, an enzyme of 32 kDa, catalyzes the oxidative conversion of heme into biliverdin and subsequently bilirubin which serves an important role in protecting cells from oxidative damage caused by free radicals (Marilena, 1997). HO-1 regulation occurs through the redox sensitive Nrf2/ARE signaling pathway. Of interest, HO-1 anchors to the endoplasmic reticulum membrane via a stretch of hydrophobic residues at its C-terminus (Schuller et al., 1998). Thus, it is expected to interact with CYPs, which are also endoplasmic reticulum-bound enzymes.

As(III), via accumulating in the mitochondria and altering cellular respiration, has been shown to stimulate the production of superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2). Using Hepa 1c1c7 cells, we have shown that As(III) alone increased HO-1 mRNA that coincided with increased cellular glutathione levels, either to compensate the oxidative stress production by As(III) or as a direct response to oxidative stress (Elbekai and El-Kadi, 2005). Of interest, As(III) decreased the TCDD-mediated induction of CYP1A1 catalytic activity, and this inhibition was further potentiated when glutathione was depleted.

Almost all studies on the As(III)-mediated effect on CYP1A1 catalytic activity suspected a role of HO-1 in the degradation of its heme group, creating a hollow functionless protein (Anwar-Mohamed et al., 2009). HO-1 activity was elevated in primary cultures of chick hepatocytes treated with As(III) (Jacobs et al., 1999). We have previously demonstrated that HO-1 mRNA was elevated and total

cellular heme content was decreased in Hepa 1c1c7 cells treated with As(III). We therefore speculated that the induction of HO-1 may contribute to the inhibition of CYP1A1 activity by As(III), despite the presence of other interplaying mechanisms. In primary cultures of chick and rat hepatocytes the effect of As(III) on CYP1A1 catalytic activity was not reversed by mesoporphyrin, an inhibitor of HO-1 (Jacobs et al., 1999). Moreover, the addition of heme increased HO-1 activity to similar levels in the absence and presence of As(III), while the decrease in CYP1A1 activity was only observed in the presence of As(III) (Jacobs et al., 1999). Thus, it was concluded that the elevated levels of HO-1 alone may not be responsible for As(III)-mediated effects on CYP1A1 catalytic activity.

Recently, however, we have shown that As(III) decreased the TCDD-mediated induction of CYP1A1 catalytic activity that coincided with increased HO-1 mRNA and decreased total cellular heme content. Upon using tin mesoporphyrin (SnMP) as an HO-1 competitive inhibitor, supplementation with external heme, treatment with hemoglobin as carbon monoxide scavenger, or transfecting HepG2 cells with siRNA for HO-1 there was a partial restoration of the inhibition of TCDD-mediated induction of CYP1A1 catalytic activity (Anwar-Mohamed and El-Kadi, 2010). Thus, these results imply that HO-1 is partially involved in the As(III)-mediated down-regulation of CYP1A1 at the catalytic activity level (Anwar-Mohamed and El-Kadi, 2010).

In human breast cancer (T-47D) cells, B[*a*]P-mediated induction of CYP1A1 mRNA was not affected by As(III) treatment although B[a]P-mediated induction of CYP1A1 protein and catalytic activity levels were decreased upon exposing the cells to As(III) (Spink et al., 2002). This decrease in CYP1A1 activity coincided with an increase in HO-1 mRNA. In the absence of a decrease in CYP1A1 mRNA levels, As(III) may have decreased the CYP1A1 protein half-life through an increase in its protein degradation. In our studies however, we have shown that As(III) had no effect on CYP1A1 protein stability (Elbekai and El-Kadi, 2007).

The uncertainties in the mechanisms involved in the modulation of CYP1A1 activity and protein level by As(III) are also accompanied by many questions regarding the effect of this metal on mRNA levels. As(III) failed to attenuate the B[*a*]P-mediated induction of CYP1A1 mRNA, yet decreased its catalytic activity (Vakharia et al., 2001b; Wu et al., 2003). In agreement with these studies, pre-treatment of human lung adenocarcinoma cells (CL3) with As(III) did not affect the B[*a*]P-mediated induction of CYP1A1 mRNA (Ho and Lee, 2002). Further discrepancies arose with the emergence of data on the effect of As(III) on XRE-driven luciferase reporter gene in Hepa-1 cells. Maier et al have shown that despite having no effect on TCDD-induced CYP1A1 mRNA, As(III) decreased luciferase activity in cells transfected with the XRE-driven luciferase reporter gene (Maier et al., 2000). The discrepancy between the effect of As(III) on CYP1A1 mRNA and its effect on the XRE-driven luciferase reporter gene was not explained.

In human HepG2, it was previously demonstrated that As(III) alone did not affect CYP1A1 mRNA but reduced the benzo[*k*]fluoranthene-induced levels upon treatment with As(III) (Bessette et al., 2005). However, similar to earlier reports, As(III) alone decreased the XRE-driven luciferase reporter activity, and inhibition of TCDD-mediated induction of CYP1A1 mRNA and XRE-dependent luciferase activity was also observed in human hepatoma Huh7 cells (Chao et al., 2006). Furthermore, it was demonstrated that the actions of As(III) on blocking CYP1A1 induction by TCDD were primarily through altering CYP1A1 transcription, possibly through inhibiting the recruitment of polymerase II to the CYP1A1 promoter, which is independent of the regulatory mechanisms initiated by As(III)-induced cell arrest (Bonzo et al., 2005). Using HepG2 cells we have also shown that As(III) decreased the TCDD-mediated induction of CYP1A1 mRNA, protein, and catalytic activity levels in a dose-dependent manner. In addition, As(III) decreased the XRE-driven luciferase reporter activity (Anwar-Mohamed and El-Kadi, 2010).

Later studies contradicted previously reported data as As(III) was able to induce AhR nuclear translocation in Hepa-1 cells with the same efficiency as TCDD (Kann et al., 2005). The increase in nuclear accumulation of the AhR resulted in an increased expression of CYP1A1 mRNA when the cells were treated with As(III) alone, and potentiation of B[*a*]P-mediated induction of CYP1A1 mRNA (Kann et al., 2005). Thus, these results imply the presence of species-specific differences in the effect of As(III) on CYP1A1 mRNA expression.

We have previously shown that As(III) alone was able to increase CYP1A1 mRNA while potentiating the TCDD-, 3-MC-, β-NF-, and B[*a*]P-mediated induction of CYP1A1 mRNA in Hepa 1c1c7 cells (Elbekai and El-Kadi, 2004). Additionally, the increase in mRNA levels was translated to an increase in CYP1A1 protein levels.

Mechanistically, As(III) alone increased CYP1A1 mRNA in Hepa 1c1c7 cells in a time- and dose-dependent manner, and this inducibility was completely abolished after the addition of the RNA polymerase inhibitor actinomycin-D, implying a requirement of *de novo* RNA synthesis (Elbekai and El-Kadi, 2008). As(III) was able also to increase the XRE-dependent luciferase reporter activity despite not increasing the AhR nuclear accumulation.

To confirm the role of the AhR in the regulation of CYP1A1 by As(III), cycloheximide, a protein synthesis inhibitor that inhibits a labile protein required for the proteolysis of the AhR (Joiakim et al., 2004), caused super-induction of the CYP1A1 mRNA (Ma et al., 2000). Similarly, [Z-Leu-Leu-Leu-CHO] (MG-132), a 26-proteasome inhibitor that stabilizes the AhR protein, caused further induction of the CYP1A1 mRNA in response to As(III) treatment. Besides its transcriptional effect, As(III) also increased the stability of CYP1A1 mRNA transcripts induced by TCDD. Thus, we concluded that As(III) increases CYP1A1 transcriptionally, through an AhR-dependent mechanism, and stabilizes CYP1A1 mRNA transcripts.

The mechanism(s) by which As(III) induces CYP1A1 mRNA transcription through the AhR are still at large. However, heavy metals may alter some cellular metabolic pathways, leading to the enhanced production of endogenous AhR ligands such as biliverdin and bilirubin (Denison and Nagy, 2003). However, despite the fact that elevated pulmonary CYP1A1 expression was associated with an increase in total plasma bilirubin concentrations, the administration of bilirubin to the lung through intra-tracheal injection did not cause any increase in CYP1A1 mRNA (Seubert et al., 2002a; Seubert et al., 2002b). Moreover, As(III)–induced oxidative stress may enhance the release of arachidonic acid from glycerolphospholipids, which has been also shown to to be an endogenous AhR ligand (Schaldach et al., 1999; Denison and Nagy, 2003). As(III) might also bind to vicinal thiols in Hsp90, disrupting the interaction between Hsp90 and AhR, causing the NLS to be exposed and subsequently causing AhR nuclear accumulation (Kann et al., 2005).

Another postulated mechanism for the As(III) mediated induction of CYP1A1 implicates post-transcriptional modifications of histones. As(III) induces phosphorylation (Li et al., 2001; He et al., 2003) and acetylation (Li et al., 2001) of histone H3 through ERK, p38, and the Akt1 pathways. In addition, histone deacetylation has been shown to be involved in the inducibility of CYP1A1 mRNA transcription in human and mouse hepatoma cells (Shibazaki et al., 2004) and HeLa cells (Nakajima et al., 2003). Thus, As(III) might induce CYP1A1 through modifying the phosphorylation and acetylation of these histones.

Although many possible mechanisms might be involved in the As(III)-mediated induction of CYP1A1 mRNA in mouse hepatoma cells, much less information is available to explain the controversial effects of this metal on CYP1A1 expression in other species (Fig. 1.4.). The summarized effects of As(III) on AhR-activity biomarkers are shown in Table 1.

Fig. 1.4 Postulated mechanisms for the effect of As(III) on AhR activity biomarker

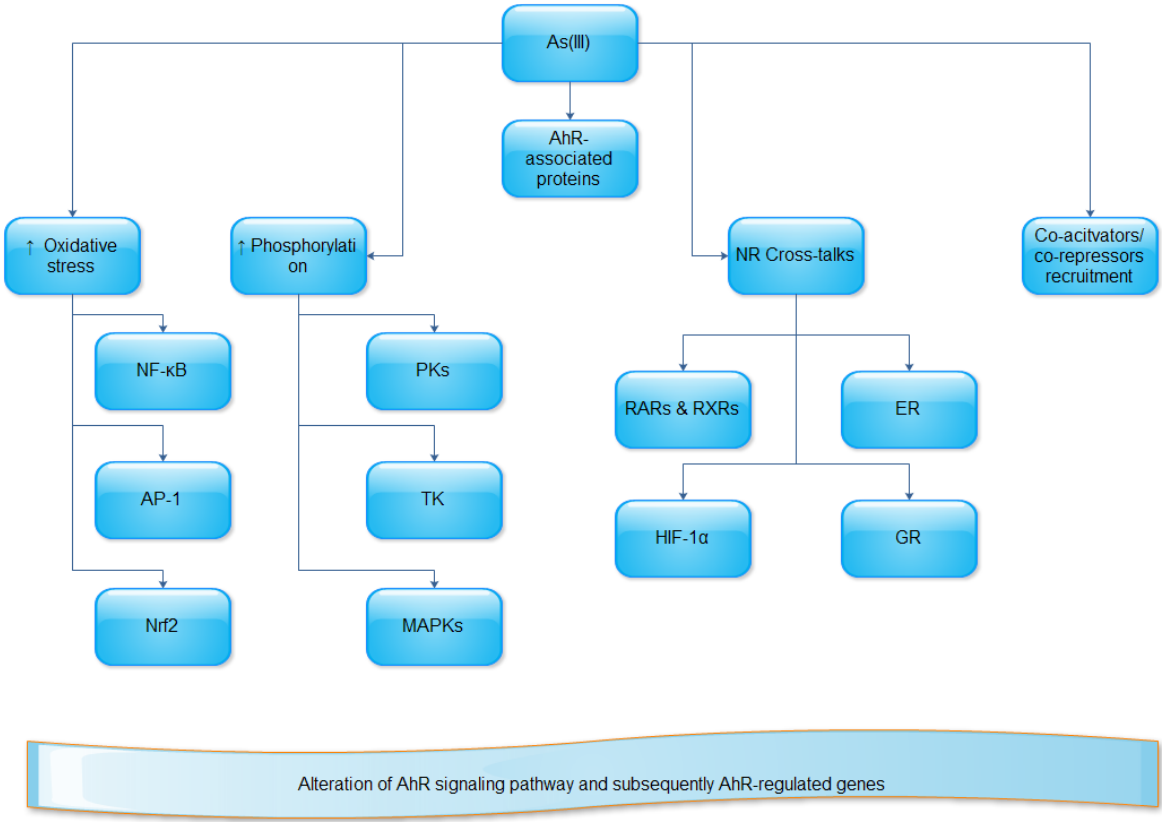

Table 1.1: The effect of As(III) on AhR-activity biomarkers

	Species (Tissue/Cell line)	Changes	Reference
In vivo	Rats (liver, lung)	↓ total hepatic CYP and CYP1A1 activities ↓ hepatic CYP1A1 activity ↑ pulmonary CYP1A1 mRNA, protein, and activity	(Siller et al., 1997; Ke et al., 2002)
	Guinea pig (kidneys, liver, lung)	↓ CYP1A1 EROD activity ↓ β-NF-mediated induction of CYP1A1 activity in kidneys, liver ↑ β-NF-mediated induction of CYP1A1 activity in the lung	(Falkner et al., 1993)
In vitro	Human (hepatocytes)	↓ PAH-mediated induction of CYP1A1 protein, and activity	(Vakharia et al., 2001a)
	Human adenocarcinoma (T-47D cells)	↔ B[*a*]P-mediated induction of CYP1A1 mRNA ↓ B[*a*]P-mediated induction of CYP1A1 protein and activity.	(Vakharia et al., 2001b; Spink et al., 2002; Wu et al., 2003)
	Human non-small-cell lung carcinoma (Cl3 cells)	↔ B[*a*]P-mediated induction of CYP1A1 mRNA	(Ho and Lee, 2002)
	Human hepatoma (HepG2 cells)	↔ CYP1A1 mRNA ↓ BKF-mediated induction of CYP1A1 mRNA ↓ TCDD-induced XRE-dependent luciferase activity ↔ CYP1A1 mRNA half-life	(Bessette et al., 2005; Bonzo et al., 2005)
	Human hepatoma (Huh7 cells)	↓ TCDD-mediated induction of CYP1A1 mRNA ↓ XRE-dependent luciferase activity	(Chao et al., 2006)
	Rat (hepatocytes)	↓ 3-MC-mediated induction of CYP1A1 mRNA, protein, and catalytic activity	(Jacobs et al., 1999)
	Mouse hepatoma (Hepa-1 cells)	↔ B[*a*]P-mediated induction of CYP1A1 activity ↔ TCDD-mediated induction of CYP1A1 mRNA ↓ TCDD-induced XRE-dependent luciferase activity	(Maier et al., 2002; Kann et al., 2005)
	Mouse hepatoma (Hepa 1c1c7 cells)	↑ AhR translocation ↑ CYP1A1 mRNA ↑↑ AhR ligand-mediated induction of CYP1A1 mRNA, protein ↓ AhR ligand-mediated induction of CYP1A1 activity ↑ XRE-dependent luciferase activity ↑ CYP1A1 mRNA half-life ↔ CYP1A1 protein stability	(Elbekai and El-Kadi, 2004; Bonzo et al., 2005; Elbekai and El-Kadi, 2005; Kann et al., 2005; Elbekai and El-Kadi, 2008)
	Chick (hepatocytes)	↓ total CYP ↓ 3-MC-mediated induction of CYP1A1 activity	(Jacobs et al., 1998)

1.7. RATIONALE, HYPOTHESES, AND OBJECTIVES

1.7.1. RATIONALE

HAHs and PAHs are widespread environmental contaminants that produce a range of toxic effects. Most of these toxic effects are mediated by the AhR, a cytosolic receptor to which these contaminants bind. By binding to the AhR, these AhR ligands induce the transcription of CYP enzymes responsible for their metabolism into toxic intermediates. The induction of phase II drug metabolizing enzymes is counterproductive to this process and as such, the toxicity of AhR ligands is regulated by a coordinated balance between the phase I and phase II drug metabolizing enzymes. Although numerous studies have examined the toxic effects of individual AhR ligands, there are relatively few reports of the combined toxic effects of AhR ligands and other environmental contaminants. In this aspect, aromatic hydrocarbon and heavy metal co-exposure generates different biological responses than is expected based on the toxicological mechanisms of each class evaluated separately. Thus, any influence of metals on the capacity of AhR ligands to induce AhR-regulated genes will influence the carcinogenicity and mutagenicity of the AhR ligands. Therefore, there is a need to understand the mechanisms driving these responses in order to dissect the exact role of AhR ligands and metals in carcinogenicity and mutagenicity.

1.7.2. HYPOTHESES

Hypothesis 1: co-exposure to As(III) and TCDD disrupts the coordinated balance of AhR-regulated genes *in vitro* and *in vivo*.

Hypothesis 2: As(III) modulation of CYPs is not limited to AhR-regulated CYPs.

Hypothesis 3: co-exposure to As(III) and TCDD *in vivo* alters AhR-regulated genes in a time-, tissue-, and AhR-regulated gene-dependent manner.

Hypothesis 4: pentavalent metabolites of As(III) differentially modulate the prototypical phase I and II AhR-regulated genes *CYP1A1* and *NQO1* through affecting their upstream signaling pathways.

1.7.3. SPECIFIC OBJECTIVES

1- To determine the possible effects of As(III) on the TCDD-mediated induction of CYP1A1 in human hepatoma HepG2 cells, and to investigate the underlying molecular mechanisms involved in this alteration.

2- To examine the potential effects of As(III) on other non-AhR-regulated CYPs using rat primary hepatocytes, and to investigate the underlying molecular mechanisms involved in these effects.

3- To examine the *in vitro-in vivo* correlations using mice liver and isolated mouse primary hepatocytes and to determine the effects of As(III) on AhR- regulated genes in extrahepatic tissues: kidney, lung, and heart.

4- To determine the effect of As(III) pentavalent metabolites on CYP1A1 and NQO1 in human hepatoma HepG2 cells, and to investigate the underlying molecular mechanisms involved in this alteration.

1.7.4. SIGNIFICANCE

Establishing the interaction between heavy metals and AhR ligands is the first step to determine the ability of heavy metals to influence the carcinogenicity and mutagenicity of AhR ligands. Identifying the mechanisms involved in the modulation of AhR-regulated genes will aid in the development of preventative strategies and new treatment modalities for AhR-ligand-mediated toxicities.

CHAPTER 2

2. MATERIALS AND METHODS

Versions of this chapter have been previously published.

2.1. CHEMICALS

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 7-ethoxyresorufin, 7-methoxyresorufin, fluorescamine, anti-goat IgG peroxidase secondary antibody, hemin, protease inhibitor cocktail, 2,6-dichlorophenolindophenol (DCPIP), dicoumarol, isothiocyanate sulforaphane (SUL), protease inhibitor cocktail, sodium arsenite ($NaAsO_2$), β-glucuronidase, arylsulfatase, chlorpromazine HCl, collagenase, collagen from rat tail, hemoglobin (Hb), and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Sigma Chemical Co. (St. Louis, MO). Tin mesoporphyrin (SnMP) and cobalt protoporphyrin (CoPP) were purchased from Frontier Scientific Inc. (Logan, UT). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). TRIzol reagent and Lipofectamine 2000 reagents were purchased from Invitrogen (Carlsbad, CA). High-Capacity cDNA Reverse Transcription Kit, SYBR® Green PCR Master Mix, human Hmox1 (HO-1) validated siRNA, Silencer® Select Negative Control #2 siRNA, and 96-well optical reaction plates with optical adhesive films were 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). AhR goat polyclonal primary antibody, Arnt goat polyclonal antibody, HSP90 mouse monoclonal primary antibody, XAP2 goat polyclonal primary antibody, CYP1A1/1A2 mouse polyclonal primary antibody, CYP3A23/3A2 mouse monoclonal primary antibody, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HSP90 mouse monoclonal primary antibody, XAP2 goat polyclonal primary antibody, and actin rabbit polyclonal primary antibodies, and anti-goat and anti-rabbit IgG peroxidase secondary antibodies, were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Cyp1b1 polyclonal primary antibody was purchased from BD Biosciences (Mississauga, ON). NQO1 rabbit polyclonal

primary antibody and HO-1 mouse monoclonal primary antibody were purchased from Abcam (Cambridge, MA). Anti-mouse IgG peroxidase secondary antibody was purchased from R&D Systems, Inc. (Minneapolis, MN). Nrf2 mouse polyclonal primary antibody and anti-mouse IgG peroxidase secondary antibody were purchased from R&D Systems (Minneapolis, MN). PXR rabbit polyclonal antibody was purchased from Novus Biologicals (Littleton, CO). Alexa Fluor 488 donkey anti-goat secondary antibody and Prolong Gold mounting media were purchased from Invitrogen (Carlsbad, CA). [γ32P]ATP was purchased from PerkinElmer Canada Inc. (Dorval, QC). Human AhR and Nrf2 validated siRNA and primers were purchased from Integrated DNA Technologies (Coralville, IA). pRL-CMV plasmid and dual luciferase assay reagents were obtained from Promega (Madison, WI). Cover glass slips 22 mm diameter (Cat. Number 72224-01) were purchased from Electron Microscopy Sciences (Hatfield, PA). [3-[(3,4-Difluorobenzyl)oxy]-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl] furan-2(5H)-one] (DFB), and [3-hydroxy-5,5-dimethyl-4-[4-(methylsulfonyl) phenyl]furan-2(5H)-one] (DFH) were generously provided by Merck Frosst Canada (Dorval, QC). Arsenic pentavalent metabolites [monomethylarsonic acid (MMA(V)), dimethylarsinic acid (DMA(V)), and trimethylarsine oxide (TMA(V))], human XRE driven luciferase reporter plasmid, and human ARE driven luciferase reporter plasmid were generously provided by Dr. X. Chris Le (University of Alberta), Dr. M. S. Denison (University of California, Davies), and Dr. S. Itoh (University of Toronto), respectively. All other chemicals and plastic wares were purchased from Fisher Scientific (Toronto, ON).

2.2. METHODS

2.2.1. CELL MODEL

To test the hypotheses raised in this proposal, human hepatoma HepG2 cells and isolated rat and mouse primary hepatocytes were utilized. The readily available human hepatoma cell line HepG2 was used in the current study for the following reasons: first, these cells have proven to be a useful model for investigations of the regulation of human CYP1A1 (Lipp et al., 1992; Kikuchi et al., 1996;

Krusekopf et al., 1997; Vakharia et al., 2001b; Kim et al., 2006); second, human hepatocytes have been shown to be one of major targets for heavy metals upon exposure (Ercal et al., 2001). Moreover, HepG2 cells are one of the most widely used human hepatoma cells and are considered a potential useful model for several toxicological studies (Dehn et al., 2004). HepG2 cells contain both Phase I (such as CYP monooxygenase enzymes, and a functional AhR) and Phase II metabolizing enzymes (such as glucuronic- and sulfate-conjugation enzymes) (Grant et al., 1988; Rodriguez-Antona et al., 2002; Westerink and Schoonen, 2007; Dvorak et al., 2008). Therefore, HepG2 cells are capable of performing the metabolic and biotransformation reactions required for detoxification of several xenobiotics (Dehn et al., 2004). Most importantly, HepG2 cells' response to dioxin is similar to that observed in primary human hepatocytes (Silkworth et al., 2005).

2.2.2. CELL CULTURE

Human hepatoma HepG2 cell line (American Type Culture Collection (ATCC) number HB-8065), was purchased from ATCC (Manassas, VA). Cells were maintained in DMEM with phenol red, supplemented with 10% heat-inactivated fetal bovine serum, 20 μM L-glutamine, 50 μg/ml amikacin, 100 IU/ml penicillin, 10 μg/ml streptomycin, 25 ng/ml amphotericin B, 0.1 mM non-essential amino acids (Invitrogen, Cat. 11140-050), and vitamin supplement solution (Invitrogen, Cat. 11120-052). Cells were grown in 75-cm^2 cell culture flasks at 37 °C in a 5% CO_2 humidified incubator.

Primary hepatocytes were isolated from male Sprague-Dawley rats or male C57Bl/6J mice and plated onto different well size (12-, 24- and 96-wells) cell culture plates. Each well of the plastic culture plates (12, 24 and 96-well, Falcon, Becton Dickinson Labware, New Jersey, U.S.A.) was coated with 1000, 500 and 125μl, respectively of Type I rat-tail collagen (50 μg/ml in 0.02 N acetic acid) overnight, 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, 50ng/ml dexamethasone phosphate, 100 IU/ml penicillin G, 10 μg/ml streptomycin, and 25ng/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%.

2.2.3. ANIMAL MODEL

Male Sprague-Dawley rats (300-350 g) and male C57BL/6J (22–30 g) mice were obtained from Charles River, Canada (Montreal, QC, Canada). Animals were group-housed under standard conditions, two rats per cage or three to five mice per cage with food and water available *ad libitum* and were maintained on a 12-h light/dark cycle. Rats and mice were treated in compliance with University of Alberta Health Sciences Animal Policy and Welfare Committee guidelines.

2.2.4. ISOLATION OF PRIMARY HEPATOCYTE

Three solutions were utilized for the isolation of rat or mouse hepatocytes as previously described (El-Kadi et al., 1997). Solution A contained (mM): NaCl 115, KCl 5, KH_2PO_4 1, HEPES 25, EGTA 0.5, 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 1 N HCl and were filtered through a 22μm membrane before use.

Rat or mouse hepatocytes were isolated by a two-step collagenase perfusion method as described previously (Seglen, 1976). Animals were anaesthetized, a midline laparotomy was performed and the portal, suprahepatic and inferior cava veins were cannulated. All tubing and solutions were maintained at 37°C and saturated with 95% O_2: 5% CO_2. For rat hepatocyte isolation, the liver was perfused via the portal vein with 400 ml of solution A, by use of a peristaltic

pump at a flow rate of 35 ml/min for 10 min, then with 300 ml of solution B at a flow rate of 30 ml/min for 10 min, until the liver appeared completely blanched and softened. For mouse hepatocyte isolation, 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, 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 filtering 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 80 μm cell strainer for rat, and 70 μm cell strainer for mouse and divided into two aliquots which were placed on ice. Once the temperature of 4°C was reached, the cells were centrifuged at 130 × *g* for 5 min for rat, and 100 × *g* for 2 min for mouse, the supernatant was aspirated and the sediment was resuspended in DMEM and re-centrifuged at 130 × *g* for 5 min for rat, and 100 × *g* for 2 min for mouse, an operation that was repeated twice. For rat hepatocytes only, the sediment was centrifuged on a 40% Percoll gradient at 2500 × g for 15 min. The supernatant was discarded and the sediment was resuspended in DMEM to obtain 0.5×10^6 cells ml^{-1}.

2.2.5. CHEMICAL TREATMENTS

Human hepatoma HepG2 cells, isolated rat hepatocytes, and isolated mouse hepatocytes were treated in serum-free medium with various concentrations of As(III) (1-10 μM) or MMA(V), DMA(V), or TMA(V) (5 μM) in the absence and presence of 1 nM TCDD, or 5 μM SUL and/or 5 μM SnMP and 80 μM hemin and/or 0.5-2 μM Hb and/or 25 μM Rif, or 1 μM CoPP. TCDD, SUL, CoPP, and SnMP were dissolved in dimethylsulfoxide (DMSO) and maintained in DMSO at −20 °C until use. As(III), MMA(V), DMA(V), TMA(V), and hemin (10 mM

stocks) were prepared freshly in double de-ionized water. Rif was dissolved in ethanol and maintained in ethanol 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 0.05% (v/v).

2.2.6. ANIMAL TREATMENT

Animals were injected intraperitoneally (i.p.) 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 i.p. The mice were divided into 4 groups. The first group (n=12) control mice received saline (0.4 mL) plus corn oil (0.4 mL). The second group (n=12) 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). Thereafter, the animals were euthanized after a single injection at 6 h (n=6) and 24 h (n=6) via cervical dislocation. Liver, heart, lung, and kidney tissues were excised, immediately frozen in liquid nitrogen, and stored at -80°C until further analysis. All experimental procedures involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee.

2.2.7. MEASUREMENT OF CELL VIABILITY

The effect of As(III), MMA(V), DMA(V), and TMA(V) on cell viability was determined using the MTT assay as described previously (Anwar-Mohamed and El-Kadi, 2009b). The MTT assay measures the conversion of MTT to formazan in living cells via mitochondrial enzymes of viable cells. In brief, HepG2 cells and isolated 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), MMA(V), DMA(V), and TMA(V) in the absence and presence of 1 nM TCDD or 25 µM Rif. 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).

2.2.8. RNA EXTRACTION AND CDNA SYNTHESIS

Total RNA from the frozen tissues or treated cells for different time points was isolated using TRIzol reagent, according to manufacturer's instructions (Invitrogen). Briefly, TRIzol reagent (600 µL) was added to each well of the six-well cell culture plates or 0.5 g tissue. Thereafter, lysate was collected into a 1.5-mL microcentrifuge tube and mixed with chloroform (120 µL), followed by centrifugation at 12,000 x *g* for 15 min at 4°C. The aqueous upper layer that contains RNA was isolated, transferred to a clean microcentrifuge tube, and isopropyl alcohol (300 µL) was added to each tube and the tubes were stored at -20°C overnight to precipitate the RNA. The RNA was collected by centrifugation at 12,000 x *g* for 10 min at 4°C and the pellet was washed by 75% ethanol in diethyl pyrocarbonate (DEPC)-treated water. The tubes were lightly vortexed to ensure the complete washing of the RNA pellet. The tubes were centrifuged for the last time at 12,000 x g for 5 min at 4°C to let the pellet settle down. The solution was aspirated out and the pellet was dried, then dissolved in DEPC-treated water. The tubes were placed in a water bath at 65°C for 10-12 min. Total RNA was quantified by measuring the absorbance at 260 nm. RNA quality was determined by measuring the 260/280 ratio. For reverse transcription-polymerase chain reaction (RT-PCR), first-strand cDNA was performed by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Total RNA (1.5 µg) from each sample was added to a mix of 2.0 µL of 10x reverse transcriptase buffer, 0.8 µL of 25x dNTP mix (100 mM), 2.0 µL of 10x 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 15 s, and finally cooled to 4°C.

2.2.9. QUANTIFICATION BY REAL-TIME POLYMERASE CHAIN REACTION (REAL-TIME PCR)

Quantitative analysis of specific mRNA expression was performed using real-time PCR by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems). The 25-µl reaction mix contained 0.1 µl of 10 µM forward primer and 0.1 µl of 10 µM reverse primer (40 nM final concentration of each primer), 12.5 µl of SYBR Green Universal Mastermix, 11.05 µl of nuclease-free water, and 1.25 µl of cDNA sample. Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. After sealing the plate with an optical adhesive cover, the thermocycling conditions were initiated at 95°C for 10 min, followed by 40 PCR cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The primers used in the current study were chosen from previously published studies and are listed in Table 2.1. A melting curve (dissociation stage) was prepared at the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product.

2.2.10. REAL-TIME PCR DATA ANALYSIS

The real time-PCR data were analyzed using the relative gene expression, i.e. ($\Delta\Delta C_T$), method as described in Applied Biosystems User Bulletin No.2 and explained further by Livak and Schmittgen (Livak and Schmittgen, 2001). Briefly, the ΔC_T values were calculated in every sample for each gene of interest as follows: $C_{T\ \text{gene of interest}} - C_{T\ \text{reporter gene}}$, with β-actin as the reporter gene. Calculation of relative changes in the expression level of one specific gene ($\Delta\Delta C_T$) was performed by subtraction of the average ΔC_T value of untreated control from each ΔC_T value from the corresponding treatment groups. Thereafter these values were put in to the equation of $2^{-\Delta\ (\Delta CT)}$. The resulting values were averaged and S.E. was calculated as follows: $2^{-\Delta\ (\Delta CT)}$ with $\Delta(\Delta C_T)$ + S.E. and $\Delta(\Delta C_T)$ – S.E., where S.E. is the standard error of the mean of the $\Delta\ (\Delta C_T)$ value.

Table 2.1: Primers and siRNA sequences

Gene	Forward primer	Reverse primer
Human *AhR*	5′-CCCTTGGAAATTCATTGCCA-3′	5′-GGAGAGGTGCTTCATATGTCGTC-3′
Human *β-actin*	5′-CTGGCACCCAGGACAATG-3′	5′-GCCGATCCACACGGAGTA-3′
Human *CYP1A1*	5′-CTATCTGGGCTGTGGGCAA-3′	5′-CTGGCTCAAGCACAACTTGG-3′
Human *HO-1*	5′-ATGGCCTCCCTGTACCACATC-3′	5′-TGTTGCGCTCAATCTCCTCCT-3′
Human *NQO1*	5′-CGCAGACCTTGTGATATTCCAG-3′	5′-CGTTTCTTCCATCCTTCCAGG-3′
Human *Nrf2*	5′-AACCACCCTGAAAGCACAGC-3′	5′-TGAAATGCCGGAGTCAGAATC-3′
Rat *β-actin*	5′-CCAGATCATGTTTGAGACCTTCAA-3′	5′-GTGGTACGACCAGAGGCATACA-3′
Rat *CYP1A1*	5′-CCAAACGAGTTCCGGCCT-3′	5′-TGCCCAAACCAAAGAGAATGA-3′
Rat *CYP1A2*	5′-CGCCCAGAGCGGTTTCTTA-3′	5′-TCCCAAGCCGAAGAGCATC-3′
Rat *CYP3A23*	5′-ATGTTCCCTGTCATCGAACAGTATG-3′	5′-TTCACAGGGACAGGTTTGCCT-3′
Rat *CYP3A2*	5′-GCTCTTGATGCATGGTTAAAGATTTG-3′	5′-ATCACAGACCTTGCCAACTCCTT-3′
Rat *HO-1*	5′-AGAGTTTCCGCCTCCAACCA-3′	5′-CGGGACTGGGCTAGTTCAGG-3′
Murine *β-actin*	5′-TATTGGCAACGAGCGGTTCC-3′	5′-GGCATAGAGGTCTTTACGGATGTC-3′

Murine *Cyp1a2*	5′-TGGAGCTGGCTTTGACACAG-3′	5′-CGTTAGGCCATGTCACAAGTAGC-3′
Murine *Cyp1b1*	5′-AATGAGGAGTTCGGGCGCACA-3′	5′-GGCGTGTGGAATGGTGACAGG-3′
Murine *Gsta1*	5′-CCCCTTTCCCTCTGCTGAAG-3′	5′-TGCAGCTTCACTGAATCTTGAAAG-3′
Murine *HO-1*	5′-GTGATGGAGCGTCCACAGC-3′	5′-TGGTGGCCTCCTTCAAGG-3′
Murine *Nqo1*	5′-GGAAGCTGCAGACCTGGTGA-3′	5′-CCTTTCAGAATGGCTGGCA-3′
h-AhR siRNA	5′-UACUUCCACCUCAGUUGGCdTdT-3′	5′-GCCAACUGAGGUGGAAGUAdTdT-3′
h-HO-1 siRNA	5′-CAAUGCAGUAUUUUUGUdTdT-3′	5′-AACAAAAAUACUGCAUUUGdAdG-3′
h-Nrf2 siRNA	5′-GUAAGAAGCCAGAUGUUAAdUdU-3′	3′-dUdUCAUUCUUCGGUCUACAATT-5′

2.2.11. PREPARATION OF CELL HOMOGENATE

Twenty-four hours after incubating the cells with treatments in six-well cell culture plates, cultured cells were washed with phosphate buffered saline (PBS) and then 0.5 ml of homogenization buffer (50 mM potassium phosphate, pH 7.4, and 1.15% KCl) was added to each well. The plates were then incubated for 24 h in a -80 °C freezer. Thawed cells were extracted and homogenized using a Kontes homogenizer at 4 °C before they were centrifuged at 10,000 × g for 20 min. The supernatant fractions were collected for determination of protein concentration using bovine serum albumin as a standard by the Lowry method (Lowry et al., 1951). The extracted cytosolic fractions were stored in a -80 °C freezer for later use in the determination of the Nqo1 and Gsta1 enzyme activities.

2.2.12. PREPARATION OF MICROSOMAL AND CYTOSOLIC PROTEIN FRACTIONS

Liver, heart, lung and kidney microsomes were prepared by differential centrifugation of homogenized tissues as previously described (Lowry et al., 1951; Lin et al., 1991; Barakat et al., 2001). Briefly, individual liver, heart, lung and kidney tissues were rapidly removed and washed in ice-cold KCl [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 homogenizing, the tissues were separated by differential ultracentrifugation at 4 ^{o}C. The first centrifugation is at around 200 × g to remove cellular debris, followed by ultracentrifugation at 9,000 × g for 10 min, the resultant S9 fraction is further subjected to ultracentrifugation at 32,000 × g for 15 min. The resulting pellet is the microsomes and the supernatant is the cytosol. After removing cytosol, the microsomal pellet is subjected to cleaning with ice cold KCl and centrifuged at the same speed of 32,000 × g for 15 min, this step was repeated twice. The final pellet was reconstituted in 0.25 M cold sucrose and the supernatant cytosols were stored at -80^{o}C. Thereafter, microsomal and cytosolic

protein concentrations were determined by the Lowry method using bovine serum albumin as a standard (Lowry et al., 1951).

2.2.13. PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

Twenty-four hours after incubation with the test compounds, cells were collected in lysis buffer containing 50 mM HEPES, 0.5 M sodium chloride, 1.5 mM $MgCl_2.6H_2O$, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 µl/ml of protease inhibitor cocktail. The cell homogenates were obtained by incubating the cell lysates on ice for 1 h, with intermittent vortexing every 10 min, followed by centrifugation at 12,000 × *g* for 10 min at 4 °C. Briefly 50 or 5 µg of cell homogenates for CYP1A1 and NQO1 analyses, respectively, and 20 µg liver, kidney lung microsomal or cytosolic proteins were resolved by denaturing gel electrophoresis, as described previously (Elbekai and El-Kadi, 2004). The samples were dissolved in 2X 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 KCl, 25 mM Tris–base). After blocking, the blots were incubated with the following antibodies: primary polyclonal mouse CYP1A1/1A2, primary polyclone rabbit NQO1, primary polyclonal rabbit Cyp1b1, primary polyclonal goat Nqo1, primary polyclonal goat Gsta1, primary polyclonal rabbit HO-1, primary polyclonal mouse Nrf2, primary polyclonal mouse HSP90, primary polyclonal rabbit actin, primary polyclonal goat XAP2, or primary polyclonal goat Gapdh for 2 h at room temperature, or primary monoclonal mouse CYP3A23/3A2 antibody overnight at 4 °C, or primary polyclonal goat AhR antibody or primary polyclonal rabbit PXR antibody overnight at 4 °C. Incubation with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody for NQO1, Cyp1b1, HO-1, PXR and actin or goat anti-mouse IgG secondary antibody for Cyp1a1/1a2, and CYP3A23/3A2, Nrf2, HSP90, or rabbit anti-goat IgG secondary antibody for AhR, Nqo1, Gsta1, XAP2, and 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, Piscataway, NJ). The intensity of the protein bands was quantified, relative to the signals obtained for actin, using ImageJ software [National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij.].

2.2.14. DETERMINATION OF CYP1A1 AND CYP1A2 ENZYME ACTIVITIES IN CELLS

CYP1A1-dependent 7-ethoxyresorufin O-deethylase (EROD), and CYP1A2-dependent 7-methoxyresorufin O-deethylase (MROD) activities were measured in intact, monolayer living cells using 7-ethoxyresorufin, and 7-methoxyresorufin as substrates, respectively, as previously described [15, 16]. Enzymatic activity was normalized for cellular protein content, which was determined using a modified fluorescent assay [17].

2.2.15. DETERMINATION OF CYP1A1 ACTIVITY USING TOTAL CELL LYSATE

CYP1A1 activity was determined according to the method described by Kennedy et al. [16]. Sub-confluent HepG2 cells (100-mm plates) were treated with As(III) (5 μM) in the absence and presence of 1 nM TCDD for 24 h. Thereafter, cells were harvested, washed in PBS, and recovered by scraping in 500 μl of 25 mM HEPES, 1.5 mM EDTA, and 10% (v/v) glycerol, pH 7.5. Cells were lysed by freeze-thawing at –80°C, and 50 μl of lysate (1–2 mg/ml of protein) was mixed with 20 μM 7-ethoxyresorufin (25 μl) in 100 mM sodium phosphate, pH 7.8, and incubated at 37°C for 15 min in 96-well plates. Reactions were started by the addition of 25 μl of 4 mM NADPH and stopped after 5 min with 150 μl of acetonitrile (resorufin production was linear with respect to time over this period). Bovine serum albumin was substituted for cell lysates in blank reactions. Enzymatic activity was normalized for cellular protein content, which was determined using a modified fluorescent assay [15].

2.2.16. DETERMINATION OF CYP3A ENZYMATIC ACTIVITY

CYP3A-dependent DFB debenzylation activity was measured in intact, monolayer living cells using DFB as a substrate, as previously described (Nicoll-Griffith et al., 2004). In brief, rat hepatocytes were incubated with As(III) (1, 5, and 10 μM) in the absence and presence of Rif (25 μM) for 48 h in 24-well plates. Thereafter, cells were washed with Krebs-Henseleit buffer containing 12.5 mM HEPES, pH 7.4, and DFB was incubated with the cells in Krebs-Henseleit at a final concentration of 60 μM. After 20 min incubation at 37 °C, aliquots (75 μl) were removed and transferred to a 96-well plate. An equal amount of 60% acetonitrile in TRIZMA base buffer (0.05 M, pH 10) was added. DFH was quantified using the Eclipse fluorescence spectrophotometer (Varian Australia PTY LTD., Australia) using an excitation wavelength of 360 nm and an emission wavelength of 440 nm. Enzymatic activity was normalized for cellular protein content, which was determined using a modified fluorescent assay (Lorenzen and Kennedy, 1993).

2.2.17. MICROSOMAL INCUBATION AND MEASURING EROD AND MROD CATALYTIC ACTIVITIES

Microsomes from different tissues and different treatments (1 mg protein/mL) were incubated in the incubation buffer (5 mM $MgCl_2.6H_2O$ dissolved in 0.5 M potassium phosphate buffer pH=7.4) at 37ºC in a shaking water bath (50 rpm). A pre-equilibration period of 5 min was performed. The reaction was initiated by the addition of 1 mM NADPH. The concentrations of substrate were 2 μM for 7-ethoxyresorufin (EROD) and 7-methoxyresorufin (MROD). After incubation at 37°C (5 min for EROD, and 10 min for MROD assays), the reaction was stopped by adding 0.5 mL of cold methanol. The amount of resorufin formed in the resulting supernatant was measured using the Baxter 96-well fluorescence plate reader using excitation and emission wavelengths of 545 and 575 nm, respectively. Formation of resorufin was linear with incubation time and protein amount. Enzymatic activities were expressed as picomoles of resorufin formed per minute and per milligram of microsomal protein.

2.2.18. DETERMINATION OF NQO1 ENZYMATIC ACTIVITY

The Nqo1 activity was determined by a continuous spectrophotometric assay to quantitate the reduction of its substrate, 2,6-dichlorophenolindophenol (DCPIP) as described previously (Preusch et al., 1991; Korashy and El-Kadi, 2006b). Briefly, 0.02 mg of cell homogenate protein and 20 µg of cytosolic protein were incubated with 1 mL of the assay buffer [40 µM DCPIP, 0.2 mM NADPH, 25 mM Tris-HCl, pH 7.8, 0.1% (v/v) Tween 20, and 0.7 mg/mL bovine serum albumin, 0 or 30 µM dicoumarol]. The rate of DCPIP reduction was monitored over 90 sec at 600 nm with an extinction coefficient (ε) of 2.1 mM^{-1} cm^{-1}. The Nqo1 activity was calculated as the decrease in absorbance per min per mg of total protein of the sample which quantitates the dicoumarol-inhibitable reduction of DCPIP.

2.2.19. DETERMINATION OF GST ACTIVITY

GST activity was determined spectrophotometrically using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate according to the method of Habig (Habig et al., 1974). Briefly, 20 µg of cytosolic or microsomal protein were incubated with 1 mM CDNB, 1 mM reduced glutathione in 0.1 M potassium phosphate buffer, pH 6.5 at 25 °C in a total volume of 1 mL. GST activity was measured as the amount of CDNB conjugate formed by recording the absorbance at 340 nm for 1.5 min with an extinction coefficient of 9600 M^{-1}/cm^{-1}. The enzyme activity was expressed as nmol/min/mg protein.

2.2.20. TRANSIENT TRANSFECTION AND LUCIFERASE ASSAY

Human hepatoma HepG2 cells and isolated mouse hepatocytes were plated onto 12-well cell culture plates. Each well of cells was transfected with 1.6 µg of XRE-driven luciferase reporter plasmid pGudLuc1.1, generously provided by Dr. M. S. Denison (University of California, Davies), ARE-driven luciferase reporter plasmid PGL3-ARE and 0.1 µg of the renilla luciferase pRL-CMV vector, used for normalization, using lipofectamine 2000 reagent according to manufacturer's instructions (Invitrogen). The luciferase assay was performed according to

manufacturer's instructions (Promega) as described previously (Elbekai and El-Kadi, 2007). In brief, after incubation with test compounds for 24 h, cells were washed with PBS, 100 µl of 1x passive lysis buffer was added into each well with continuous shaking for at least 20 min, and then the content of each well was collected separately in 1.5 ml microcentrifuge tubes. Enzyme activities were determined using a Dual-Luciferase reporter assay system (Promega). Quantification was performed using a TD-20/20 luminometer (Turner BioSystems, Sunnyvale CA).

2.2.21. AHR, HO-1, AND NRF2 SIRNA TRANSFECTION IN HUMAN HEPATOMA HEPG2 CELLS

HepG2 cells were plated onto 24-well cell culture plates. Each well of cells was transfected with HO-1 and Nrf2 siRNA at a concentration of 20 and 150 nM, respectively using INTERFERin reagent according to manufacturer's instructions (Polyplus). AhR siRNA sequence was sense: 5′-UACUUCCACCUCAGUUGGCdTdT-3′ and antisense: 5′-GCCAACUGAGGUGGAAGUAdTdT-3′. HO-1 siRNA sequences were sense: 5′-CAA AUG CAG UAU UUU UGU Utt-3′, and antisense: 5′-AAC AAA AAU ACU GCA UUU Gag-3′. Nrf2 siRNA sequences were sense, 5′-GUAAGAAGCCAGAUGUUAAdUdU-3′, and antisense, 3′-dUdUCAUUCUUCGGUCUACAATT-5′. Transfection efficiency was determined using real-time PCR post-transfection at 6, 12, and 24 h. Therefore, cells were treated 6 h post-transfection for 6 h to determine mRNA levels, or 24 h to determine protein and catalytic activity levels.

2.2.22. DETERMINATION OF MRNA HALF-LIVES

The half-life of CYP1A1 mRNA was analyzed by an Act-D-chase assay. Cells were pre-treated with 1 nM TCDD for 12 h. Cells were then washed and incubated with 5 µg/ml Act-D, to inhibit further RNA synthesis, immediately before treatment with 5 µM As(III). Total RNA was extracted at 0, 1, 3, 6, and 12 h after incubation with As(III). Real-time PCR reactions were performed using

SYBR® Green PCR Master Mix (Applied Biosystems). The fold change in the level of CYP1A1 (target gene) between treated and untreated cells, corrected by the level of ß-actin, was determined using the following equation: Fold change = 2-Δ (ΔCt), where ΔCt = Ct(target) - Ct(ß-actin) and Δ(ΔCt) = ΔCt(treated) - ΔCt(untreated).

2.2.23. PREPARATION OF NUCLEAR AND CYTOSOLIC EXTRACTS

Nuclear and cytosolic extracts were prepared from treated primary hepatocytes or HepG2 cells as previously described (Denison et al., 2002). All nuclear and cytoplasmic protein extractions were performed on ice with ice-cold reagents. Protease inhibitors were added to reagents before use, and the NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL) was utilized to obtain nuclear and cytosolic fractions. The fractions were then stored at –80°C until further analyses. Protein concentrations for the nuclear extracts were determined using the method of Lowry (Lowry et al., 1951).

2.2.24. ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

To visualize the ability of As(III) to alter the transformation and subsequent DNA binding of the AhR, a complementary pair of synthetic oligonucleotides containing the sequence 5′-GATCTGGCTCTTCTCACGCAACTCCG-3′ and 5′-GATCCGGAGTTGCGTGAGAAGAGCCA-3′, corresponding to the XRE binding site, were synthesized and radiolabeled with [γ32P]ATP as previously described (Denison et al., 2002) and used as a DNA probe in all experiments. Binding reactions using aliquots of 20 μg nuclear extracts and excess radiolabeled oligonucleotides were allowed to proceed for 15 min at 20 °C in a buffer containing 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 25 mM HEPES, 0.5 μg poly(dI–dC), and 0.4–0.8 mM KCl. To determine the specificity of binding to the oligonucleotide, nuclear extracts were preincubated at room temperature for 20 min with 100-fold molar excess of unlabeled XRE or 0.6 μg of anti-ARNT antibody (Santa Cruz Biotechnology, Inc.) before adding the γ32P-labeled probe.

Protein-DNA complexes were separated under non-denaturing conditions on a 4% polyacrylamide gel using 1× TBE (90 mM of Tris borate, 90 mM of boric acid, 4 mM of EDTA) as a running buffer. The gels were dried and the protein–DNA complexes were visualized by autoradiography.

2.2.25. IMMUNOPRECIPITATION OF AHR, HSP90, AND NRF2 PROTEINS

AhR, HSP90, and Nrf2 proteins were immunoprecipitated from cytosolic and nuclear fractions (400 - 500 μg protein) using antibodies bound to protein A/G sepharose overnight at 4 °C. The AhR, HSP90, Nrf2, and XAP2 were visualized by Western blot analysis using anti-AhR goat polyclonal antibody, anti-HSP90 mouse monoclonal antibody, and anti-XAP2 goat polyclonal antibody. Primary antibodies were detected with either peroxidase-conjugated rabbit anti-goat IgG secondary antibody for AhR and XAP2, or peroxidase conjugated goat anti-mouse IgG secondary antibody for HSP90. The bands were visualized with the enhanced chemiluminescence method according to manufacturer's instructions (Amersham, Arlington Heights, IL).

2.2.26. DETERMINATION OF TOTAL CELLULAR HEME CONTENT

After a 24-h incubation period with test compounds, cells were pelleted and boiled in 2.0 M oxalic acid for 30 min followed by resuspension in cold PBS and centrifugation at 14,000 g for 15 min. The supernatant was then removed and the fluorescence of protoporphyrin IX was assayed using the Eclipse fluorescence spectrophotometer (Varian Australia Pty Ltd., Mulgrave, Victoria Australia) using an excitation wavelength of 405 nm and an emission wavelength of 600 nm. Background was determined by measuring the fluorescence in the absence of cells. Cellular heme content was calculated as a percentage of serum-free medium-treated cells after normalization of cellular heme content with cellular protein, which was determined using the method of Lowry (Lowry et al., 1951).

2.2.27. MEASURING OF SERUM HB LEVELS

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

2.2.28. IMMUNOCYTOCHEMICAL ANALYSIS OF AHR LOCALIZATION

Plated cells on collagen coated cover glass slips were treated for 1 h with either vehicle or 1 µM Hb. Treated cells were fixed using 3.7% (w/v) paraformaldehye 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 time, and then permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature. Cells were then washed twice with PBS, 10 min each, before being incubated with blocking buffer (1X 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. Cells were then washed three times with PBS, 10 min each time, before being incubated with Alexa Fluor 488 (green) donkey anti-goat secondary antibody diluted in blocking buffer (1: 2000). Cells were then washed three times with PBS, 10 min each time, and thereafter DNA was stained with DAPI (blue) diluted in PBS (1: 15000). The stained cover slips were rinsed with double deionized water to remove excess

DAPI. Cover slips were then dried on a paper towel and 3-5 µL of Prolong Gold mounting media was added to each cover slip before being mounted on a microscope glass slip. Images were obtained using spinning disk confocal, integrated by Quorum Technologies (Guelph, ON) on an Olympus IX-81 stand with a Yokogawa CSU-X11 confocal scan unit, with a 100X/1.4NA lens obtaining image pixel size of 109nm.

2.2.29. DETERMINATION OF ROS

To determine the degree of ROS formation induced by As(III), MMA(V), DMA(V), or TMA(V), a fluorometric assay, utilizing the unique intracellular oxidation of 2′,7′-dichlorofluorescein diacetate (DCF-DA), was used. Cells grown for 24 h in 96-well plates were pretreated with 5 µM DCF-DA 2 h prior to the addition of the metals. After 12 h incubation with the metals in the dark, fluorescence was measured using a Synergy H4 Microplate Reader (Biotek Instruments) set to 37℃. Measurements were made using a 485/20 nm excitation and a 528/20 nm emission filter pair and a gain sensitivity setting of 55%; readings were made from the bottom of the well.

2.2.30. COMPETITIVE LIGAND BINDING ASSAY

Ligand binding was performed using the hydroxyapatite (HAP) assay as previously described (Denison et al., 1986) with slight modifications. Specifically, untreated guinea pig cytosolic protein was diluted to 2 mg/ml in MEDG (25 mM 3-(N-morpholino) propanesulfonic acid, pH 7.5, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiotreitol, 10% [v/v] glycerol). Aliquots of 100 µl were incubated in the presence of 2 nM [^{3}H]TCDD alone (total binding), 2 nM [^{3}H]TCDD and 200 nM 2,3,7,8-tetrachlorodibenzofuran (TCDF) (100 fold excess of competitor, non-specific binding) or 2 nM [^{3}H]TCDD in the presence of increasing concentrations of SB (20 µM, 100 µM, and 200 µM). All chemicals stocks were prepared in DMSO, in which DMSO content in reactions was adjusted to 2% (v/v) where necessary. After 1.5 h incubation at room temperature, reactions were further incubated with 250 µl of hydroxyapatite

suspension for additional 30 min with gentle vortexing every 10 min. Thereafter, reactions were washed three times with 1 ml MEGT buffer (25 mM 3-(N-morpholino)propanesulfonic acid, pH 7.5, 1 mM ethylenediaminetetraacetic acid, 10% [v/v] glycerol, 0.5% [v/v] Tween 80). The HAP pellets were transferred to 4 ml scintillation vials, scintillation cocktail was added and the samples were counted in a scintillation counter.

2.2.31. STATISTICAL ANALYSIS

The comparative analysis of the results from various experimental groups with their corresponding controls was performed using SigmaStat for Windows (Systat Software, CA, USA). 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 Student-Newman-Keuls post hoc test was carried out to assess statistical significance between treatment groups compared to TCDD. For mRNA half-lives statistical significance was assessed using two-way ANOVA followed by the Student-Newman-Keuls post hoc test. The differences were considered significant when $P < 0.05$.

CHAPTER 3

3. Results

Versions of this chapter have been previously published.

3.1. ARSENITE DOWN-REGULATES CYTOCHROME P450 1A1 AT TRANSCRIPTIONAL AND POST-TRANSLATIONAL LEVELS IN HUMAN HEPG2 CELLS

3.1.1. EFFECT OF CO-EXPOSURE TO AS(III) AND TCDD ON CELL VIABILITY

To determine the non-toxic concentrations of As(III) to be utilized in the current study, HepG2 cells were exposed for 24 h with increasing concentrations of As(III) (1 – 10 μM) in the absence and presence of 1 nM TCDD, thereafter cytotoxicity was assessed using the MTT assay. Figure 3.1.1 shows that As(III) at concentrations of 1 – 10 μM in the presence and absence of 1 nM TCDD did not affect cell viability. Therefore, all subsequent studies were conducted using concentrations of 1 – 10 μM.

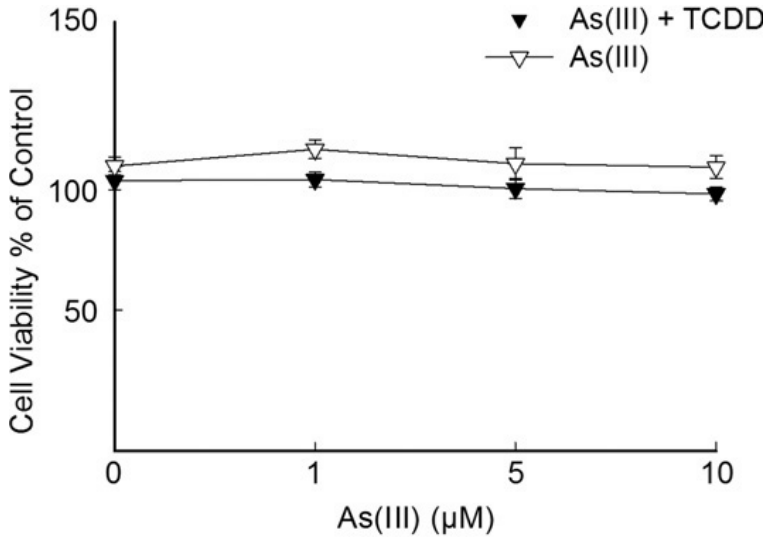

Fig. 3.1.1 Effect of As(III) on cell viability.
HepG2 cells were treated for 24 h with As(III) (0, 5, and 10 μM) in the absence and presence of 1 nM TCDD. Cell cytotoxicity was determined using the MTT assay. Data are expressed as percentage of untreated control (which is set at 100%) ± SE (n = 8). (+) $P < 0.05$, compared to control (concentration = 0 μM); (*) $P < 0.05$, compared to respective TCDD treatment.

3.1.2. CONCENTRATION-DEPENDENT EFFECT OF CO-EXPOSURE TO AS(III) AND TCDD ON INDUCIBLE CYP1A1 MRNA

To examine the effect of co-exposure to As(III) and TCDD on CYP1A1 mRNA, HepG2 cells were treated with various concentrations of As(III) in the presence of 1 nM TCDD (Fig. 3.1.2A). Thereafter, CYP1A1 mRNA was assessed using real-time PCR. TCDD alone caused 40-fold increase in CYP1A1 mRNA 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 by 10-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 by 25-fold (Fig. 3.1.2A).

3.1.3. CONCENTRATION-DEPENDENT EFFECT OF CO-EXPOSURE TO AS(III) AND TCDD ON CYP1A1 PROTEIN AND CATALYTIC ACTIVITY

To examine whether the observed inhibition of the TCDD-mediated induction of CYP1A1 mRNA by As(III) is further translated to the protein and activity levels, HepG2 cells were treated for 24 h with increasing concentrations of As(III) in the presence of 1 nM TCDD. Figures 3.1.2B and 3.1.2C show that TCDD alone caused 80-fold and 40-fold increases in CYP1A1 protein and catalytic activity, respectively. Of interest, As(III) decreased the TCDD-mediated induction of CYP1A1 protein and catalytic activity levels in a dose-dependent manner. This inhibitory effect of As(III) on the CYP1A1 protein and catalytic activity levels is in concordance with the observed effect at the mRNA level, in which the initial significant inhibition took place at 1 μM As(III) and reached maximal inhibition at 10 μM (Fig. 3.1.2B and 3.1.2C).

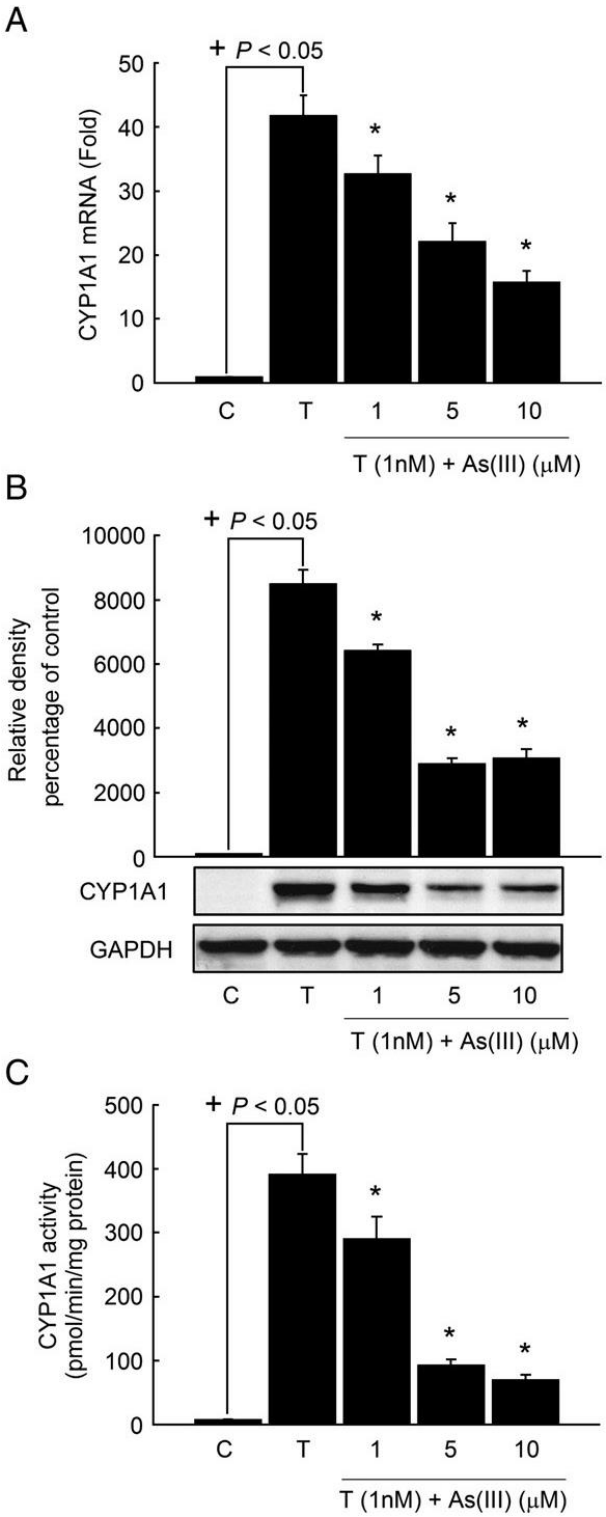

Fig. 3.1.2 Effect of As(III) on CYP1A1 mRNA, protein, and catalytic activity in HepG2 cells.

HepG2 cells were treated with increasing concentrations of As(III) in the presence of 1 nM TCDD for 6 h for mRNA or 24 h for protein and catalytic activity. **A,** First-strand cDNA was synthesized from total RNA (1 μg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. **B,** Protein (50 μg) was separated on a 10% SDS-PAGE. CYP1A1 protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to GAPDH signals which were used as loading control. One of three representative experiments is shown. **C,** 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 mean ± SE (n = 6). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

3.1.4. TRANSCRIPTIONAL INHIBITION OF CYP1A1 GENE BY AS(III)

HepG2 cells were transiently transfected with the XRE-driven luciferase reporter gene in order to study the effect of As(III) on the AhR-dependent transcriptional activation. Luciferase activity results showed that 5 μM As(III) alone inhibited the constitutive expression of the luciferase activity (Fig. 3.1.3). On the other hand, 1 nM TCDD alone caused a significant increase of luciferase activity by 280-fold as compared to control. Interestingly, co-treatment with As(III) and TCDD significantly decreased the TCDD-mediated induction of luciferase activity by 65% (Fig. 3.1.3).

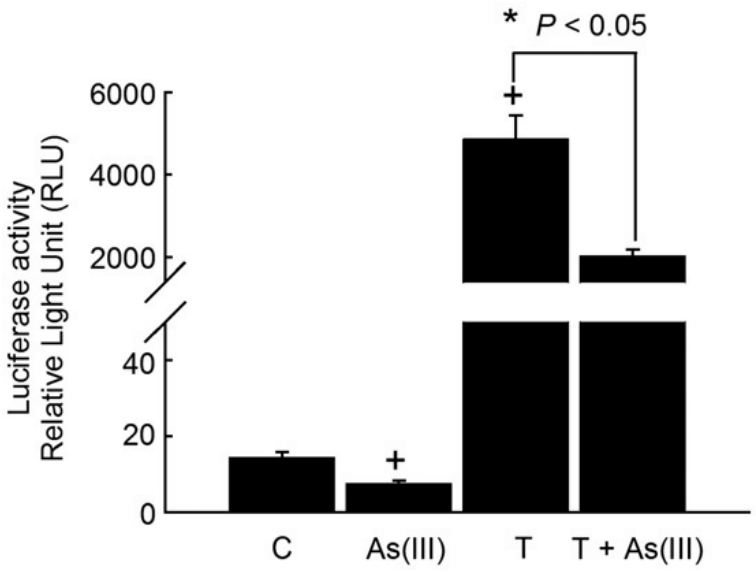

Fig. 3.1.3 Effect of As(III) on luciferase activity.
HepG2 cells were transiently transfected with the XRE-luciferase transporter plasmid pGudLuc6.1. Cells were treated with vehicle, TCDD (1 nM), As(III) (5 μM), TCDD (1 nM) + As(III) (5 μM) for 24 h. Cells were lysed and luciferase activity was measured according to manufacturer's instruction. Luciferase activity is reported as relative light units. Values are presented as mean ± SE (n = 6). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

3.1.5. POST-TRANSCRIPTIONAL MODIFICATION OF CYP1A1 MRNA BY AS(III)

In order to examine the effect of As(III) on the CYP1A1 mRNA stability, we performed the Act-D chase experiment on intact viable HepG2 cells. The level of mRNA expression is not only a function of the transcription rate, but is also dependent on the elimination rate, through processing or degradation. If As(III) decreases CYP1A1 mRNA via decreasing its stability, a decrease in half-life would be expected to take place. Figure 3.1.4 shows that CYP1A1 mRNA decayed with a half-life of 4.6 $\pm$ 0.6 h. Furthermore, As(III) did not alter CYP1A1 mRNA half-life, which was 4.9 $\pm$ 0.8 h (Fig. 3.1.4).

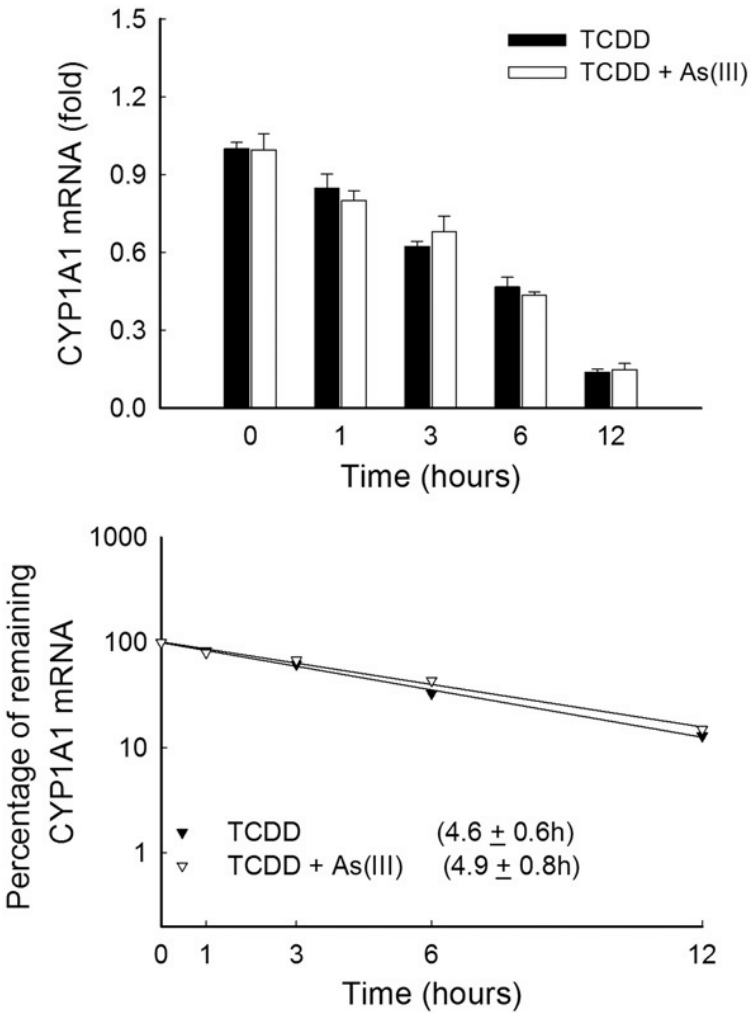

Fig. 3.1.4 Effect of As(III) on CYP1A1 mRNA half-life using real-time PCR. HepG2 cells were grown to 90% confluence in six-well cell culture plates, and then treated with 1 nM TCDD for 12 h. The cells were then washed and incubated in fresh medium containing 5 μM As(III) plus 5 μg/ml Act-D, a RNA synthesis inhibitor. First-strand cDNA was synthesized from total RNA (1 μg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. mRNA decay curves were analyzed individually, and the half-life was estimated from the slope of a straight line fitted by exponential regression analysis ($r^2 \geq 0.90$) to a semilog plot of mRNA amount, expressed as a percent of treatment at time = 0 h (maximum, 100%) level, versus time. The half-lives obtained from three independent experiments were then used to calculate the mean half-life (mean ± SE, $n = 3$). (*) $P < 0.05$ compared with TCDD.

3.1.6. THE EFFECT OF SUPPLEMENTING NADPH ON AS(III)-MEDIATED DECREASE OF CYP1A1 ACTIVITY.

To investigate whether the As(III)-mediated decrease of CYP1A1 activity is through decreasing intracellular NADPH, HepG2 cells were treated for 24 h with 5 μM As(III) with or without 1 nM TCDD and the CYP1A1 activity was assessed in total cellular lysate in the presence of NADPH. Figure 5 shows that TCDD alone caused 180-fold increase in CYP1A1 catalytic activity. On the other hand, As(III) decreased the TCDD-mediated induction of CYP1A1 catalytic activity level by 3.5-fold in total cell lysate supplemented with NADPH (Fig. 3.1.5). This inhibitory effect of As(III) on the CYP1A1 catalytic activity level in total cell lysate was similar to that observed at the catalytic activity levels in intact cells.

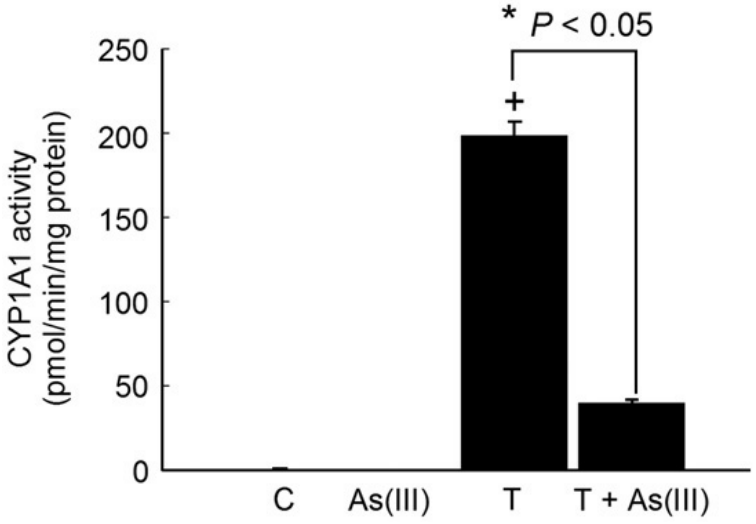

Fig. 3.1.5 Effect of supplementing NADPH on As(III)-mediated decrease of CYP1A1 activity.

HepG2 cells were treated with 5 μM As(III) in the absence and presence of 1 nM TCDD for 24 h. Thereafter, total cell lysates were prepared as described under *Materials and Methods*. CYP1A1 activity was measured in total cell lysate using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SE (n = 8). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment.

3.1.7. THE EFFECT OF SNMP ON THE POST-TRANSLATIONAL MODIFICATION OF CYP1A1 CATALYTIC ACTIVITY BY AS(III)

The fact that As(III) inhibited the TCDD-mediated induction of CYP1A1 at the catalytic activity level more than what is observed on 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 As(III) and 1 nM TCDD in the presence and absence of 5 μM SnMP. Our results showed that As(III) in the presence of 1 nM TCDD was able to increase HO-1 mRNA by 15-fold compared to control (Fig. 3.1.6A). TCDD alone did not significantly alter the HO-1 mRNA. As expected, SnMP alone increased HO-1 mRNA by ~ 0.5-fold; however, when the cells were co-exposed to As(III) and TCDD in the presence of SnMP, the As(III)-mediated induction of HO-1 mRNA was not altered by the SnMP treatment.

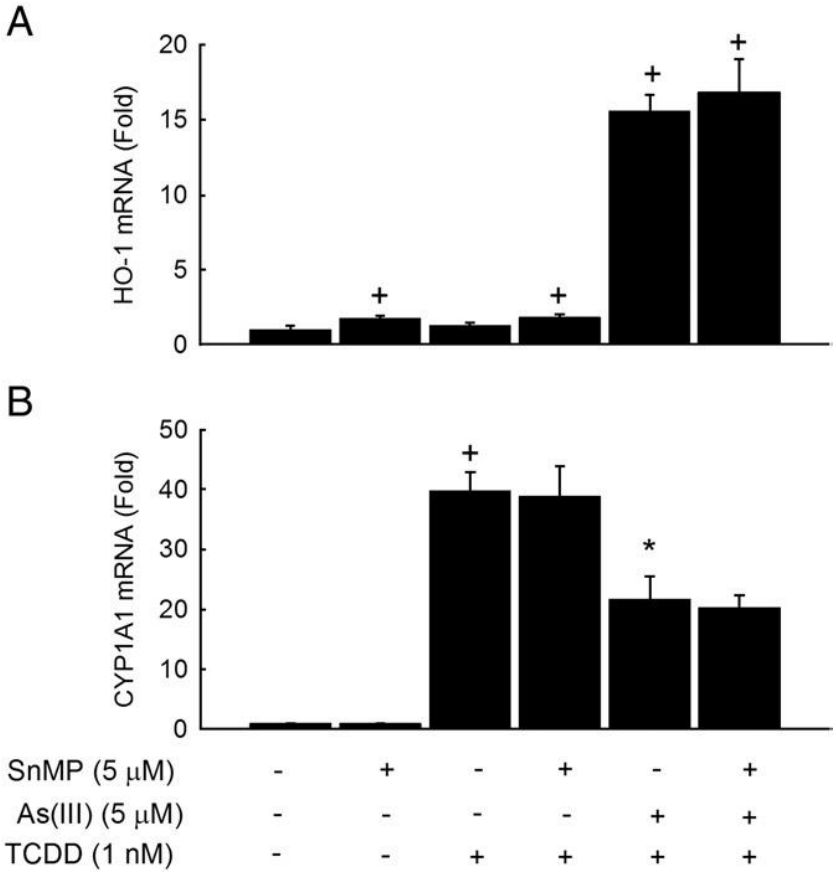

Fig. 3.1.6 Effect of SnMP on HO-1 and CYP1A1 mRNA levels in the presence of As(III).

HepG2 cells were treated with 5 μM of As(III) and 1 nM TCDD in the presence and absence of 5 μM SnMP for 6 h for HO-1 and CYP1A1 mRNA. **A and B,** First-strand cDNA was synthesized from total RNA (1 μg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment.

Although SnMP did not significantly alter the HO-1 mRNA when concomitantly used with As(III) and TCDD, there was still a possibility that SnMP might reverse the inhibitory effect of As(III) on CYP1A1 catalytic activity through inducing CYP1A1 mRNA levels. Therefore, we examined the effect of SnMP on CYP1A1 mRNA levels. SnMP alone or in the presence of TCDD or As(III) plus TCDD did not affect CYP1A1 mRNA levels with all treatments, thus eliminating this possibility (Fig. 3.1.6B). 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, As(III) at the concentration of 5 μM decreased the TCDD-mediated induction of CYP1A1 catalytic activity. Intriguingly, SnMP partially reversed the As(III)-mediated decrease in CYP1A1 activity. Upon treating the cells with SnMP in the presence of both As(III) and TCDD, there was a partial restoration of the As(III)-mediated down-regulation of CYP1A1 catalytic activity induced by TCDD (Fig. 3.1.7A). In spite of being successful in partially reversing the As(III)-mediated decrease in CYP1A1 activity through inhibiting HO-1, SnMP was unable to completely restore the CYP1A1 activity.

To examine whether the effect of As(III) is specific for CYP1A1 we investigated the effect of As(III) on CYP1A2 activity. In addition, we determined if the effect mediated by As(III) could be reversed by SnMP. Our results demonstrated that TCDD alone increased the CYP1A2 catalytic activity levels by 4-fold, while SnMP alone, or in the presence of TCDD, did not affect CYP1A2 activity. In contrast, 5 μM As(III) was able to decrease the TCDD-mediated induction of CYP1A2 catalytic activity by 2.5-fold. On the other hand, when the cells were co-exposed to SnMP in the presence of TCDD plus As(III), there was a partial restoration of the As(III)-mediated decrease of the TCDD-mediated induction of CYP1A2 catalytic activity (Fig. 3.1.7B).

The ability of As(III) to inhibit CYP1A1 activity more than inhibiting its mRNA prompted further investigation. Therefore, we hypothesized that the decrease in

TCDD-mediated induction of CYP1A1 activity is attributed to decreased total cellular heme content. Our results showed that the treatments of TCDD alone, SnMP alone or TCDD plus SnMP did not affect total cellular heme content. On the other hand, 5 μM As(III) in the presence of 1 nM TCDD caused a 0.4-fold decrease in the total cellular heme content (Fig. 3.1.7C). Incongruously, when the cells were treated with SnMP in the presence of TCDD plus As(III) there was almost a complete restoration of total cellular heme content levels that were decreased by As(III) (Fig. 3.1.7C). Importantly, with SnMP alone or in the presence of TCDD, fluorescence readings were similar to that of untreated cells, eliminating the possibility that SnMP might act to restore the total heme content through a false positive reading.

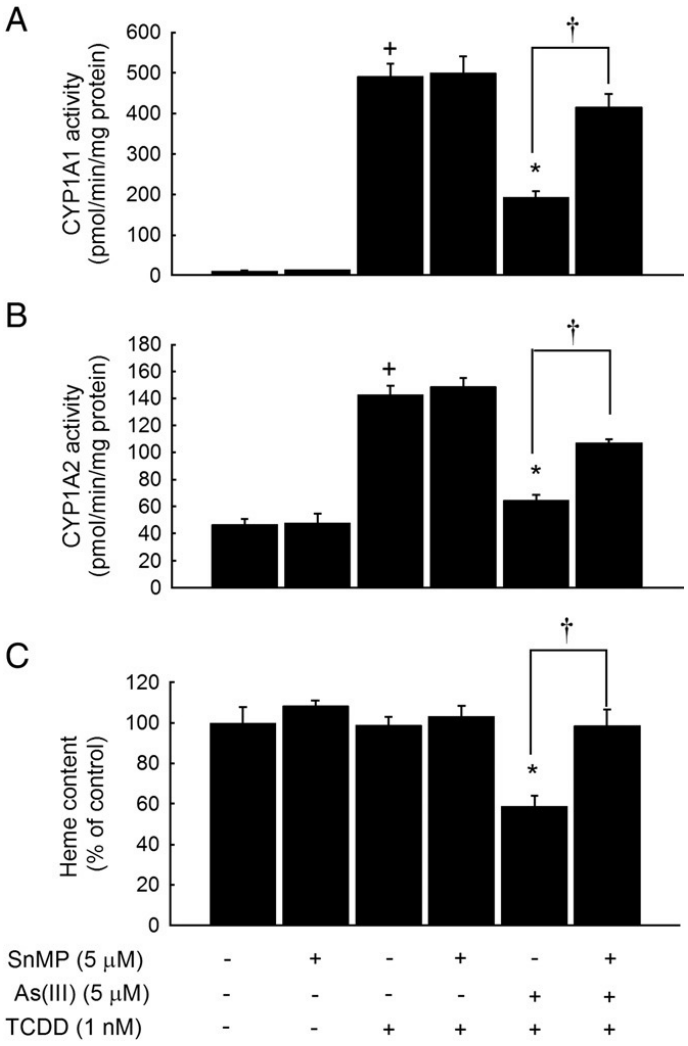

Fig. 3.1.7 Effect of SnMP as a competitive inhibitor of HO-1 on CYP1A1 and CYP1A2 catalytic activities and total cellular heme content levels in the presence of As(III).

HepG2 cells were treated with 5 μM As(III) and 1 nM TCDD in the presence and absence of 5 μM SnMP for 24 h. **A and B,** CYP1A1 and CYP1A2 activities were measured in intact living cells treated with 5 μM As(III) and 1 nM TCDD in the presence and absence of 5 μM SnMP for 24 h. CYP1A1 and CYP1A2 activities were measured using 7-ethoxyresorufin and 7-methoxyresorufin as a substrates, respectively. Values are presented as mean ± SE (n = 8). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment; (†) $P < 0.05$, compared to respective As(III) + TCDD treatment. **C,** Total heme content in HepG2 cells treated with 5 μM As(III) and 1 nM TCDD in the presence and absence of 5 μM SnMP for 24 h. The conversion of heme to protoporphyrin IX by oxalic acid was measured fluorometrically. Cellular heme content was calculated as a percent of serum-free medium treated cells following normalization with cellular protein. (*) $P < 0.05$, compared to respective TCDD treatment; (†) $P < 0.05$, compared to respective As(III) + TCDD treatment.

3.1.8. THE EFFECT OF EXOGENOUS HEME AND THE CARBON MONOXIDE (CO) SCAVENGER, HEMOGLOBIN (HB) ON AS(III)-MEDIATED DECREASE OF CYP1A1 ACTIVITY.

In an attempt to examine whether the presence of external heme will restore the As(III)-mediated decrease of CYP1A1 activity, HepG2 cells were co-exposed to 5 μM As(III) and 1 nM TCDD in the presence and absence of 80 μM hemin, a precursor of heme. Our results showed that hemin alone did not affect CYP1A1 activity. On the other hand, hemin significantly potentiated the TCDD-mediated induction of CYP1A1 activity. Interestingly, the addition of hemin partially restored the As(III)-mediated decrease of CYP1A1 activity by 2- fold compared to As(III) plus TCDD (Fig. 3.1.8A).

To examine the possibility that As(III) acts to decrease the TCDD-mediated induction of CYP1A1 activity through liberating CO as a byproduct of heme degradation, HepG2 cells were co-exposed to 5 μM As(III) and 1 nM TCDD in the presence and absence of 100 μM Hb. Our results showed that Hb alone was able to induce the CYP1A1 activity by 9-fold compared to control. Similarly, TCDD alone induced the CYP1A1 catalytic activity by 47-fold which is further induced by Hb to 60-fold compared to control (Fig. 3.1.8B). Interestingly, the treatment with Hb partially restored the As(III)-mediated decrease of CYP1A1 activity (Fig. 3.1.8B).

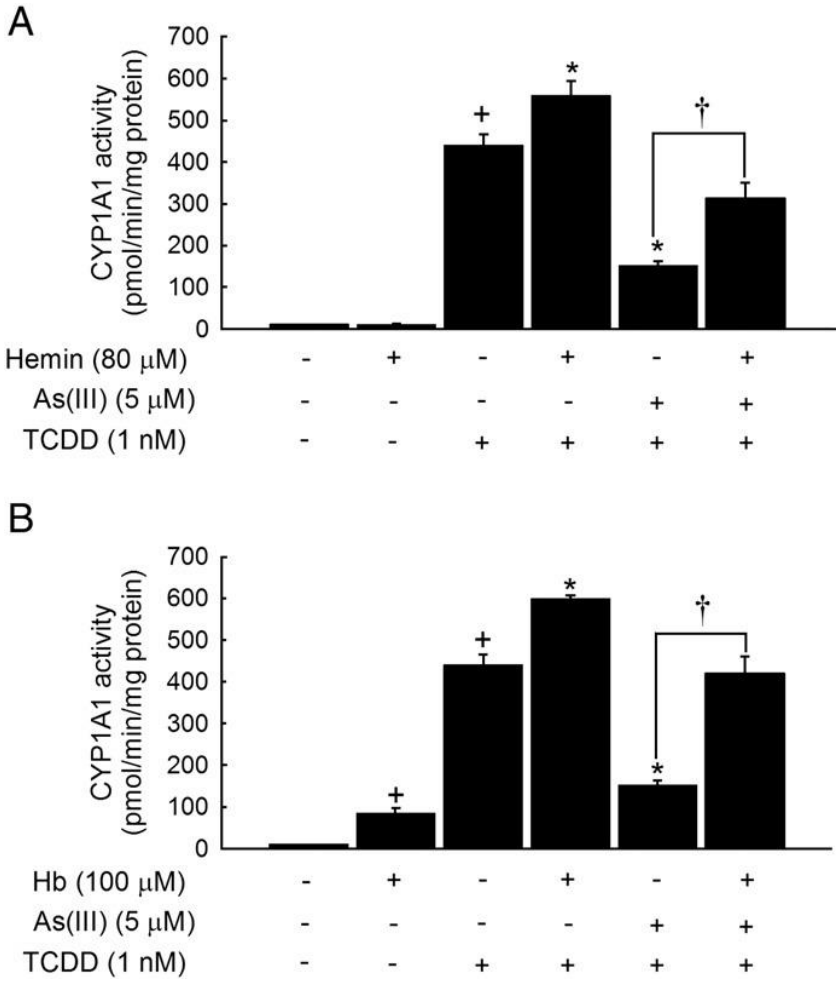

Fig. 3.1.8 Effect of supplementing external heme and the carbon monoxide (CO) scavenger, hemoglobin (Hb) on As(III)-mediated decrease of CYP1A1 activity.

HepG2 cells were treated with 5 μM As(III) and 1 nM TCDD in the presence and absence of 80 μM hemin for 24 h, or 100 μM Hb. **A,** CYP1A1 activity was measured in intact living cells treated with 5 μM As(III) and 1 nM TCDD in the presence and absence of 80 μM hemin for 24 h. CYP1A1 activity was measured using 7-ethoxyresorufin as a substrates. **B**, CYP1A1 activity was measured in intact living cells treated with 5 μM As(III) and 1 nM TCDD in the presence and absence of 100 μM Hb for 24 h. CYP1A1 activity was measured using 7-ethoxyresorufin as a substrates. Values are presented as mean ± SE (n = 8). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment; (†) $P < 0.05$, compared to respective As(III) + TCDD treatment.

3.1.9. THE EFFECT OF HO-1 SIRNA ON AS(III)-MEDIATED INHIBITION OF CYP1A1 CATALYTIC ACTIVITY

We took a genetic approach to confirm whether or not HO-1 is involved in the As(III)-mediated decrease of the TCDD-mediated induction of CYP1A1 catalytic activity. For this purpose, HepG2 cells were transfected with human HO-1 siRNA or Silencer negative control siRNA for 6 h, and then the cells were treated with 5 μM As(III) in the presence and absence of 1 nM TCDD. Our results showed that HO-1 siRNA significantly decreased HO-1 mRNA by 0.8-fold as compared to control (Fig. 3.1.9A). On the other hand, As(III) was able to increase HO-1 mRNA levels, in the absence and presence of TCDD, to reach 25-fold compared to control. When the cells were transfected with HO-1 siRNA, and then treated with As(III) in the presence or absence of TCDD there was a statistically significant decrease in HO-1 mRNA by 20-fold. Furthermore, the silencer select negative control siRNA did not affect the inducible level of HO-1 mRNA by As(III), eliminating the possibility that the inhibitory effects of HO-1 siRNA might have been due to any toxicity. To test the selectivity of the siRNA for HO-1, we determined the CYP1A1 mRNA levels in cells transfected with siRNA for HO-1. Figure 3.1.9A shows that CYP1A1 mRNA levels were not altered by the HO-1 siRNA or silencer select negative control siRNA transfections. Thus, the observed effects on the CYP1A1 catalytic activity levels are solely through knocking down HO-1.

To confirm whether the knockdown of HO-1 mRNA is further translated to functional protein, we examined HO-1 protein expression levels. As(III) at the concentration of 5 μM was able to increase the HO-1 protein by 22-fold. On the contrary HO-1 siRNA completely abolished the As(III)-mediated induction of HO-1 protein (Fig. 3.1.9B). Figure 10A shows that CYP1A1 mRNA levels were not affected by the HO-1 siRNA or silencer select negative control siRNA transfections. Thus, the observed effects on the CYP1A1 catalytic activity levels are solely through knocking down HO-1.

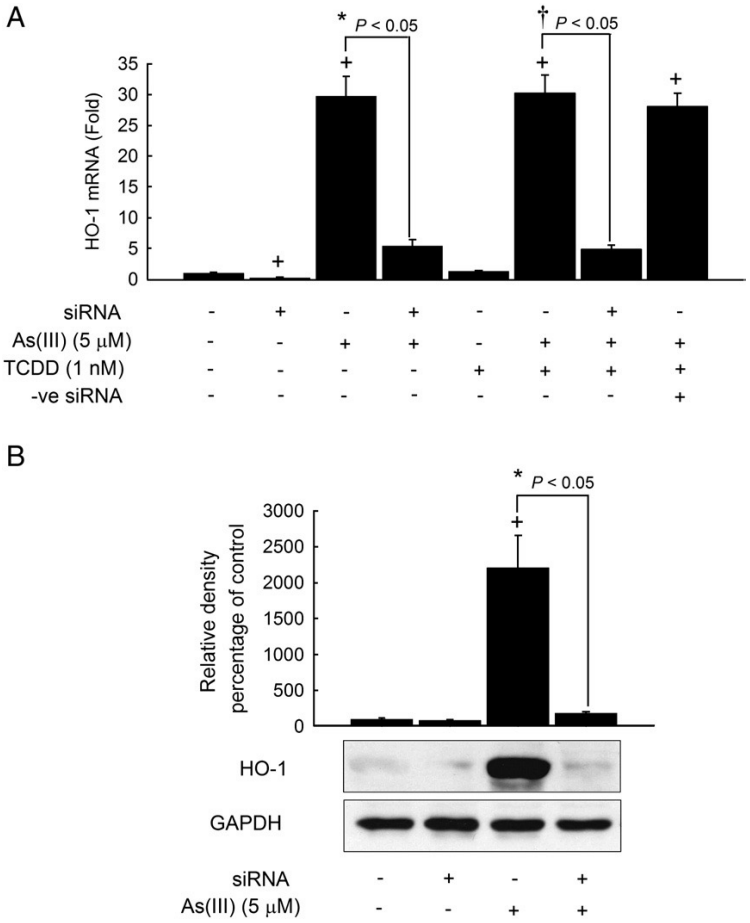

Fig. 3.1.9 Effect of HO-1 siRNA on As(III)-mediated induction of HO-1 mRNA and protein.

HepG2 cells were transiently transfected with 20 nM HO-1 siRNA (siRNA) or 20 nM Silencer select negative control siRNA (-ve siRNA) for 6 h, and thereafter cells were treated with vehicle, TCDD (1 nM), As(III) (5 μM), or TCDD (1 nM) + As(III) (5 μM) for 6 h for HO-1 mRNA or 24 h for HO-1 protein. **A,** First-strand cDNA was synthesized from total RNA (1 μg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (+) $P < 0.05$, compared to respective control; (*) $P < 0.05$, compared to respective As(III) treatment; (†) $P < 0.05$, compared to respective As(III) + TCDD treatment. **B,** Protein (50 μg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C and then incubated with a primary HO-1 antibody overnight at 4°C, followed by 4 h incubation with secondary antibody at room temperature. HO-1 protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to GAPDH signals, which was used as loading control. One of three representative experiments is shown. (+) $P < 0.05$, compared to respective control; (*) $P < 0.05$, compared to respective As(III) treatment.

Looking at CYP1A1 catalytic activity, As(III) alone or in the presence of HO-1 siRNA did not affect CYP1A1 catalytic activity (Fig. 3.1.10B). TCDD alone increased the CYP1A1 catalytic activity by 50-fold, whereas As(III) significantly decreased the TCDD-mediated induction of CYP1A1 catalytic activity by ~ 0.8-fold. Interestingly, when HepG2 cells were transfected with HO-1 siRNA and then co-exposed to As(III) and TCDD, As(III) decreased the TCDD-induced catalytic activity by ~ 0.2-fold, compared to control, and was unable to maintain the same inhibitory effect on CYP1A1 catalytic activity when compared to non-transfected cells (Fig. 3.1.10B). Moreover, when HepG2 cells were transfected with silencer select negative control siRNA and then co-exposed to As(III) and TCDD, As(III) decreased the TCDD-mediated induction of CYP1A1 catalytic activity by ~ 0.8-fold, similar to that of non-transfected cells.

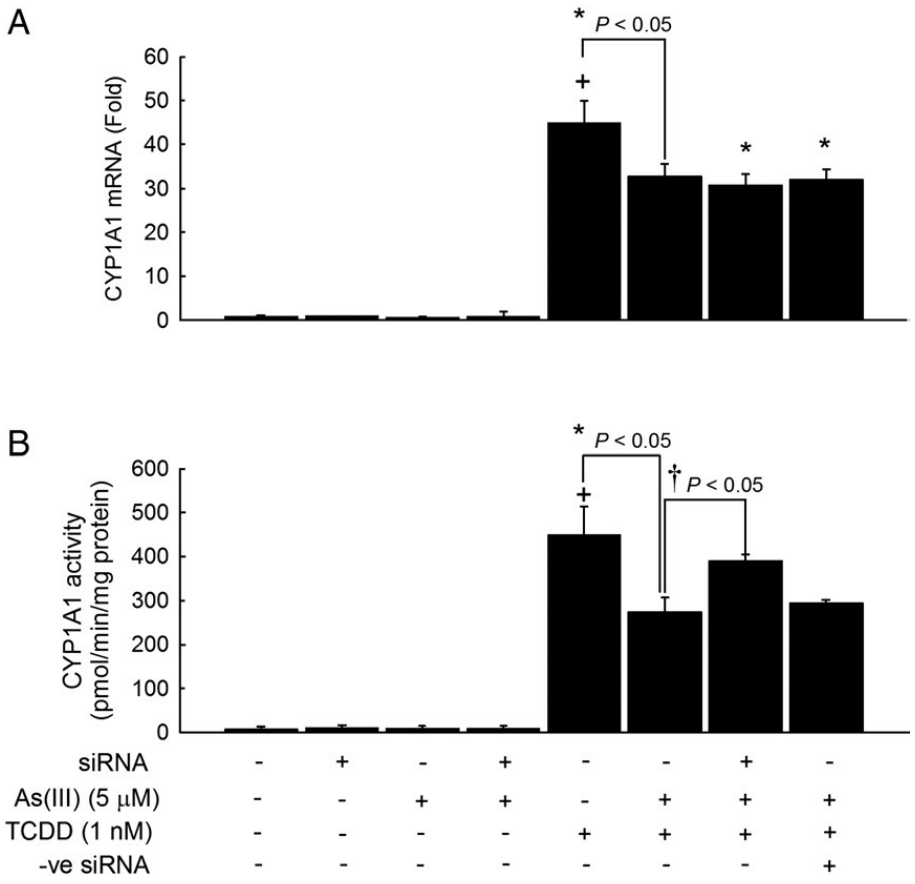

Fig. 3.1.10 Effect of HO-1 siRNA on As(III)-mediated inhibition of CYP1A1 mRNA and catalytic activity.

HepG2 cells were transiently transfected with 20 nM HO-1 siRNA (siRNA) or 20 nM Silencer select negative control siRNA (-ve siRNA) for 6 h, and thereafter cells were treated with vehicle, TCDD (1 nM), As(III) (5 μM), or TCDD (1 nM) + As(III) (5 μM) for 6 h for CYP1A1 mRNA or 24 h for CYP1A1 catalytic activity. **A,** First-strand cDNA was synthesized from total RNA (1 μg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective As(III) treatment; (†) $P < 0.05$, compared to respective As(III) + TCDD treatment. **B,** CYP1A1 activity was measured in intact living cells transiently transfected with 20 nM HO-1 siRNA (siRNA) or 20 nM Silencer select negative control siRNA (-ve siRNA) for 6 h and thereafter treated with 5 μM As(III), 1 nM TCDD, or 5 μM As(III) + 1 nM TCDD for 24 h. CYP1A1 activity was measured using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SE ($n = 6$). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment; (†) $P < 0.05$, compared to respective As(III) + TCDD treatment.

3.1.10. THE EFFECT OF THE HO-1 INDUCER, COPP, ON TOTAL CELLULAR HEME CONTENT, AND CYP1A1 MRNA AND CATALYTIC ACTIVITY.

In order confirm the role of HO-1 in the down-regulation of CYP1A1 at the catalytic activity level, we used CoPP, which is a potent HO-1 inducer, to simulate the As(III)-mediated effect on CYP1A1 activity. Our results showed that 1 μM CoPP in the absence and presence of 1 nM TCDD was able to significantly induce HO-1 mRNA by 37-fold compared to control (Fig. 3.1.11A). On the contrary, when the cells were transfected with HO-1 siRNA prior to the treatment by CoPP in the presence of 1 nM TCDD, the HO-1 mRNA levels were significantly decreased by ~ 9-fold compared to 1 nM TCDD + 1 μM CoPP treatment (Fig. 3.1.11A). In agreement with HO-1 mRNA data, treatment with 1 μM CoPP in the absence and presence of 1 nM TCDD was able to decrease the total cellular heme content to 0.6-fold, compared to control (Fig. 3.1.11B). Thus, these results imply that CoPP, through inducing HO-1, decreases the total cellular heme content. The treatment with CoPP (1 μM) did not alter the TCDD-mediated induction of CYP1A1 mRNA levels. Moreover, the knockdown of HO-1 mRNA did not affect the TCDD-mediated induction of CYP1A1 mRNA (Fig. 3.1.12A).

Interestingly, CoPP at the concentration of 1 μM was able to significantly decrease the TCDD-mediated induction of CYP1A1 activity by 3-fold (Fig. 3.1.12B). On the other hand when the cells were transfected with HO-1 siRNA and then treated with TCDD in the presence of 1 μM CoPP there was a complete restoration of the TCDD-mediated induction of CYP1A1 catalytic activity (Fig. 3.1.12B).

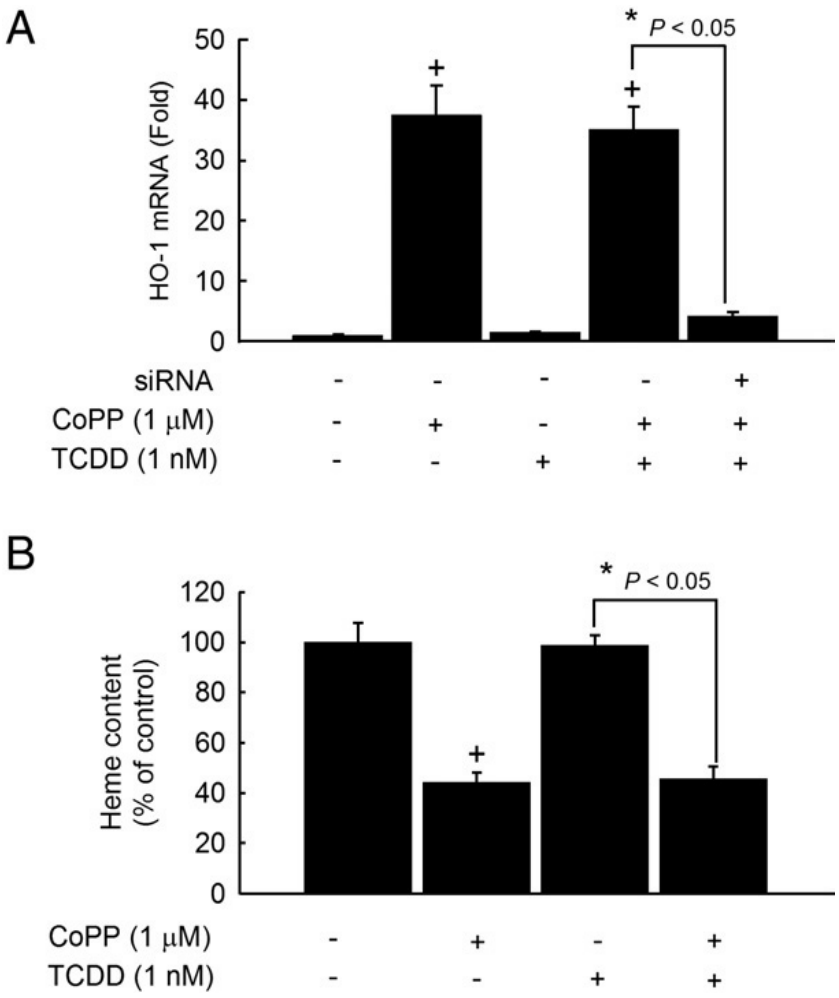

Fig. 3.1.11 Effect of HO-1 siRNA on CoPP-mediated induction of HO-1 mRNA and COPP-mediated inhibition of total cellular heme content.
HepG2 cells were transiently transfected with 20 nM HO-1 siRNA (siRNA) or 20 nM Silencer select negative control siRNA (-ve siRNA) for 6 h, and thereafter cells were treated with vehicle, TCDD (1 nM), CoPP (1 μM), or TCDD (1 nM) + CoPP (1 μM) for 6 h for HO-1 mRNA or 24 h for HO-1 protein. **A,** First-strand cDNA was synthesized from total RNA (1 μg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective CoPP + TCDD treatment. **B,** Total heme content in HepG2 cells treated with 1 μM CoPP in the absence and presence of 1 nM TCDD for 24 h. The conversion of heme to protoporphyrin IX by oxalic acid was measured fluorometrically. Cellular heme content was calculated as a percent of serum-free medium treated cells following normalization with cellular protein. (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective CoPP + TCDD treatment.

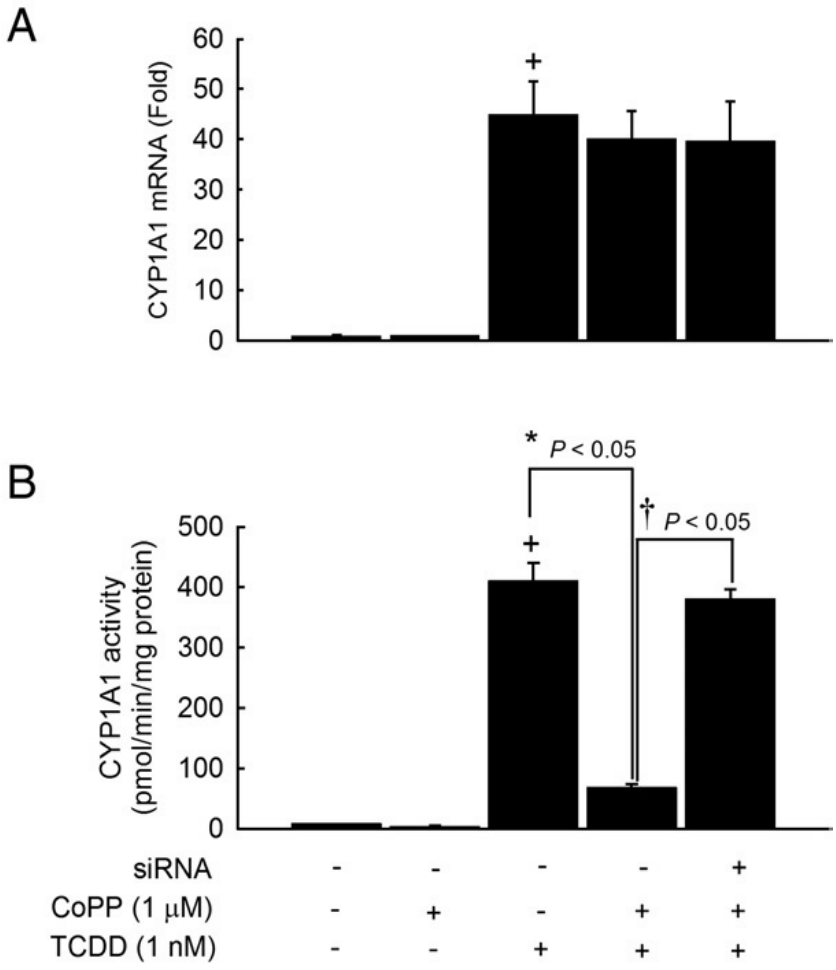

Fig. 3.1.12 Effect of HO-1 siRNA on CoPP-mediated inhibition of CYP1A1 mRNA and catalytic activity levels.

HepG2 cells were transiently transfected with 20 nM HO-1 siRNA (siRNA) or 20 nM Silencer select negative control siRNA (-ve siRNA) for 6 h, and thereafter cells were treated with vehicle, TCDD (1 nM), CoPP (1 μM), or TCDD (1 nM) + CoPP (1 μM) for 6 h for CYP1A1 mRNA or 24 h for CYP1A1 catalytic activity. **A,** First-strand cDNA was synthesized from total RNA (1 μg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (+) $P < 0.05$, compared to control. **B,** CYP1A1 activity was measured in intact living cells transiently transfected with 20 nM HO-1 siRNA (siRNA) or 20 nM Silencer select negative control siRNA (-ve siRNA) for 6 h and thereafter treated with vehicle, TCDD (1 nM), CoPP (1 μM), TCDD (1 nM) + CoPP (1 μM) for 24 h. CYP1A1 activity was measured using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SE ($n = 8$). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment; (†) $P < 0.05$, compared to respective CoPP + TCDD treatment.

3.2. INHIBITION OF HEME OXYGENASE-1 PARTIALLY REVERSES THE ARSENITE-MEDIATED DECREASE OF CYTOCHROME P450 1A1 (CYP1A1), CYP1A2, CYP3A23, AND CYP3A2 CATALYTIC ACTIVITY IN ISOLATED RAT HEPATOCYTES

3.2.1. EFFECT OF CO-EXPOSURE TO AS(III) AND TCDD ON CELL VIABILITY

To determine the non-toxic concentrations of As(III) to be utilized in the current study, rat hepatocytes were exposed for 24 h to increasing concentrations of As(III) (1 – 50 μM) in the absence and presence of 1 nM TCDD. Thereafter, cytotoxicity was assessed using the MTT assay. Figure 3.2.1 shows that As(III) at concentrations of 1 – 10 μM in the presence of 1 nM TCDD or 25 μM Rif did not affect cell viability. On the other hand, As(III) at concentrations of 25 and 50 μM decreased cells viability by 65% and 75%, respectively. Therefore, all subsequent studies were conducted using concentrations of 1-10 μM.

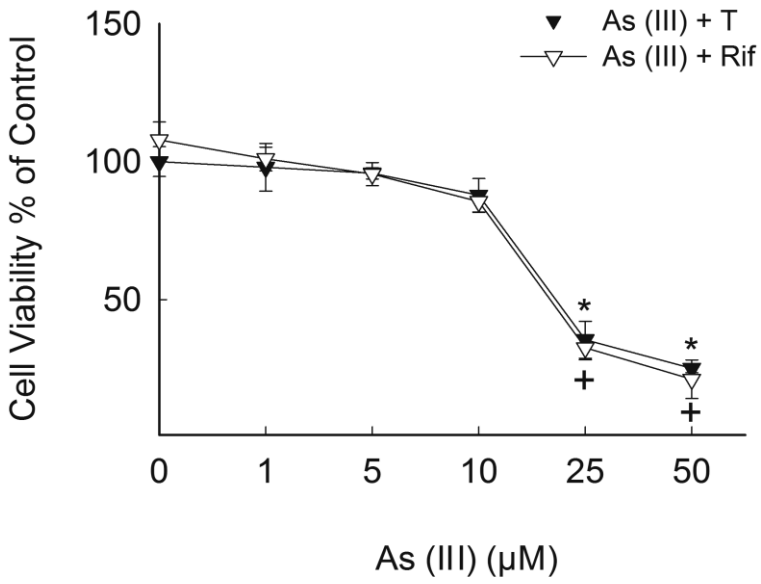

Fig. 3.2.1 Effect of As(III) on cell viability.
Freshly isolated rat hepatocytes were treated for 24 h with As(III) (0, 1, 5, 10, 25 and 50 μM) in the presence of 1 nM TCDD or 25 μM Rif. Cell cytotoxicity was determined using the MTT assay. Data are expressed as percentage of untreated control (which is set at 100%) ± SE (n = 8). (+) $P < 0.05$, compared to control (concentration = 0 μM); (*) $P < 0.05$, compared to respective TCDD treatment.

3.2.2. EFFECT OF CO-EXPOSURE TO AS(III) ON TCDD-INDUCED CYP1A1 AND CYP1A2 MRNA, PROTEIN, AND CATALYTIC ACTIVITY LEVELS

To examine the effect of co-exposure to As(III) and TCDD on CYP1A1 and CYP1A2 mRNA levels, rat hepatocytes were treated for 6 h with As(III) (1-10 μM) in the presence of 1 nM TCDD (Fig. 3.2.2A). Thereafter, CYP1A1 and CYP1A2 mRNA levels were assessed using real-time PCR. TCDD alone caused 2600% and 800% increases in CYP1A1 and CYP1A2 mRNA levels, respectively. On the other hand, when the cells were co-exposed to As(III) and TCDD, As(III) decreased the TCDD-mediated induction of both CYP1A1 and CYP1A2 mRNA levels in a dose-dependent manner. Initially, As(III) at the concentration of 1 μM caused a non-significant decrease in TCDD-mediated induction of CYP1A1 and CYP1A2 mRNA levels by 15% and 20%, respectively. The maximum inhibition took place at the highest concentration tested, 10 μM, which caused a decrease in the TCDD-mediated induction of CYP1A1 and CYP1A2 mRNA levels by 65% and 80%, respectively (Fig. 3.2.2A).

To examine whether the observed inhibition of the TCDD-mediated induction of CYP1A1 and CYP1A2 mRNAs by As(III) is further translated to the protein and activity levels, rat hepatocytes were treated for 24 h with increasing concentrations of As(III) in the presence of 1 nM TCDD. Figure 3.2.2B shows that TCDD alone caused 1800% increase in CYP1A1/1A2 protein. Interestingly, As(III) decreased the TCDD-mediated induction of CYP1A1/1A2 protein in a dose-dependent manner (Fig. 3.2.2B). As(III) at the concentration of 1 μM caused a non-significant decrease in TCDD-mediated induction of CYP1A1/1A2 protein expression levels by 8% (Fig. 3.2.2B). The maximum inhibition took place at the highest concentration tested, 10 μM, which caused a decrease in the TCDD-mediated induction of CYP1A1/1A2 protein expression levels by 48% (Fig. 3.2.2B). Of interest, As(III) decreased the TCDD-mediated induction of CYP1A1 and CYP1A2 catalytic activity levels in a dose-dependent manner, yet with a

more pronounced decrease in the activity levels than at the protein expression levels. For example, at the catalytic activity level, TCDD alone significantly induced CYP1A1 and CYP1A2 catalytic activities by 3300% and 600%, respectively. Of interest, when primary cells were co-exposed to As(III) and TCDD, As(III) significantly decreased the TCDD-mediated induction of CYP1A1 and CYP1A2 catalytic activities at 1, 5, and 10 μM As(III) by 60%, 86%, and 94%, respectively, for CYP1A1, and by 55%, 75%, and 90%, respectively, for CYP1A2 (Fig. 3.2.2C).

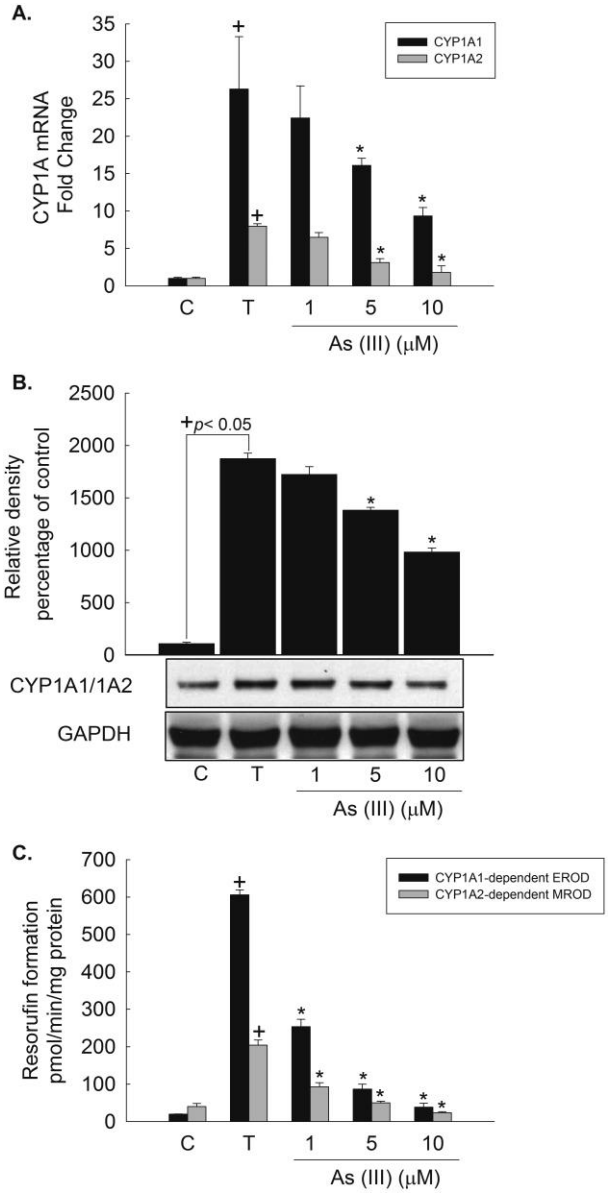

Fig. 3.2.2 Effect of As(III) on CYP1A1 and CYP1A2 mRNA, protein, and catalytic activity in isolated rat hepatocytes.

Freshly isolated rat hepatocytes were treated with As(III) (1, 5, and 10 μM) in the presence of 1 nM TCDD for 6 h for mRNA or 24 h for protein and catalytic activity. **(A)** CYP1A1 and CYP1A2 mRNA levels were quantified using RT-PCR and normalized to β-actin. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. **(B)** CYP1A1/2 protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to GAPDH signals, which was used as loading control. One of three representative experiments is shown. **(C)** CYP1A1-dependent EROD and CYP1A2-dpendent MROD activities were measured in intact living cells. Activities were measured using 7-ethoxyresorufin and 7-methoxyresorufin as substrates, respectively. Values are presented as mean ± SE. (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

3.2.3. EFFECT OF CO-EXPOSURE TO AS(III) ON RIF-INDUCED CYP3A23 AND CYP3A2 MRNA, PROTEIN, AND CATALYTIC ACTIVITY LEVELS.

To examine the effect of co-exposure to As(III) and Rif on CYP3A23 and CYP3A2 mRNA levels, rat hepatocytes were treated for 24 h with As(III) (1-10 μM) in the presence of 25 μM Rif (Fig. 3.2.3A). Thereafter, CYP3A23 and CYP3A2 mRNA levels were assessed using real-time PCR. Rif alone caused 215% increase in both CYP3A23 and CYP3A2 mRNA levels. When the cells were co-exposed to As(III) and Rif, As(III) decreased the Rif-mediated induction of both CYP3A23 and CYP3A2 mRNA levels in a dose-dependent manner (Fig. 3.2.3A). Initially, As(III) at the concentration of 1 μM caused a non-significant decrease in Rif-mediated induction of CYP3A23 and CYP3A2 mRNA levels by 10% and 4%, respectively. The maximum inhibition took place at the highest concentration tested, 10 μM, which caused a decrease in the Rif-mediated induction of CYP3A23 and CYP3A2 mRNA levels by 60% and 65%, respectively (Fig. 3.2.3A).

In order to determine whether the observed inhibition of the Rif-mediated induction of CYP3A23 and CYP3A2 mRNAs by As(III) is further translated to the protein and activity levels, rat hepatocytes were treated for 48 h with As(III) (1-10 μM) in the presence of 25 μM Rif. Figure 3.2.3B shows that Rif alone caused 263% increase in CYP3A protein. As(III) at the concentration of 1 μM did not affect Rif-mediated induction of CYP3A protein. The maximum inhibition took place at the highest concentration tested, 10 μM, which caused a decrease in the Rif-mediated induction of CY3A protein expression level by 55% (Fig. 3.2.3B). At the catalytic activity level, Rif alone significantly induced CYP3A catalytic activity by 160%. Of interest, when primary cells were co-exposed to As(III) and Rif, As(III) decreased the Rif-mediated induction of CYP3A catalytic activity in a dose-dependent manner, yet with a more pronounced decrease in the activity levels than in the protein expression levels (Fig. 3.2.3C). For example,

As(III) significantly decreased the Rif-mediated induction of CYP3A catalytic activity at 1, 5, and 10 μM As(III) by 55%, 75%, and 91%, respectively (Fig. 3.2.3C).

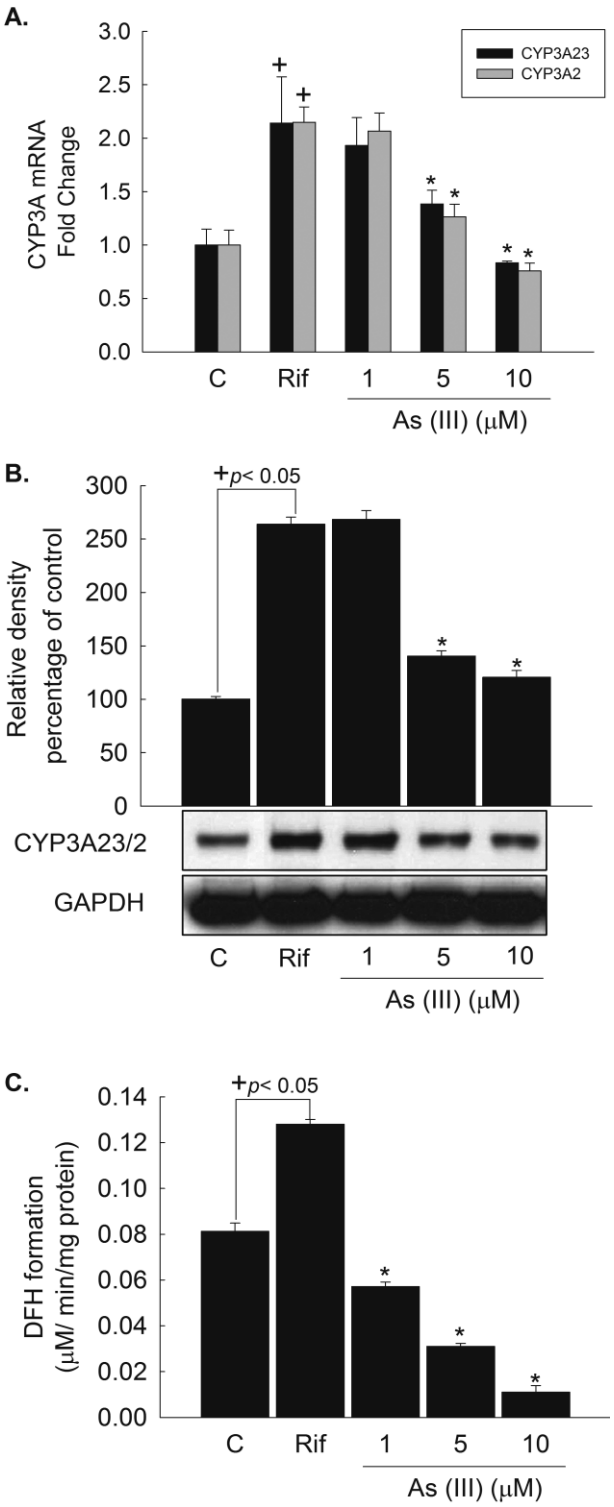

Fig. 3.2.3 Effect of As(III) on CYP3A23 and CYP3A2 mRNA, protein, and catalytic activity in isolated rat hepatocytes.

Freshly isolated rat hepatocytes were treated with As(III) (1, 5, and 10 μM) in the presence of 25 μM Rif for 24 h for mRNA or 48 h for protein and catalytic activity. **(A)** CYP3A23 and CYP3A2 mRNA levels were quantified using RT-PCR and normalized to β-actin. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. **(B)** CYP3A protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to GAPDH signals, which was used as loading control. One of three representative experiments is shown. **(C)** CYP3A activity was measured in intact living cells. Activities were measured using DFB as a substrate. Values are presented as mean ± SE. (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective rifampin (Rif) treatment.

3.2.4. EFFECT OF CO-EXPOSURE TO AS(III) AND TCDD ON THE LEVELS OF AHR PROTEIN AND PXR PROTEIN.

In an effort to determine whether As(III) interferes with the nuclear translocation of AhR to the nucleus, we examined the potential effect of As(III) on TCDD-induced translocation of AhR to the nucleus using Western blot analysis. For this purpose, rat hepatocytes were treated with vehicle, As(III), TCDD, and TCDD plus As(III) for 2 h, followed by extraction of nuclear extracts. Our results showed that As(III) alone did not affect the nuclear accumulation of AhR. On the other hand, TCDD increased the nuclear accumulation of AhR by 266% (Fig. 3.2.4A). Interestingly, we found that As(III) was able to significantly inhibit the TCDD-induced nuclear accumulation of AhR by 47%, suggesting that As(III) inhibits the nuclear accumulation through either decreasing the AhR nuclear translocation or through increasing its degradation.

To determine whether As(III) inhibited the AhR nuclear accumulation due to increasing its degradation, we examined the effect of co-exposure to As(III) and TCDD on the AhR protein using total cell lysate of rat hepatocytes treated for 24 h. Our results showed that AhR is not altered by As(III) treatment (Fig. 3.2.4B). On the other hand, TCDD in the absence and presence of As(III) decreased the total cellular AhR by 80% and 78%, respectively (Fig. 3.2.4A).

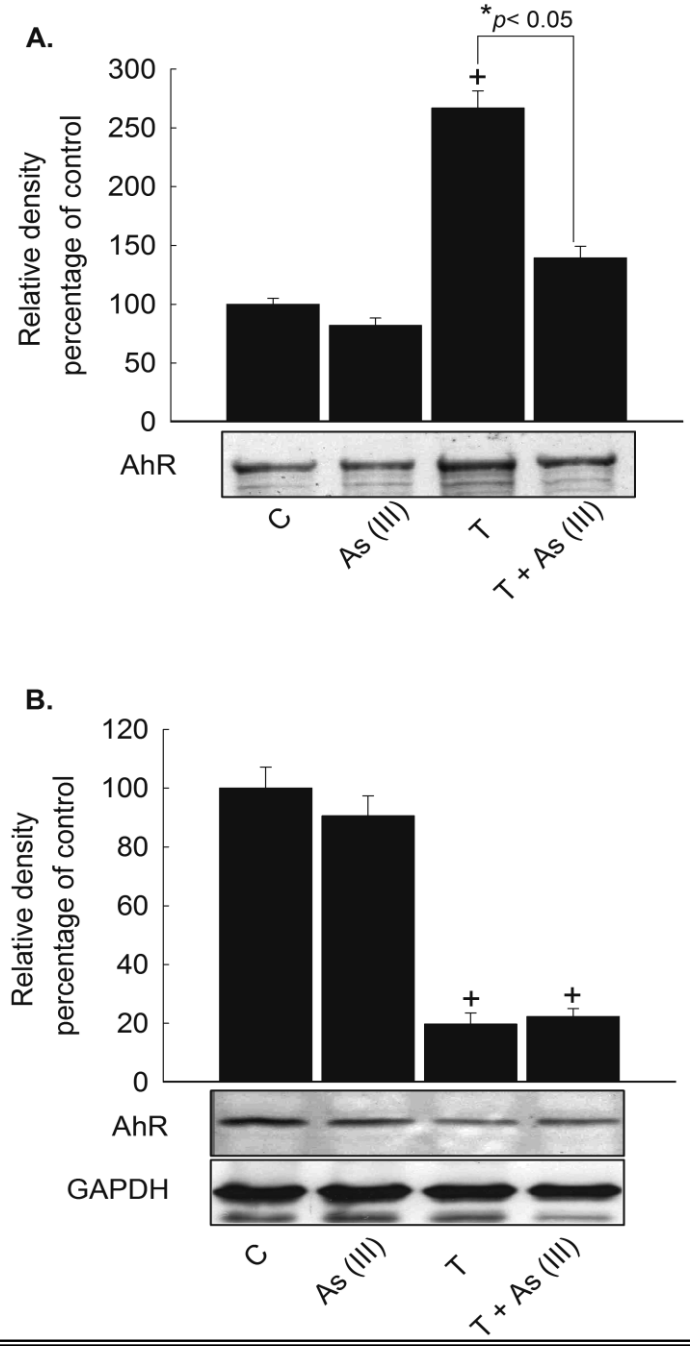

Fig. 3.2.4 Effect of co-exposure to As(III) and TCDD on AhR protein.
Freshly isolated rat hepatocytes were treated for 2 h for nuclear protein **(A)** or for 24 h for total cell lysate **(B)** with vehicle or 5 μM As(III) in the absence and presence of 1 nM TCDD. AhR protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to GAPDH signals (not shown), which was used as loading control. One of three representative experiments blots is shown. (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T).

In an attempt to investigate whether As(III) interferes with the nuclear translocation of PXR to the nucleus, we examined the potential effect of As(III) on Rif-induced translocation of PXR to the nucleus using Western blot analysis. For this purpose, rat hepatocytes were treated with vehicle, As(III), Rif, and Rif plus As(III) for 2 h, followed by extraction of nuclear extracts. Our results showed that As(III) alone significantly decreased the nuclear accumulation of PXR by 78% compared to control (Fig. 3.2.5A). In contrast Rif increased the nuclear accumulation of PXR by 317% compared to control (Fig. 3.2.5A). Importantly, we found that As(III) was also able to significantly inhibit the Rif-induced nuclear accumulation of PXR by 83%, suggesting that As(III) inhibits the nuclear accumulation through either decreasing the PXR nuclear translocation or through increasing its degradation. To test whether As(III) inhibited the PXR nuclear accumulation due to increasing its degradation, we examined the effect of co-exposure to As(III) and Rif on the PXR protein using total cell lysate of rat hepatocytes treated for 24 h. Our results showed that PXR protein was not affected by any of the treatments tested (Fig. 3.2.5B).

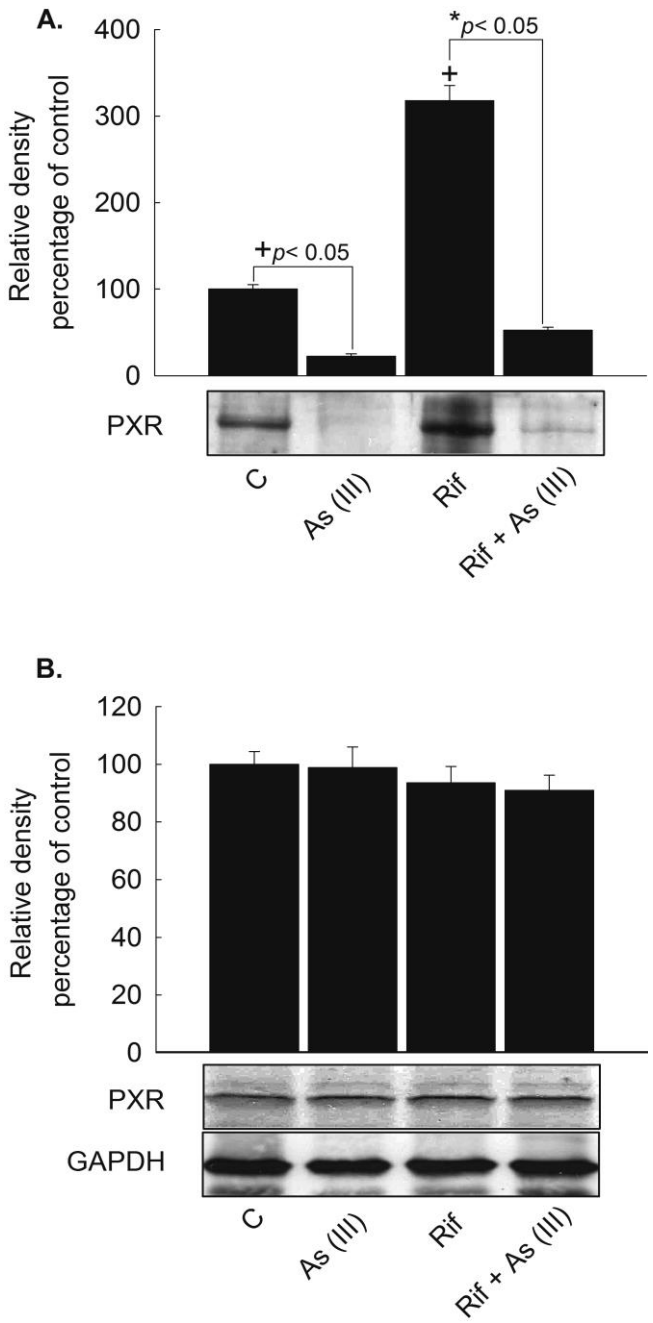

Fig. 3.2.5 Effect of co-exposure to As(III) and TCDD on PXR protein.
Freshly isolated rat hepatocytes were treated for 2 h for nuclear protein **(A)** or for 24 h for total cell lysate **(B)** with vehicle or 5 μM As(III) in the absence and presence of 25 μM Rif. PXR protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to GAPDH signals (not shown), which was used as loading control. One of three representative experiments blots is shown. (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective rifampin (Rif).

3.2.5. TRANSCRIPTIONAL INHIBITION OF CYP1A1 AND CYP1A2 GENE EXPRESSION BY AS(III).

To examine the possibility that As(III) interferes with the nuclear binding of the transformed AhR to the XRE, we examined the potential effect of As(III) on TCDD-induced translocation of AhR to the nucleus and the subsequent binding to XRE, the promoter sequence of both CYP1A1 and CYP1A2, by EMSA. For this purpose, rat hepatocytes were treated with vehicle, As(III), TCDD, or As(III) plus TCDD for 2 h, followed by extraction of nuclear extracts. Extracts from vehicle- and TCDD-treated cells were used as negative and positive controls, respectively. Figure 3.2.6A shows that As(III) alone did not induce AhR/Arnt/XRE complex formation, as shown by the intensity of the bands. In contrast, TCDD significantly increased AhR/Arnt/XRE binding (Lane 3). In addition, As(III) partially decreased the TCDD-induced nuclear formation of AhR/Arnt/XRE complex (lane 4). The specificity of the TCDD-induced AhR/Arnt heterodimer binding to XRE was confirmed by competition assay using 100-fold M excess of unlabelled XRE (Lane 5) and 0.6 μM Anti-Arnt antibody (super-shift); although no super-shift was seen, a loss of signal in the presence of antibody was achieved (Lane 6).

The ability of As(III) to inhibit the nuclear accumulation of AhR with a subsequent decrease in its binding to the XRE prompted us to investigate its effect on the AhR associated proteins HSP90 and XAP2. Therefore, we immunoprecipitated AhR protein from cytosolic and nuclear fractions to examine its localization, and to examine whether As(III) would affect the binding of AhR to HSP90 or XAP2 in these fractions. If As(III) was able to inhibit the nuclear accumulation of AhR, then we would expect it would reside in the cytoplasm since As(III) did not cause any degradation of total AhR protein. Our results show that As(III) decreased the nuclear accumulation of AhR in the absence and presence of TCDD (Fig. 3.2.6B). Importantly, HSP90 and XAP2 proteins coprecipitated with AhR from the cytosolic fraction, and coprecipitation was intensified by exposure to As(III), indicating that As(III) was able to stabilize the binding of AhR to HSP90 and XAP2 in the absence and presence of TCDD (Fig.

3.2.6B). As expected, almost no HSP90 or XAP2 coprecipitated with AhR from the nuclear fractions (Fig. 3.2.6B).

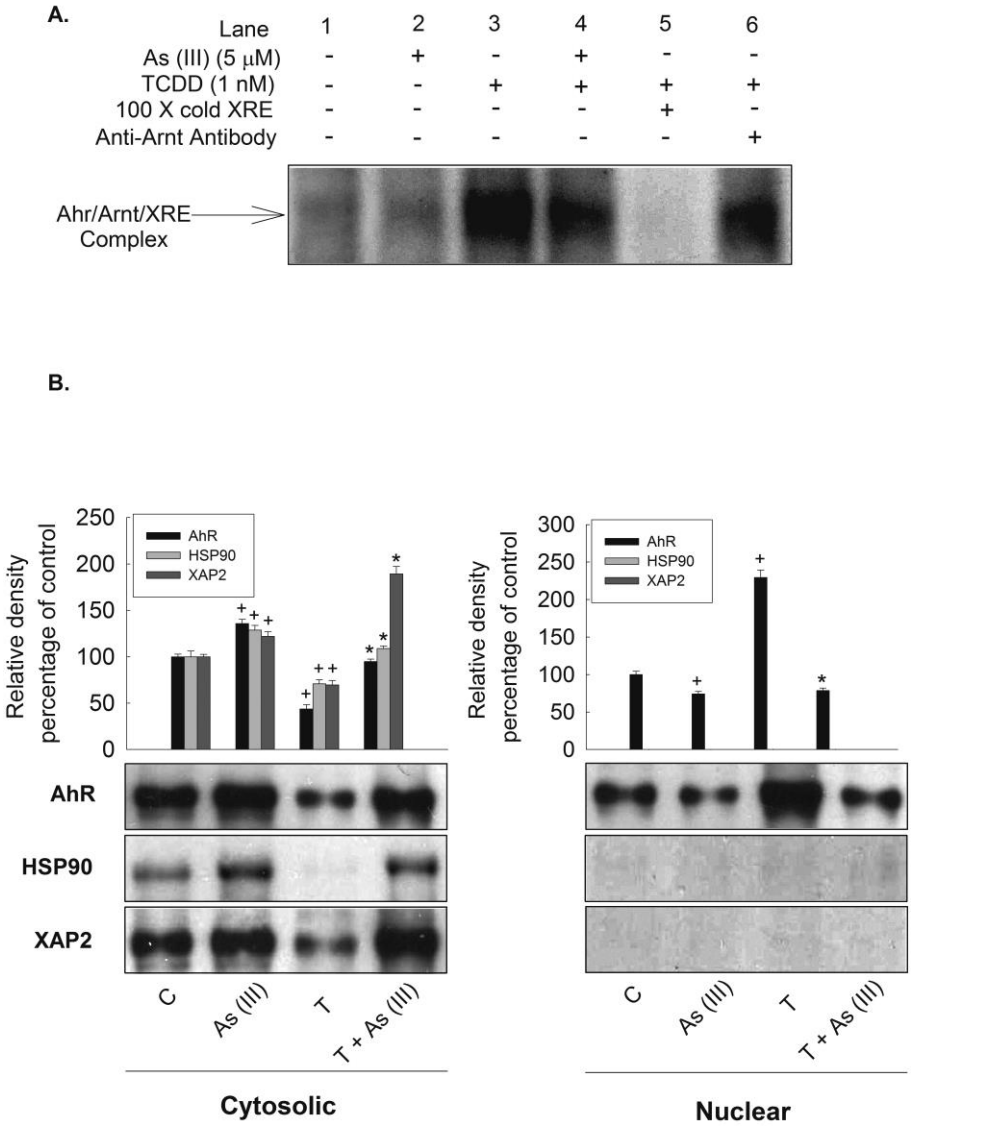

Fig. 3.2.6 Effect of As(III) on AhR/Arnt/XRE binding and AhR/HSP90/XAP2 binding.

(A) Nuclear proteins were mixed with [γ^{32}P]-labeled XRE, and the formation of AhR/ARNT/XRE complexes was analyzed by EMSA. The specificity of binding was determined by incubating the protein treated with TCDD with 100-fold molar excess of cold XRE or 0.6 μM Anti-Arnt antibody. The arrow indicates the specific shift representing the AhR/Arnt/XRE complex. This pattern of AhR alteration was observed in three separate experiments, and only one blot is shown. **(B)** AhR protein was immunoprecipitated from cytosolic and nuclear fractions of treated primary hepatocytes. Immunoprecipitates were then analyzed by Western blot analyses. AhR, HSP90, or XAP2 proteins were detected using the enhanced chemiluminescence method. One of three representative experiments is shown as blots. (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T).

3.2.6. EFFECT OF CO-EXPOSURE TO AS(III) AND TCDD OR RIF ON HO-1 MRNA AND TOTAL CELLULAR HEME CONTENT.

The fact that the effect of As(III) on the activity level was more pronounced than the mRNA or protein expression levels prompted us to probe the role of HO-1 in As(III)-mediated inhibition of the TCDD- and Rif-mediated induction of CYP1A1/1A2 and CYP3A23/3A2. Therefore, we examined the effect of As(III) on HO-1 mRNA, a rate-limiting enzyme of heme degradation. For this purpose, rat hepatocytes were treated with 5 μM As(III) in the absence and presence of 1 nM TCDD or 25 μM Rif. Thereafter, HO-1 mRNA was measured using real-time PCR. Figure 3.2.7A shows that As(III) alone increased HO-1 mRNA levels by 1000% compared to control. On the other hand, TCDD or Rif alone did not alter HO-1 mRNA level. Co-exposure to As(III) and TCDD or Rif significantly increased the HO-1 mRNA levels by 950% and 920%, respectively, which was not significantly different from As(III) alone (Fig. 3.2.7A).

To further illustrate the role of HO-1 in the decreased catalytic activity of P450s, we measured the total cellular heme content. Our results showed that treatment of cells with TCDD or Rif alone did not affect total cellular heme content. On the other hand, 5 μM As(III) alone or in the presence of 1 nM TCDD or 25 μM Rif caused a 36%, 47%, 50% decrease in the total cellular heme content, respectively (Fig. 3.2.7B).

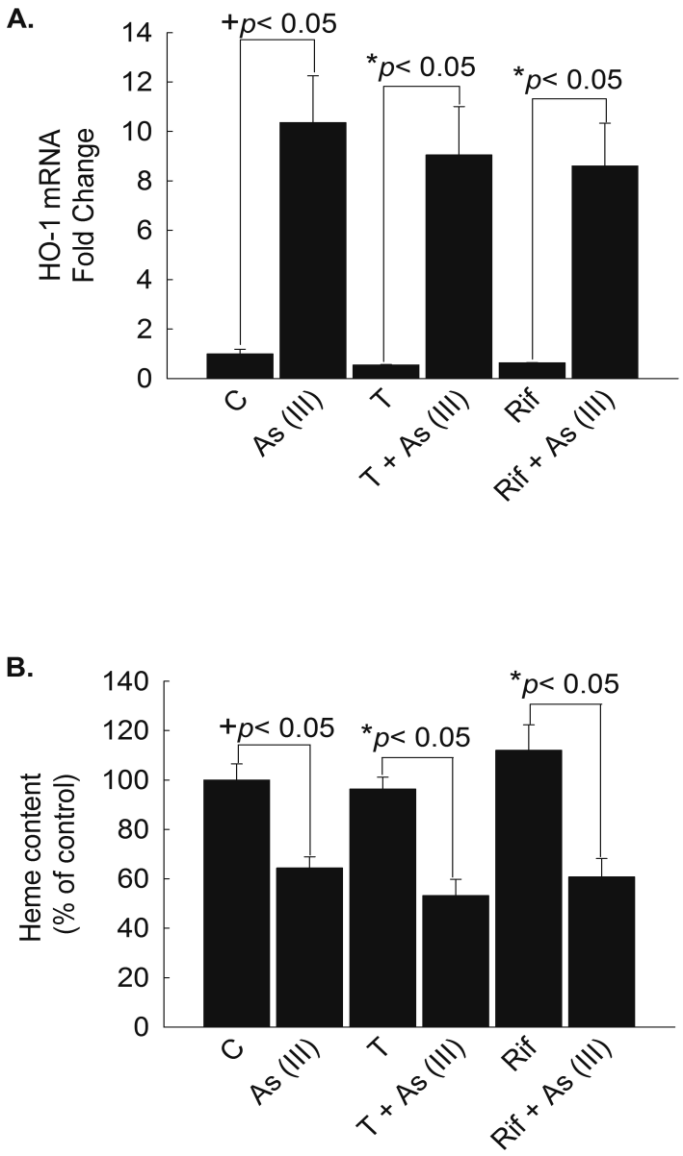

Fig. 3.2.7 Effect of As(III) on HO-1 mRNA and total cellular heme content. **(A)** HO-1 mRNA levels were quantified using RT-PCR and normalized to β-actin. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. **(B)** Total heme content in freshly isolated rat hepatocytes was calculated by measuring the conversion of heme to protoporphyrin IX by oxalic acid which was measured fluorometrically. Cellular heme content was calculated as a percent of serum-free medium treated cells following normalization with cellular protein. (+) $P < 0.05$, compared to control (C) (concentration = 0 μM); (*) $P < 0.05$, compared to respective inducer.

3.2.7. THE EFFECT OF SNMP AND EXOGENOUS HEME ON THE POST-TRANSLATIONAL MODIFICATION OF CYP1A1, CYP1A2, AND CYP3A CATALYTIC ACTIVITY BY AS(III)

The fact that As(III)-mediated inhibition of CYP1A1, CYP1A2 and CYP3A at the catalytic activity level is associated with elevated HO-1 mRNA and decreased total cellular heme content prompted us to investigate the possible outcome of inhibiting HO-1 using SnMP, or supplying external heme. For this purpose rat hepatocytes were co-exposed to 5 μM As(III) and 1 nM TCDD or 25 μM Rif in the absence and of 5 μM SnMP. Our results showed that SnMP alone did not alter the CYP1A1, CYP1A2, or CYP3A catalytic activities (Fig. 3.2.8A, 3.2.8B, and 3.2.8C). Similarly, the induced CYP1A1, CYP1A2, and CYP3A catalytic activities were not affected by SnMP treatment. On the other hand, As(III) at the concentration of 5 μM decreased the inducible CYP1A1, CYP1A2, and CYP3A catalytic activities by 40%, 51%, and 80%, respectively. Upon treating the cells with SnMP in the presence of both As(III) and the respective inducer, there was a partial restoration of the As(III)-mediated inhibition of CYP1A1, CYP1A2, and CYP3A catalytic activities to 70%, 83%, and 84%, respectively compared to TCDD alone (Fig. 3.2.8A, 3.2.8B, and 3.2.8C).

In an attempt to examine whether the presence of external heme will restore the As(III)-mediated decrease of P450 activity, rat hepatocytes were co-exposed to 5 μM As(III) and 1 nM TCDD or 25 μM Rif in the absence and of 80 μM hemin, a precursor of heme. Our results showed that hemin alone did not affect CYP1A1, CYP1A2, or CYP3A catalytic activities (Fig. 3.2.8A, 3.2.8B, and 3.2.8C). Similar to SnMP, the induced CYP1A1, CYP1A2, and CYP3A catalytic activities were not affected by hemin treatment. Upon supplementing the cells with hemin in the presence of both As(III) and the respective inducer, there was a partial restoration of the As(III)-mediated inhibition of CYP1A1, CYP1A2, and CYP3A catalytic activities to 50%, 68%, and 64%, respectively compared to TCDD alone (Fig. 3.2.8A, 3.2.8B, and 3.2.8C).

With the fact that SnMP and heme were able to partially restore the As(III)-mediated inhibition of CYP1A1, CYP1A2, and CYP3A catalytic activities, there was still a possibility that SnMP or heme might reverse the inhibitory effect of As(III) on these P450s catalytic activities by inducing their mRNA levels. Therefore, we examined the effect of SnMP and heme on CYP1A1, CYP1A2, CYP3A23, and CYP3A2 mRNA levels. SnMP and heme alone or in the presence of As(III), TCDD, Rif, As(III) plus TCDD, or As(III) plus Rif did not affect any of the P450s mRNA levels with all treatments, thus eliminating this possibility (Fig. 3.2.9A, 3.2.9B, 3.2.9C, 3.2.9D).

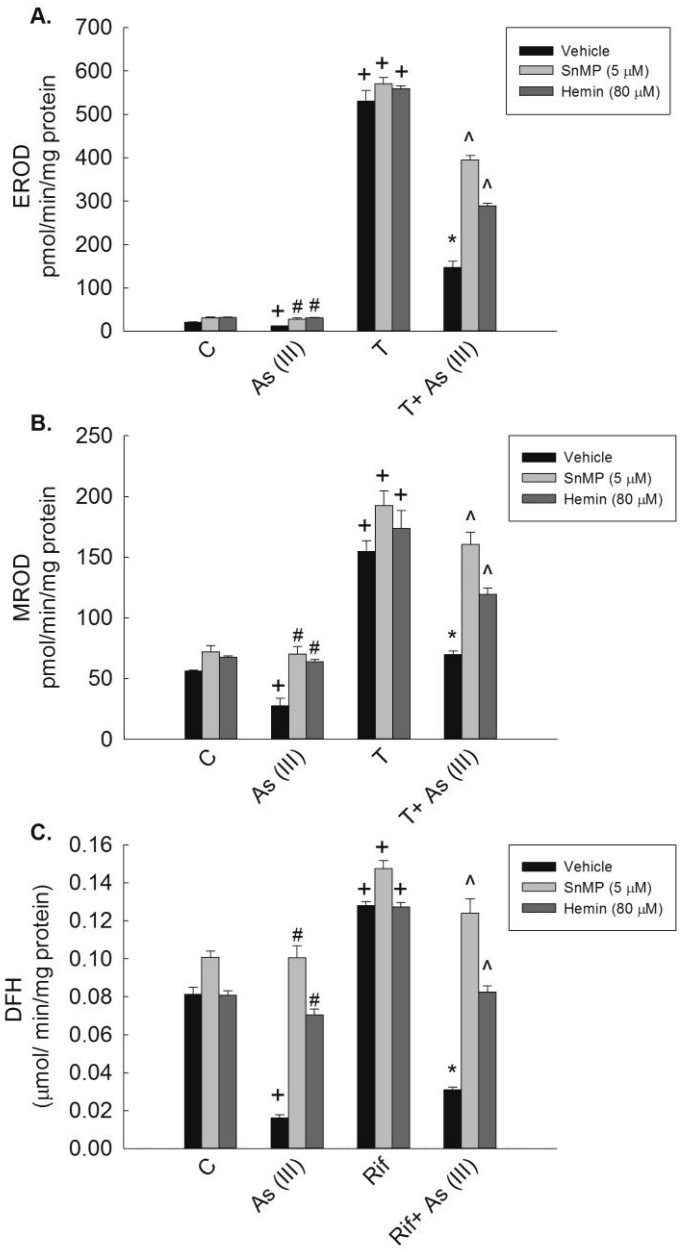

Fig. 3.2.8 Effect of SnMP as a competitive inhibitor of HO-1, or hemin, as heme precursor, on CYP1A1, CYP1A2, and CYP3A catalytic activities.
(A) EROD activity was measured using 7-ethoxyresorufin as a substrate. **(B)** MROD activity was measured using 7-methoxyresorufin as a substrate. **(C)** CYP3A activity was using DFB as a substrate. Values are presented as mean ± SE. (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective inducer treatment, (#) $P < 0.05$ compared to As(III) only treatment, (^) $P < 0.05$ compared to As(III) + respective inducer treatment.

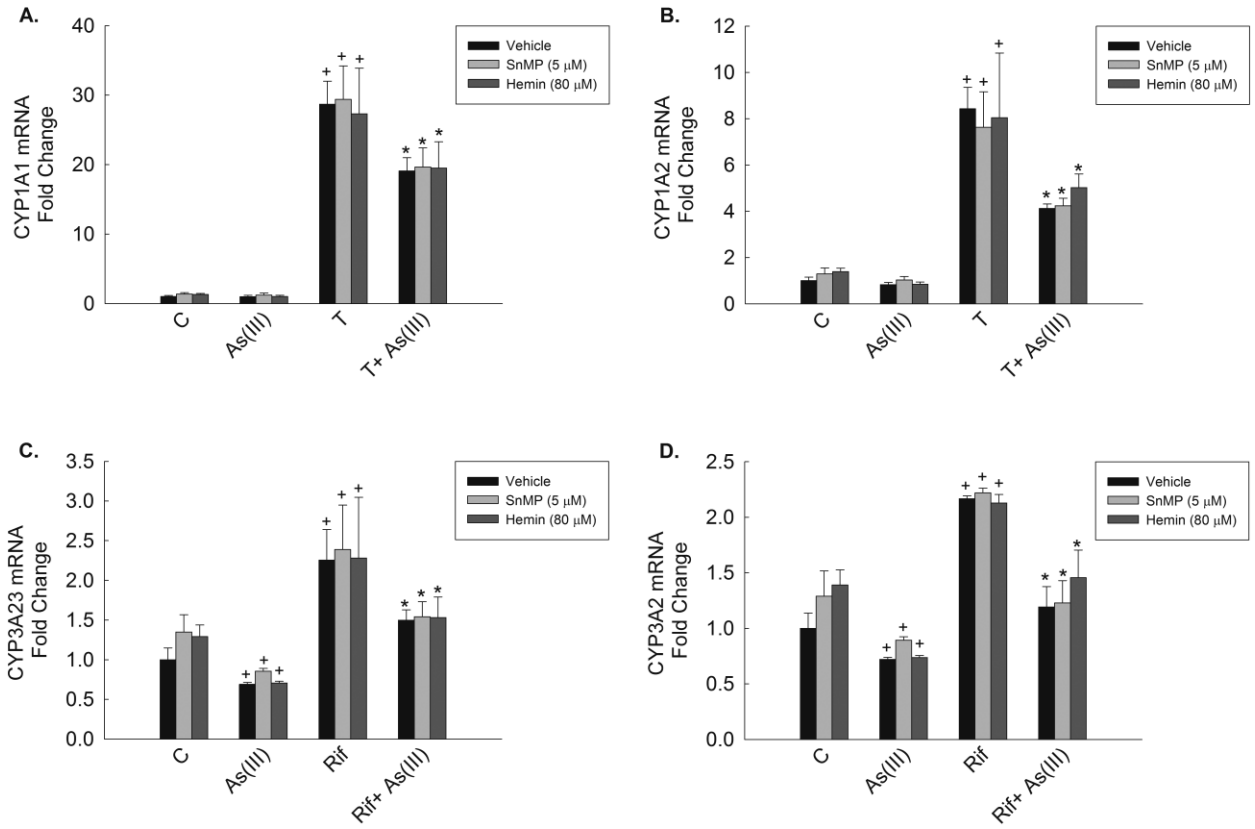

Fig. 3.2.9 Effect of SnMP as a competitive inhibitor of HO-1, or hemin, as a heme precursor on CYP1A1, CYP1A2, and CYP3A mRNA levels.

(A) CYP1A1 **(B)** CYP1A2 **(C)** CYP3A23 **(D)** CYP3A2mRNA levels were quantified using RT-PCR and normalized to β-actin. Values are presented as mean ± SE. (+) $P < 0.05$, compared to respective control treatment (C); (*) $P < 0.05$, compared to respective inducer treatment.

3.3. DIFFERENTIAL MODULATION OF ARYL HYDROCARBON RECEPTOR REGULATED ENZYMES BY ARSENITE IN THE KIDNEY, LUNG, AND HEART OF C57BL/6 MICE

3.3.1. DIFFERENTIAL EXPRESSION OF AHR-REGULATED GENES AND HO-1 IN THE KIDNEY, LUNG, AND HEART

All of the genes examined were constitutively expressed in the kidney, lung and heart at different levels (Fig. 3.3.1 and Fig. 3.3.2). Cyp1a1 expression differed greatly across the extrahepatic organs with the lung, being the highest expressing tissue followed by the kidney and lastly the heart (Fig. 3.3.1A). Similarly, Cyp1a2 expression showed a similar trend to that of Cyp1a1, with the lung being the highest expressing tissue followed by the kidney and lastly the heart (Fig. 3.3.1B). To the contrast to Cyp1a1 and Cyp1a2, Cyp1b1 was highly expressed in both kidney and lung, while the heart was the least organ to express Cyp1b1 (Fig. 3.3.1C). The phase II AhR-regulated genes showed a different expression pattern across tissues than phase I AhR-regulated genes. For example, Nqo1 gene expression was the highest in the kidney followed by the heart and lastly the lung (Fig. 2A). On the contrary, Gsta1 expression was the highest in the lung followed by the kidney and lastly the heart (Fig. 3.3.2B). HO-1 is mainly regulated by the nuclear factor-erythroid 2 p45-related factor 2 (Nrf2)/antioxidant responsive element (ARE) signaling pathway, and its expression was the highest in the kidney followed by the lung and the heart (Fig. 3.3.2C).

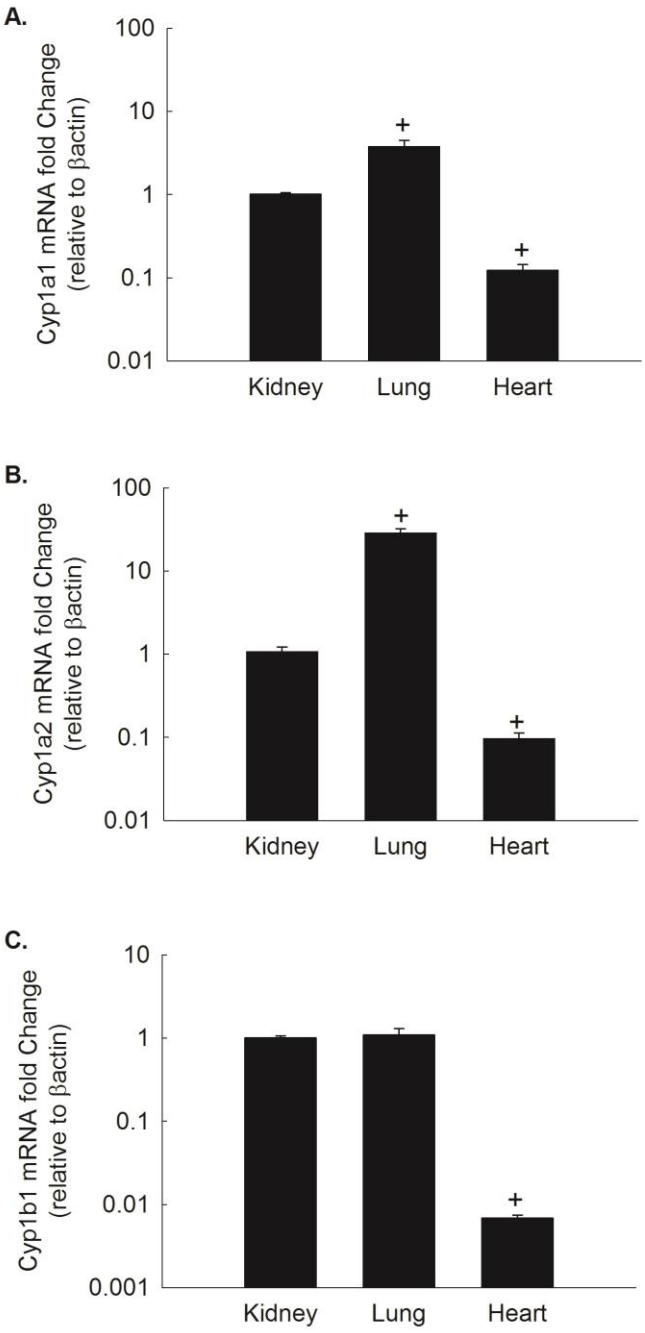

Fig. 3.3.1 Constitutive expression of phase I AhR-regulated genes in different tissues.

Total RNA was isolated from kidney, lung, and heart of 6 h time point control animals. The relative expression of Cyp1a1 **(A)**, Cyp1a2 **(B),** and Cyp1b1 **(C)** was determined by reverse transcription followed by real-time PCR. The data were analyzed using the relative gene expression method. The data were normalized to the endogenous reference gene β-actin and relative to a calibrator (kidney). Results are presented as mean ± S.E.M. (*n*=6).

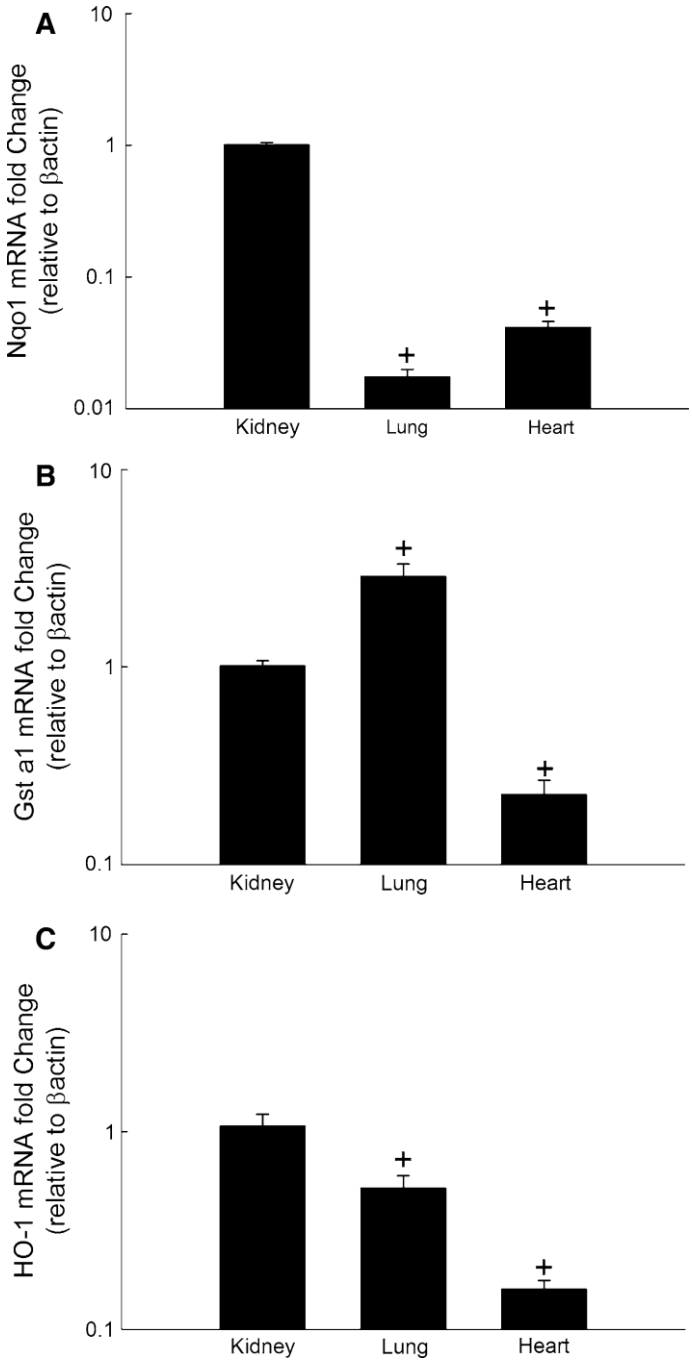

Fig. 3.3.2 Constitutive expression of phase II AhR-regulated genes and HO-1 in different tissues.

Total RNA was isolated from kidney, lung, and heart of 6 h time point control animals. The relative expression of Nqo1 **(A)**, Gsta1 **(B)**, and HO-1 **(C)** was determined by reverse transcription followed by real-time PCR. The data were analyzed using the relative gene expression method. The data were normalized to the endogenous reference gene β-actin and relative to a calibrator (kidney). Results are presented as mean ± S.E.M. (*n*=6).

3.3.2. EXPRESSION OF CYP1A1, CYP1A2, CYP1B1, NQO1, GSTA1, AND HO-1 MRNA IN THE KIDNEY, LUNG, AND HEART

To examine the constitutive expression of different AhR-regulated genes in a single tissue, total RNA was isolated from kidney, lung, and heart of male C57Bl/6 mice, and steady-state mRNA for all tested genes were determined by real-time PCR. All of the examined genes were found to be constitutively expressed in the kidney, lung and heart at different levels (Fig. 3.3.3A**,** 3.3.3B and 3.3.3C). Furthermore, Cyp1a2 was the lowest expressed genes in the three examined tissues and was considered as a calibrator.

In the kidney, Nqo1 was the most highly expressed gene, 6250-fold higher than calibrator (Fig. 3.3.3A). Cyp1a1 and HO-1 were also highly expressed genes with 250- and 2200-fold higher than calibrator, respectively (Fig. 3.3.3A). Cyp1b1 and Gsta1 were moderately expressed genes with 72- and 22-fold higher than calibrator, respectively (Fig. 3.3.3A). In contrast to the kidney, lung Nqo1 was low expressed with 2.4-fold higher than calibrator. However, Cyp1a1 and HO-1 were the most highly expressed genes with 1313- and 305-fold higher than calibrator, respectively (Fig. 3.3.3B). Lung Cyp1b1 and Gsta1 were low to moderate expressed genes with 1.4- and 3.2-fold higher than calibrator, respectively (Fig. 3.3.3B). Similar to the kidney, heart Nqo1 was a highly expressed gene with 6618-fold higher than calibrator (Fig. 3.3.3C). Furthermore, Cyp1a1 and HO-1 were also highly expressed genes with 2013- and 779-fold higher than calibrator, respectively (Fig. 3.3.3C). Cyp1b1 and Gsta1 were moderately expressed with 120- and 83-fold higher than calibrator, respectively (Fig. 3.3.3C).

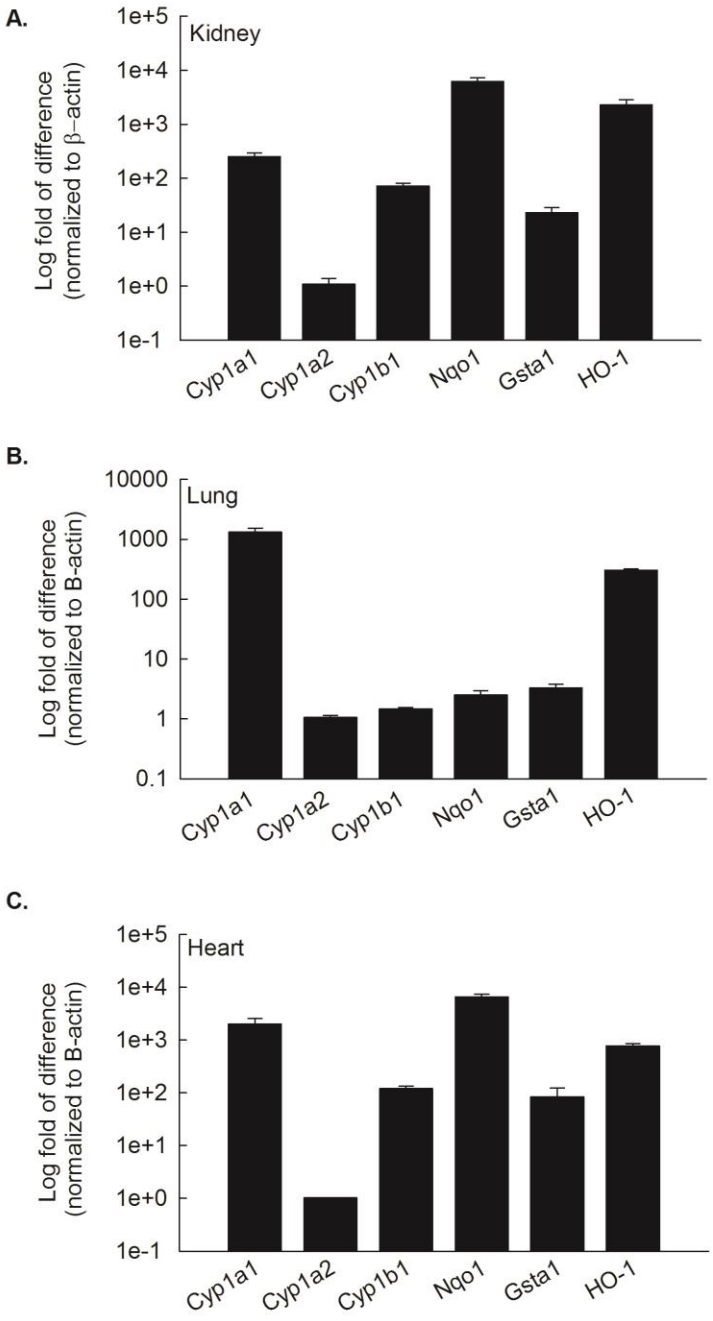

Fig. 3.3.3 Constitutive expression of AhR-regulated and HO-1 genes in the kidney, lung, and heart.

Total RNA was isolated from extrahepatic tissues 6 h and 24 h after treatment, and the relative expression of these genes in the kidney (A), lung (B), and heart (C) were determined by reverse transcription followed by real-time PCR. The data were analyzed using the relative gene expression method, which were normalized to the endogenous reference gene β-actin, and relative to a calibrator. The lowest expressed gene in each tissue was used as a calibrator. Results are presented as mean ± S.E.M. (*n*=6).

3.3.3. EFFECT OF CO-EXPOSURE TO AS(III) AND TCDD ON CYP1A1 AND CYP1A2 MRNA IN THE KIDNEY, LUNG, AND HEART OF C57BL6 MICE

At 6 h and 24h, As(III) alone significantly induced lung Cyp1a1 mRNA levels by 10- and 0.80-fold, respectively compared to control (Fig. 3.3.4A and 3.3.4B). In kidney and heart, As(III) alone failed to cause any significant changes in Cyp1a1 mRNA levels at 6 h and 24 h (Fig. 3.3.4A and 3.3.4B). TCDD alone significantly induced Cyp1a1 mRNA levels at 6 h in the kidney, lung, and heart by 880-, 180-, and 420-fold, respectively, and at 24 h by 270-, 80-, and 415-, respectively, compared to control (Fig. 3.3.4A and 3.3.4B). When animals were co-exposed to As(III) and TCDD, As(III) at 6 h significantly inhibited the TCDD-mediated induction of Cyp1a1 mRNA in the kidney and heart by 612- and 148-fold, respectively, compared to TCDD alone. On the other hand, As(III) at 6 h significantly potentiated the TCDD-mediated induction of Cyp1a1 mRNA in the lung by 109-fold compared to TCDD alone (Fig. 3.3.4A). As(III) at 24 h significantly potentiated kidney and lung Cyp1a1 mRNA levels by 719- and 23-fold, respectively, compared to TCDD alone, while it did not alter the TCDD-mediated induction of Cyp1a1 mRNA in the heart (Fig. 3.3.4B).

At 6 h and 24h, As(III) alone failed to significantly affect Cyp1a2 mRNA levels in kidney, lung, or heart (Fig. 3.3.5A and 3.3.5B). TCDD alone significantly induced Cyp1a2 mRNA levels at 6 h in the kidney, lung, and heart by 40-, 0.80-, and 12-fold, respectively, and at 24 h by 13-, 0.80-, and 12-fold, respectively, compared to control (Fig. 3.3.5A and 3.3.5B). When animals were co-exposed to As(III) and TCDD, As(III) at 6 h significantly inhibited the TCDD-mediated induction of Cyp1a2 mRNA in the kidney and heart by 25- and 8.50-fold, respectively, compared to TCDD alone. On the other hand, As(III) at 6 h did not alter the TCDD-mediated induction of Cyp1a2 mRNA in the lung (Fig. 3.3.5A). In contrast, As(III) at 24 h significantly potentiated kidney Cyp1a2 mRNA by 7-fold, compared to TCDD alone, while it did not alter the TCDD-mediated induction of Cyp1a2 mRNA in the lung and heart (Fig. 3.3.5B).

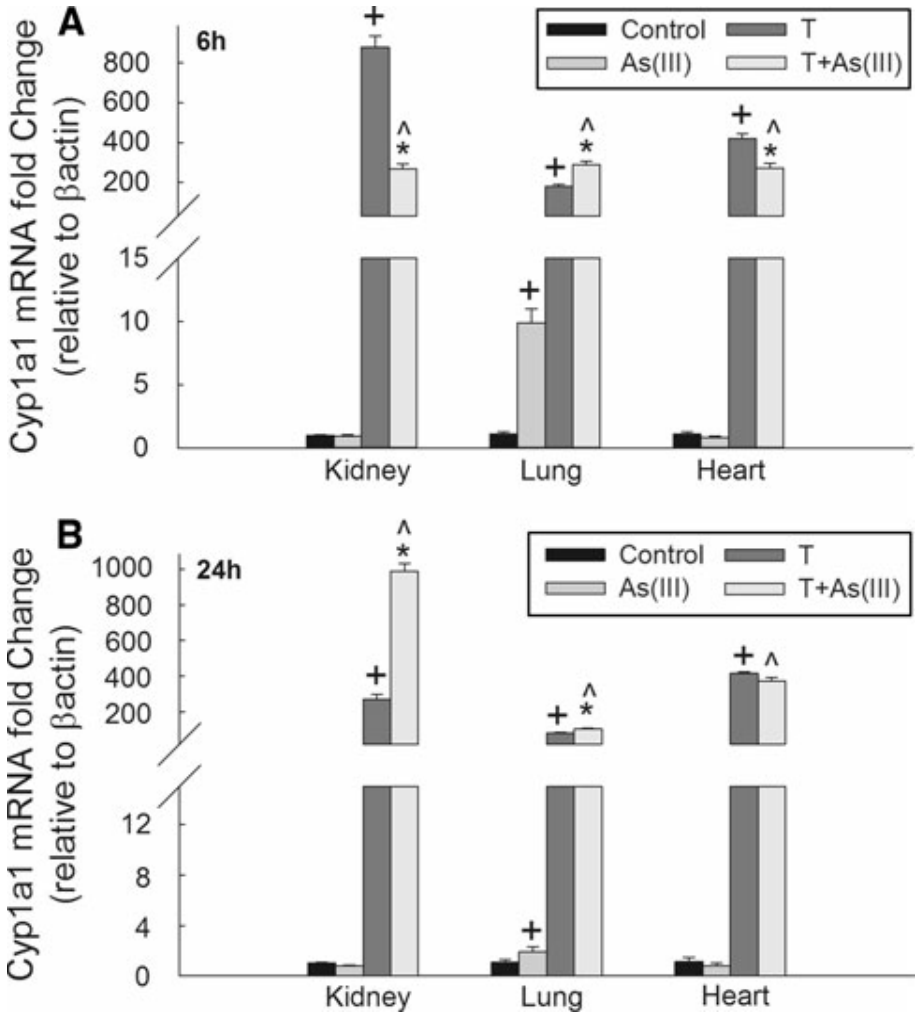

Fig. 3.3.4 Effect of co-exposure to As(III) and TCDD on Cyp1a1 mRNA in the kidney, lung, and heart of C57Bl6 mice.

Animals were injected i.p. with 12.5 mg/kg As(III) in the absence and presence of 15 µg/kg TCDD for 6h **(A)** and 24h **(B)**. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from kidney and the expression of Cyp1a1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. (+) $P < 0.05$, compared to control (untreated animals); (*) $P < 0.05$, compared to respective TCDD treatment; (^)$P < 0.05$, compared to respective As(III) treatment.

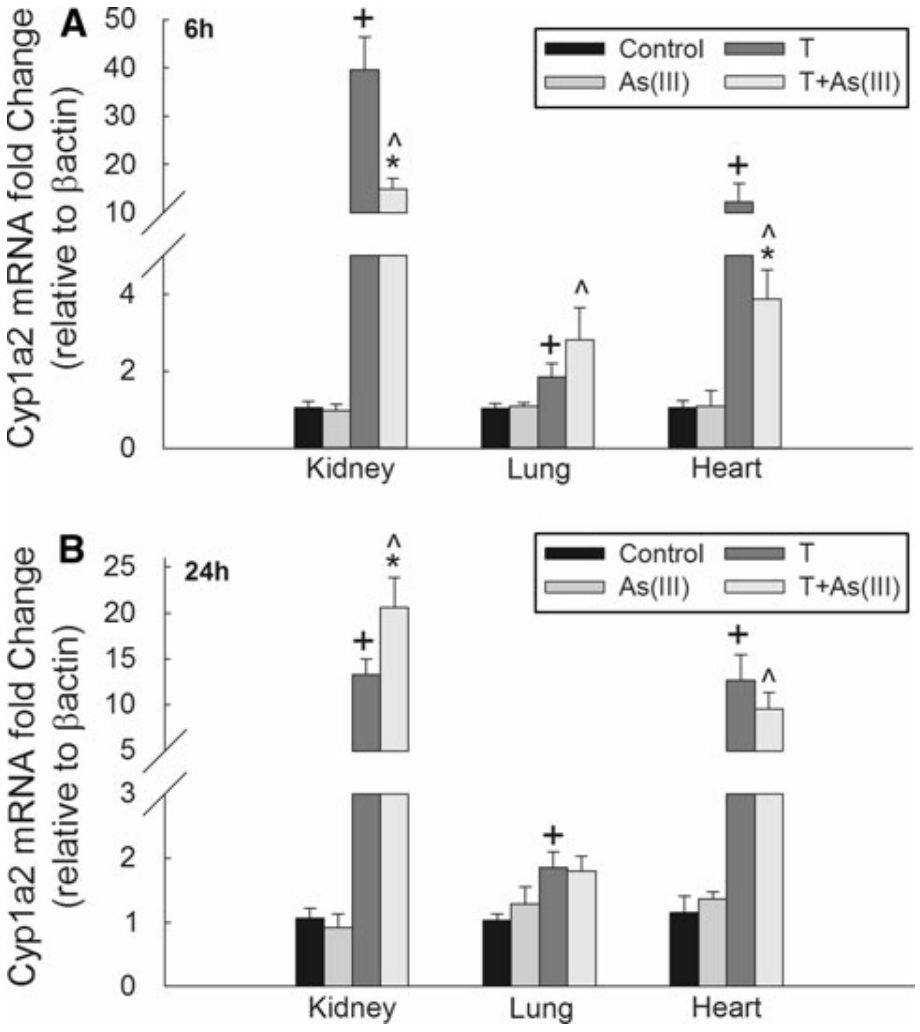

Fig. 3.3.5 Effect of co-exposure to As(III) and TCDD on Cyp1a2 mRNA in the kidney, lung, and heart of C57Bl6 mice.

Animals were injected i.p. with 12.5 mg/kg As(III) in the absence and presence of 15 µg/kg TCDD for 6 h **(A)** and 24 h **(B)**. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from kidney and the expression of Cyp1a2 was measured using real-time PCR. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. (+) $P < 0.05$, compared to control (untreated animals); (*) $P < 0.05$, compared to respective TCDD treatment; (^)$P < 0.05$, compared to respective As(III) treatment.

3.3.4. EFFECT OF CO-EXPOSURE TO AS(III) AND TCDD ON CYP1A PROTEIN EXPRESSION AND EROD AND MROD CATALYTIC ACTIVITY LEVELS IN THE KIDNEY AND LUNG OF C57BL/6 MICE

Our results showed that As(III) alone significantly induced lung Cyp1a protein expression levels by 2.30-fold compared to control (Fig. 3.3.6). TCDD alone significantly induced kidney and the lung Cyp1a protein expression levels by 7.50- and 11-fold, respectively, compared to control (Fig. 3.3.6). On the other hand when animals were co-exposed to As(III) and TCDD, As(III) significantly inhibited TCDD-mediated induction of kidney Cyp1a protein expression levels by 7-fold, while on the other hand it significantly potentiated the TCDD-mediated induction of lung Cyp1a protein expression levels by 138-fold compared to TCDD alone (Fig. 3.3.6).

At the catalytic activity levels, As(III) alone significantly inhibited the kidney EROD activity by 0.50-fold, while it significantly induced the lung EROD activity by 7-fold (Fig 3.3.7A). Similarly As(III) alone significantly inhibited the kidney MROD activity by 0.70-fold, while it significantly induced the lung MROD activity by 2.20-fold, respectively, compared to control (Fig 3.3.7B). TCDD alone significantly induced kidney and lung EROD activities by 11.50- and 13-, and kidney and lung MROD activities by 14- and 0.80-fold, respectively, compared to control (Fig. 3.3.7A and 3.3.7B). When animals were co-exposed to As(III) and TCDD, As(III) significantly inhibited TCDD-mediated induction of kidney EROD catalytic activity by 5.50-fold, while on the other hand it potentiated the TCDD-mediated induction of lung EROD by 127-fold respectively, compared to TCDD alone (Fig. 3.3.7A). Moreover, As(III) significantly inhibited the TCDD-mediated induction of kidney MROD catalytic activity by 21-fold, while on the other hand it potentiated the TCDD-mediated induction of lung MROD catalytic activity by 21-fold, respectively, compared to TCDD alone (Fig. 3.3.7B).

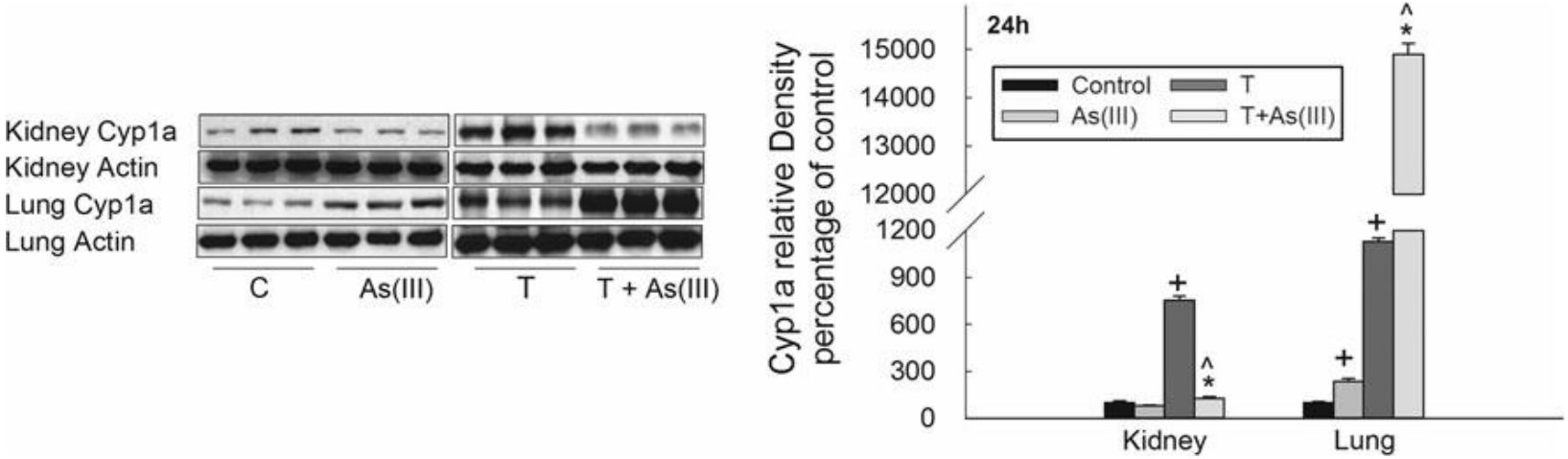

Fig. 3.3.6 Effect of co-exposure to As(III) and TCDD on Cyp1a protein expression levels in the kidney and lung of C57Bl/6 mice.

Kidney and lung microsomal proteins were isolated after 24 h of treatment. 30 μg of microsomal proteins were separated on a 10% SDS-PAGE. Proteins were detected using the enhanced chemiluminescence method. The graph represents the relative amount of protein normalized to β-actin signals (mean ± SEM, n = 6), and the results are expressed as percentage of the control values taken as 100%. (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment; (^)$P < 0.05$, compared to respective As(III) treatment.

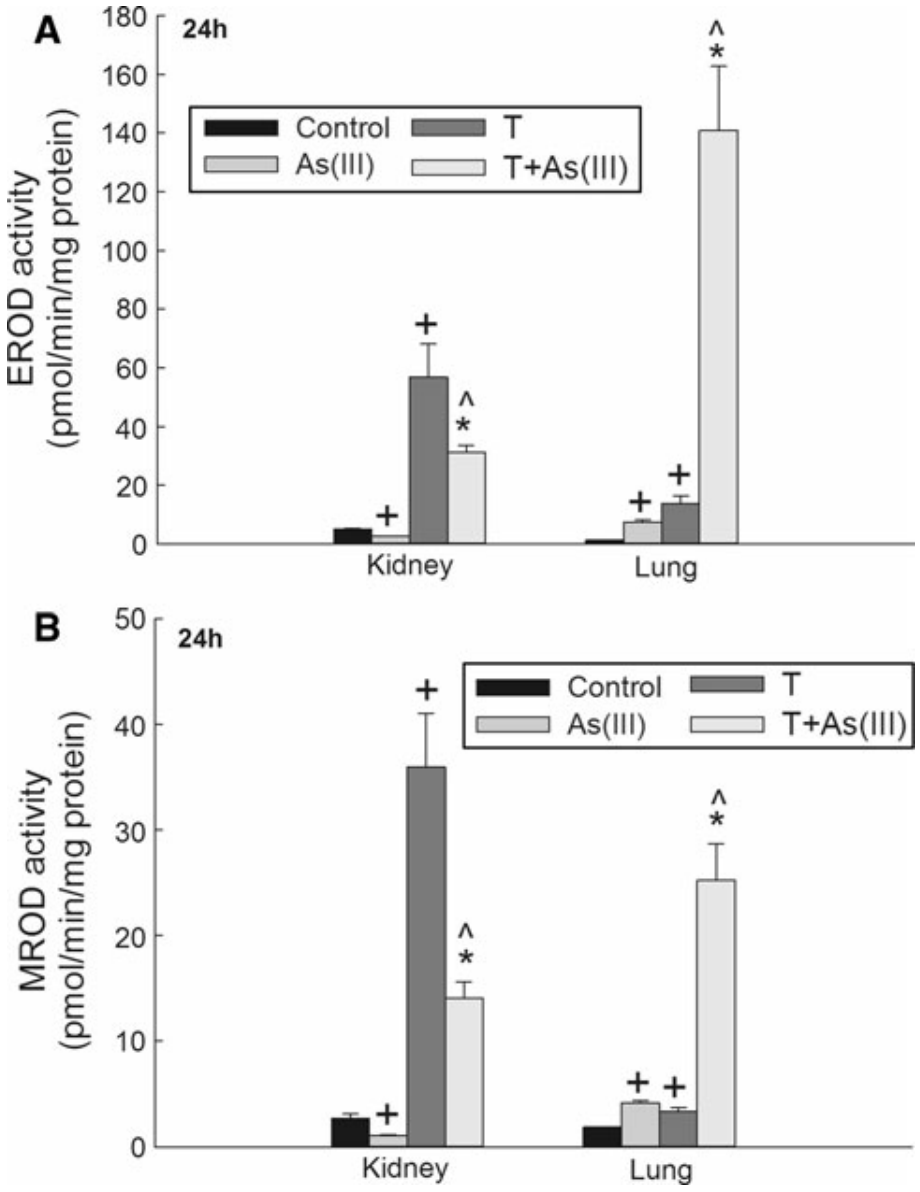

Fig. 3.3.7 Effect of co-exposure to As(III) and TCDD on EROD and MROD catalytic activity levels in the kidney and lung of C57Bl/6 mice.

Kidney and lung microsomal protein was isolated after 24 h of treatment. EROD and MROD activities were measured using 7-ethoxyresorufin and 7-methoxyresorufin as substrates, respectively. The reaction was started by the addition of 1 mM NADPH and lasted for 5 min for EROD and 10 min for MROD. The reaction was terminated by the addition of ice cold acetonitrile. Values are presented as mean ± SEM ($n = 6$). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment; (^)$P < 0.05$, compared to respective As(III) treatment.

3.3.5. EFFECT OF CO-EXPOSURE TO AS(III) AND TCDD ON NQO1 AND GSTA1 MRNA IN THE KIDNEY, LUNG, AND HEART OF C57BL6 MICE

At 6 h, As(III) alone significantly induced the kidney, lung, and heart Nqo1 mRNA levels by 3-, 4-, and 2-fold, respectively compared to control (Fig. 3.3.8A). At 24 h, As(III) alone significantly induced the kidney, lung, and heart Nqo1 mRNA levels by 1.50-, 1.40-, and 1.60-fold, respectively, compared to control (Fig. 3.3.8B). TCDD alone significantly induced Nqo1 mRNA levels at 6 h in the kidney, lung, and heart by 1.80-, 7.70-, and 1.70-fold, respectively, and at 24 h by 2-, 2-, and 1.60-fold, respectively, compared to control (Fig. 3.3.8A and 3.3.8B). When animals were co-exposed to As(III) and TCDD, As(III) at 6 h significantly potentiated the TCDD-mediated induction of Nqo1 mRNA in the kidney, lung, and heart by 4-, 5.70-, and 2-fold, respectively, compared to TCDD alone (Fig. 3.3.8A). Similarly, As(III) at 24 h significantly potentiated the kidney, lung, and heart Nqo1 mRNA levels by 2.29-, 1.30-, and 0.50-fold, respectively, compared to TCDD alone (Fig. 3.3.8B).

At 6 h, As(III) alone significantly induced the kidney, lung, and heart Gsta1 mRNA levels by 598-, 31-, and 49-fold, respectively compared to control (Fig. 3.3.9A). At 24 h, As(III) alone significantly induced Kidney, lung, and heart Gsta1 mRNA levels by 206-, 7-, and 10-fold, respectively, compared to control (Fig. 3.3.9B). TCDD alone significantly induced Gsta1 mRNA levels at 6 h in the kidney, lung, and heart by 8.90-, 2.20-, and 2.50-fold, respectively, respectively, compared to control (Fig. 3.3.9A). At 24 h, TCDD alone significantly induced Gsta1 mRNA levels in kidney and heart by 6.20- and 2.90-fold, respectively, compared to control, while it did not alter lung Gsta1 mRNA levels (Fig. 3.3.9B). When animals were co-exposed to As(III) and TCDD, As(III) at 6 h significantly potentiated the TCDD-mediated induction of Gsta1 mRNA in the kidney, lung, and heart by 890-, 27-, and 91-fold, respectively, compared to TCDD alone (Fig. 3.3.9A). Similarly, As(III) at 24 h significantly potentiated the kidney, lung, and

heart Gsta1 mRNA levels by 344-, 4.60-, and 21-fold, respectively, compared to TCDD alone (Fig. 3.3.9B).

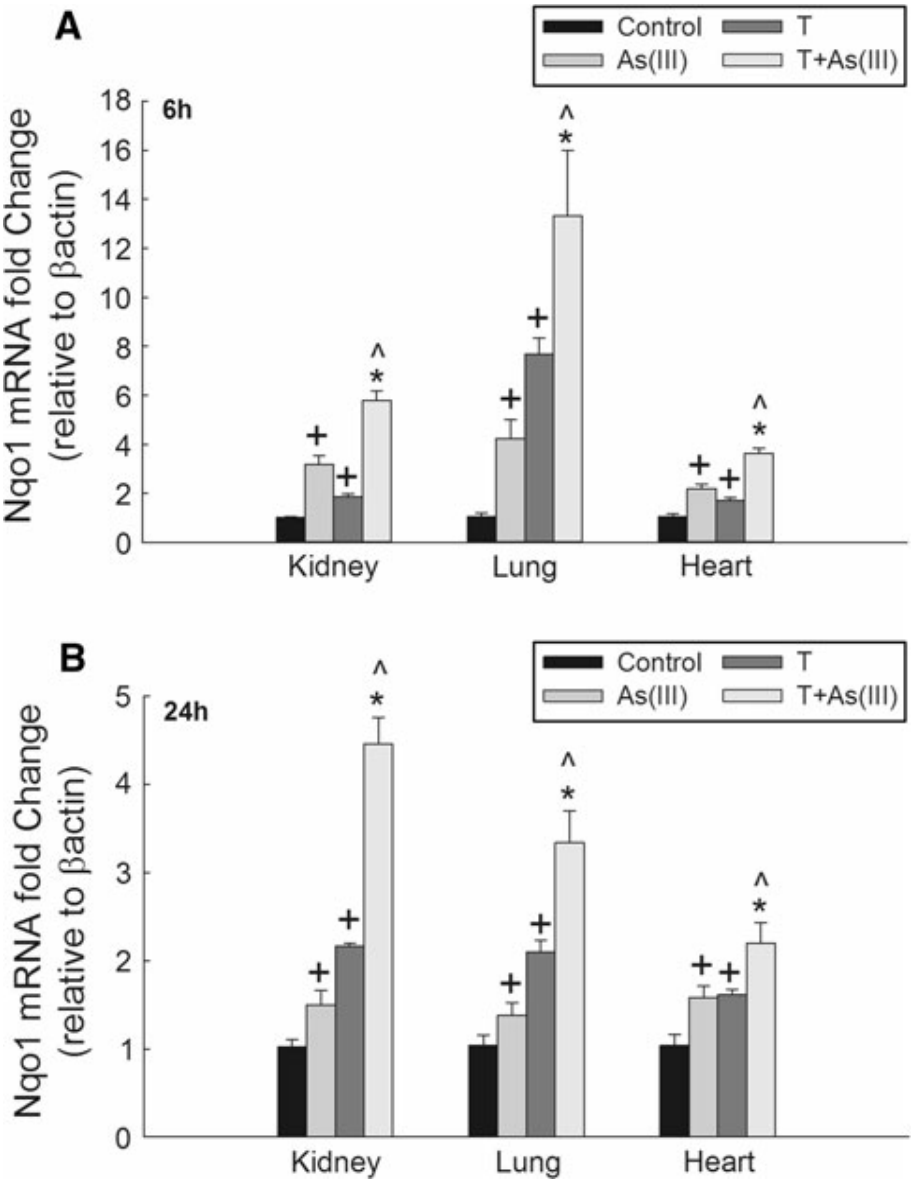

Fig. 3.3.8 Effect of co-exposure to As(III) and TCDD on Nqo1 mRNA in the kidney, lung, and heart of C57Bl6 mice.

Animals were injected i.p. with 12.5 mg/kg As(III) in the absence and presence of 15 µg/kg TCDD for 6 h **(A)** and 24 h **(B)**. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from kidney and the expression of Nqo1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. (+) $P < 0.05$, compared to control (untreated animals); (*) $P < 0.05$, compared to respective TCDD treatment; (^)$P < 0.05$, compared to respective As(III) treatment.

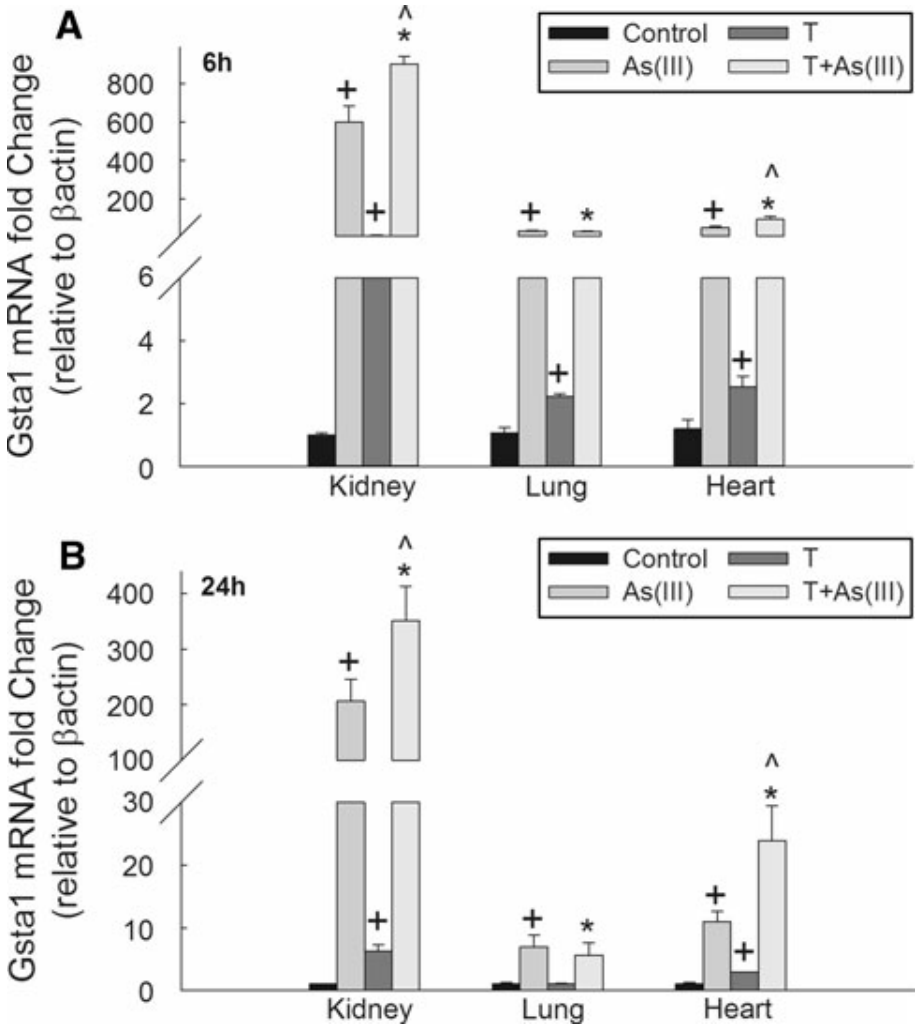

Fig. 3.3.9 Effect of co-exposure to As(III) and TCDD on Gsta1 mRNA in the kidney, lung, and heart of C57Bl6 mice.

Animals were injected i.p. with 12.5 mg/kg As(III) in the absence and presence of 15 μg/kg TCDD for 6 h **(A)** and 24 h **(B)**. First-strand cDNA was synthesized from total RNA (1.5 μg) extracted from kidney and the expression of Gsta1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. (+) $P < 0.05$, compared to control (untreated animals); (*) $P < 0.05$, compared to respective TCDD treatment; (^)$P < 0.05$, compared to respective As(III) treatment.

3.3.6. EFFECT OF CO-EXPOSURE TO AS(III) AND TCDD ON NQO1 AND GSTA1 PROTEIN EXPRESSION AND CATALYTIC ACTIVITY LEVELS IN THE KIDNEY AND LUNG OF C57BL6 MICE

As(III) alone significantly induced the kidney and lung Nqo1 protein expression levels by 6.50- and 6-fold, respectively, and kidney and lung Gsta1 protein expression levels by 10- and 5-fold, respectively, compared to control (Fig. 3.3.10A and 3.3.10B). TCDD alone significantly induced kidney and lung Nqo1 protein expression levels by 0.60- and 5-fold, respectively, and kidney and lung Gsta1 protein expression levels by 3.40- and 2.30, respectively, compared to control (Fig. 3.3.10A and 3.3.10B). When animals were co-exposed to As(III) and TCDD, As(III) significantly potentiated the TCDD-mediated induction of kidney and lung Nqo1 protein expression levels by 6.80- and 7.50-fold, respectively, and kidney and lung Gsta1 protein expression levels by 13- and 0.32-fold, respectively, compared to TCDD alone (Fig. 3.3.10A and 3.3.10B).

At the catalytic activity levels, As(III) alone significantly induced the kidney and lung Nqo1 activity by 1.95- and 1.95-fold, respectively, compared to control (Fig. 3.3.11A). Similarly As(III) alone significantly induced kidney and lung Gsta1 activity by 1.60- and 2-fold, respectively, compared to control (Fig. 3.3.11A). TCDD alone significantly induced lung Nqo1 activity by 1.50-fold, compared to control, while it did not alter the kidney Nqo1 activity (Fig. 3.3.11A). Furthermore, TCDD alone significantly induced kidney and lung Gsta1 activities by 1.20- and 1.80-fold, respectively, compared to control (Fig. 3.3.11B). When animals were co-exposed to As(III) and TCDD, As(III) significantly potentiated the TCDD-mediated induction of the kidney and lung Nqo1 catalytic activities by 2- and 0.50-fold, respectively, compared to TCDD alone. Additionally, As(III) significantly potentiated the TCDD-mediated induction of kidney Gsta1 catalytic activity by 0.32-fold, compared to TCDD alone, while it did not alter the TCDD-mediated induction of lung Gsta1 catalytic activity (Fig. 3.3.11B).

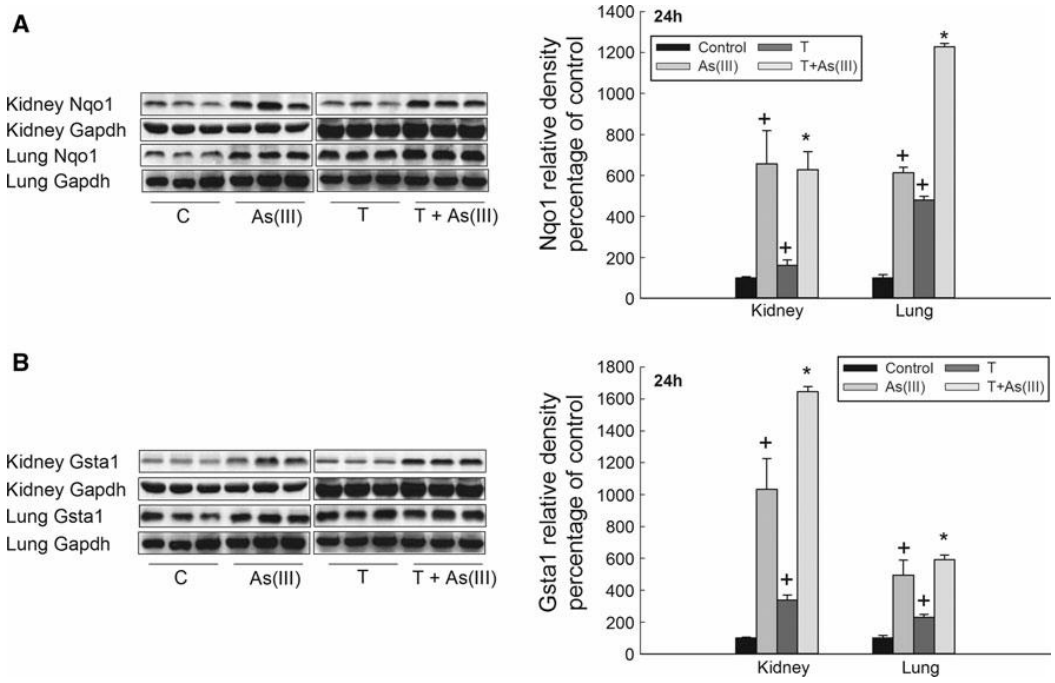

Fig. 3.3.10 Effect of co-exposure to As(III) and TCDD on Nqo1 and Gsta1 protein expression levels in the kidney and lung of C57Bl/6 mice.

Kidney and lung cytosolic proteins were isolated after 24 h of treatment. 5 or 30 µg of cytosolic proteins for Nqo1 and Gsta1, respectively, were separated on a 10% SDS-PAGE. Proteins were detected using the enhanced chemiluminescence method. The graph represents the relative amount of protein normalized to Gapdh signals (mean ± SEM, n = 6), and the results are expressed as percentage of the control values taken as 100%. (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment; (^)$P < 0.05$, compared to respective As(III) treatment.

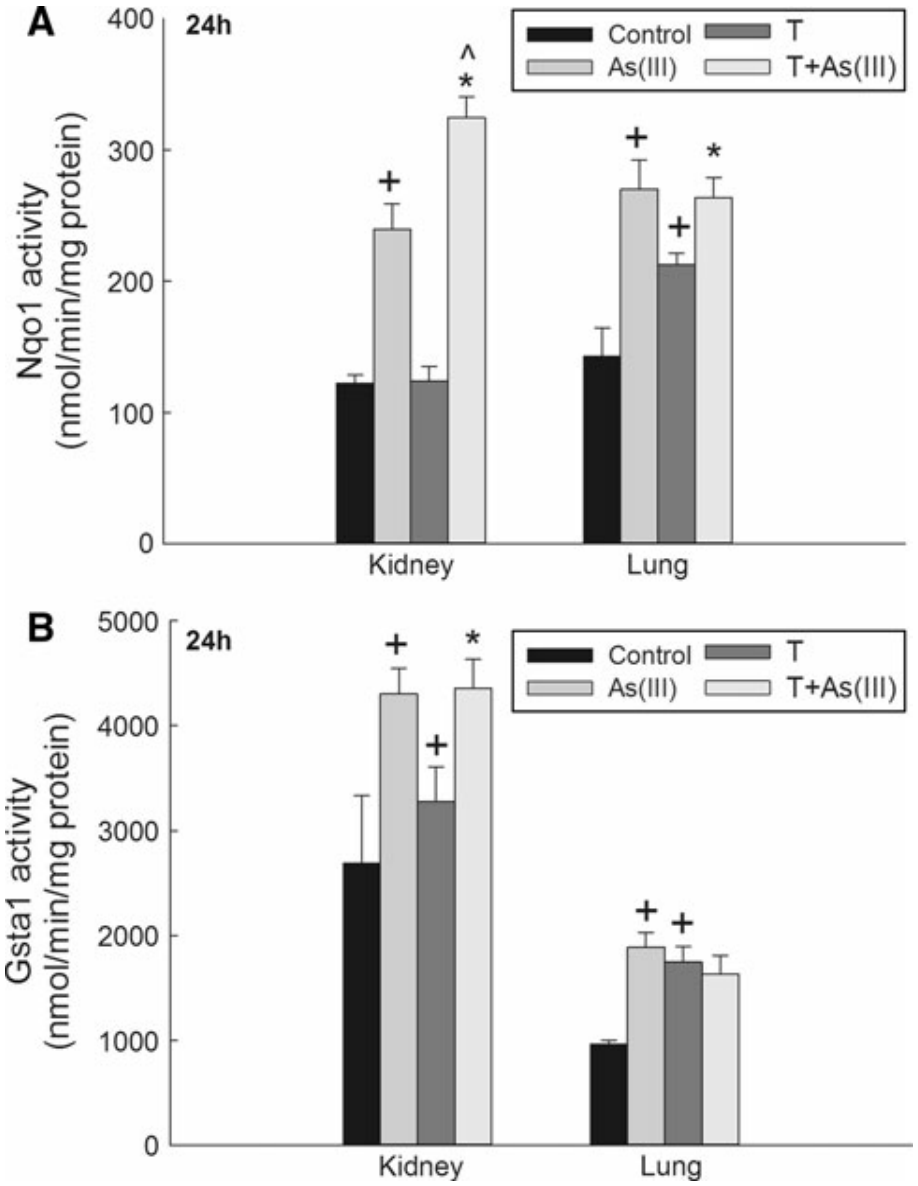

Fig. 3.3.11 Effect of co-exposure to As(III) and TCDD on Nqo1 and Gsta1 catalytic activity levels in the kidney and lung of C57Bl/6 mice.
Kidney and lung cytosolic protein was isolated after 24 h of treatment. Nqo1 enzyme activity was determined spectrophotometrically using DCPIP as substrate, and dicoumarol as specific Nqo1 inhibitor. Gsta1 activity was determined spectrophotometricaly using CDNB as a substrate as described under Materials and Methods. Values are presented as mean ± SEM ($n = 6$). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment; (^)$P < 0.05$, compared to respective As(III) treatment.

3.3.7. EFFECT OF CO-EXPOSURE TO AS(III) AND TCDD ON HO-1 MRNA LEVELS IN THE KIDNEY, LUNG, AND HEART

Our results demonstrated that As(III) alone was able to induce HO-1 mRNA levels in the kidney, lung, and heart after 6 h of treatment by 120-, 53-, 234-fold, respectively, compared to control (Fig. 3.3.12A). Importantly, As(III) alone after 24 h of treatment significantly induced lung HO-1 mRNA levels by 2.20-fold, while it did not significantly affect HO-1 mRNA levels in the kidney or heart (Fig. 3.3.12B). TCDD alone, after 6 h of treatment, significantly induced HO-1 mRNA levels in the heart only by 2-fold, compared to control (Fig. 3.3.12A). When the animals were co-exposed to As(III) and TCDD for 6 h, HO-1 mRNA levels were not significantly different from those of As(III) alone (Fig. 3.3.12A), and this induction was not significantly different from that of As(III) alone. At 24 h, however, HO-1 mRNA levels were significantly induced in the kidney, lung and heart by 1.70-, 2-, and 2.50-fold, respectively, compared to control (Fig. 3.3.12B).

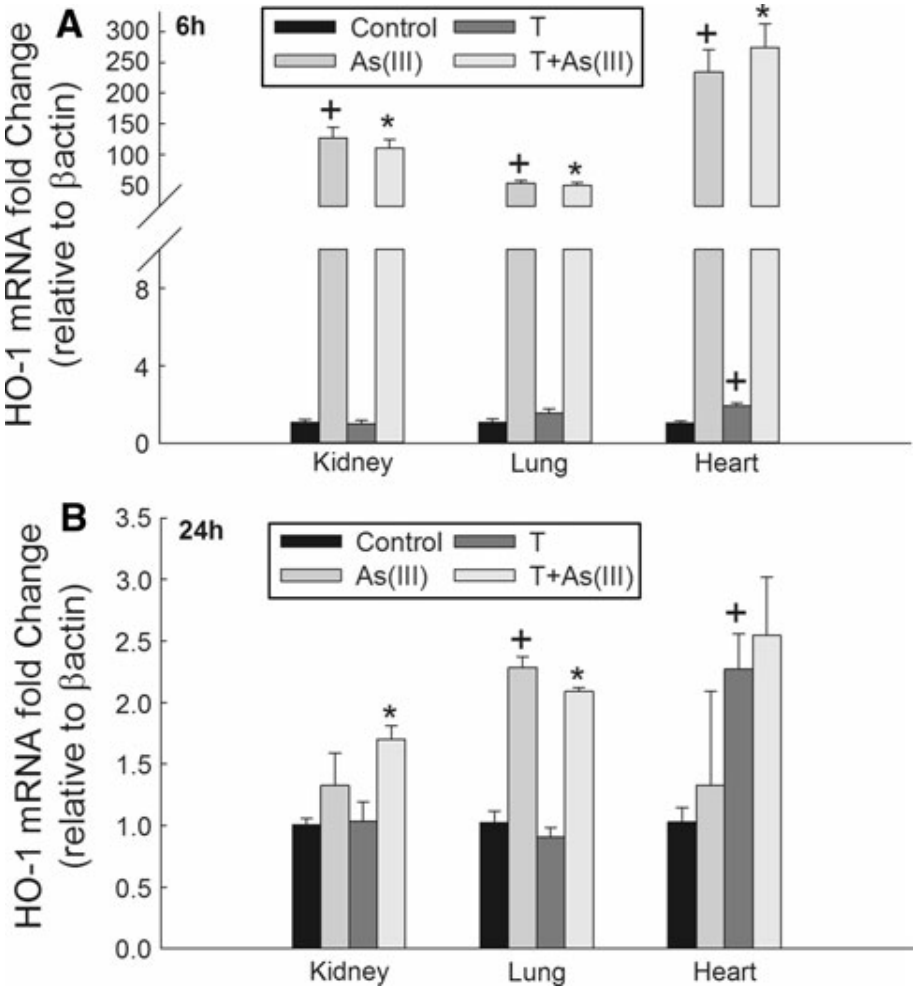

Fig. 3.3.12 Effect of co-exposure to As(III) and TCDD on HO-1 mRNA in the kidney, lung, and heart of C57Bl6 mice.

Animals were injected i.p. with 12.5 mg/kg As(III) in the absence and presence of 15 µg/kg TCDD for 6 h **(A)** and 24 h **(B)**. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from kidney and the expression of HO-1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. (+) $P < 0.05$, compared to control (untreated animals); (*) $P < 0.05$, compared to respective TCDD treatment.

Table 3.1: Summary of the effects of As(III) and TCDD on the expression of AhR-regulated genes in kidney, lung and heart

Treatment	Gene	Kidney		Lung		Heart	
		6h	24h	6h	24h	6h	24h
As(III)	Cyp1a1	↔mRNA	↔mRNA, ↔protein, ↓activity	↑mRNA	↑mRNA, ↑protein, ↑activity	↔ mRNA	↔ mRNA
	Cyp1a2	↔mRNA	↔mRNA, ↔protein, ↓activity	↑mRNA	↔ mRNA, ↑protein, ↑activity	↔ mRNA	↔mRNA
	Nqo1	↑mRNA	↑mRNA, ↑protein, ↑activity	↑mRNA	↑mRNA, ↑protein, ↑activity	↑ mRNA	↑mRNA
	Gsta1	↑mRNA	↑mRNA, ↑protein, ↑activity	↑mRNA	↑mRNA, ↑protein, ↑activity	↑mRNA	↑mRNA
	HO-1	↑mRNA	↔ mRNA	↑mRNA	↑mRNA	↑ mRNA	↔ mRNA
TCDD	Cyp1a1	↑mRNA	↑mRNA, ↑protein, ↑activity	↑mRNA	↑mRNA, ↑protein, ↑activity	↑mRNA	↑ mRNA
	Cyp1a2	↑mRNA	↑mRNA, ↑protein, ↑activity	↑mRNA	↑mRNA, ↑protein, ↑activity	↑mRNA	↑ mRNA
	Nqo1	↑mRNA	↑mRNA, ↑protein, ↔activity	↑mRNA	↑mRNA, ↑protein, ↑activity	↑mRNA	↑ mRNA
	Gsta1	↑mRNA	↑mRNA, ↑protein, ↑activity	↑mRNA	↔mRNA, ↑protein, ↑activity	↑mRNA	↑ mRNA
	HO-1	↔mRNA	↔mRNA	↔mRNA	↔mRNA	↑ mRNA	↑ mRNA
As(III) +	Cyp1a1	↓mRNA	↑↑mRNA, ↓protein, ↓activity	↑↑mRNA	↑↑mRNA, ↑↑protein, ↑↑activity	↓ mRNA	↔ mRNA
TCDD	Cyp1a2	↓mRNA	↑↑mRNA, ↓protein, ↓activity	↔mRNA	↔mRNA, ↑↑protein, ↑↑activity	↓ mRNA	↔ mRNA
	Nqo1	↑↑mRNA	↑↑mRNA, ↑↑protein, ↑↑activity	↑↑mRNA	↑↑mRNA, ↑↑protein, ↑↑activity	↑ mRNA	↑ mRNA
	Gsta1	↑↑mRNA	↑↑mRNA, ↑↑protein, ↑↑activity	↑↑mRNA	↑↑mRNA, ↑↑protein, ↔activity	↑ mRNA	↑ mRNA
	HO-1	↑↑mRNA	↑↑mRNA	↑↑mRNA	↑↑mRNA	↑ mRNA	↔ mRNA

(↑) increase; (↓) decrease; (↔) no change; (↑↑) potentiation

3.4. DIFFERENTIAL MODULATION OF CYTOCHROME P450 1A1 (CYP1A1) BY ARSENITE *IN VIVO* AND *IN VITRO* IN C57BL/6 MICE

3.4.1. EFFECT OF CO-EXPOSURE TO AS(III) AND TCDD ON CYP1A1, CYP1A2, AND CYP1B1 MRNA LEVELS IN THE LIVER OF C57BL/6 MICE

At 6 h and 24h, As(III) alone failed to significantly affect Cyp1a1, Cyp1a2, or Cyp1b1 mRNA levels in the liver (Fig. 3.4.1A, 3.4.1B, and 3.4.1C). 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 (Fig. 3.4.1A, 3.4.1B, and 3.4.1C). 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 (Fig. 3.4.1A, 3.4.1B, and 3.4.1C). When animals were co-exposed 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, while there was no effect of this co-exposure on Cyp1b1 mRNA levels at 6 h (Fig. 3.4.1A, 3.4.1B, and 3.4.1C). 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, while it did not affect the TCDD-mediated induction of Cyp1a2 or Cyp1b1 mRNA levels in the liver (Fig. 3.4.1A, 3.4.1B, and 3.4.1C).

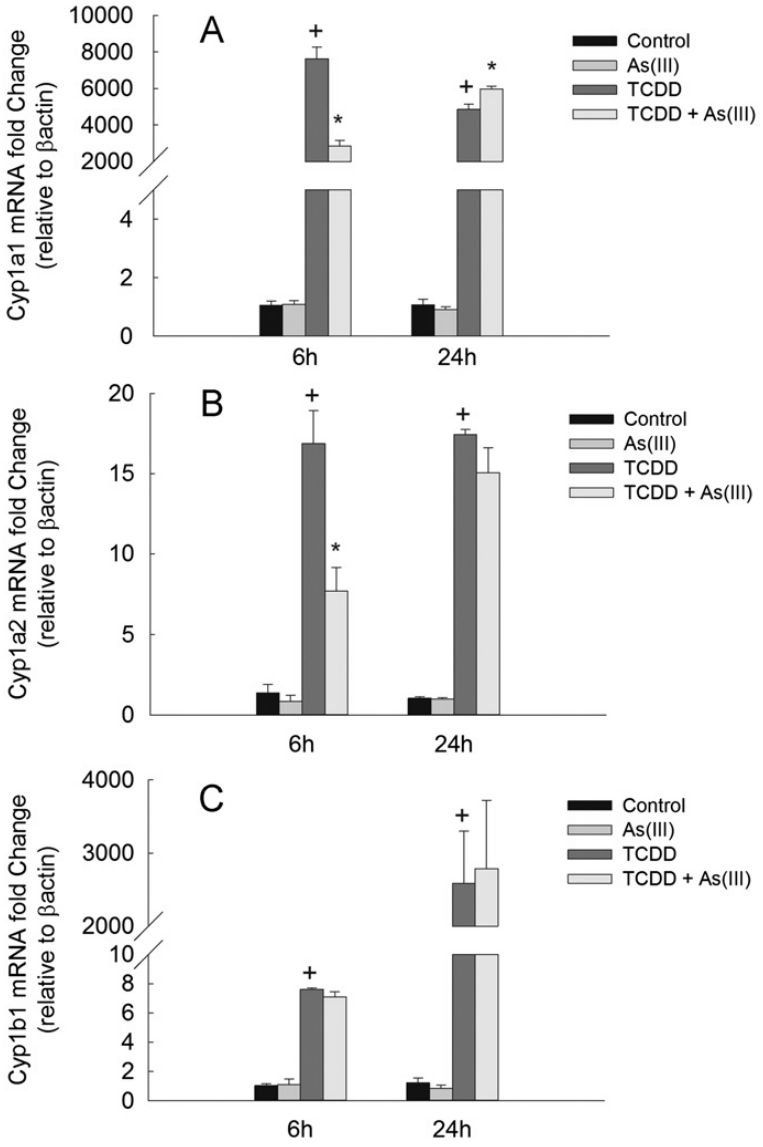

Fig. 3.4.1 Effect of co-exposure to As(III) and TCDD on liver Cyp1a1, Cyp1a2, and Cyp1b1 mRNA in C57BL/6 mice.

Animals were injected i.p. with 12.5 mg/kg As(III) in the absence and presence of 15 µg/kg TCDD for 6 h and 24 h. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from liver and the expression of Cyp1a1, Cyp1a2, and Cyp1b1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. (+) $P < 0.05$, compared to control (untreated animals); (*) $P < 0.05$, compared to respective TCDD treatment.

3.4.2. EFFECT OF CO-EXPOSURE 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. 3.4.2A). 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. 3.4.2A). On the other hand when animals were co-exposed 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. 3.4.2A).

At the catalytic activity levels, As(III) alone did not significantly affect EROD or MROD activity in the liver (Fig. 3.4.2B and 3.4.2C). TCDD alone significantly induced EROD and MROD activities in the liver by 5.8- and 6.4-fold, respectively, compared to control (Fig. 3.4.2B and 3.4.2C). When animals were co-exposed 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 (Fig. 3.4.2B and 3.4.2C).

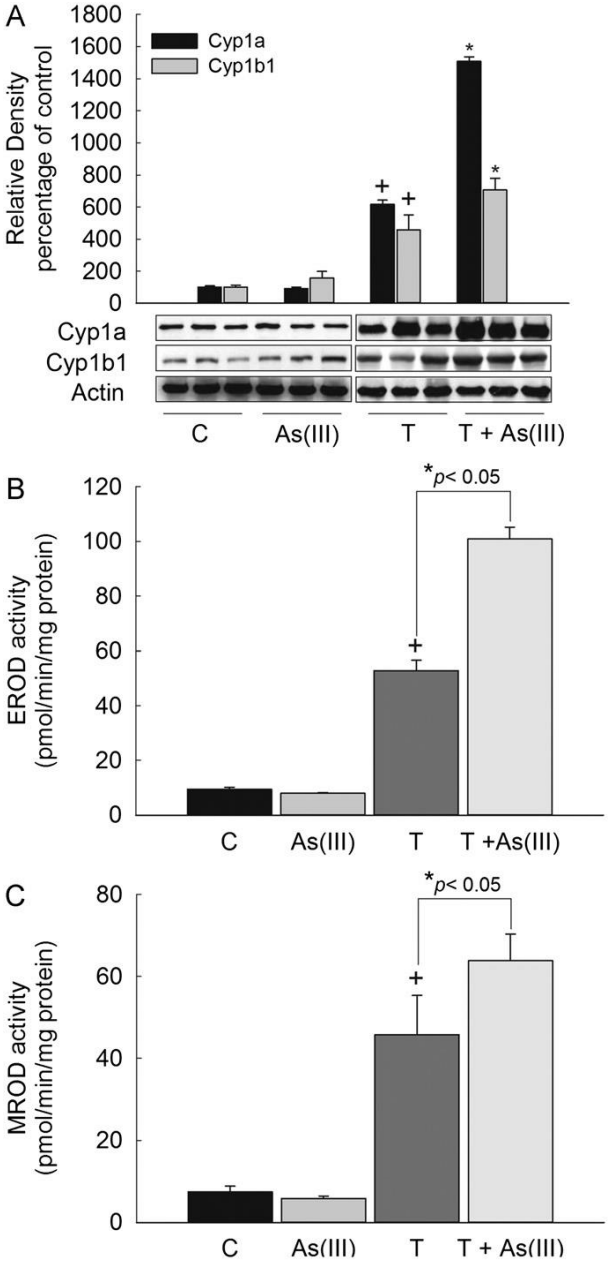

Fig. 3.4.2 Effect of co-exposure to As(III) and TCDD on liver Cyp1a and Cyp1b1 protein expression levels, EROD and MROD activities in C57BL/6 mice.

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

3.4.3. EFFECT OF CO-EXPOSURE 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, while at 24 h it did not affect its mRNA levels (Fig. 3.4.3). TCDD alone at 6 h or 24 h did not significantly affect HO-1 mRNA levels in the liver compared to control (Fig. 3.4.3). When animals were co-exposed 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. 3.4.3). 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. 3.4.3).

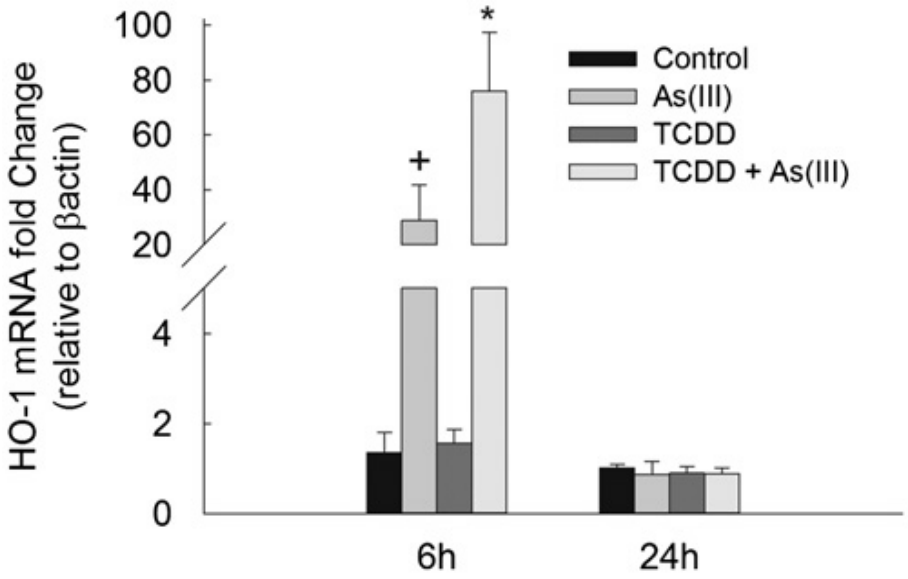

Fig. 3.4.3 Effect of co-exposure to As(III) and TCDD on liver HO-1 mRNA in C57BL/6 mice.

Animals were injected i.p. with 12.5 mg/kg As(III) in the absence and presence of 15 µg/kg TCDD for 6 h and 24 h. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from liver and the expression of HO-1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. (+) $P < 0.05$, compared to control (untreated animals); (*) $P < 0.05$, compared to respective TCDD treatment.

3.4.4. EFFECT OF CO-EXPOSURE TO AS(III) AND TCDD ON CELL VIABILITY

To determine the non-toxic concentrations of As(III) to be utilized in the current 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 MTT assay. Figure 3.4.4 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. 3.4.4). Therefore, all subsequent studies were conducted using the concentrations of 1 – 10 µM.

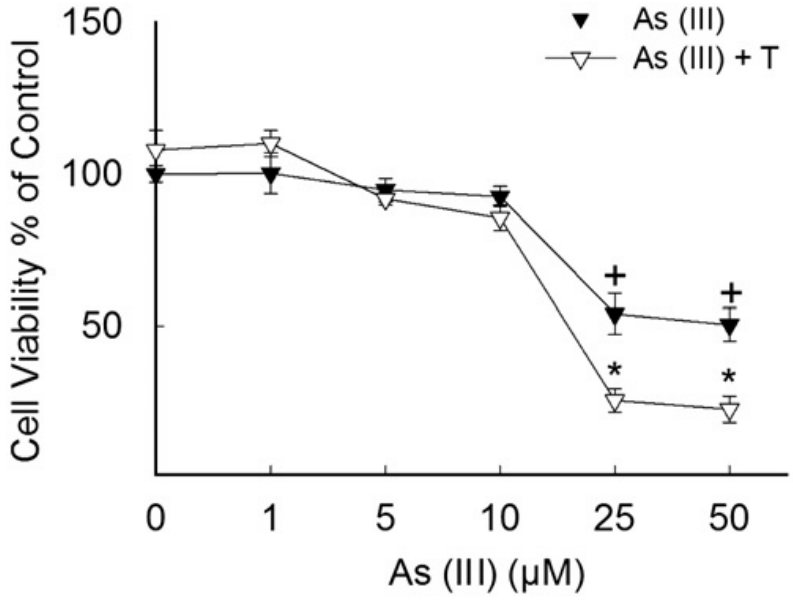

Fig. 3.4.4 Effect of As(III) on cell viability.
Isolated hepatocytes were treated for 24 h with As(III) (0, 5, 10, 25, and 50 μM) in the absence and presence of 1 nM TCDD. Cell cytotoxicity was determined using MTT assay. Data are expressed as percentage of untreated control (which is set at 100%) ± SE (n = 8). (+) $P < 0.05$, compared to control (concentration = 0 μM); (*) $P < 0.05$, compared to respective TCDD treatment.

3.4.5. TIME- AND CONCENTRATION-DEPENDENT EFFECT OF CO-EXPOSURE TO AS(III) AND TCDD ON CYP1A1 MRNA LEVELS IN ISOLATED HEPATOCYTES

To examine the effect of co-exposure to As(III) and TCDD on Cyp1a1 mRNA, isolated hepatocytes were treated with various concentrations of As(III) in the presence of 1 nM TCDD (Fig. 3.4.5A). Thereafter, Cyp1a1 mRNA was assessed using real-time PCR. TCDD alone caused 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. 3.4.5A).

To better understand the kinetics of Cyp1a1 mRNA in response to the co-exposure 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). Figure 3.4.5B 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. 3.4.5B). In contrast, when isolated hepatocytes were co-exposed to As(III) and TCDD, there was a significant decrease in the Cyp1a1 mRNA levels that occurred as early as 3 h by 3.3-fold, compared to TCDD alone. Similarly, As(III) significantly decreased the TCDD-mediated induction of Cyp1a1 mRNA levels at 6 h, 12 h, and 24 h by -1.9-, -2.5-, -2.7-fold, respectively, compared to TCDD alone (Fig. 3.4.5B).

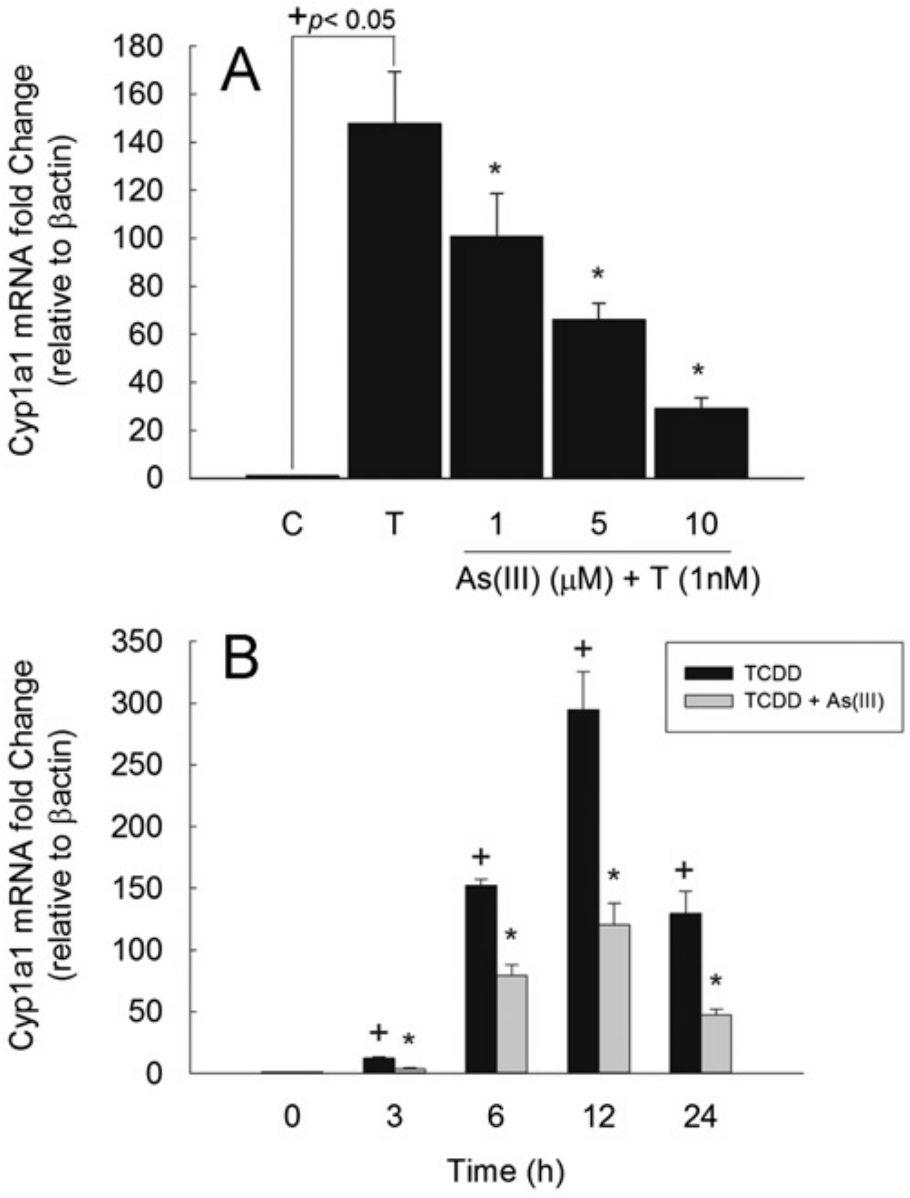

Fig. 3.4.5 Concentration- and time-dependent effect of As(III) on CYP1A1 mRNA in isolated hepatocytes.

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

3.4.6. CONCENTRATION-DEPENDENT EFFECT OF CO-EXPOSURE 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. Figure 3.4.6A and 3.4.6B 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 (Fig. 3.4.6A and 3.4.6B).

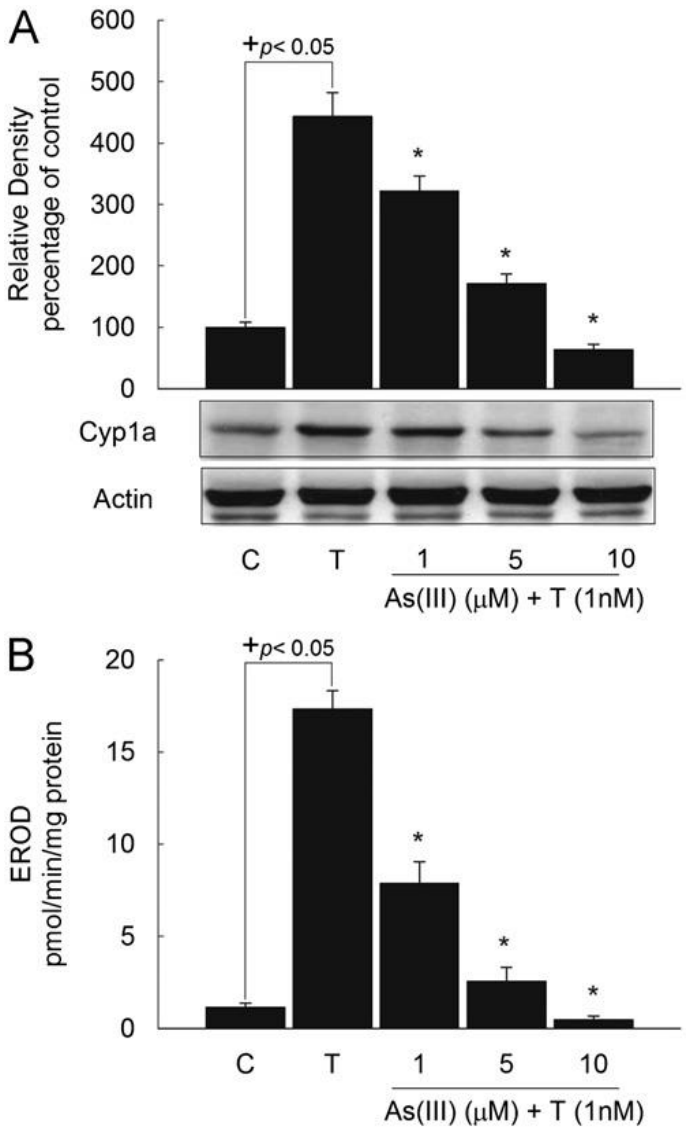

Fig. 3.4.6 Effect of As(III) on Cyp1a protein and catalytic activity in isolated hepatocytes.

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 on a 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C and then incubated with a primary Cyp1a antibody for 24 h at 4°C, followed by 1 h incubation with secondary antibody at room temperature. Cyp1a protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to actin signals, which was used as loading control. One of three representative experiments is shown. **(B)** Cyp1a1 activity was measured in intact living cells treated with increasing concentrations of 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 mean ± SE (n = 6). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

3.4.7. TRANSCRIPTIONAL AND POST-TRANSLATIONAL INHIBITION OF CYP1A BY AS(III)

Isolated hepatocytes were transiently transfected with the XRE-driven luciferase reporter gene in order 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. 3.4.3). On the other hand, 1 nM TCDD alone caused a significant increase of luciferase activity by 12.8-fold as compared to control (Fig. 3.4.7A). Interestingly, co-treatment with As(III) and TCDD significantly decreased the TCDD-mediated induction of luciferase activity by -2.4-fold (Fig. 3.4.7A).

The fact that As(III) inhibited the TCDD-mediated induction of Cyp1a1 at the catalytic activity level more than inhibiting its mRNA or protein levels prompted us to investigate the possible effect of As(III) on HO-1 mRNA levels. For this purpose isolated hepatocytes were co-exposed 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. 3.4.7B).

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 co-exposed 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, there was a partial restoration of the As(III)-

mediated down-regulation of Cyp1a1 catalytic activity induced by TCDD (Fig. 3.4.7C). In spite of being successful in partially reversing the As(III)-mediated decrease in Cyp1a1 activity through inhibiting HO-1, SnMP was unable to completely restore Cyp1a1 activity, implying the presence of a transcriptional mechanism in addition to the post-translational modification.

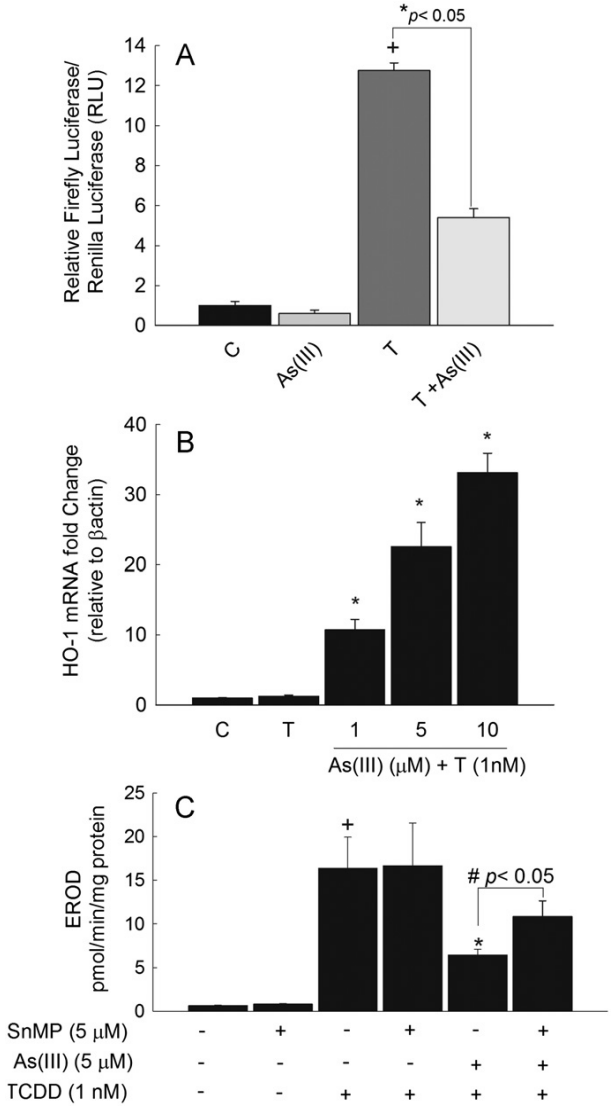

Fig. 3.4.7 Effect of As(III) on luciferase activity (A), HO-1 mRNA in isolated hepatocytes (B), and the effect of SnMP as a competitive inhibitor of HO-1 on Cyp1a1 catalytic activity (C). **(A)** Hepatocytes were transiently transfected with the XRE-luciferase transporter plasmid pGudLuc1.1 and renilla luciferase plasmid pRL-CMV plasmid. Cells were treated with vehicle, As(III) (5 μM), TCDD (1 nM), or TCDD (1 nM) + As(III) (5 μM) for 24 h. Cells were lysed and luciferase activity was measured according to manufacturer's instructions. Luciferase activity is reported relative to renilla activity. **(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 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. **(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 (C) (concentration = 0 μM); (*) $P < 0.05$, compared to respective TCDD (T) treatment; (#) $P < 0.05$, compared to respective TCDD + As(III) treatment.

3.4.8. 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. 3.4.8A). On the other hand, TCDD alone did not significantly affect serum Hb levels. Interestingly, when animals were co-exposed to As(III) and TCDD, As(III) increased serum Hb levels by 2.1-fold, compared to TCDD alone (Fig. 3.4.8A).

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. 3.4.8B). As expected, TCDD alone significantly induced luciferase activity by 32-fold, compared to control. Interestingly, when isolated hepatocytes were co-exposed to Hb and TCDD, Hb significantly potentiated the TCDD-mediated induction of luciferase activity by 1.3-fold, compared to TCDD alone (Fig. 3.4.8B).

In order to correlate the *in vivo* effects with those observed at the *in vitro* levels we tried to mimic the *in vivo* situation *in vitro*. For this purpose, isolated hepatocytes were treated with As(III) for 5 hours 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 the slow poke reactor (data not shown). In addition, we needed to remove As(III) from the medium to rule out any direct effect of As(III). 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 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 co-exposure to As(III) and TCDD (Fig. 3.4.7A). Importantly, when cells were treated with As(III) and then co-exposed to TCDD and Hb, there was a significant potentiation to the XRE-driven luciferase activity by 1.5-fold, compared to TCDD alone (Fig. 3.4.8C).

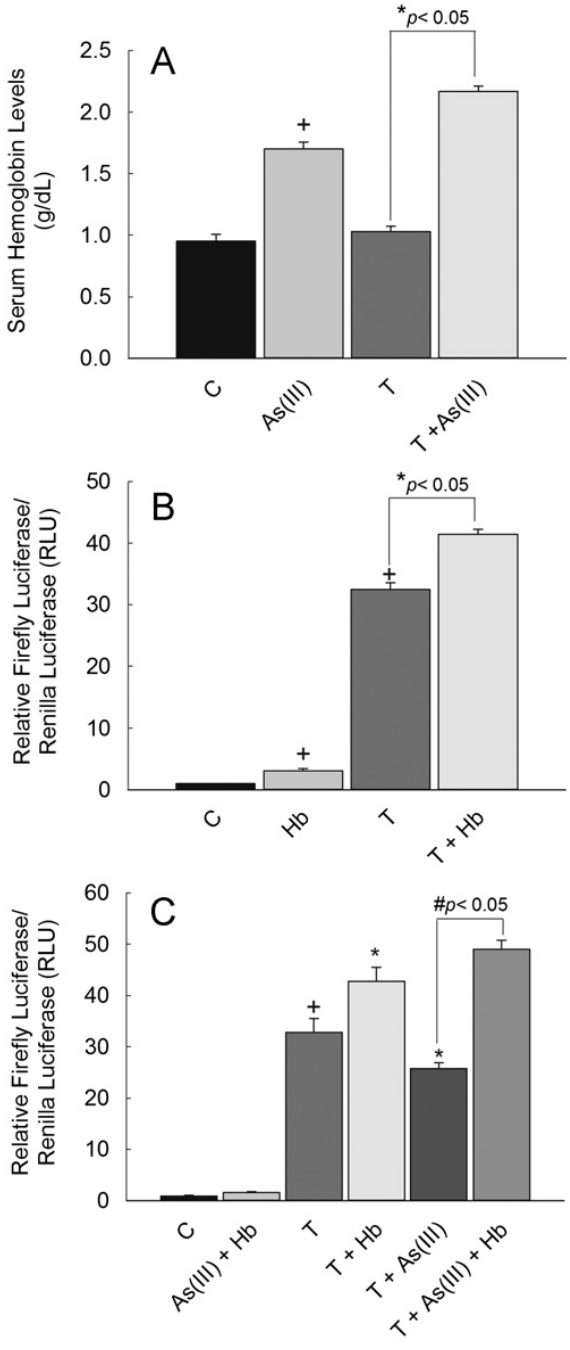

Fig. 3.4.8 Effect of As(III) on serum Hb levels in vivo and the effect of Hb on luciferase activity in isolated hepatocytes.

(A) Animals were injected i.p. 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 serum Hb levels were measured as described under Materials and Methods section. **(B)** Hepatocytes were transiently transfected with the XRE-luciferase transporter plasmid pGudLuc1.1 and renilla luciferase plasmid pRL-CMV plasmid. Cells were treated with vehicle, Hb (1 µM), TCDD (1 nM), TCDD (1 nM) + Hb (1 µM) for 24 h. Cells were lysed and luciferase activity was measured according to manufacturer's instruction. Luciferase activity is reported relative to renilla activity. **(C)** Hepatocytes were transiently transfected with the XRE-luciferase transporter plasmid pGudLuc1.1 and renilla luciferase plasmid pRL-CMV plasmid. Cells were treated with vehicle, As(III) (5 µM), TCDD (1 nM), TCDD (1 nM) + As(III) (5 µM) for 5 h. Cells were then washed and further incubated with Hb for additional 24 hours. Cells were lysed and luciferase activity was measured according to manufacturer's instruction. Luciferase activity is reported relative to renilla activity. Values are presented as mean ± SE (n = 6). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment; (#) $P < 0.05$, compared to respective TCDD + As(III) treatment.

3.4.9. 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 through 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. For this purpose, 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. 3.4.9A). 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 confirmation to the DNA-binding form (Fig. 3.4.9A). In order 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 in the Materials and Methods section. Figure 3.4.9B 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.

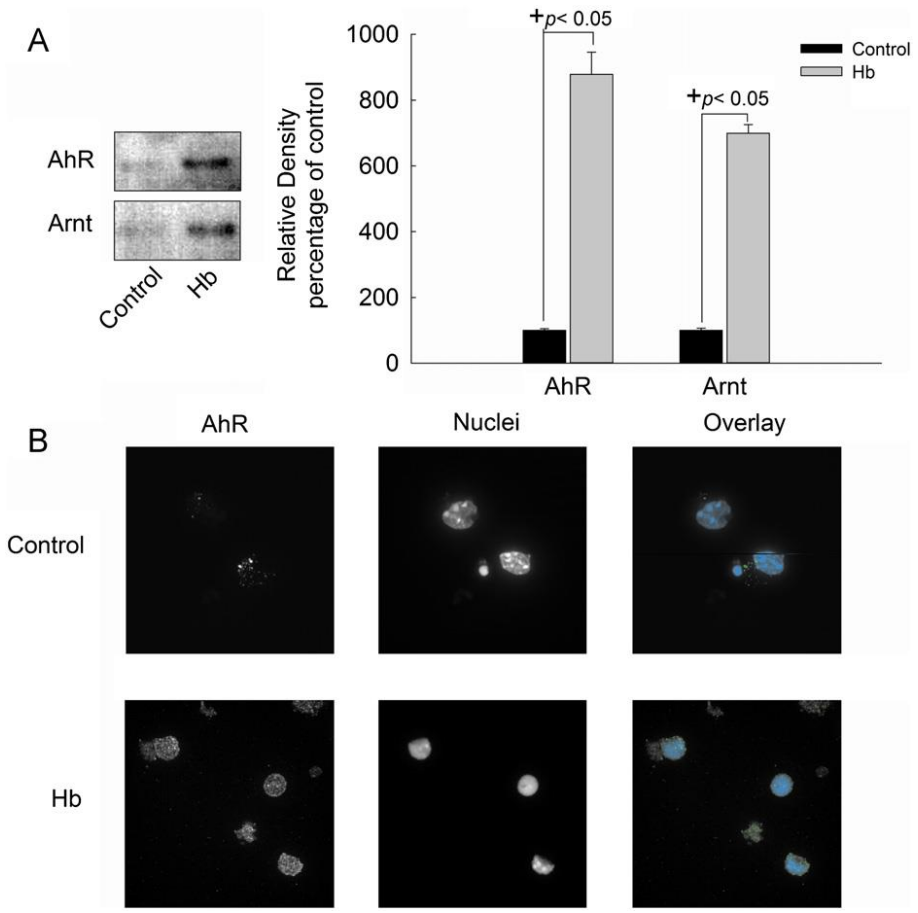

Fig. 3.4.9 Effect of Hb on AhR subcellular localization in isolated hepatocytes.

(A) AhR protein was immunoprecipitated from nuclear fractions of treated isolated hepatocytes. Immunoprecipitates were then analyzed by Western blot analyses. 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); (*) $P < 0.05$, compared to respective TCDD (T). **(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, thereafter fixed and stained as described in Materials and Methods section. The magnification of each micrograph is identical. Cells were all stained at the same time and the results are representative of three independent experiments.

3.5. METHYLATED PENTAVALENT ARSENIC METABOLITES ARE BIFUNCTIONAL INDUCERS AS THEY INDUCE CYTOCHROME P450 1A1 (CYP1A1) AND NAD(P)H: QUINONE OXIDOREDUCATSE (NQO1) THROUGH AHR- AND NRF2-DEPENDENT MECHANISMS.

3.5.1. EFFECT OF EXPOSURE TO MMA(V), DMA(V), AND TMA(V) IN THE ABSENCE AND PRESENCE OF TCDD ON CYP1A1 MRNA

To examine the effect of co-exposure to MMA(V), DMA(V), and TMA(V) in the absence and presence of TCDD on CYP1A1 mRNA, HepG2 cells were treated with 5 μM of MMA(V), DMA(V), or TMA(V) in the absence and presence of 1 nM TCDD (Fig. 3.5.1A and 3.5.1B). Thereafter, CYP1A1 mRNA was assessed using real-time PCR. The concentrations of MMA(V), DMA(V), and TMA(V) used hereafter were chosen to match the tested concentration of sodium arsenite As(III) we previously used on HepG2 cells or mouse and rat primary hepatocytes (Isoherranen et al., 2003; Anwar-Mohamed and El-Kadi, 2010). MMA(V), DMA(V), and TMA(V) alone significantly induced CYP1A1 mRNA by 4.68-, 5.55-, and 6.73-fold, respectively, compared to control (Fig. 3.5.1A). TCDD alone significantly induced CYP1A1 mRNA levels by 24.64-fold, compared to control, that was further potentiated by MMA(V), DMA(V), and TMA(V) to 35.55-, 36.57-, and 38.56-fold, respectively, compared to control (Fig. 3.5.1B).

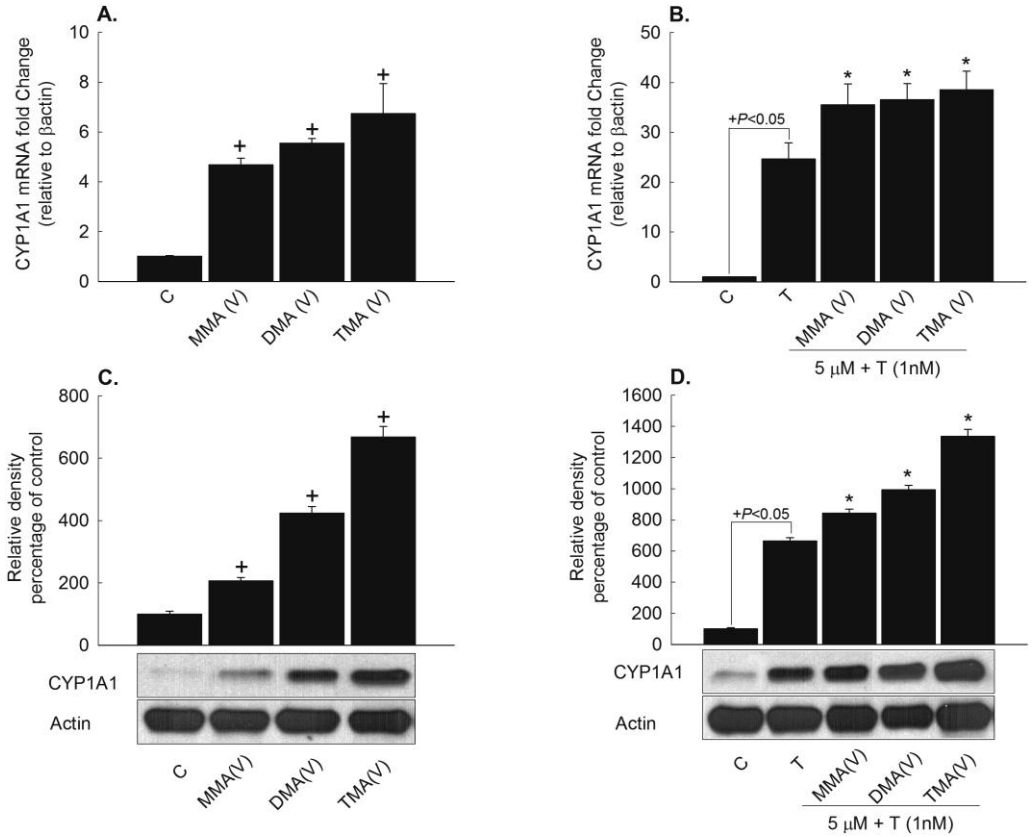

Fig. 3.5.1 Effect of exposure to MMA(V), DMA(V), and TMA(V) in the absence (A and C) and presence of TCDD (B and D) on CYP1A1 mRNA and protein.

(A and C) HepG2 cells were treated with 5 μM MMA(V), DMA(V), or TMA(V) in the absence and presence of 1 nM TCDD for 6 h. First-strand cDNA was synthesized from total RNA (1.5 μg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. Values are presented as mean ± SE (n = 6). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment. **(B and D)** HepG2 cells were treated with 5 μM MMA(V), DMA(V), or TMA(V) in the absence and presence of 1 nM TCDD for 24 h. Protein (50 μg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C and then incubated with a primary CYP1A1 antibody for 2 h at 4°C, followed by 1 h incubation with secondary antibody at room temperature. CYP1A1 protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to actin signals, which was used as loading control. One of three representative experiments is shown. Values are presented as mean ± SE (n = 6). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

3.5.2. EFFECT OF EXPOSURE TO MMA(V), DMA(V), AND TMA(V) IN THE ABSENCE AND PRESENCE OF TCDD ON CYP1A1 PROTEIN AND CATALYTIC ACTIVITY

To investigate whether the observed induction of CYP1A1 mRNA levels in response to MMA(V), DMA(V), and TMA(V) in the absence and presence of TCDD is further reflected at the protein and activity levels, HepG2 cells were treated for 24 h with 5 μM MMA(V), DMA(V), or TMA(V) in the absence and presence of 1 nM TCDD. Figure 3.5.1C shows that MMA(V), DMA(V), and TMA(V) were able to significantly induce CYP1A1 protein expression levels by 2.06-, 4.24-, and 6.68-fold, respectively, compared to control. Furthermore, MMA(V), DMA(V), and TMA(V) were able to significantly increase CYP1A1 catalytic activity by 2.63-,3.52-, and 4.27-fold, respectively compared to control (Fig. 3.5.2A). On the other hand, TCDD alone significantly induced CYP1A1 protein and catalytic activity levels by 6.63- and 31.20-fold, respectively, compared to control (Fig. 3.5.1D and 3.5.2B). When HepG2 cells were co-exposed to MMA(V), DMA(V), or TMA(V) in the presence of 1 nM TCDD, there was further potentiation to CYP1A1 protein levels to reach 8.43-, 9.92-, and 13.35-fold, respectively, compared to control (Fig. 3.5.1D). Similarly, MMA(V), DMA(V), and TMA(V) potentiated the TCDD-mediated induction of CYP1A1 activity to reach 40.60-, 51.61-, and 58.60-fold, respectively, compared to control (Fig. 3.5.2B).

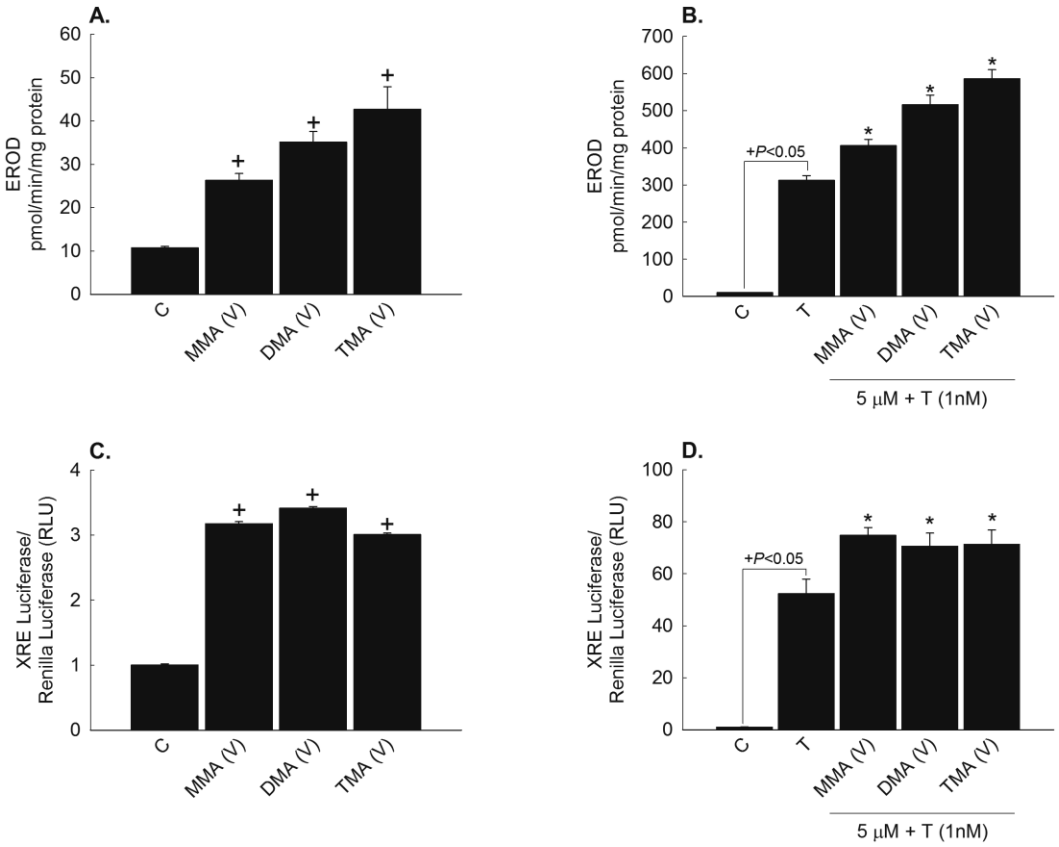

Fig. 3.5.2 Effect of exposure to MMA(V), DMA(V), and TMA(V) in the absence (A and C) and presence of TCDD (B and D) on CYP1A1 catalytic activity levels and XRE-driven luciferase reporter activity.

(A and C) HepG2 cells were treated with 5 μM MMA(V), DMA(V), or TMA(V) in the absence and presence of 1 nM TCDD for 24 h. CYP1A1 activity was measured in intact living cells treated with MMA(V), DMA(V), or TMA(V), in the absence and presence of 1 nM TCDD for 24 h. CYP1A1 activity was measured using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SE (n = 8). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment. **(B and D)** HepG2 cells were transiently transfected with 200 nM Silencer select negative control siRNA in addition to XRE-luciferase reporter plasmid pGudLuc6.1 for 6 h, and thereafter cells were treated with 5 μM MMA(V), DMA(V), or TMA(V) in the absence and presence of 1 nM TCDD for 24 h. Cells were lysed and luciferase activity was measured according to manufacturer's instruction. Luciferase activity is reported as relative light unit. Values are presented as mean ± SE (n = 6). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

3.5.3. EFFECT OF EXPOSURE TO MMA(V), DMA(V), AND TMA(V) ON XRE-DRIVEN LUCIFERASE REPORTER ACTIVITY IN THE ABSENCE AND PRESENCE OF TCDD

To determine if the observed effect upon co-exposure to MMA(V), DMA(V), and TMA(V) in the absence and presence of TCDD on CYP1A1 is occurring through an AhR-dependent mechanism, HepG2 cells were transiently co-transfected with the XRE-driven luciferase reporter plasmid and its normalizing control construct, renilla luciferase, in the presence of –ve control siRNA. Luciferase activity results showed that MMA(V), DMA(V), and TMA(V) alone significantly induced XRE-driven luciferase activity by 3.17-, 3.41-, and 3.00-fold, respectively, compared to control (Fig. 3.5.2C). TCDD alone significantly induced XRE-driven luciferase activity by 52.34-fold, compared to control (Fig. 3.5.2D). On the other hand, co-exposure to MMA(V), DMA(V), or TMA(V) and TCDD significantly potentiated XRE-driven luciferase reporter activity to 74.76-, 70.62-,71.32-fold, respectively, compared to control (Fig. 3.5.2C).

3.5.4. EFFECT OF EXPOSURE TO MMA(V), DMA(V), AND TMA(V) IN THE ABSENCE AND PRESENCE OF TCDD OR SUL ON NQO1 MRNA

To examine the ability of MMA(V), DMA(V), and TMA(V) to modulate NQO1 gene expression, HepG2 cells were treated with 5 µM MMA(V), DMA(V), or TMA(V) in the absence and presence of 1 nM TCDD or 5 µM SUL. Thereafter, NQO1 mRNA was assessed using real-time PCR. Initially, MMA(V), DMA(V), and TMA(V) alone significantly induced NQO1 mRNA levels by 5.04-, 4.68-, 3.78-fold, respectively compared to control (Fig. 3.5.3A). TCDD alone significantly induced NQO1 mRNA levels by 4.85-fold, compared to control, that was further potentiated by MMA(V), DMA(V), and TMA(V) to 8.37-, 7.48-, and 7.41-fold, respectively, compared to control (Fig. 3.5.3B). Similarly, SUL alone significantly induced NQO1 mRNA levels by 5.09-fold, compared to control, that was further potentiated by MMA(V), DMA(V), and TMA(V) to 7.35-, 8.31-, and 9.10-fold, respectively, compared to control (Fig. 3.5.3B).

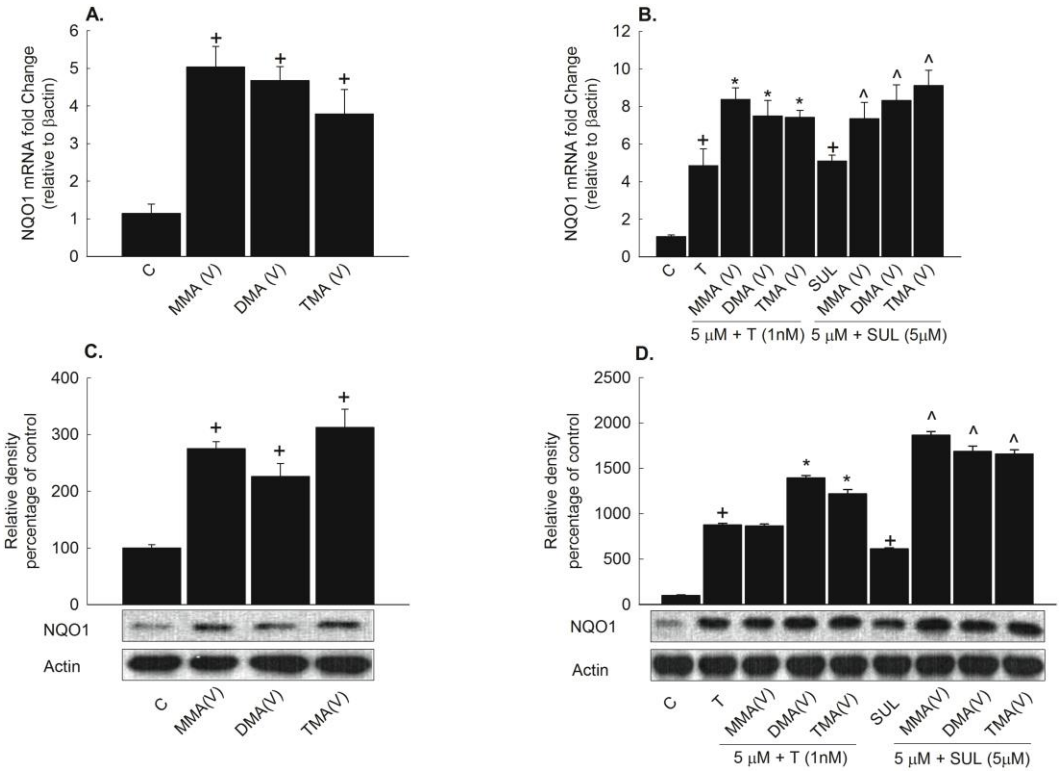

Fig. 3.5.3 Effect of exposure to MMA(V), DMA(V), and TMA(V) in the absence (A and C) and presence of TCDD or SUL (B and D) on NQO1 mRNA and protein.

(A and C) HepG2 cells were treated with 5 μM MMA(V), DMA(V), or TMA(V) in the absence and presence of 1 nM TCDD or 5 μM SUL for 6 h. First-strand cDNA was synthesized from total RNA (1.5 μg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. Values are presented as 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 sulforaphane treatment (SUL). **(B and D)** HepG2 cells were treated with 5 μM MMA(V), DMA(V), or TMA(V) in the absence and presence of 1 nM TCDD or 5 μM SUL for 24 h. Protein (5 μg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C and then incubated with a primary NQO1 antibody for 24 h at 4°C, followed by 1 h incubation with secondary antibody at room temperature. NQO1 protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to actin signals, which was used as loading control. One of three representative experiments is shown. Values are presented as 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 SUL treatment.

3.5.5. EFFECT OF EXPOSURE TO MMA(V), DMA(V), AND TMA(V) IN THE ABSENCE AND PRESENCE OF TCDD OR SUL ON NQO1 PROTEIN AND CATALYTIC ACTIVITY

To examine whether the observed induction of NQO1 mRNA levels in response to MMA(V), DMA(V), and TMA(V) in the absence and presence of TCDD or SUL is further reflected at the protein and activity levels, HepG2 cells were treated for 24 h with 5 μM MMA(V), DMA(V), or TMA(V) in the absence and presence of 1 nM TCDD or 5 μM SUL. Figure 3C shows that MMA(V), DMA(V), and TMA(V) were able to significantly induce NQO1 protein expression levels by 2.75-, 2.25-, and 3.13-fold, respectively, compared to control. Furthermore, MMA(V), DMA(V), and TMA(V) were able to significantly increase NQO1 catalytic activity levels by 1.93-,1.73-, and 1.51-fold, respectively compared to control (Fig. 3.5.4A). On the other hand, TCDD alone significantly induced NQO1 protein and catalytic activity levels by 8.76- and 2.44-fold, respectively, compared to control (Fig. 3.5.3D and 3.5.4B). When HepG2 cells were co-exposed MMA(V), DMA(V), or TMA(V) and TCDD, there was further potentiation of NQO1 protein levels by DMA(V) and TMA(V) but not MMA(V) to reach 13.95-, and 12.18-fold, respectively, compared to control (Fig. 3.5.3D). Similarly, MMA(V), DMA(V), and TMA(V) potentiated the TCDD-mediated induction of NQO1 activity to reach 3.74-, 3.63-, and 3.52-fold, respectively, compared to control (Fig. 3.5.4B). SUL alone significantly induced NQO1 protein and catalytic activity levels by 6.12- and 2.39-fold, respectively, compared to control (Fig. 3.5.3D and 3.5.4B). When HepG2 cells were co-exposed to MMA(V), DMA(V), or TMA(V) in the presence of 5 μM SUL, there was further potentiation of NQO1 protein levels by MMA(V), DMA(V), and TMA(V) to reach 18.65-, 16.86-, and 16.57-fold, respectively, compared to control (Fig. 3.5.3D). Moreover, MMA(V), DMA(V), and TMA(V) potentiated the SUL-mediated induction of NQO1 activity to reach 4.48-, 4.23-, and 4.1-fold, respectively, compared to control (Fig. 3.5.4B).

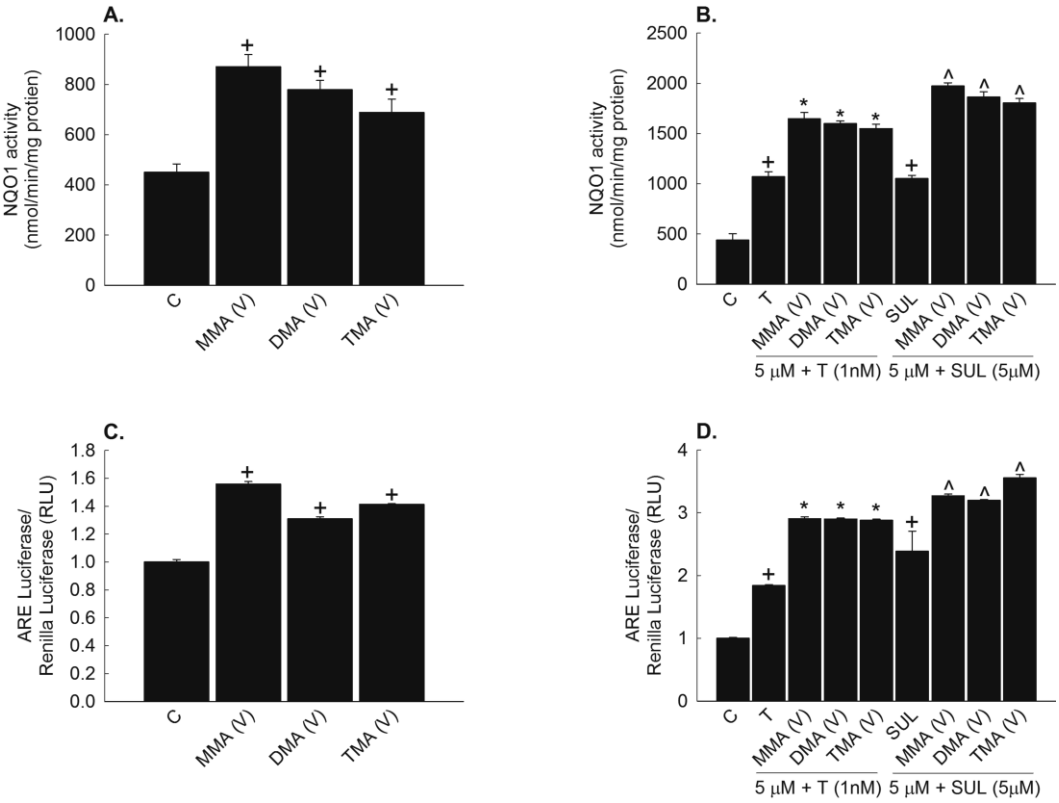

Fig. 3.5.4 Effect of exposure to MMA(V), DMA(V), and TMA(V) in the absence (A and C) and presence of TCDD or SUL (B and D) on NQO1 catalytic activity levels and ARE-driven luciferase reporter activity.
(A and C) HepG2 cells were treated with 5 μM MMA(V), DMA(V), or TMA(V) in the absence and presence of 1 nM TCDD or 5 μM SUL for 24 h. NQO1 enzyme activity was determined spectrophotometrically using DCPIP as substrate. Values are presented as mean ± SE (n = 6). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment; (^) $P < 0.05$, compared to respective SUL treatment. **(B and D)** HepG2 cells were transiently transfected with 200 nM Silencer select negative control siRNA in addition to ARE-luciferase reporter plasmid PGL3-ARE for 6 h, and thereafter cells were treated with 5 μM MMA(V), DMA(V), or TMA(V) in the absence and presence of 1 nM TCDD for 24 h. Cells were lysed and luciferase activity was measured according to manufacturer's instruction. Luciferase activity is reported as relative light unit. Values are presented as 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 SUL treatment.

3.5.6. EFFECT OF EXPOSURE TO MMA(V), DMA(V), AND TMA(V) ON ARE-DRIVEN LUCIFERASE REPORTER ACTIVITY IN THE ABSENCE AND PRESENCE OF TCDD OR SUL

To determine if the observed effect upon co-exposure to MMA(V), DMA(V), and TMA(V) in the absence and presence of TCDD or SUL on NQO1 is occurring through an Nrf2-dependent mechanism, HepG2 cells were transiently co-transfected with ARE-driven luciferase reporter plasmid and its normalizing control construct, renilla luciferase, in the presence of –ve control siRNA. Luciferase activity results showed that MMA(V), DMA(V), and TMA(V) alone significantly induced ARE-driven luciferase activity by 1.55-, 1.30-, and 1.41-fold, respectively, compared to control (Fig. 3.5.4C). TCDD alone significantly induced ARE-driven luciferase activity by 1.83-fold, compared to control (Fig. 3.5.4D). On the other hand, co-exposure to MMA(V), DMA(V), or TMA(V) and TCDD significantly potentiated ARE-driven luciferase activity to 2.90-, 2.90-, 2.88-fold, respectively, compared to control (Fig. 3.5.4D). Similarly, SUL alone significantly induced ARE-driven luciferase activity by 2.38-fold, compared to control (Fig. 3.5.4D). On the other hand, co-exposure to MMA(V), DMA(V), or TMA(V) and SUL significantly potentiated ARE-driven luciferase activity to 3.27-, 3.19-, 3.55-fold, respectively, compared to control (Fig. 3.5.4D).

3.5.7. OXIDATIVE STRESS INDUCED BY AS(III), MMA(V), DMA(V), AND TMA(V)

In order to investigate whether organic arsenicals typified by MMA(V), DMA(V), and TMA(V) induce oxidative stress, we investigated their effect in HepG2 cells by measuring intracellular ROS based on quantification of the oxidation-activated fluorescent dye DCF-DA. The oxidation of DCF to its fluorescent probe was measured 12 h after cells were treated with 5 μM As(III), MMA(V), DMA(V), or TMA(V) (Fig. 3.5.5A). As(III) increased DCF fluorescence by 5-fold compared to control while MMA(V), DMA(V), and TMA(V) increased DCF fluorescence by 13-, 17-, and 24-fold respectively compared to control (Fig. 3.5.5A).

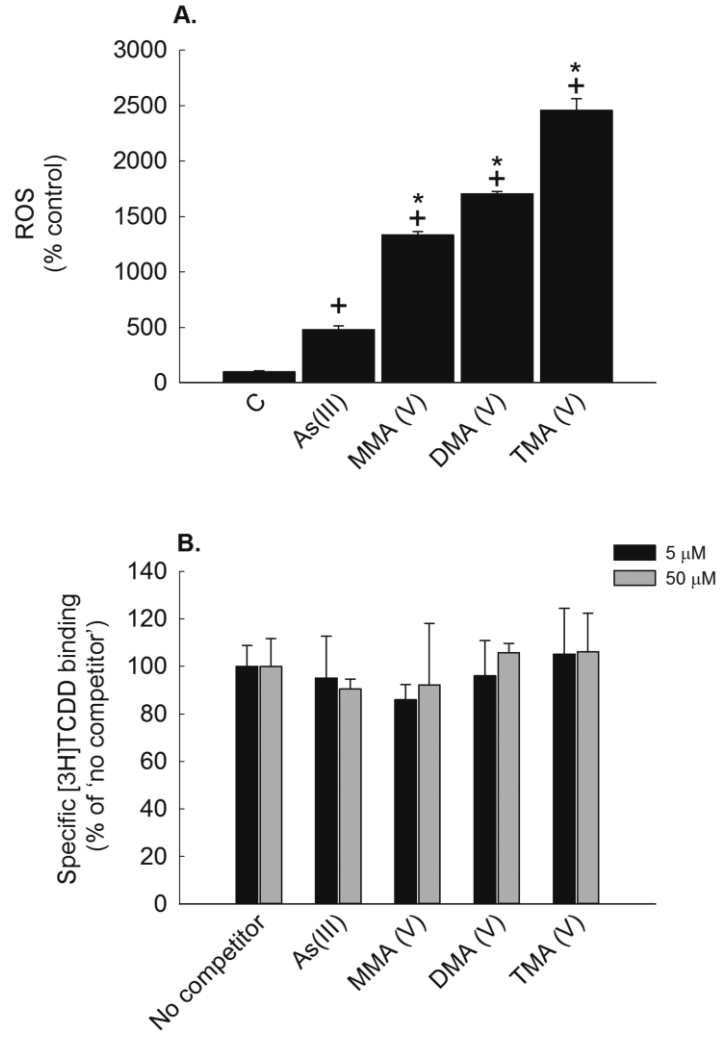

Fig. 3.5.5 Induction of oxidative stress by As(III), MMA(V), DMA(V), and TMA(V) as measured by the formation of the fluorescent form of DCF (A); AhR-ligand binding assay for MMA(V), DMA(V), and TMA(V) in guinea pig liver cytosol (B).

(A) For the DCF-DA assay, HepG2 cells were treated with 5 μM MMA(V), DMA(V), or TMA(V) for 12 h in the presence of DCF-DA. Fluorescence was then measured using excitation and emission wavelengths of 485 and 535 nm, respectively. Data are expressed as mean fluorescence intensity/mg protein ± SE (n = 8). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to As(III) treatment. **(B)** Untreated guinea pig hepatic cytosol (2 mg/ml) was incubated with 2 nM [^{3}H]-TCDD alone (total binding), 2 nM [^{3}H]-TCDD and 200 nM TCDF (100 fold excess of competitor) (non-specific binding), or 2 nM [^{3}H]-TCDD in the presence of 5 or 50 μM As(III), MMA(V), DMA(V), or TMA(V) and the samples analyzed by the hydroxyapatite assay as described under Materials and Methods. Values were adjusted for non-specific binding and expressed as % specific binding relative to the absence of a competitor ligand. Values are presented as mean ± SEM, n=9. (*) $P < 0.05$ compared to [^{3}H]-TCDD.

3.5.8. INDUCTION OF CYP1A1 BY MMA(V), DMA(V), AND TMA(V) IS THROUGH AN AHR LIGAND-INDEPENDENT MECHANISM

The fact that MMA(V), DMA(V), and TMA(V) increased the prototypical downstream target of AhR, CYP1A1, while decreasing the total AhR protein levels, mimicking the effect of TCDD, prompted us to examine these metabolites as potential AhR ligands. To establish whether or not MMA(V), DMA(V), or TMA(V) are direct ligands for the AhR, a ligand competition binding assay using hydroxyapatite was performed (Fig. 3.5.5B). The total binding is the overall binding of [^{3}H]-TCDD to cytosolic protein. However part of this binding is non-specific, i.e. not through the AhR, or not through the ligand-binding center of the AhR. To account for this non-specific binding, reactions are conducted in the presence of 100-fold excess of competitor. TCDF was chosen rather than TCDD because of its higher solubility as TCDD would not be soluble at 200 nM. Therefore, the difference between total and non-specific binding is specific binding of [^{3}H]-TCDD to the AhR. Our results demonstrated that neither As(III), nor MMA(V), DMA(V), or TMA(V) at the concentrations of 5 μM and 50 μM were able to significantly displace [^{3}H]-TCDD (Fig. 3.5.5B). Thus, activation of the AhR and its subsequent nuclear localization upon exposure to MMA(V), DMA(V), and TMA(V) is occurring through a ligand-independent mechanism.

3.5.9. EFFECT OF AHR AND NRF2 SIRNAS ON AHR AND NRF2 MRNA AND PROTEIN EXPRESSION LEVELS

We took a genetic approach to examine whether or not the induction of *CYP1A1* and *NQO1* by MMA(V), DMA(V), and TMA(V) is AhR- and Nrf2-dependent, respectively. SiRNAs against human AhR and human Nrf2 were employed. Furthermore, in order to confirm the knockdown of AhR and Nrf2 we measured their mRNA and protein expression levels 24 h and 48 h post-transfection, respectively. Our results demonstrated that AhR siRNA was able to significantly decrease AhR mRNA and protein expression levels by -4.45- and -4.34-fold, respectively, compared to control (Fig. 3.5.6A and 3.5.6B). Silencer select negative control was unable to alter AhR at the mRNA or protein expression levels (Fig. 3.5.6A and 3.5.6B). Similarly, Nrf2 siRNA significantly decreased Nrf2 mRNA and protein expression levels by -2.85- and -9.7-fold, respectively, compared to control, while on the other hand silencer select negative control was unable to alter Nrf2 mRNA or protein expression levels (Fig. 3.5.6C and 3.5.6D).

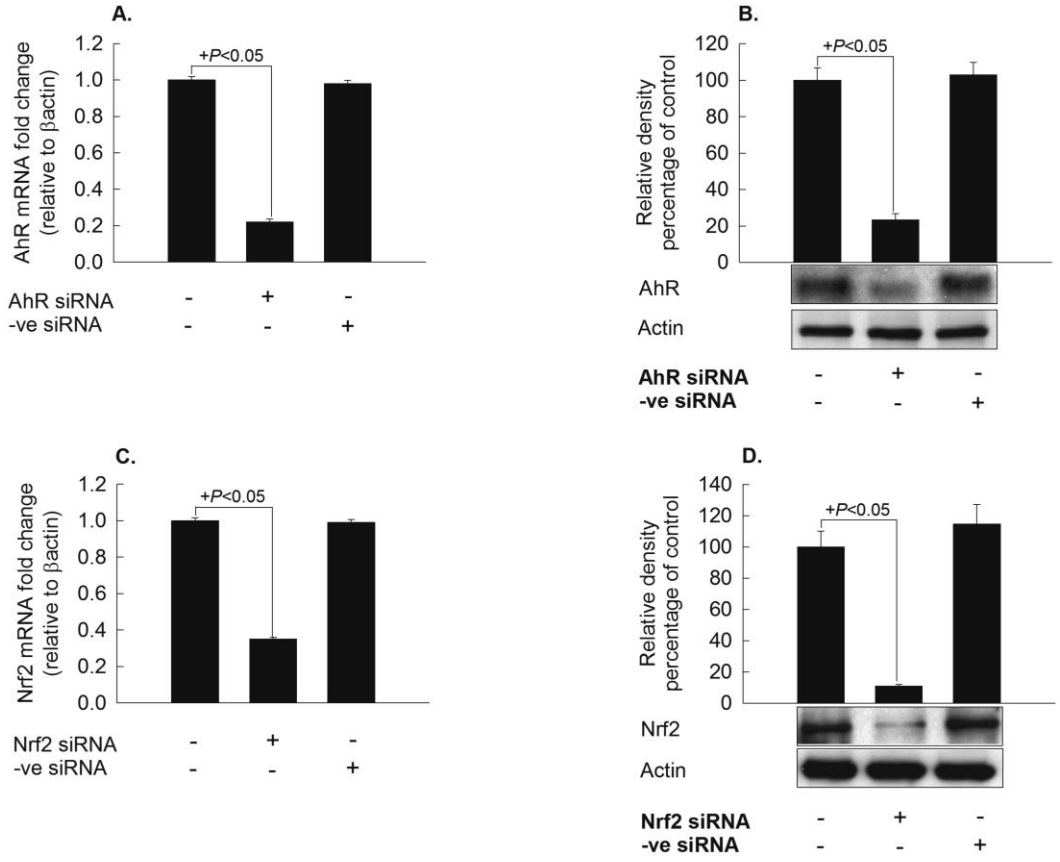

Fig. 3.5.6 Effect of AhR and Nrf2 siRNAs on AhR mRNA and protein (A and B) and Nrf2 mRNA and protein (C and D) expression levels.

(A and B) HepG2 cells were transiently transfected with 200 nM AhR, Nrf2, or Silencer select negative control siRNAs (-ve siRNA) for 24 h, and thereafter AhR and Nrf2 mRNAs were quantified using RT-PCR and normalized to β-actin housekeeping gene. Triplicate reactions were performed for each experiment and the values represent mean of fold change ± SE (n = 4). (+) $P < 0.05$ compared with control. **(C and D),** HepG2 were transiently transfected with 200 nM AhR, Nrf2, or -ve siRNA for 48 h, thereafter AhR and Nrf2 proteins were quantified using Western blot analyses and normalized to actin housekeeping gene. Values are presented as mean ± SE ($n = 4$). (+) $P < 0.05$, compared to corresponding siRNA alone.

3.5.10. EFFECT OF AHR KNOCKDOWN ON MMA(V), DMA(V), AND TMA(V) INDUCED CYP1A1 PROTEIN EXPRESSION LEVELS

To further examine whether or not the induction of CYP1A1 is AhR dependent or not we tested the effect of AhR knockdown on CYP1A1 protein expression levels. For this purpose, HepG2 cells were transfected with AhR siRNA or scrambled (–ve siRNA) for 24 h. Thereafter, transfected cells were treated with 5 μM As(III), MMA(V), DMA(V), TMA(V), or 1 nM TCDD for additional 24 h (Fig. 3.5. 7).

Our results showed that in the presence of –ve siRNA As(III) alone significantly lowered CYP1A1 protein by -2.30-fold compared to control. On the other hand, MMA(V), DMA(V), TMA(V), and TCDD in the presence of –ve siRNA were able to significantly induce CYP1A1 protein expression levels by 4.04-, 7.36-, 11.92-, and 91.87-fold, respectively, compared to control (Fig. 3.5.7). Upon the knockdown of the AhR, As(III)-mediated inhibition of CYP1A1 protein expression levels was further potentiated, while the ability of MMA(V), DMA(V), TMA(V), and TCDD to induce CYP1A1 protein expression levels was significantly decreased, compared to the respective –ve siRNA treatments (Fig. 3.5.7).

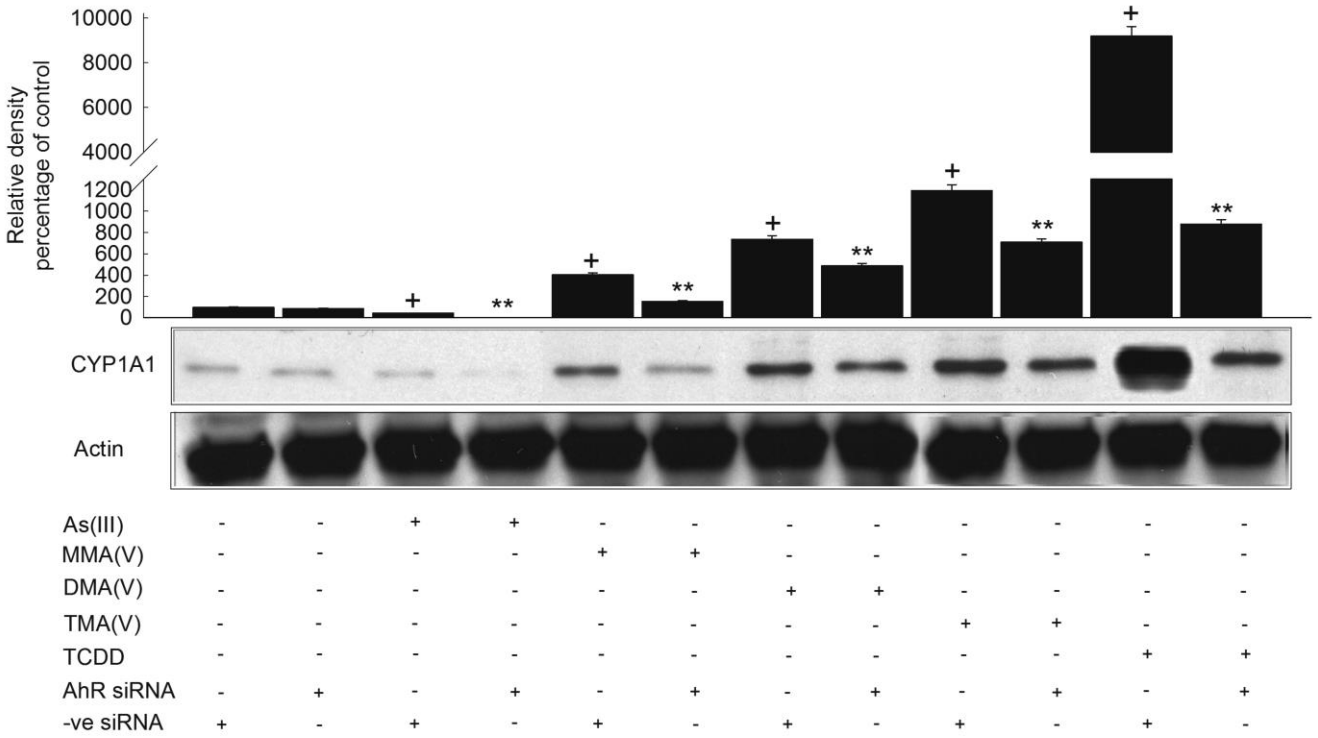

Fig. 3.5.7 Effect of AhR knockdown on MMA(V), DMA(V), and TMA(V) induced CYP1A1 protein expression levels.
HepG2 cells were transiently transfected with 200 nM AhR, or -ve siRNA for 24 h, and thereafter treated with either 5 μM As(III), MMA(V), DMA(V), TMA(V) or 1 nM TCDD for an additional 24 h. CYP1A1 protein was quantified using Western blot analyses and normalized to actin. Values are presented as mean ± SEM (n = 4). (+) $P < 0.05$, compared to control; (**) $P < 0.05$, compared to respective –ve siRNA treatment.

3.5.11. EFFECT OF NRF2 KNOCKDOWN ON MMA(V), DMA(V), AND TMA(V) INDUCED NQO1 PROTEIN EXPRESSION LEVELS

Similar to their effect on CYP1A1 protein expression levels in the absence of AhR, it was of importance to us to determine if similar effects would occur on the NQO1 protein expression levels in the absence of Nrf2. For this purpose, HepG2 cells were transfected with Nrf2 siRNA or –ve siRNA for 24 h. Thereafter, transfected cells were treated with 5μM As(III), MMA(V), DMA(V), TMA(V), or SUL for additional 24 h (Fig. 3.5.8).

Our results showed that in the presence of –ve siRNA As(III), MMA(V), DMA(V), TMA(V), and SUL were able to significantly induce NQO1 protein expression levels by 1.97-, 4.96-, 5.82-, 7.79-, and 14.55-fold, respectively, compared to control (Fig. 3.5.8). Upon the knockdown of the Nrf2, the ability of As(III), MMA(V), DMA(V), TMA(V), and TCDD to induce NQO1 protein expression levels was significantly decreased, compared to the respective –ve siRNA treatments (Fig. 3.5.8).

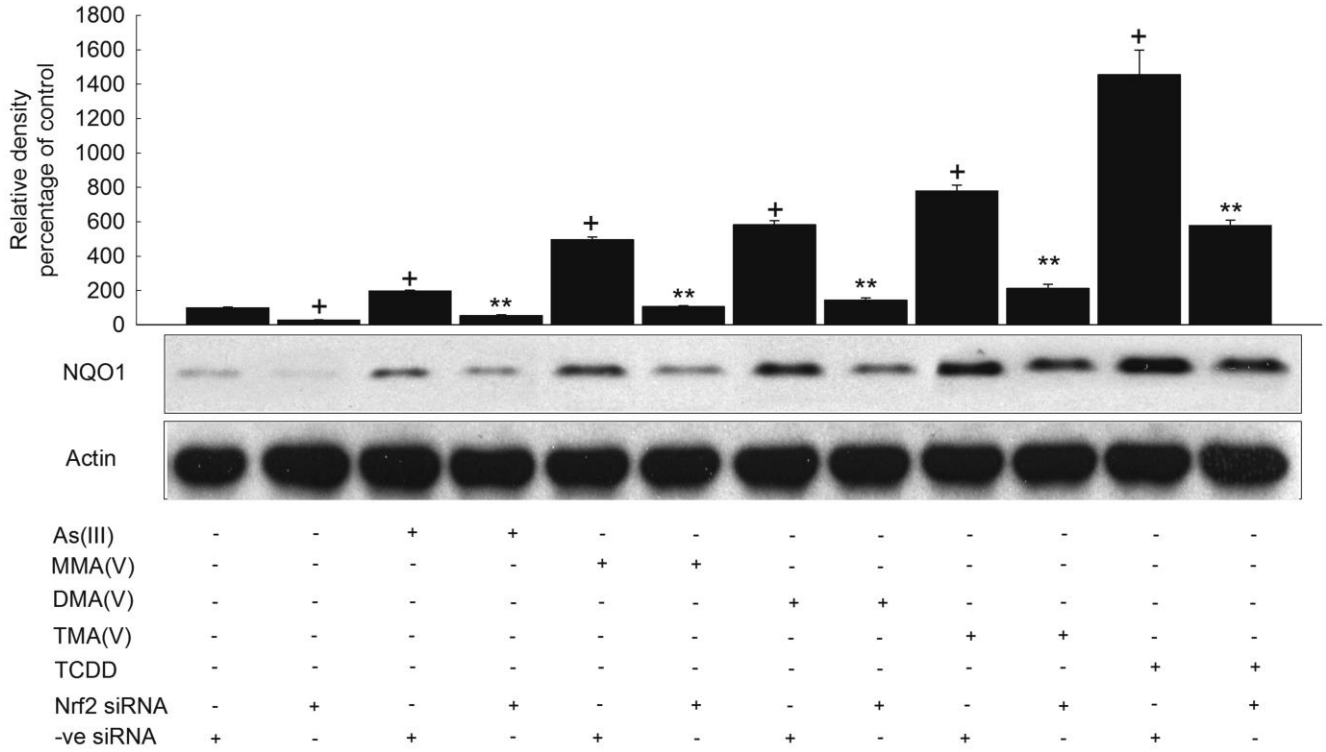

Fig. 3.5.8 Effect of Nrf2 knockdown on MMA(V), DMA(V), and TMA(V) induced NQO1 protein expression levels.
HepG2 cells were transiently transfected with 200 nM Nrf2, or -ve siRNA for 24 h, and thereafter treated with either 5 μM As(III), MMA(V), DMA(V), TMA(V), or SUL for an additional 24 h. NQO1 protein was quantified using Western blot analyses and normalized to actin. Values are presented as mean ± SEM (n = 4). (+) $P < 0.05$, compared to control; (**) $P < 0.05$, compared to respective –ve siRNA treatment.

3.5.12. EFFECT OF EXPOSURE TO MMA(V), DMA(V), AND TMA(V) ON TOTAL AHR AND NRF2 PROTEINS

To determine whether the effects of MMA(V), DMA(V), or TMA(V) on CYP1A1 and NQO1 are through affecting protein levels of both AhR and Nrf2 we examined the effect of MMA(V), DMA(V), and TMA(V) on AhR and Nrf2 protein levels. If MMA(V), DMA(V), or TMA(V) increase CYP1A1 gene expression levels through activating AhR one would expect lowered protein levels as activation of AhR also initiates its degradation process. Inversely, activation of Nrf2 and its subsequent dissociation from Keap1 stabilizes it and prevents it from being degraded. In this regard our results showed that MMA(V), DMA(V), and TMA(V), significantly decreased AhR protein levels by -4-, -10-, and -20-fold, respectively, as compared to control (Fig. 3.5.9A). TCDD alone significantly decreased AhR protein levels by -12-fold compared to control (Fig. 9A). In contrast to the situation with AhR, MMA(V), DMA(V), and TMA(V) significantly increased Nrf2 protein levels by 6.63-, 8.43-, and 10-fold, respectively, compared to control (Fig. 3.5.9B). Similarly, SUL alone significantly increased Nrf2 protein levels by 13.35-fold compared to control (Fig. 3.5.9B).

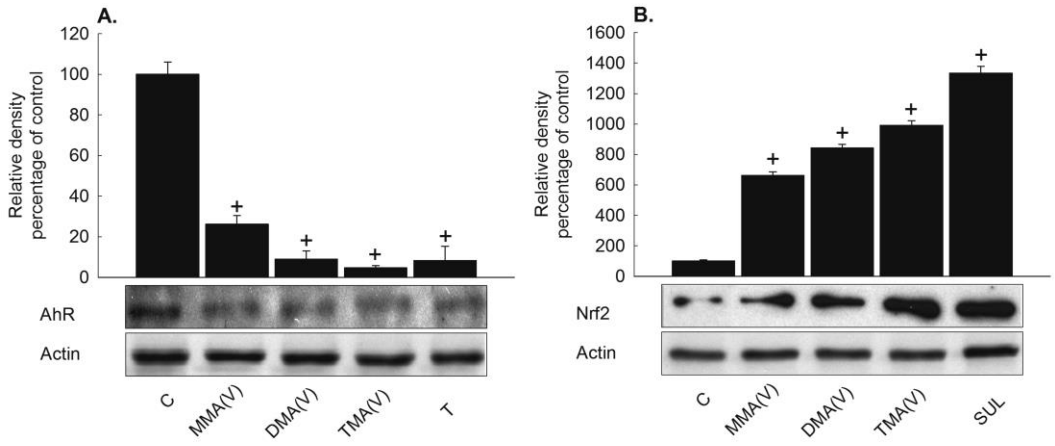

Fig. 3.5.9 Effect of MMA(V), DMA(V), and TMA(V) on total AhR (A) and Nrf2 (B) protein levels.

HepG2 cells were treated with 5 μM MMA(V), DMA(V), or TMA(V) in the absence and presence of 1 nM TCDD or 5 μM SUL for 24 h. Protein (100 μg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C and then incubated with a primary AhR or Nrf2 antibody for 24 h at 4°C, followed by 1 h incubation with secondary antibody at room temperature. AhR and Nrf2 proteins were detected using the enhanced chemiluminescence method. The intensity of bands was normalized to actin signals, which was used as loading control. One of six representative experiments blots is shown. Values are presented as mean ± SE (n = 6). (+) $P < 0.05$, compared to control (C).

3.5.13. EFFECT OF EXPOSURE TO MMA(V), DMA(V), AND TMA(V) ON THE LOCALIZATION OF AHR, HSP90, AND NRF2 PROTEINS

With the ability of MMA(V), DMA(V), and TMA(V) to induce *CYP1A1* through an AhR-dependent and a ligand-independent mechanisms in addition to inducing *NQO1* through an Nrf2-dependent mechanism, we sought to determine the effect of these metabolites on the cellular localization of AhR and Nrf2, and the cytosolic levels of HSP90. For this purpose, HepG2 cells were treated with vehicle, As(III), MMA(V), DMA(V), TMA(V), and TCDD for 3 h, or As(III), MMA(V), DMA(V), TMA(V), and SUL for 6 h followed by extraction of cytosolic and nuclear extracts. Thereafter, AhR and Nrf2 proteins were immunoprecipitated from cytosolic and nuclear fractions to examine their cellular localization, while HSP90 was immunoprecipitated from cytosolic extracts to determine its expression levels.

Our results showed that As(III) alone did not significantly affect the localization of AhR. On the other hand, MMA(V), DMA(V), TMA(V) and TCDD increased the nuclear accumulation of AhR by 7.82-, 7.83-, 8.90-, and 7.91-fold respectively, compared to control (Fig. 3.5.10A). This induced nuclear accumulation of AhR by MMA(V), DMA(V), TMA(V), and TCDD coincided with a significant decrease in cytosolic AhR levels by -7.5-, -2.77-, -4.95-, and -12.57-fold, respectively, compared to control (Fig. 3.5.10A). Looking at the cytosolic levels of HSP90, our results demonstrated that As(III) alone increases cytosolic HSP90 protein expression levels by 1.84-fold, compared to control. Importantly, MMA(V), DMA(V), TMA(V), and TCDD significantly decreased cytosolic HSP90 protein expression levels by -2.27-, -2.43-, -2.04-, and -2.77-fold, respectively, compared to control (Fig. 3.5.10B). Furthermore, As(III), MMA(V), DMA(V), TMA(V), and SUL increased nuclear accumulation of Nrf2 protein by 3.17-, 6.33-, 6.04-, 7.68-, 11.42-fold, respectively compared to control (Fig. 3.5.10C). This induced nuclear accumulation of Nrf2 by As(III), MMA(V), DMA(V), TMA(V), and SUL coincided with a significant decrease in cytosolic

Nrf2 levels by-3.23-, -7.61-, -7.50-, -5.65-, and -4.50-fold, respectively, compared to control (Fig. 3.5.10C).

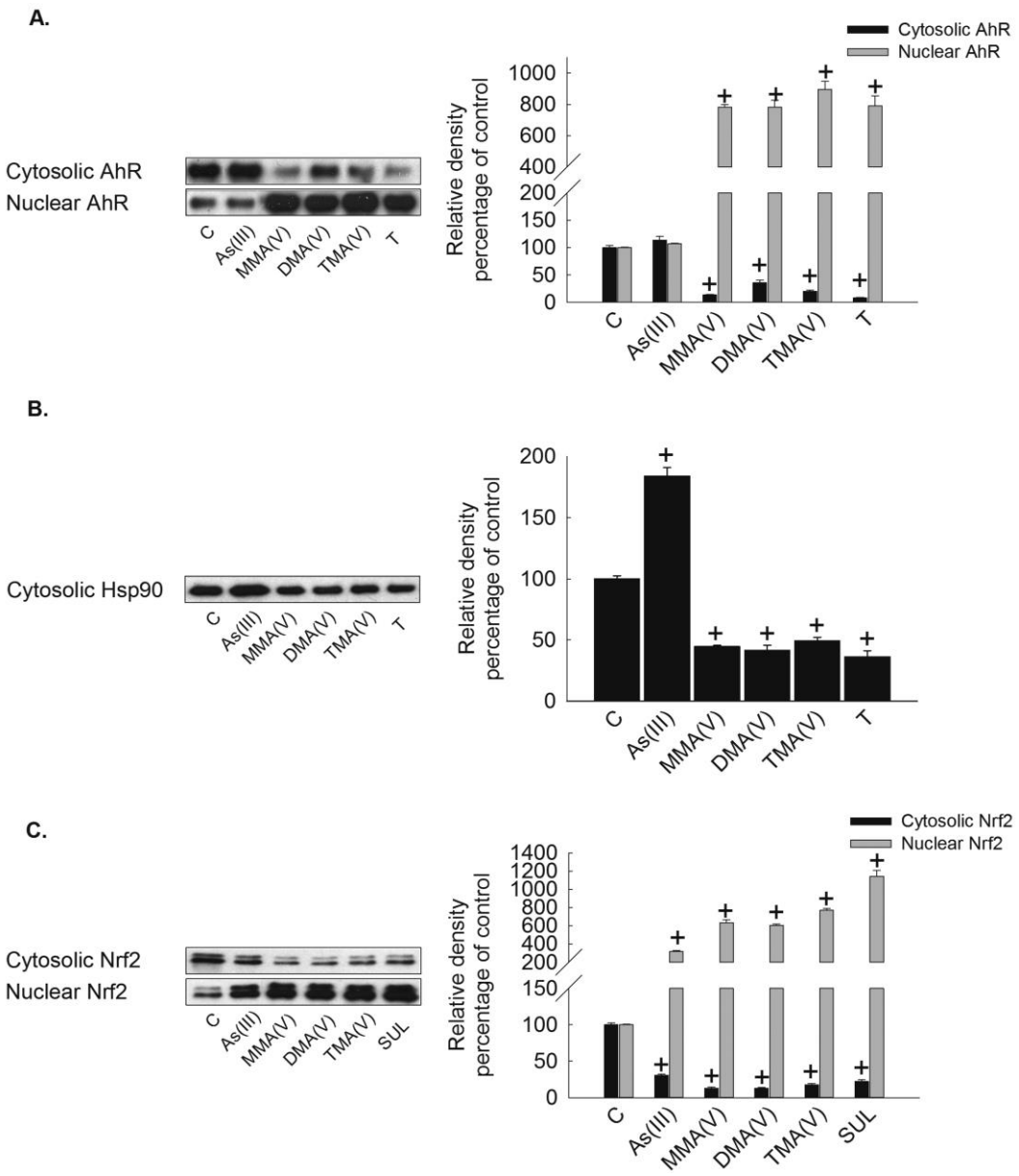

Fig. 3.5.10 Effect of exposure to MMA(V), DMA(V), and TMA(V) on the localization of AhR (A), HSP90 (B), and Nrf2 (C) proteins.

AhR and Nrf2 proteins were immunoprecipitated from cytosolic and nuclear fractions of treated HepG2 cells. HSP90 was immunoprecipitated from cytosolic fractions of treated HepG2 cells. Immunoprecipitates were then analyzed by Western blot analyses. AhR, HSP90, or Nrf2 proteins were detected using the enhanced chemiluminescence method. One of three representative experiments blots is shown. (+) $P < 0.05$, compared to control (C).

CHAPTER 4

4. Discussion

Versions of this chapter have been previously published.

4.1. ARSENITE DOWN-REGULATES THE CYTOCHROME P450 1A1 AT TRANSCRIPTIONAL AND POST-TRANSLATIONAL LEVELS IN HUMAN HEPG2 CELLS

The mechanism(s) by which As(III) exhibits its anti-cancer effects are still at large and undeniably occurring via multiple mechanisms. One of the proposed mechanisms by which As(III) mediates its anti-cancer effects is through inhibiting the gene expression of *CYP1A1*.

In the current study we have shown that As(III) inhibits the TCDD-mediated induction of CYP1A1 at mRNA, protein and catalytic activity levels in a dose-dependent manner. Our findings are in agreement with previous studies showing that As(III)-mediated decrease in CYP1A1 activity was accompanied by either a decrease or no change in the CYP1A1 mRNA levels. For example, it was shown that As(III) was able to reduce benzo[k]fluoranthene- and TCDD-induced CYP1A1 mRNA in HepG2 and Huh7 cells, respectively (Bessette et al., 2005; Chao et al., 2006). An explanation offered to explain this phenomenon is that As(III) blocks the recruitment of polymerase II to the CYP1A1 promoter, thus inhibiting its transcription (Bonzo et al., 2005). In contrast, data from our laboratory have shown that As(III) differentially up-regulates *Cyp1a1* gene expression and causes further potentiation of the TCDD-mediated induction of Cyp1a1 mRNA in murine hepatoma Hepa 1c1c7 cells (Elbekai and El-Kadi, 2007). The controversy between the effect of As(III) on the human HepG2 and mouse Hepa 1c1c7 cells could be attributed to the mechanistic differences in the regulation of CYP1A1 gene expression upon treatment by TCDD (Ramadoss et al., 2004; Ramadoss and Perdew, 2005). Factors that could be responsible for these species-specific characteristics of AhR functions, and subsequently CYP1A1 inducibility, could be summarized in three major components: the nuclear translocation, transcription initiation via remodeling of chromatin, and finally proteasomal degradation of the AhR (Anwar-Mohamed et al., 2009). For example, it has been shown that in Hepa 1c1c7 cells the co-activator CREB-binding protein (CBP) is recruited to the CYP1A1 promoter region post-treatment

with TCDD, reaching its peak at 4 h, and this coincided with the recruitment of AhR and polymerase II, while there was no recruitment of p300 (Suzuki and Nohara, 2007). In contrast, in HepG2, p300 recruitment is increased in response to TCDD to reach its peak between 4 – 12 h, while CBP recruitment unaffected (Suzuki and Nohara, 2007).

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 gene expression. Our findings are in agreement with previous findings showing that As(III) decreases luciferase activity in cells transfected with the XRE-driven luciferase reporter gene in HepG2 and Huh7 cells (Bessette et al., 2005; Chao et al., 2006). Being able to decrease the *CYP1A1* gene expression through a transcriptional mechanism, As(III) was also suspected to participate in decreasing CYP1A1 mRNA stability. Our results showed that As(III) was unable to significantly alter CYP1A1 half-life. These results suggest that As(III) is acting through a transcriptional mechanism to inhibit the *CYP1A1* gene expression.

The ability of As(III) to inhibit the CYP1A1 at the activity level more than that observed on the mRNA or protein expression levels raised the question whether there is a post-translational modification that might have occurred to the CYP1A1 apoprotein. Mounting evidence suggests a role of HO-1 in the As(III)-mediated decrease in CYP1A1 catalytic activity levels (Falkner et al., 1993; Jacobs et al., 1999; Bessette et al., 2009). In the current study we have shown that As(III) increases HO-1 mRNA and protein levels. HO-1, an enzyme of 32 kDa, catalyzes the oxidative conversion of heme into biliverdin which serves an important role in protecting cells from oxidative damage, such as that by free radicals (Marilena, 1997). HO-1 anchors to the endoplasmic reticulum membrane via a stretch of hydrophobic residues at the C-terminus (Schuller et al., 1998). Thus, it is expected to interact with CYP450s which are also endoplasmic reticulum-bound enzymes. The fact that As(III) induces HO-1 with a consequent decrease in the heme pool

could result in the failure to form a functioning CYP1A1 protein. Moreover, the apoprotein would be more susceptible to proteasomal degradation (Anwar-Mohamed et al., 2009).

The role of HO-1 in the down-regulation of CYP1A1 at the catalytic activity level was supported by a number of pieces of evidence. At first, this inhibitory effect of As(III) was also observed on the CYP1A2 catalytic activity. Secondly, As(III) decreased the total cellular heme content in HepG2 cells. These results are in agreement with previously published studies demonstrating that As(III) decreases the total cellular heme content in HepG2 and other cell lines (Falkner et al., 1993; Jacobs et al., 1998; Jacobs et al., 1999; Vakharia et al., 2001a; Bessette et al., 2009). Thus, As(III)-mediated decrease of CYP1A1 is not exclusive; rather it affects the total heme pool, causing a general inhibitory effect on all CYP450s. Thirdly, an HO-1 inhibitor, SnMP, partially restored the As(III)-mediated down-regulation of CYP1A1 and CYP1A2 catalytic activities, and total cellular heme content. The observed effect of SnMP on the As(III)-mediated decrease in CYP1A1 and CYP1A2 catalytic activities and total cellular heme content levels was solely through competitively inhibiting HO-1 protein and not through altering HO-1 or CYP1A1 mRNAs.

Despite the fact that As(III) decreased *CYP1A1* gene expression through a transcriptional mechanism, there was still a persistent inhibition of the CYP1A1 catalytic activity that was in concordance with the inhibition occurring at the mRNA and protein expression levels. Our result is in agreement with previous studies showing that As(III), independent of its action on CYP1A1 mRNA levels, decreases CYP1A1 catalytic activity, total CYP450 content, and total cellular heme content (Falkner et al., 1993; Jacobs et al., 1998; Jacobs et al., 1999; Vakharia et al., 2001a; Bessette et al., 2009). In contrast, a previous study using primary cultured chick hepatocytes has shown that SnMP was unable to reverse the action of As(III) on CYP1A1 catalytic activity (Jacobs et al., 1999). These contradictory results may be explained by cell and species differences.

Because of the potential non-specific effects of pharmacological inhibitors, siRNA was used to confirm the role of HO-1 in the down-regulation of CYP1A1 catalytic activity by As(III). Our results showed that HO-1 mRNA and protein were successfully knocked down in HepG2 cells. Interestingly, As(III)-mediated decrease in CYP1A1 catalytic activity was partially reversed upon transfection with HO-1 siRNA. In agreement with our results, it has been recently shown that in HepG2 cells, HO-1 knockdown partially reverses the effect of As(III) on the benzo-k-fluoranthene-induced CYP1A1 catalytic activity level (Bessette et al., 2009) .

Despite the fact that As(III)-mediated inhibition of CYP1A1 catalytic activity is in part through degrading its heme content, we determined whether or not CoPP, a known HO-1 inducer, will behave similarly. Our results demonstrated that CoPP caused an induction of HO-1 and a decrease in the total cellular heme content. Looking at the effect of CoPP on the CYP1A1 mRNA, our results demonstrated that 1 µM CoPP was unable to cause any significant alteration of CYP1A1 mRNA while it decreased the TCDD-mediated induction of CYP1A1 activity. In addition, transfection with HO-1 siRNA, completely restored the TCDD-mediated induction of CYP1A1 catalytic activity that was initially inhibited by CoPP. Taken together, these results further confirm the role of HO-1 in the modulation of CYP1A1 activity.

It has been previously reported that a deficiency in glucose-6-phosphate dehydrogenase (G6PD) decreases the ability of the cells to generate NADPH (Scott et al., 1991). In addition, previous studies have demonstrated that G6PD was inhibited in different brain regions of As(III) exposed male Wistar rats (Mishra and Flora, 2008). Therefore, we examined whether As(III) decreases the cellular level of NADPH which is required for CYP1A1 activity. Our results showed that As(III) preserved its inhibitory effect on the TCDD-mediated induction of CYP1A1 despite supplementing the enzymatic system with excessive

NADPH. Therefore, the effect of As(III) on the TCDD-mediated induction of CYP1A1 activity does not involve an effect on intracellular NADPH levels.

In order to decipher the role of HO-1 in the As(III)-mediated decrease of CYP1A1 activity we used hemin, a precursor of heme. If As(III) decreases the TCDD-mediated induction of CYP1A1 activity through degrading its heme, then supplying heme will restore the TCDD-mediated induction of CYP1A1 activity. Our results demonstrated that treatment with hemin caused partial restoration of the As(III)-mediated decrease of CYP1A1 activity. In contrast to our results, previous reports have shown that exogenous heme was incapable of restoring the As(III)-mediated decrease in CYP1A1 activity in primary cultures of chick and rat hepatocytes (Jacobs et al., 1998; Jacobs et al., 1999). These contradictions may be explained by the differences in the cells used and the differences in the concentrations of heme used. In our study we used 80 μM hemin (Hintze and Theil, 2005) while in the other studies 2 - 5 μM were used (Jacobs et al., 1998; Jacobs et al., 1999).

HO-1 catalyzes the degradation of heme to CO, biliverdin, and free iron (Abraham et al., 1988). CO is known to bind and inhibit heme-containing proteins such as CYP1A1 (Dulak and Jozkowicz, 2003). Therefore, it was of importance to examine whether the As(III)-mediated decrease of CYP1A1 activity is due to CO generation. In the current study, we have shown that Hb was able to restore the As(III)-mediated decrease of CYP1A1 activity, suggesting that CO produced by HO-1 activity participated in lowering CYP1A1 activity. In addition, Hb is a heme-containing protein, and may protect the CYP1A1 degradation by serving as a substrate for HO-1. In agreement with our results, previous studies have shown that Hb, as CO scavenger, was able to induce CYP2E1 activity in primary human hepatocytes (Yao et al., 2009) and E47 cells without altering protein levels (Gong et al., 2004).

In conclusion, the present study demonstrated that As(III) down-regulates *CYP1A1* gene expression through transcriptional and post-translational mechanisms. Moreover, HO-1 is partially involved in the As(III)-mediated down-regulation of CYP1A1 at the catalytic activity level as supplementation with SnMP, heme, and hemoglobin, in addition to knocking-down HO-1 all caused partial restoration of the CYP1A1 activity.

4.2. INHIBITION OF HEME OXYGENASE-1 PARTIALLY REVERSES THE ARSENITE-MEDIATED DECREASE OF CYTOCHROME P450 1A1 (CYP1A1), CYP1A2, CYP3A23, AND CYP3A2 CATALYTIC ACTIVITY IN ISOLATED RAT HEPATOCYTES

The current study provides the first evidence that As(III) decreases CYP1A1, CYP1A2, CYP3A23 and CYP3A2 at mRNA, protein, and catalytic activity levels in freshly isolated rat primary hepatocytes. Furthermore, inhibiting As(III)-mediated induction of HO-1 partially restores the enzymatic activity of these P450s that was initially decreased by As(III), confirming a direct role for HO-1 in the inhibition of P450s.

Although the effect of As(III) on P450s' activities does not always parallel its effect on the expression of their corresponding mRNA or protein levels, almost all studies have reported a decrease in P450s' catalytic activities in hepatic and extra-hepatic tissues and cells in response to As(III) (Anwar-Mohamed et al., 2009). Thus, it is apparent that As(III) may have a direct effect on the function of P450s, independent of its effect on their transcriptional regulation. Hence, multiple, but common, underlying mechanisms may be involved in its effect. In the current study, we investigated the effect of As(III) on CYP1A1, CYP1A2, CYP3A23, and CYP3A2 and the possible underlying mechanisms.

Firstly, we investigated the effect of As(III) on CYP1A1 and CYP1A2 at the mRNA, protein and catalytic activity levels. Our results demonstrated that As(III) decreased both the constitutive and the TCDD-mediated induction of CYP1A1 and CYP1A2 at the mRNA, protein and catalytic activity levels (Fig. 3.2.2A, 3.2.2B, and 3.2.2C). Similar effects of As(III) have been previously reported in rodents (Albores et al., 1989; Jacobs et al., 1999). For example, in studies using Wistar rats, As(III) decreased total hepatic P450s content and monoxygenase activities of several P450s including CYP1A1 and CYP2B1 (Siller et al., 1997). Similarly, studies on primary cultures of chick and rat hepatocytes showed that

As(III) decreased total P450s and 3-methylcholanthrene-mediated induction of CYP1A1 activity in chick hepatocytes. Moreover, As(III) decreased CYP1A, CYP2B, and CYP3A at the mRNA, protein, and catalytic activity in rat hepatocytes (Jacobs et al., 1998; Jacobs et al., 1999). The effect of As(III) was also tested in primary human hepatocytes in which As(III) decreased PAH-mediated induction of CYP1A2 but not CYP1A1 at mRNA levels, while it decreased protein and catalytic activity levels of both isozymes in these cells (Vakharia et al., 2001a).

To examine whether the observed effect of As(III) is exclusive for the AhR regulated genes *CYP1A1* and *CYP1A2*, we examined its effect on another P450 subfamily, CYP3A, which is under the control of PXR. Our results showed that As(III) decreased both constitutive and Rif-mediated induction of CYP3A23 and CYP3A2 at the mRNA, protein and catalytic activity levels. In agreement with our findings, previous studies have also shown that As(III) decreases dexamethasone-mediated induction of CYP3A23 mRNA, protein, and catalytic activity in primary rat hepatocytes (Jacobs et al., 1999). However, these studies utilized dexamethasone which primarily induces the glucocorticoid receptor (GR), rather than PXR (Bock, 2011).

The fact that As(III) inhibited the transcription of CYP1A1 and CYP1A2 that are under the transcriptional control of AhR, and CYP3A23 and CYP3A2 which are under the control of PXR, prompted us to investigate the effects of As(III) on AhR and PXR proteins. It has been previously demonstrated that TCDD increases AhR nuclear accumulation in rat primary hepatocytes (Xu et al., 2000). In agreement with these findings, our results showed that TCDD increased the nuclear accumulation of AhR by 266%. Importantly, As(III) decreased the TCDD-induced AhR nuclear accumulation, implying the presence of a transcriptional mechanism.

Previous data have shown that liganded AhR is ubiquinated prior to its degradation by the 26S proteasomal pathway (Pollenz, 2002). To determine whether or not the decrease in AhR nuclear accumulation is due to the increase of the degradation of AhR protein by As(III), we examined the effect of As(III) on AhR protein levels at 24 h. Our results showed that As(III) alone did not affect cellular AhR levels. Similarly, we investigated the effects of As(III) on PXR protein. In the current study we demonstrated that As(III) inhibited the constitutive and Rif-induced nuclear accumulation of PXR. Furthermore, As(III) alone or in the presence of Rif did not affect total cellular PXR levels after 24 h of exposure.

The ability of As(III) to inhibit *CYP1A1* and *CYP1A2* gene expression with a decreased nuclear accumulation of AhR prompted us to investigate the effect of As(III) on the transcriptional level. For this reason we performed EMSA using nuclear extracts of rat primary hepatocytes. In concordance with our nuclear AhR protein results, our EMSA results showed that As(III) inhibited the AhR translocation to the nucleus and its subsequent binding to XRE (Fig. 3.2.6A).

In order to investigate the mechanism behind the inhibition of AhR nuclear accumulation and the subsequent binding to XRE we immunoprecipitated AhR protein from cytosolic and nuclear fractions of treated primary hepatocytes. Our results demonstrated that As(III) treatments were associated with preferential localization of the AhR in the cytosol over the nucleus. Furthermore, As(III) stabilized the binding of cytosolic AhR to HSP90 and XAP2 which could be the mechanism by which As(III) inhibits the AhR nuclear accumulation.

The fact that As(III) inhibited CYP1A1, CYP1A2, and CYP3A at the activity levels more than the mRNA or protein expression levels raised the question of whether there is a post-translational modification that might have occurred to their apoproteins. Mounting evidence suggests the role of HO-1 in the As(III)-mediated decrease of P450s' catalytic activity (Bessette et al., 2009; Anwar-Mohamed and

El-Kadi, 2010). HO-1, an enzyme of 32 kDa, catalyzes the oxidative conversion of heme into biliverdin and subsequently bilirubin which serves an important role in protecting cells from oxidative damage caused by free radicals (Marilena, 1997). HO-1 regulation occurs through the redox sensitive Nrf2/ARE signaling pathway. Of interest, HO-1 anchors to the endoplasmic reticulum membrane via a stretch of hydrophobic residues at its C-terminus (Schuller et al., 1998). Thus, it is expected to interact with P450s, which are also endoplasmic reticulum-bound enzymes. However, in order for this degradation to occur, P450 protein unfolding should precede heme degradation. Our results showed that HO-1 mRNA levels are elevated in all treatments receiving As(III). This elevation in HO-1 mRNA coincided with a decrease in total cellular heme content in the same treatments. In this regard, almost all studies on the As(III)-mediated effect on P450 catalytic activity implicated a role of HO-1 in the degradation of its heme group, creating a hollow functionless protein (Anwar-Mohamed and El-Kadi, 2010). For example, HO-1 activity was elevated in primary cultures of chick hepatocytes treated with As(III) (Jacobs et al., 1999). We therefore speculated that the induction of HO-1 may contribute to the inhibition of CYP1A1, CYP1A2, and CYP3A catalytic activity levels by As(III), despite the presence of other interplaying mechanisms.

In order to decipher the role of HO-1 in the As(III)-mediated decrease of CYP1A1, CYP1A2, and CYP3A catalytic activity levels we used two approaches. We investigated whether treatment of cells with (1) an inhibitor of HO-1, SnMP or (2) supplementing with external heme would restore As(III)-mediated decrease of CYP1A1, CYP1A2, and CYP3A catalytic activities. If As(III) decreases the TCDD- and Rif-mediated induction of CYP1A1, CYP1A2 and CYP3A activities through degrading its heme via HO-1, then inhibiting HO-1 or supplying heme will restore the TCDD- and Rif-mediated induction of CYP1A1, CYP1A2, and CYP3A catalytic activity levels. Our results showed that inhibiting HO-1 using SnMP partially restores the As(III)-mediated decrease of CYP1A1, CYP1A2 and CYP3A catalytic activities. In contrast to our findings, it has been previously shown that in the primary cultures of chick hepatocytes the effect of As(III) on

CYP1A1 catalytic activity was not reversed by SnMP (Jacobs et al., 1999). The contradictions between this study and the previous study could be explained by species differences or SnMP concentration differences as the previous study used 0.4 nM of SnMP while in the current study we used 5 μM.

Furthermore, upon using hemin as heme precursor, there was also a partial restoration of the As(III)-mediated decrease of CYP1A1, CYP1A2, and CYP3A catalytic activity levels. In contrast to our results, previous reports have shown that exogenous heme was incapable of restoring the As(III)-mediated decrease of CYP1A1 activity in primary cultures of chick and rat hepatocytes (Jacobs et al., 1998; Jacobs et al., 1999). These contradictions may be explained by the differences in the concentrations of heme used, in our study we used 80 μM hemin (Anwar-Mohamed and El-Kadi, 2010) while in the other studies 2.5 to 10 μM were used (Jacobs et al., 1998; Jacobs et al., 1999). The restoration of P450 activity in response to SnMP or heme could be explained by two important mechanisms. Firstly, SnMP, through inhibiting HO-1, restores P450 activities and this would protect P450 heme content. Secondly, free heme provided externally would act to distract HO-1 from degrading bound heme since free heme is readily available for it to degrade.

In conclusion, the present study demonstrated that As(III) down-regulates CYP1A1, CYP1A2, CYP3A23, and CYP3A2 through transcriptional and post-translational mechanisms. Moreover, HO-1 is partially involved in the As(III)-mediated down-regulation of these P450s at the catalytic activity level as treatment with an HO-1 inhibitor, SnMP, or supplementing with external heme caused partial restoration of the CYP1A1, CYP1A2, CYP3A23, and CYP3A2 catalytic activities.

4.3. DIFFERENTIAL MODULATION OF ARYL HYDROCARBON RECEPTOR-REGULATED ENZYMES BY ARSENITE IN THE KIDNEY, LUNG, AND HEART OF C57BL/6 MICE

In the current study we have demonstrated that As(III) modulates the constitutive and TCDD-inducible AhR-regulated enzymes in a time-, tissue-, and AhR-regulated enzyme-specific manner. Furthermore, the concentrations of As(III) and TCDD utilized in the current study were selected based on previous *in vivo* studies that used the same concentrations of both As(III) and TCDD on C57BL/6 mice (Mehra and Kanwar, 1980; Hu et al., 1999; Uno et al., 2008; Wong et al., 2010).

Our findings showed that Cyp1a1, Cyp1a2, Cyp1b1, Nqo1, Gsta1 and HO-1 are constitutively expressed in all the examined tissues. Furthermore, Nqo1 and HO-1 were amongst the highest constitutively expressed genes, followed by Cyp1a1, Cyp1b1, Gsta1 and lastly Cyp1a2 in the kidney of C57Bl/6 mice. In the lung of C57Bl/6 mice, the highest constitutively expressed gene was Cyp1a1 followed by HO-1 while Cyp1a2, Cyp1b1, Nqo1, and Gsta1 were expressed at the same level. Lastly in the heart the expression of these genes from high to low was, Nqo1> Cyp1a1> HO-1> Cyp1b1> Gsta1> Cyp1a2. In agreement with our findings, previous studies have demonstrated that Cyp1a1 and Cyp1b1 were the highest expressed genes in male C57Bl/6 mice and male Sprague–Dawley rats kidney, lung, and heart (Shimada et al., 2002; Elsherbiny et al., 2010). Similarly, Cyp1a2 constitutive expression was the lowest in all tested tissues, which is in agreement with previously published studies (Shimada et al., 2002; Zordoky et al., 2008). Therefore, Cyp1a2 was set as the calibrator in these tissues. The tissue-specific expression of AhR-regulated enzymes examined in the current study and other previous studies has been a subject of discrepancies (Jaiswal, 2000; McMahon et al., 2001; Shimada et al., 2003; Frericks et al., 2007). Several factors may have led to these discrepancies. The first factor is the use of a conventional PCR technique or Northern blot analyses in most of these studies, which may be insensitive because of the low expression levels of AhR-regulated genes,

especially in extrahepatic tissues such as the heart (Jaiswal, 2000; McMahon et al., 2001; Shimada et al., 2003). Secondly, most of the previous studies focused on one tissue without giving comparative information regarding the other tissues. Therefore, it was necessary to examine the expression of multiple AhR-regulated enzymes simultaneously in different organs by a sensitive technique, such as the real-time PCR technique. Our findings showed that the AhR-regulated enzymes Cyp1a1, Cyp1a2, Nqo1, and Gsta1 in addition to HO-1 are constitutively expressed in all the examined tissues. The lung had the highest constitutive expression of Cyp1a1, Cyp1a2, and Gsta1 followed by the kidney and lastly the heart. The kidney had the highest constitutive expression of Nqo1 and HO-1. Generally, the heart had the lowest level of constitutive expression of all tested genes except for Nqo1 where it came second after the kidney.

In the current study As(III) significantly increased the Cyp1a1 mRNA in lung but not in the kidney or heart of C57Bl6 mice. Furthermore, As(III) failed to alter Cyp1a2 mRNA in all tested tissues. At the protein and catalytic activity levels As(III) induced lung Cyp1a protein expression levels with a subsequent increase in EROD and MROD catalytic activities while it inhibited the kidney EROD and MROD catalytic activities. The limitation of minimal protein quantities that could be extracted from heart samples hindered us from measuring protein expression and catalytic activities of AhR-regulated genes in this organ. Our results are in line with previous reports which showed that As(III) was able to induce Cyp1a1 mRNA levels in liver- and lung-derived cell lines, and lung tissues of C57BL/6 mice (Elbekai and El-Kadi, 2007; Wu et al., 2009). Similarly and in agreement with our results As(III) also failed to cause similar induction of Cyp1a1 mRNA levels in the kidney of C57Bl/6 mice (Seubert et al., 2002b). We report here for the first time that As(III) did not significantly alter Cyp1a1 mRNA levels in the heart. Importantly, there have been no previous attempts to investigate the effects of As(III) on Cyp1a2 expression. In the current study it was demonstrated that As(III) alters Cyp1a2 mRNA, protein, and catalytic activity levels in the kidney,

lung, and heart of C57Bl/6 mice in a pattern different from that of Cyp1a1. Thus the effect of As(III) on Cyp1a1 cannot be generalized to include Cyp1a2.

Previous reports from our lab have demonstrated that As(III) potentiated the TCDD-mediated induction of Cyp1a1 mRNA and protein expression levels in the mouse liver derived cell line Hepa 1c1c7 cells after 6 h of treatment (Elbekai and El-Kadi, 2007). However, it was never reported before if As(III) would affect TCDD-mediated induction of Cyp1a1 and Cyp1a2 mRNA, protein, or catalytic activity levels in the kidney, lung, or heart of C57Bl/6 mice. Therefore, we examined the effect of As(III) on the TCDD-mediated induction of Cyp1a1 and Cyp1a2 at the mRNA, protein and catalytic activity levels. As(III) after 6 h of treatment significantly inhibited the TCDD-mediated induction of the kidney and heart Cyp1a1 and Cyp1a2 mRNA levels while it significantly potentiated the TCDD-mediated induction of lung Cyp1a1 but not Cyp1a2 mRNA levels. On the contrary, As(III) after 24 h of treatment significantly potentiated the TCDD-mediated induction of kidney and lung Cyp1a1 and kidney Cyp1a2 mRNA levels. At the protein and catalytic activity levels, As(III) significantly inhibited the kidney Cyp1a while it significantly potentiated lung Cyp1a protein expression levels, which were subsequently translated to their EROD and MROD catalytic activity levels.

The differences between the results of mRNA expression obtained after 6 h and 24 h of treatment are multifactorial. Therefore, we hypothesize that the effects observed at 6 h is the direct effect of non-metabolized As(III) in the form of sodium arsenite. However, the effect after 24 h is an indirect effect that could have arisen due to other metabolic and kinetic changes that are summarized as follows: first, the distribution and elimination half-life of As(III) from these organs is 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 (Birnbaum, 1986; Hughes et al., 1999). Second, As(III) is metabolized in these organs to produce mono- and di-methyl arsenate and arsenite metabolites, which in turn will have

differential effects on these enzymes than the parent compound, sodium arsenite, especially at the mRNA and protein stability levels (Cui et al., 2008). 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 (He et al., 2007). Fourth, As(III) might have affected other physiological processes or even triggered other physiological modulators such as bilirubin and billiverdin which are also AhR-ligands (Denison and Nagy, 2003). In the current study we have demonstrated that As(III) alone or in the presence of TCDD was able to induce HO-1 mRNA levels as early as 6 h in all tested tissues. However, this induction was either completely abolished or reduced after 24 h of treatment. We have previously demonstrated that there is an inverse correlation between HO-1 and CYP1A1 activity (Anwar-Mohamed et al., 2009; Anwar-Mohamed and El-Kadi, 2010). In this study the correlation between HO-1 and EROD and MROD catalytic activities was proportional in the lung as As(III) potentiated the TCDD-mediated induction of Cyp1a mRNA, protein, and catalytic activity levels. This can be explained by the fact that As(III) is known to bind to Hb from red blood cells, causing hemolysis and increasing plasma Hb levels (Klimecki and Carter, 1995). Therefore, it is possible that As(III)-liberated Hb might have inhibited the inhibitory effect of HO-1 on Cyp1a1 and Cyp1a2 catalytic activities in the lung. Interestingly, the main excretion route of As(III) and its metabolites is the kidney (Vahter and Concha, 2001); therefore, in the kidney the inhibitory effect of As(III) will persist as As(III) gets eliminated and plasma Hb does not reach the kidney.

With regard to AhR-regulated phase II enzymes, As(III) alone significantly induced Nqo1 and Gsta1 mRNA, protein, and catalytic activities in the kidney, lung, and heart. Importantly, the co-exposure to As(III) and TCDD significantly potentiated the induction of kidney Nqo1 mRNA after 6 h and 24 h of treatment, lung Nqo1 after 24 h of treatment, and heart Nqo1 and Gsta1 mRNA after 6 h and 24 h of treatment. As(III) is not known to be an AhR ligand, yet it has been shown

be an oxidative stress inducer (Elbekai and El-Kadi, 2005). The regulation of Nqo1 and Gsta1 involves, in addition to the AhR-XRE pathway, the Nrf2-antioxidant response element (ARE) pathway. However, the induction of Gsta1 mRNA levels by As(III) in the absence and presence of TCDD was much greater than the induction of Nqo1 mRNA. It is still unknown if mouse Gsta1 regulation involves, in addition to the AhR-XRE and Nrf2-ARE pathways, an additional pathway. To the contrast, potential regulatory elements have been identified in the human *GSTA1* promoter, including AP-1 and AP-2 consensus sequence and a glucocorticoid response element (Whalen and Boyer, 1998). Thus, it might be a possibility that the effect of As(III) on Gsta1 might be mediated through a different pathway.

In conclusion, the present study demonstrates for the first time that As(III) modulates constitutive and TCDD-induced AhR-regulated genes in a time-, tissue-, and AhR-regulated enzyme-specific manner. Furthermore, the effect on one of these enzymes could not be generalized to other enzymes even if it is in one organ, as there are multiple factors that could interplay to cause differential effects.

4.4. DIFFERENTIAL MODULATION OF CYTOCHROME P450 1A1 (CYP1A1) BY ARSENITE *IN VIVO* AND *IN VITRO* IN C57BL/6 MICE

In the current 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 the current 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 (Mehra and Kanwar, 1980; Hu et al., 1999; Elbekai and El-Kadi, 2004; Uno et al., 2008; Wong et al., 2010).

In the present study As(III) by itself did not affect liver Cyp1a1, Cyp1a2, or Cyp1b1 at the mRNA, protein or catalytic activity levels. 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 C57BL6 mice liver (Seubert et al., 2002b). There is one difference between our study and the previously published study in which As(III) was administered subcutaneously, in a dose closer to what we used in the current study (11 mg/kg) (Seubert et al., 2002b). Importantly, in the same study EROD activity was not affected at 48 h 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 h 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 guinea pig (Falkner et al., 1993). Furthermore, in Wistar rats As(III) decreased total hepatic P450 content and monoxygenase activities of several P450s including CYP1A1 (Siller et al., 1997). 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 effect of As(III) on the TCDD-mediated induction of Cyp1a1 and Cyp1a2 mRNA between the 6 h and 24 h time points might be due to multiple factors. We hypothesized that the effects observed at 6 h are due to the direct effect of non-metabolized 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 (Birnbaum, 1986; Hughes et al., 1999). Second, As(III) is metabolized in the liver to mono-, di-, and tri-methyl arsenates and arsenites, which in turn will have differential effects on Cyp1a1 and Cyp1a2 mRNA, protein, and catalytic activity levels (Cui et al., 2008). 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 (He et al., 2007). 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 (Denison and Nagy, 2003). 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 (Klimecki and Carter, 1995).

In order to test our hypothesis that the effects of As(III) on the TCDD-mediated induction of Cyp1a1 and Cyp1a2 mRNA levels at 6 h are in fact due to the direct

effect of As(III), we measured As(III) levels in 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 (Maier et al., 2000). Similarly, studies on primary cultures of chick and rat hepatocytes showed that As(III) decreased total P450 and 3-MC-mediated induction of CYP1A1 activity in chick hepatocytes, and CYP1A1 mRNA, protein and catalytic activity in rat hepatocytes (Jacobs et al., 1998; Jacobs et al., 1999; Anwar-Mohamed and El-Kadi, 2010). The effect of As(III) was also tested in primary human hepatocytes in which As(III) decreased PAH-mediated induction of CYP1A2 at the mRNA levels, while it decreased protein and catalytic activity levels of CYP1A1 and CYP1A2 (Vakharia et al., 2001a). 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 through increasing HO-1 (Elbekai and El-Kadi, 2004).

The ability of As(III) to inhibit the Cyp1a1 at the activity level more than that observed on the mRNA or protein expression levels in isolated hepatocytes raised the question of whether there is a post-translational modification of the Cyp1a1

protein that might have occurred. Evidence from our laboratory and others suggest a role of HO-1 in the As(III)-mediated decrease in Cyp1a1 catalytic activity levels in Hepa 1c1c7 cells, HepG2 cells, and rat isolated hepatocytes (Falkner et al., 1993; Jacobs et al., 1999; Bessette et al., 2009; Anwar-Mohamed and El-Kadi, 2010; Anwar-Mohamed et al., 2011). In the current study we have shown that As(III) increases HO-1 mRNA levels. Thus it is plausible that As(III), through 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 (Anwar-Mohamed et al., 2009). However, this might have not occurred at the *in vivo* level due to the fact that HO-1 was not persistently elevated, and thus its effect could not be seen at the activity level.

In order to dissect the role of HO-1 in the As(III)-mediated decrease of 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 through 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 of 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 (Anwar-Mohamed and El-Kadi, 2010).

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 levels. In this regard, previous studies have shown that i.p.

administered As(III) had a terminal half-life of 9 h, with more than 90% of the dose being eliminated within 24 h (Hughes et al., 1999). Also, the distribution of As(III) might have contributed to this differential expression (Hughes et al., 1999). 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 (Hughes et al., 1999). However, at the *in vitro* level, As(III) or its metabolites are persistently present in the cells throughout the treatment time-course. Thus, the effect we see *in vitro* is either for As(III) or 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 (Klimecki and Carter, 1995). 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 (Anwar-Mohamed and El-Kadi, 2010). 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 the current study we have demonstrated that Hb increases XRE-driven luciferase activity through 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 (Baumann et al., 1983). The complex is then internalized by the hepatocytes through a receptor-mediated endocytosis (Kino et al., 1982). 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 (Denison and Nagy, 2003). 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 (Dulak and Jozkowicz, 2003). 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 (Yao et al., 2009), and E47 cells without altering protein levels (Gong et al., 2004).

In conclusion, the present 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 which are not present *in vitro*.

4.5. METHYLATED PENTAVALENT ARSENIC METABOLITES ARE BIFUNCTIONAL INDUCERS AS THEY INDUCE CYTOCHROME P450 1A1 (CYP1A1) AND NAD(P)H: QUINONE OXIDOREDUCATSE (NQO1) THROUGH AHR- AND NRF2-DEPENDENT MECHANISMS

It is well established in humans as well as in other mammalian species that As(III) undergoes biomethylation, yielding mono-, di-, and possibly trimethylated metabolites. The biomethylation has been recently attributed to a specific methyltransferase, the arsenic methyl transferase (AS3MT), which utilizes *S*-adenosylmethionine as a methyl group donor (Zakharyan et al., 1999; Styblo et al., 2000; Thomas et al., 2004). The first methylation step to As(III) yields MMA(V). According to the metabolic scheme postulated by Cullen in 1984 (Anwar-Mohamed et al., 2012), MMA(V) is then reduced to MMA(III), before it gets further methylated to yield DMA(V). Similarly, a reduction step to DMA(V) to yield DMA(III) precedes the third methylation step to yield TMA(V).

In the present study we have demonstrated that methylated pentavalent arsenic metabolites induce both *CYP1A1* and *NQO1* through AhR- and Nrf2-dependent mechanisms. Furthermore, the concentrations of these metabolites were chosen to match those of inorganic As(III) that were used in our previous *in vitro* studies (Isoherranen et al., 2003; Anwar-Mohamed and El-Kadi, 2010). Moreover, these metabolites do not pose any cytotoxicity to HepG2 cells at the concentrations tested (data not shown). In addition, the concentrations tested of both TCDD and SUL utilized in the current study were chosen based on previous studies that used the same concentrations for both inducers on HepG2 cells without posing cytotoxicity (Anwar-Mohamed and El-Kadi, 2009a; Anwar-Mohamed and El-Kadi, 2009b; Abdelhamid et al., 2010).

In the current study we hypothesized that MMA(V), DMA(V), and TMA(V) are bifunctional inducers in HepG2 cells as they mediate the induction of *CYP1A1* through activating an AhR signaling pathway, and they mediate the induction of

NQO1 through activating both AhR and Nrf2 signaling pathways. Thus the objectives of the current study were: (i) to examine the effect of MMA(V), DMA(V), and TMA(V) on the constitutive and inducible expression levels of both *CYP1A1* and *NQO1*; (ii) to examine the differences between inorganic As(III) and these organic metabolites especially on *CYP1A1;* and (iii) to explore the underlying mechanisms involved in this modulation.

The regulation of *CYP1A1* gene expression involves activation of a cytosolic transcriptional factor, the AhR, as the first step in a series of molecular events promoting *CYP1A1* transcription and translation processes (Denison et al., 1989). In the present study we have shown that MMA(V), DMA(V), and TMA(V) significantly induced the constitutive expression of CYP1A1 at the mRNA, protein, and catalytic activity levels while potentiating the TCDD-mediated induction of CYP1A1 at the mRNA, protein, and catalytic activity levels. Furthermore, MMA(V), DMA(V), and TMA(V) increased XRE-driven luciferase reporter activity in the absence and presence of TCDD.

The fact that MMA(V), DMA(V), and TMA(V) increased *CYP1A1* expression while we and others have shown previously that inorganic As(III) decreases CYP1A1 expression and AhR nuclear accumulation in HepG2 cells (Anwar-Mohamed et al., 2008; Anwar-Mohamed et al., 2009) raised the question of what could be the key difference(s) between these metabolites and As(III) that caused these completely opposite responses. For this purpose we conducted a series of experiments to examine these differences. First, we measured ROS generation in HepG2 cells in response to As(III), MMA(V), DMA(V), and TMA(V) using DCFDA. Our results showed that MMA(V), DMA(V), and TMA(V) increased DCF fluorescence more than inorganic As(III). In this regard, it has been shown previously that activation of AhR is associated with increased ROS production, possibly through the induction of CYP1A1 (Elshenawy et al., 2013). Therefore, it is plausible that the increased ROS production in response to MMA(V), DMA(V), and TMA(V) over inorganic As(III) could be in part due to AhR activation.

Second, and to confirm the requirement of AhR in *CYP1A1* induction by MMA(V), DMA(V), and TMA(V), we knocked down AhR in HepG2 cells using siRNA. Our results showed that MMA(V)-, DMA(V)-, and TMA(V)-mediated induction of CYP1A1 protein expression levels is AhR-dependent. Third, and to confirm that MMA(V), DMA(V), and TMA(V) activate the AhR, we measured its cellular localization in response to these metabolites. Our results showed that pentavalent arsenic metabolites induce nuclear accumulation of AhR protein which coincided with decreased cytoplasmic localization and a decrease in total AhR protein levels. In this regard, previous reports have shown that AhR protein is significantly decreased in cell culture and in numerous tissues following exposure to various AhR ligands (Pollenz, 2002). To determine whether or not MMA(V), DMA(V), and TMA(V) are AhR ligands, we measured their ability to bind to AhR in guinea pig liver cytosol. Our results demonstrated that MMA(V), DMA(V), and TMA(V) are not AhR ligands. However, there have been several lines of evidence supporting the presence of a ligand-independent regulation of the AhR. For example, in the current study we have shown that MMA(V), DMA(V), and TMA(V) decrease cytosolic HSP90 levels while As(III) increases its level. Previous studies have shown that although HSP90 is required to maintain AhR in a ligand binding conformation, disrupting this binding either by high salt or by decreasing its expression renders the AhR in a state that is indistinguishable from its ligand-activated form in terms of nuclear accumulation and XRE binding (Amara et al., 2013; El-Sherbeni et al., 2013). Thus, it is possible that through decreasing cytosolic HSP90 levels, MMA(V), DMA(V), and TMA(V) induced a ligand-independent activation of the AhR. Another example of the ligand-independent activation of the AhR comes from the use of the AhR antagonist 3′-methoxy-4’-aminoflavone (MNF), that can block the induction of *CYP1A1* by TCDD; it was shown that omeprazole can induce *CYP1A1* despite the presence of this antagonist (Ma and Whitlock, 1996). This specific mechanism has been shown to be tyrosine kinase-dependent as it was blocked by herbimycin A, while it is not independent of the AhR, as the nuclear accumulation of the DNA binding of the AhR was observed (Ma and Whitlock, 1996).

The marked increase in CYP1A1 mRNA, protein, and catalytic activity levels in response to MMA(V), DMA(V), and TMA(V) seems to be methylation-dependent. As such, increasing methylation increased CYP1A1 mRNA, protein, and catalytic activity levels. One possibility could have been that increasing methylation increases the availability of these metabolites intracellularly. However, it was previously demonstrated that increasing methylation in fact hinders the metabolites from being available intracellularly and thus it eliminates this possibility (Dopp et al., 2004).

In the current study we have demonstrated that MMA(V), DMA(V), and TMA(V) significantly induced the constitutive expression of NQO1 at the mRNA, protein, and catalytic activity levels while potentiating the TCDD- and SUL-mediated induction of NQO1 at the mRNA, protein, and catalytic activity levels. *NQO1* gene expression can be induced through two separate regulatory elements associated with its 5′-flanking region. The first pathway includes activation of the AhR (Anwar-Mohamed and El-Kadi, 2009a), and the second pathway involves activation of the Nrf2. As such, the increased expression of *NQO1* gene expression in response to oxidative stress caused by agents such as SUL, tert-butylhydroquinone (t-BHQ), As(III), and H_2O_2 occurs through this signaling pathway (Itoh et al., 1997). It was of importance for us to examine if the increased *NQO1* expression is due to AhR signaling pathway activation alone or is it also due to Nrf2 signaling pathway activation. Thus we examined the effect of MMA(V), DMA(V), and TMA(V) on the ARE-driven luciferase reporter activity. Our results demonstrated that MMA(V), DMA(V), and TMA(V) all increased ARE-driven luciferase reporter activity in the absence and presence of TCDD and SUL. To date, there has been no study examining the effect of MMA(V), DMA(V), and TMA(V) on Nrf2 activation or nuclear accumulation. However, it has been previously shown that MMA(III) induces Nrf2 signaling pathway activation and nuclear accumulation of Nrf2 in the human breast carcinoma cell line MDA-MB-231 (Wang et al., 2008).

The observation that MMA(V), DMA(V), and TMA(V) induced ARE-driven luciferase reporter activity and subsequently *NQO1* prompted us to investigate the effect of Nrf2 knockdown on the NQO1 protein expression levels. Interestingly, our results demonstrated that the knockdown of Nrf2 significantly decreased the ability of MMA(V), DMA(V), and TMA(V) to induce NQO1 protein expression levels, implying that Nrf2 is essential for MMA(V), DMA(V), and TMA(V) to induce *NQO1*. Lastly, MMA(V), DMA(V), and TMA(V) induced nuclear accumulation of Nrf2 which coincided with decreased cytoplasmic localization and increased total Nrf2 protein levels. It has been well documented that activation of Nrf2 and its subsequent release from its tethering Keap1 protein complex protects it from being degraded through the 26S proteasomal pathway (Anwar-Mohamed et al., 2013). Thus, the postulated mechanism by which these metabolites activate ARE seems to start by generation of ROS either directly or through activating the AhR signaling pathway. Following this step, the increased ROS generation activates Nrf2 with a subsequent nuclear accumulation, and thus increased *NQO1* expression.

In conclusion, the present study demonstrates for the first time that methylated pentavalent arsenic metabolites are bifunctional inducers as they increase *CYP1A1* gene expression through activating the AhR/XRE signaling pathway and they increase *NQO1* gene expression through activating the Nrf2/ARE signaling pathway in addition to the AhR/XRE pathway.

4.6. GENERAL DISCUSSION AND CONCLUSION

Heavy metals and AhR ligands are carcinogenic environmental co-contaminants. Quantitatively, the extent of human exposure to mixtures of AhR ligands in general and dioxin specifically in addition to heavy metals is currently unknown. However, these complex mixtures may pose a biological effect that is different than what is expected from each class separately due to possible synergistic or

antagonistic interactions via multiple mechanisms. With what is known about AhR ligands, their toxicity may be modified by two main mechanisms: competition for their sites of action and/or metabolizing enzymes, or by influencing their metabolism by the various metabolic activating or detoxifying enzymes. Therefore, we undertook extensive studies to investigate the effect of As(III) and its methylated pentavalent metabolites on the modulation of the AhR-regulated genes: CYP1A1, CYP1A2, CYP1B1, NQO1, and GSTA1. In our first study, we reported the first evidence that As(III) down-regulates the expression of CYP1A1 by affecting its transcriptional level with a further decrease in its catalytic activity levels. In addition, the increased inhibitory effect of As(III) on the TCDD-mediated induction of CYP1A1 catalytic activity is merely attributed to the As(III)-mediated induction of HO-1, which subsequently leads to the formation of a hollow functionless CYP1A1 protein. In the second study, our aims were to investigate the possible effects of As(III) on constitutive and inducible expression levels of CYP1A1/1A2 and CYP3A23/3A2 at the mRNA, protein, and catalytic activity levels in rat primary hepatocytes; to investigate the effect of As(III) on the transcription factors AhR and PXR; and to test the possible role of HO-1 in the decreased activity of P450s in response to As(III) treatment. We observed that As(III) inhibited both the AhR-regulated CYP1A1 and CYP1A2 in addition to the PXR-regulated CYP3A23 and CYP3A2 at the mRNA, protein, and catalytic activity levels. Furthermore, As(III) inhibited the nuclear accumulation of both transcription factors. Importantly, As(III) caused retention of AhR that is bound to XAP-2 and HSP90 in the cytosol, providing a possible mechanism by which As(III) inhibits *CYP1A1* transcription. Lastly, HO-1 was partially involved in the As(III)-mediated down-regulation of these P450s at the catalytic activity level, as treatment with an HO-1 inhibitor, SnMP, or supplementing with external heme, caused partial restoration of the CYP1A1, CYP1A2, CYP3A23, and CYP3A2 catalytic activities.

In the third study, we examined the co-exposure to As(III) and TCDD on the extrahepatic tissues, lung, kidney, and heart of C57Bl/6 mice. In this regard,

As(III) alone inhibited Cyp1a1 and Cyp1a2 in the kidney, while it induced their levels in the lung and did not affect their mRNA levels in the heart. As(III) also induced Nqo1 and Gsta1 in all tested tissues. Upon co-exposure to As(III) and TCDD, As(III) inhibited the TCDD-mediated induction of Cyp1a1 in the kidney and heart and Cyp1a2 in the kidney and heart, while it potentiated TCDD-mediated induction of Cyp1a1 in the lung, and Nqo1 and Gsta1 in the kidney and lung. In conclusion, the present study demonstrates for the first time that As(III) modulates constitutive and TCDD-induced AhR-regulated genes in a time-, tissue-, and AhR-regulated enzyme-specific manner.

In the fourth study, and based on our observation at the in vivo level that As(III) behaves differentially in different organs we tested the effect of co-exposure to As(III) and TCDD on livers of C57Bl/6 mice. We observed that As(III) inhibits the TCDD-mediated induction of Cyp1a1 mRNA levels at 6 h post treatment while it potentiates its levels at 24 h post treatment. In addition, As(III) increased the serum Hb levels in animals treated for 24 h. Therefore, we conducted a series of experiments using isolated mouse hepatocytes, in which we 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. Therefore, we concluded that Hb is an in vivo-specific modulator of the AhR signaling pathway.

In the fifth study, it was of importance for us to see if As(III) metabolites will behave differently than their parent compound with regard to both AhR and Nrf2 signaling pathways. Therefore, we examined the possible effects of methylated pentavalent arsenic metabolites, namely MMA(V), DMA(V), and TMA(V) using HepG2 cells. Our results demonstrated that, unlike their parent compound, methylated pentavalent metabolites induce CYP1A1 mRNA, protein, and catalytic activity levels. Furthermore they induce NQO1 mRNA, protein, and

catalytic activity levels. Importantly, MMA(V), DMA(V), and TMA(V) all induced nuclear accumulation of AhR while As(III) did not have any effect on AhR cellular localization. The increase in AhR nuclear accumulation was also accompanied by a decrease in total AhR protein levels, which is in line with the fact that activated AhR is more susceptible to proteasomal degradation than inactive AhR. The activation of AhR and subsequently the increased expression of CYP1A1 could therefore be attributed to a ligand-independent mechanism as these metabolites were shown not to be AhR ligands. However, the induction is AhR-dependent as the knockdown of AhR using siRNA significantly inhibited MMA(V), DMA(V), and TMA(V)-mediated induction of CYP1A1, confirming the requirement of AhR in this regulation. Another possible mechanism is highlighted by the fact that MMA(V), DMA(V), and TMA(V) all decreased total cytosolic HSP90 protein levels. In this regard and despite its necessity in preserving a high-affinity ligand binding confirmation for the AhR, lowering HSP90 protein expression levels participates in the ligand-independent activation of the AhR, thus the term ligand-independent mechanism. At the Nrf2 level, MMA(V), DMA(V), and TMA(V) all increased the nuclear accumulation of Nrf2 with a subsequent stabilization of its total protein levels. Opposite to the AhR, activation of Nrf2 with subsequent liberation from its tethering Keap1 protects it from proteasomal degradation. The increase in Nrf2 nuclear accumulation could be attributed to an increase in oxidative stress as all metabolites including As(III) increased DCF fluorescence, which is a surrogate marker for increased ROS production. Thus, we concluded that MMA(V), DMA(V), and TMA(V) are bifunctional inducers as they induce CYP1A1 through activating the AhR signaling pathway and NQO1 through activating both the AhR and the Nrf2 signaling pathways.

Although the effect of metals on the metabolism of xenobiotics has not been tested, it would seem that the combination of metals and AhR ligands causes an imbalance in the regulation of both phase I and phase II xenobiotic metabolizing enzymes. Thus, there is a need to evaluate the biological response of metal–AhR

ligand combinations as it would seem that the combination confers a response that is different than expected based on the toxicological mechanisms of each class evaluated separately.

The therapeutic benefits of As(III) in treating malignancies versus its carcinogenecity is a solid proof of the hypothesis that As(III) acts differentially than its biomethylated metabolites. Furthermore, As(III) and/or its metabolites together with the combination of AhR ligands such as dioxin will impose different biological and toxicological responses based on the overall balance between their effect on the AhR signaling pathway which ultimately leads to the production of genotoxic metabolites and their effect on the Nrf2 signaling pathway which ultimately counteracts the effect of AhR. Therefore, future studies are required to determine the effect(s) of As(III) and its biomethylated metabolites on the AhR and Nrf2 activity to quantify their effects on the mutagenicity/carcinogenecity associated with different environmental pollutants.

4.6.1. FUTURE OBJECTIVES:

(1) To determine the effect of acute and chronic co-exposure to As(III) and its metabolites in addition to AhR ligands on the expression of AhR- and Nrf2-regulated genes *in vivo*,

(2) To characterize the role of NF-kB and AP-1 signalling pathways in the modulation of AhR-regulated genes by As(III) and its metabolites,

(3) To examine the effect of As(III) and its metabolites on histone unwinding and the possible role in AhR inhibition, and the possible physical interation of As(III) metabolites with Nrf2,

(4) To determine the effect of heavy metals in the absence and presence of AhR ligands on AhR and Nrf2 regulated phase III transporters *in vivo*

(5) To determine the role of co-activator and co-repressor proteins in the interactions between heavy metals, the AhR and AP-1 and/or NF-kB signaling pathways, and

(6) To identify those sets of genes mediating the cross-talk between the AhR and Nrf2, AP-1, and NF-kB**.**

CHAPTER 5

5. References

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