UNIVERSITY OF ALBERTA

MODULATION OF ARYL HYDROCARBON RECEPTOR-REGULATED GENES BY MERCURY

By

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EDMONTON, ALBERTA

This work is dedicated to my parents

&

my wife

With all love and respect

Issa

ABSTRACT

Aryl hydrocarbon receptor (AhR) ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and metals, such as mercury (Hg), are environmental co-contaminants with multiple biological consequences. Therefore, the objectives of the current dissertation were to: 1) examine the potential effect of co-exposure to Hg and TCDD on the expression of the AhR-regulated phase I and phase II genes in HepG2 cells, isolated mouse hepatocytes, and *in vivo* in the liver, kidney, lung, and heart of C57Bl/6J mice, and 2) to explore the molecular mechanisms involved in this modulation. *In vitro,* Hg^{2+} significantly inhibited the TCDD-mediated induction of CYP1A1 at the mRNA, protein, and catalytic activity levels. Furthermore, co-exposure to Hg^{2+} and TCDD significantly decreased the TCDD-mediated induction of AhR-dependent luciferase reporter gene expression. At the post-translational level, Hg^{2+} significantly decreased the protein half-life, and increased the expression of heme oxygenase-1 (HO-1), which coincided with further decrease in the CYP1A1 catalytic activity levels. With regard to NQO1, Hg^{2+} increased its expression at the mRNA, protein, and activity levels in the absence and presence of both NQO1 inducers, TCDD and Sulforaphane (SUL), which coincided with increased nuclear accumulation of Nrf2 protein. Hg^{2+} was able to induce the antioxidant responsive element (ARE)-dependent luciferase reporter gene expression in an Nrf2 dependent mechanism. *In vivo,* Hg^{2+} differentially modulated phase I and phase II AhR-regulated genes at the constitutive and inducible levels. Interestingly, Hg^{2+} increased serum hemoglobin (Hb) levels in

mice treated for 24 h with Hg^{2+}. Upon treatment of isolated hepatocytes with Hb alone, there was an increase in the AhR-dependent luciferase activity with a subsequent increase in Cyp1a1 protein and catalytic activity levels. In conclusion, the present study demonstrates that exposure to Hg^{2+} and TCDD *in vitro* may decrease TCDD-mediated carcinogenicity by decreasing the induction of CYP1A1 and increasing the induction of NQO1. However; at the *in vivo* level, Hg^{2+} modulates the constitutive and inducible AhR-regulated genes in a time-, tissue- and, AhR-regulated enzyme specific manner. In addition, at the *in vitro* level HO-1 and Nrf2 are involved in the modulation of CYP1A1 and NQO1 respectively, while Hb was implicated as an *in vivo* specific modulator of Cyps.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3MC	3-Methylcholanthrene
7ER	7-Ethoxyresorufin
Act-D	Actinomycin D
AHH	Aryl hydrocarbon hydroxylase
AhR	Aryl hydrocarbon receptor
AhRR	AhR repressor
ALDH3	Aldehyde dehydrogenase 3
ANOVA	Analysis of variance
AP-1	Activated protein-1
ARE	Antioxidant responsive element
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATCC	American type culture collection
ATP	Adenosine triphosphate
AUBPs	AU binding proteins
B[a]P	Benzo[a]pyrene
B[a]PDE	Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide
bHLH	Basic-helix-loop-helix
βNF	β-naphthoflavone
CAR	Constitutive Androstane Receptor
CBP	CREB-binding protein
CDNB	1-chloro-2,4-dinitrobenzene
cDNA	Complementary DNA

CHX	Cycloheximide
CYP or Cyp	Cytochrome P450
CYP1A1 or Cyp1a1	Cytochrome P450 1A1
CYP1A2 or Cyp1a2	Cytochrome P450 1a2
CYP1B1 or Cyp 1b1	Cytochrome P450 1b1
CYP2S1	Cytochrome P450 2S1
DCPIP	2,6-dichlorophenolindophenol
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EMSA	Electrophoretic mobility shift assay
EROD	7-Ethoxyresorufin O-deethylase
FXR	Farnesoid X Receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GR	Glucocorticoid Receptor
GSH	Glutathione
GSTA1	Glutathione S-transferase A1
HAH	Halogenated aromatic hydrocarbon
Hb	Hemoglobin
HDAC	Histone deacetylase
HepG2	Human hepatoma HepG2
Hepa 1c1c7	Murine hepatoma Hepa 1c1c7
Hg	Mercury

Hg^{2+}	Mercury chloride
HSP90	Heat shock protein 90-kDa
i.p.	Intraperitoneal
JNK	c-JUN N-terminal kinase
Keap1	Kelch-like ECH associating protein 1
LD50	Median lethal dose
LXR	Liver X Receptor
MAPK	Mitogen-Activated Protein Kinase
MeHg	Methylmercury
mRNA	Messenger RNA
MRE	Metal responsive element
MRP	Multi-drug resistance protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NF-κB	Nuclear factor kappa B
NQO1	NAD(P)H:quinine oxidoreductase 1
Nrf2	Nuclear factor erythroid 2-related factor 2
PAH	Polycyclic aromatic hydrocarbon
PAS	Per-ARNT-Sim
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PKC	Protein kinase C
P23	23-kDa heat shock protein

PPAR	Peroxisome Proliferator-Activated Receptor
PXR	Pregnane X Receptor
RLU	Relative light unit
RT	Reverse transcription
RXR	Retinoid X Receptor
siRNA	Short interference RNA
SnMP	tin-mesoprophyrene
SUL	Sulforaphane
$t_{1/2}$	Half-life
TCDD	2,3,7,8,-Tetrachlorodibenzo-*p*-dioxin
UGT1A6	Uridine diphosphate glucuronosyltransferase 1A6
XAP2	Hepatitis B virus X-associated protein 2
XMEs	Xenobiotic metabolizing enzymes
XRE	Xenobiotic responsive element

CHAPTER 1- INTRODUCTION

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1.1 ARYL HYDROCARBON RECEPTOR

1.1.1. Historical Background

The aryl hydrocarbon receptor (AhR) is a captivating and interesting protein. Early studies on the regulation of cytochrome P450s (CYPs) demonstrated the first observations of the stimulation of a mixed function oxidase enzyme activity in response to benzo[a]pyrene (BaP), an environmental toxicant (Nebert and Bausserman, 1970). At that time this enzyme was called B[a]P hydroxylase (Nebert and Gielen, 1972). Later, the nomenclature of this enzyme as B[a]P hydroxylase was changed to aryl hydrocarbon hydroxylase (AHH), since the enzyme from cell cultures or from mammalian liver microsomes was able to convert a variety of PAHs to phenolic derivatives, and is not specific for B[a]P (Nebert and Bausserman, 1970).

Poland and co-workers were the first to identify the presence of a small pool of high affinity stereospecific binding sites (receptors) that were responsible for the reported changes in enzyme activities induced by PAHs and halogenated aromatic hydrocarbons (HAHs) (Poland and Glover, 1973; Poland *et al.*, 1976). Their studies demonstrated that this receptor is found in the cytosolic fraction of C57BL/6 mice, and furthermore it reversibly binds to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most potent inducer of AHH activity 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 HAHs and PAHs to the 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 conformation change in this receptor, allowing it to shuttle into the nucleus, bind to DNA, and subsequently initiate the transcription process (Landers and Bunce, 1991). Later studies showed that the TCDD-receptor complex is a large molecule with a Stokes radius of 6.6 nm, as determined by gel filtration on calibrated columns, that sediments at 5.0 S on glycerol gradients (Poland and Kende, 1976; Carlstedt-Duke *et al.*, 1981). Since that time, this receptor has been known as the AhR.

1.1.2. Molecular Characterization of the AhR

AhR is a cytosolic ligand-activated transcriptional factor that belongs to the basic-helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) family of transcription proteins that are involved in the regulation of cell differentiation and proliferation (Whitelaw *et al.*, 1993; Kerzee and Ramos, 2001). Members of the bHLH family also include the AhR nuclear translocator (ARNT), *Drosophila* circadian rhythm protein period (Per), and the *Drosophila* neurogenic protein single minded (Sim) (Schmidt *et al.*, 1993; Schmidt and Bradfield, 1996; Schmidt *et al.*, 1996). These proteins are characterized by the presence of a Per-ARNT-Sim (PAS) domain, a homology region of approximately 250 amino acids (Fig. 1.1).

The *AhR* gene is composed of 11 exons of approximately 30 kb of DNA, where the sequences encoding the bHLH domain are contained within exon 2; the PAS domain is encoded by exons 3-9, whereas the binding domain is encoded by exons 7 and 8 (Schmidt and Bradfield, 1996; Shimada *et al.*, 1996). Sequence analysis studies on the murine *AhR* gene revealed the presence of DNA

recognition sites for several transcription factors, such as the xenobiotic responsive element (XRE), metal responsive element (MRE), glucocorticoid responsive element, and activator protein-1 (AP-1) binding sites (Garrison and Denison, 2000), suggesting that these regulatory elements may affect *AhR* gene expression.

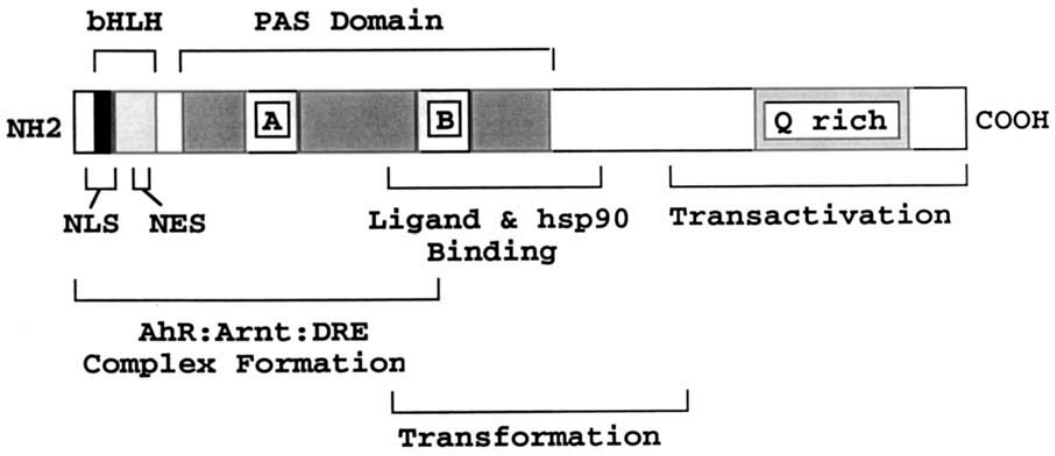

Figure 1.1. Functional and structural domains of AhR. The N-terminal region of the AhR consists of the bHLH region that functions in dimerization with ARNT, DNA binding, HSP 90 interaction and possesses sequences responsible for AhR nuclear localization (NLS) and nuclear export (NES). Next to the bHLH region, the PAS domain is localized and consists of two structural repeats (PAS A-B). PAS A functions in AhR dimerization with ARNT, whereas PAS B functions in AhR ligand and HSP90 binding. The C-terminal region of AhR protein consists of a glutamine-rich region (Q-rich region) that responsible for the transactivation of AhR (Denison et al 2002).

1.1.3. AhR signaling pathway

The AhR is a member of HLH/PAS family of transcription factors. 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 associates with ARNT (Nebert and Duffy, 1997). The whole complex then acts as a transcription factor that binds to a specific DNA recognition sequence, termed the XRE, located in the promoter region of a number of AhR-regulated genes. Among these genes are those encoding a number of drug metabolizing enzymes, including four phase I enzymes [cytochrome P450 lAl (CYPlAl), CYP1A2, CYPlBl, and CYP2Sl] and four phase II enzymes [NAD(P)H: quinone oxidoreductase-1 (NQOl), glutathione S-transferase Al (GSTAl), cytosolic aldehyde dehydrogenase-3 and UDP-glucuronosyltransferase 1A6 (UGT1A6) (Nebert and Duffy, 1997; Rivera et al., 2002) (Figure 1.2)].

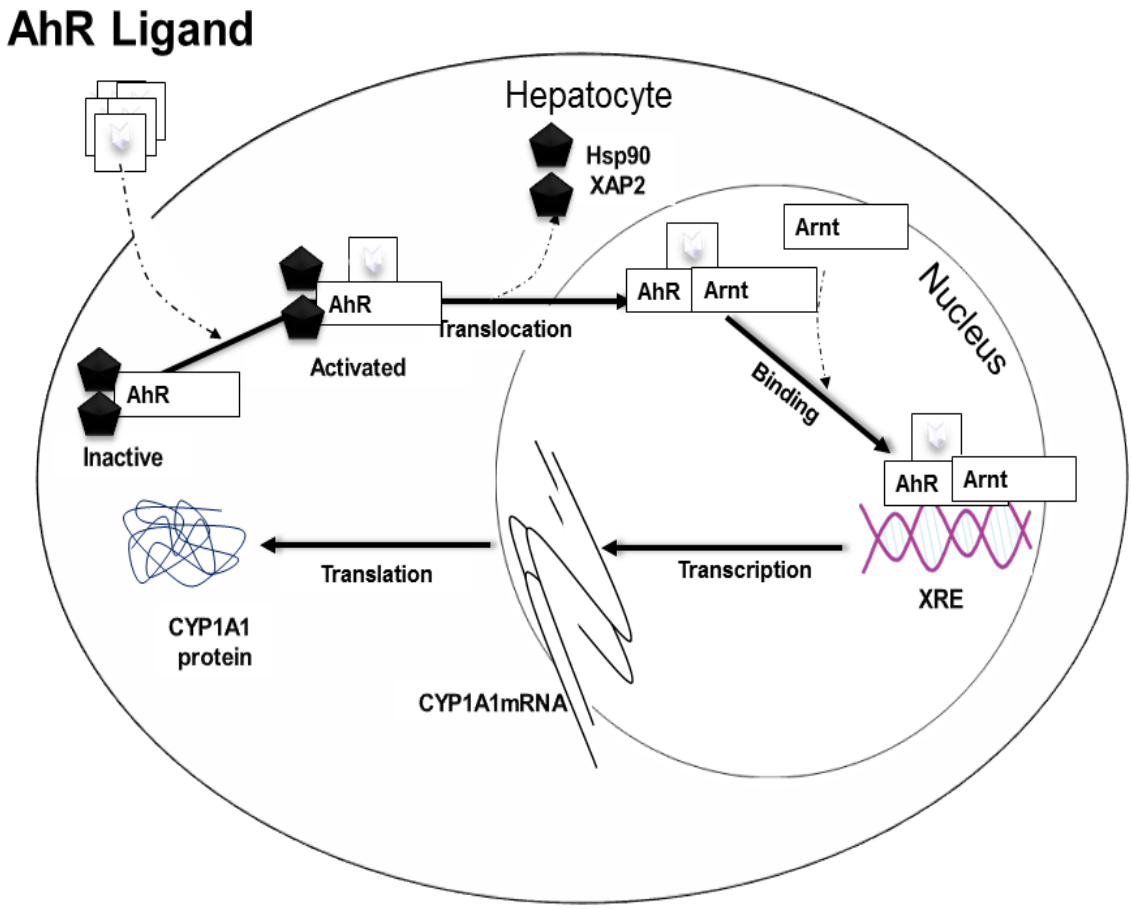

Figure 1.2. A working model for the AhR signalling pathway. AhR is found inactive in the cytoplasm in combination with two HSP90, the co-chaperone p23 and a XAP2. Upon ligand binding, AhR gets activated and the liganded AhR complex is translocated to the nucleus where it heterodimerizes with a structurally related nuclear protein called ARNT. The activation/transformation of ligand-AhR-ARNT complex leads to binding to its specific DNA sequence called XRE upstream of the AhR-regulated genes, including CYP1A1. A negative feedback regulation of the AhR pathway is carried out through the AhRR. AhRR can exert the negative feedback regulation on the AhR pathway by competing with AhR for ARNT and formation of inactive AhRR-ARNT transcriptional complexes on XRE. After transcription, AhR is exported out of the nucleus to the cytosol where it degraded by the ubiquitin-proteasomal pathway (Denison et al 2011).

The AhR protein contains several domains critical for its function. The bHLH motif located in the N-terminal of the protein contains two functionally distinct and highly conserved domains (Fukunaga *et al.*, 1995). The first is the basic-region which is mainly involved in the binding of AhR to DNA. The second is the helix-loop-helix domain which is embroiled in protein-protein interactions. The AhR protein contains two Per-ARNT-Sim (PAS) domains, PAS-A and PAS-B. These PAS domains are involved in secondary interactions with other PAS containing proteins, for example AhR and 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).

While 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). It is thought that prostaglandin E synthase 3 stabilizes 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-terminal of AhR (Whitelaw *et al.*, 1994; Kerzee and Ramos, 2001).

1.1.4. Tissue and Cellular Expression of AhR

The AhR protein content is known to vary across tissues and cell types at various developmental stages (Swanson and Bradfield, 1993; Hahn, 1998). Generally, AhR protein is expressed in most if not all, tissues of mammalian and a number of non-mammalian vertebrates, with the highest mRNA and protein levels found in the lung, liver, kidney, and placenta, whereas lower levels are expressed in the heart (Carver *et al.*, 1994; Takahashi *et al.*, 1996; Walker *et al.*, 1997; Hahn, 1998; Garrison and Denison, 2000; Bradshaw *et al.*, 2002; Mehrabi *et al.*, 2002). Although AhR and ARNT are expressed in a largely co-ordinated manner across the tissues, low expression levels of ARNT protein in particular tissues could decrease the sensitivity of that organ to AhR ligands (Carver *et al.*, 1994). Moreover, the effects of AhR agonists vary between different species and strains of the same species. For example, the median lethal dose (LD50) of TCDD varies over 5000-fold among different species of animals, where it ranges from 1 μg/kg for guinea pig, the most sensitive animal, to more than 5000 μg/kg for hamster, the most resistant animal (Poland and Knutson, 1982). The main reason behind the different responses of AhR ligands between species is the species-specific biochemical and physiological characteristics including the variation of binding affinities of AhR between species (Denison *et al.*, 2011). In this context, several studies demonstrated that human AhR contains a point mutation in its ligand-binding domain that decreases its binding affinity to TCDD by around 10-fold compared to the responsive C57BL/6 mouse AhR (Harper *et al.*, 1988; Ramadoss and Perdew, 2004). In addition, recent microarray

gene expression analysis has revealed dramatic species-specific AhR-related differences between TCDD-treated hepatocytes obtained from wild type C57BL/6 mice and transgenic C57BL/6 mice in which the AhR has been selectively replaced by humanized AhR (Flaveny *et al.*, 2010). The different response of AhR agonists between strains of the same species is exemplified by 2 different mouse strains. C57BL/6 mice were found to be a high-responsive strain of mice to TCDD when compared to DBA/2 mice which are considered as a low-responsive strain. The difference in response between the two strains to TCDD was attributed to a polymorphism of the AhR protein of DBA/2 mice that affected its binding affinity to TCDD (Ema *et al.,* 1994).

1.2. AhR-REGULATED GENES

The importance of the toxicological sequences of AhR ligands arises from the ability of these ligands to induce a battery of genes, in hepatic and extra-hepatic tissues, by activating the AhR (Spink *et al.,* 2002). In addition to their ability to activate the AhR, several PAHs and HAHs are also substrates for phase I AhR regulated enzymes such as CYPlAl, CYP1A2, and CYPlBl (Spink *et al.,* 2002). Such an interaction would result in the formation of diol-epoxides capable of forming covalent adducts when these genotoxic metabolites interact with guanines in critical genes, thus initiating tumorigenesis and other toxicological consequences (Spink *et al.,* 2002). In contrast, activation of phase II AhR-regulated enzymes such as NQOl counteracts this process by detoxifying the

mutagenic and carcinogenic metabolites formed by phase I enzymes (Spink *et al.*, 2002).

1.2.1. Phase I AhR-Regulated Genes

The CYPs are single polypeptide membrane-bound heme proteins that play an essential role in the oxidative metabolism of a great variety of xenobiotic and endogenous compounds (Nebert and Russell, 2002). Within CYPs, the heme-group is coordinated 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 of the human and in animal hepatic and extrahepatic organs (Spink *et al.*, 2002).

CYPs are generally classified based on the primary amino acid sequences of the purified CYP enzyme (Nelson, 2006; Sim and Ingelman-Sundberg, 2006). Members in a gene family generally should share more than 40% amino acid sequence identity. Mammalian members of the same subfamily have a 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 each individual member (Nelson, 2006; Sim and Ingelman-Sundberg, 2006). In addition, italicized font is usually used to refer to the gene representing the enzyme (for example, *CYP1A1).* However, italicized small letters are used to describe mouse enzymes (for example, Cyp1al).

It is well established 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). Among 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. CYPlAl is primarily an extra-hepatic enzyme since its hepatic 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 both the 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 three 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.

1.2.1.1. CYP1A1

Among the AhR-regulated CYPs, CYPIA1 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 induction was discovered from the observation that PAHs induce their own metabolism (Whitlock, 1999). CYP1A1 is capable of producing polar, toxic, or even carcinogenic metabolites from various AhR ligands, including PAHs and HAHs. Dissection of the mammalian *CYP1A1* gene revealed multiple regulatory elements which may modulate its expression. The first is the XRE, in which several XRE sequences were localized approximately 1 kb upstream of the transcription start site in all mammalian *CYP1A1* genes (Hines *et al.*, 1988). Secondly, there is the presence of three glucocorticoid responsive elements (GREs) in the human, rat, and mouse *Cyplal* gene, which explains the modulation of PAHs-induced CYP1A1 by glucocorticoids (Monostory *et al.*, 2005). Thirdly, there is a negative regulatory element (NRE), located between -560 and -831, which inhibits the constitutive expression of CYPlAl, due to an interaction with the nuclear transcription factor Oct-1 (Hines *et al.*, 1988).

CYP1A1 participates in the process of metabolic activation of several PAHs such as B[a]P. The process of activation of B[a]P starts with the combined action of CYP1A1 and CYP1B1 that results in the formation of an epoxide. Subsequently, the formed epoxide is subjected to hydrolysis by epoxide hydrolase enzymes to a dihydrodiol intermediate, which is further metabolized by CYP1A1 and CYP1B1 to the ultimate reactive species, benzo[*a*]pyrene-7,8-

dihydrodiol-9,10-epoxide (B[a]PDE) (Fig.1.3) (Singh *et al.*, 2006). B[a]PDE can be found as two diastereoisomers, namely, *anti*-B[a]PDE and *syn*-B[a]PDE and each of these diastereoisomers can be resolved into (+)- and (-)-enantiomers. Notably, both enantiomers can covalently react with the N2 exocyclic amino group of guanine by cis- or trans-addition via the C10 position and to a lesser extent the N6 and N4 exocyclic amino groups of adenine and cytosine, respectively, forming DNA adducts. It has been shown that the isomer with the 7*R*,8*S*,9*S*,10*R*-configuration, (+)-*anti*-B[a]PDE, is the ultimate carcinogenic metabolite of B[*a*]P that can bind to DNA and induce mutations, DNA breakage, and cancer (Fig.1.3) (Singh *et al.*, 2006).

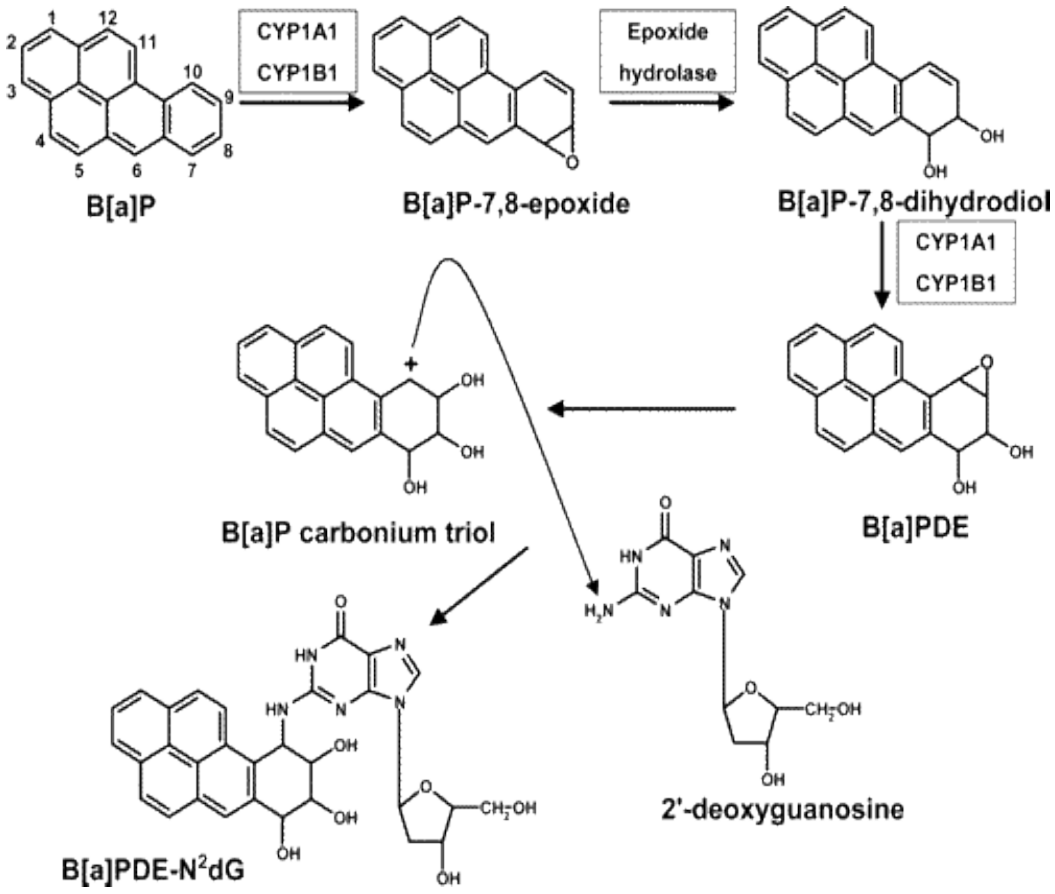

Figure 1.3. Role of CYP1A1 in B[a]P metabolic activation. CYP1A1 and CYP1B1 oxidize B[a]P, which results in the formation of an epoxide. Subsequently, the formed epoxide is subjected to hydrolysis by epoxide hydrolase enzyme to a dihydrodiol intermediate, which is further metabolized by CYP1A1 and CYP1B1 to the ultimate reactive species, benzo[*a*]pyrene-7,8- dihydrodiol-9,10-epoxide (B[a]PDE) that can covalently react with the N2 exocyclic amino group of guanine, forming DNA adducts (Singh *et al.*, 2006).

On the contrary, later studies demonstrated the beneficial role of Cyp1a1 in detoxification of oral B[a]P using *Cyp1a1* knockout mice (Uno *et al.*, 2004). This protective property of Cyp1a1 was found to be dependent on several factors such as the tissue in question and the route of administration (Nebert *et al.*, 2004). However, the role of Cyp1a1 in metabolic activation of B[a]P is mainly a first step in the detoxification process by introducing a polar group that can be further conjugated by phase II XMEs to be eliminated from body. Therefore, if these reactive metabolites can be conjugated by phase II XMEs, the body will eliminate the PAHs. Conversely, if the balance between the activation (by CYP1A1) and detoxification (by phase II XMEs such as GST) is disturbed, several harmful effects of the reactive metabolites will appear, including mutations and cancer development. In agreement with this hypothesis, the effect of decreased activity of GST enzyme and increased activity of CYP1A1 due to polymorphism can be considered as a major risk factor for the formation of B[a]P-DNA adducts in human leukocytes of healthy smokers (Lodovici *et al.*, 2004).

1.2.1.2. CYP1A2

Besides the CYP1A1, CYP1A2 is an important phase I enzyme involved in the metabolic activation of several procarcinogens including PAHs. CYP1A2 is constitutively expressed in human liver and is further induced by AhR agonists such as TCDD (Kohle and Bock, 2007). CYP1A2 catalyzes the *O*-dealkylation of 7-ethoxyresorufin and 7-methoxyresorufin (Liu *et al.*, 2001; Uchida *et al.*, 2002)

and it is involved in the metabolic activation of aromatic amines and the metabolism of several common compounds such as caffeine, acetaminophen, theophylline, phenacetin, imipramine, and propranolol (Brosen, 1995; Tonge *et al.*, 1998; Obase *et al.*, 2003; Cheng *et al.*, 2006). Moreover, CYP1A2 has been correlated with different cancers such as human tobacco-related cancers (Smith *et al.*, 1996). Furthermore, the constitutive expression of Cyp1a2 in wild type mouse liver but not in AhR knockout mice strongly suggests that Cyp1a2 is under AhR regulation (Quattrochi *et al.*, 1998; Uchida *et al.*, 2002). This was supported by the observations that TCDD markedly induced CYP1A2 in primary human hepatocytes through AhR-dependent mechanisms (Zhang *et al.*, 2006). However, an AhR-independent regulation of CYP1A2 has been also reported previously (Quattrochi *et al.*, 1998), in which a sequence analysis study of the human *CYP1A2* gene revealed the presence of two sequences homologus to the binding site of the AP-1, in addition to the XRE (Quattrochi *et al.*, 1998).

1.2.1.3. CYP1B1

CYP1B1 is constitutively expressed in extra-hepatic organs and is highly induced by PAHs and TCDD through an AhR-dependent mechanism (McFadyen *et al.*, 2001; Shimada and Fujii-Kuriyama, 2004). It is responsible for the metabolic activation of several PAHs to their reactive intermediates (Shimada and Fujii-Kuriyama, 2004). In this regard, several lines of evidence demonstrated the correlation between CYP1B1 and breast cancer development (Spink *et al.*, 1998; Ragin *et al.*, 2010). The ability of CYP1B1 to metabolize 17β-estradiol to 4-

hydroxylated products is one of the postulated mechanisms of CYP1B1 involvement in breast cancer development (Spink *et al.*, 1998). These metabolites are recognized as potent carcinogens with high affinity for estrogen receptors and can cause breast cancer in women (Liehr and Ricci, 1996; Spink *et al.*, 1998).

It has been shown that CYP1B1 expression is controlled by both transcriptional and post-translational mechanisms (Murray *et al.*, 2001). CYP1B1 can metabolize a range of toxic and carcinogenic chemicals and endogenous substances, and thus plays a critical role in the metabolic bioactivation of numerous procarcinogens such as PAHs and HAHs. Several studies demonstrated that the constitutive and inducible expressions of CYP1B1 mRNA do not correlate with the expression of AhR mRNA. In addition, the constitutive Cyp1b1 mRNA and protein were expressed in ARNT-deficient murine hepatoma cells as compared to wild-type (WT) cells (Eltom *et al.*, 1999). These results suggested that other mechanisms possibly contribute to the regulation of CYP1B1, including non-AhR-mediated pathways and/or post-transcriptional mechanisms.

1.2.1.4. CYP2S1

It was thought previously that TCDD inducibility is primarily restricted to the CYP1 family members. However; other work has identified for the first time a novel CYP, CYP2S1, in human (Rylander *et al.*, 2001) and mice (Rivera *et al.*, 2002), which exhibited a 10-fold induction of its mRNA levels upon exposure to TCDD (Rivera *et al.*, 2002). The sequences of amino acids in the human and mouse CYP2S1 revealed a 70% sequence identity (Saarikoski *et al.*,

2005); Saarikoski et al., 2005). Further studies showed that the promoter regions of both human and mouse *CYP2S1* contain XRE core sequences, suggesting that AhR and ARNT mediate the induction of CYP2S1 by TCDD through binding to XRE, in a manner typical of that of the CYP1 family (Rivera *et al.*, 2002). Moreover, it was later demonstrated that CYP2S1 is involved in the process of metabolic activation of B[a]P (Bui *et al.,* 2009). It is constitutively expressed in several tissues such as lung, stomach, and intestine; however, lower levels were found in liver (Rylander *et al.*, 2001).

1.2.2. Phase II AhR-Regulated Genes

Phase II AhR-regulated enzymes play an essential role in the detoxification of xenobiotics and carcinogenic metabolites (Lee and Johnson, 2004; Xu *et al.*, 2005). These enzymes, with the exception of NQO1 and ALDH3, catalyze conjugation reactions which are necessary for xenobiotic metabolism or further metabolism of phase I enzyme products (Swinney *et al.,* 2006). Several studies have demonstrated a complex regulation of these genes, in which the transcriptional activation of these genes is regulated by both XRE and the antioxidant responsive element (ARE) (Friling *et al.,* 1990; Chen and Kunsch, 2004; Miao *et al.,* 2005; Xu *et al.,* 2005).

1.2.2.1. NQO1

Early in 1958, quinone oxidoreductase was first identified by Lars Ernster in the rat liver cytosol and was designated as DT diaphorase, currently known as

NQO1 (Ernster *et al.*, 1962). NQO1 is a homo-dimer flavoprotein with multiple cytoprotective functions. It has long been considered as an important chemopreventive enzyme that catalyzes the reduction and detoxification of various exogenous and endogenous quinines (Nioi and Hayes, 2004; Dinkova-Kostova and Talalay, 2010). NQO1 catalyzes the two-electron reduction of several environmental contaminants, electrophilic and endogenous compounds (Jaiswal, 2000). To date three different forms of NQOs that has been identified. Among these forms, NQO1 is the most extensively studied enzyme. 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). Mounting evidence has demonstrated a direct association between the inhibition of NQO1 activities and increased risk of carcinogenesis (Nioi and Hayes, 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; Amara *et al.*, 2012a). *NQO1* gene expression is inducible by a wide range of xenobiotics, such as PAHs and HAHs (Jaiswal, 2000), antioxidants, such as *tert-butyl* hydroquinone (tBHQ) (Danson *et al.*, 2004; Nioi and Hayes, 2004; Park *et al.*, 2004), and metals, such as arsenite, mercury and lead (Kann *et al.*, 2005; Amara and El-Kadi, 2011; Korashy and El-Kadi, 2012). Up to the present time, analysis of the 5'-flanking region of the *NQO1* gene has demonstrated the existence of several cis-acting regulatory elements that mediate the transcriptional activation of

NQO1 gene, such as XRE, ARE, nuclear factor-KB (NF-κB), and AP-1 (Yao and O'Dwyer, 1995; Rushmore and Kong, 2002; Park *et al.*, 2004). *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 of *NQO1* gene expression in response to oxidative stress caused by agents such as isothiocyanate sulforaphane (SUL), 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 the ARE (Ma *et al.*, 2004; Korashy *et al.*, 2007) (Fig.1.5.).

It is well established that NQO1 protects against quinone toxicity (Fig. 1.5), in that quinones can readily undergo single-electron reduction reactions, catalysed, for example, by cytochrome P450 reductase (P450 reductase). This results in production of reactive semiquinone intermediates which in turn rearrange to yield an unpaired electron on an oxygen atom (Fig. 1.5). Moreover,

these molecules can form adducts directly with cellular macromolecules including DNA, and are thereby carcinogenic (Joseph and Jaiswal, 1994).

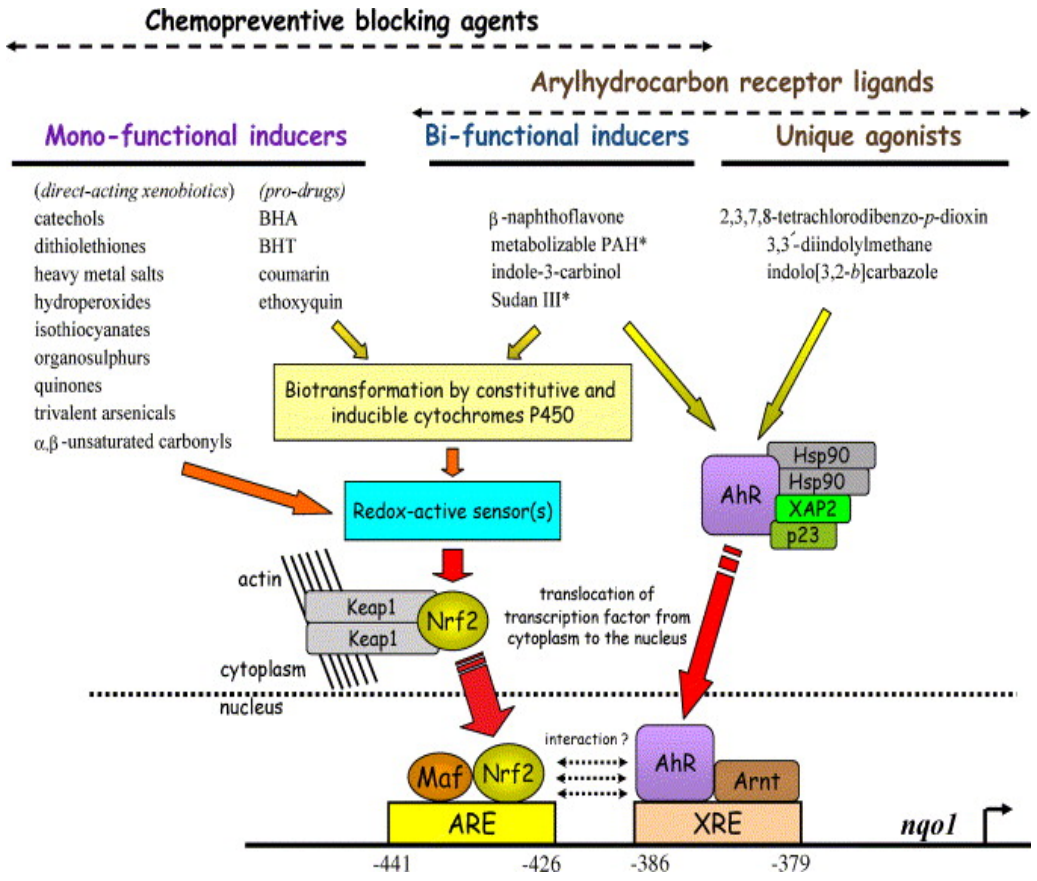

Figure 1.4. Regulation of the NQO1 gene by mono- and bi-functional inducing agents.
Xenobiotics that induce NQO1 enzyme activity in Hepa-1c1c7 cells are listed according to the pathways they are believed to stimulate. The transcription factors and the enhancers in the 5′-flanking region of mouse Nqo1 that they activate are presented in the context of the oxidoreductase gene. In addition, the possible interaction between Nrf2 and AhR is shown. (Nioi and Hayes, 2004).

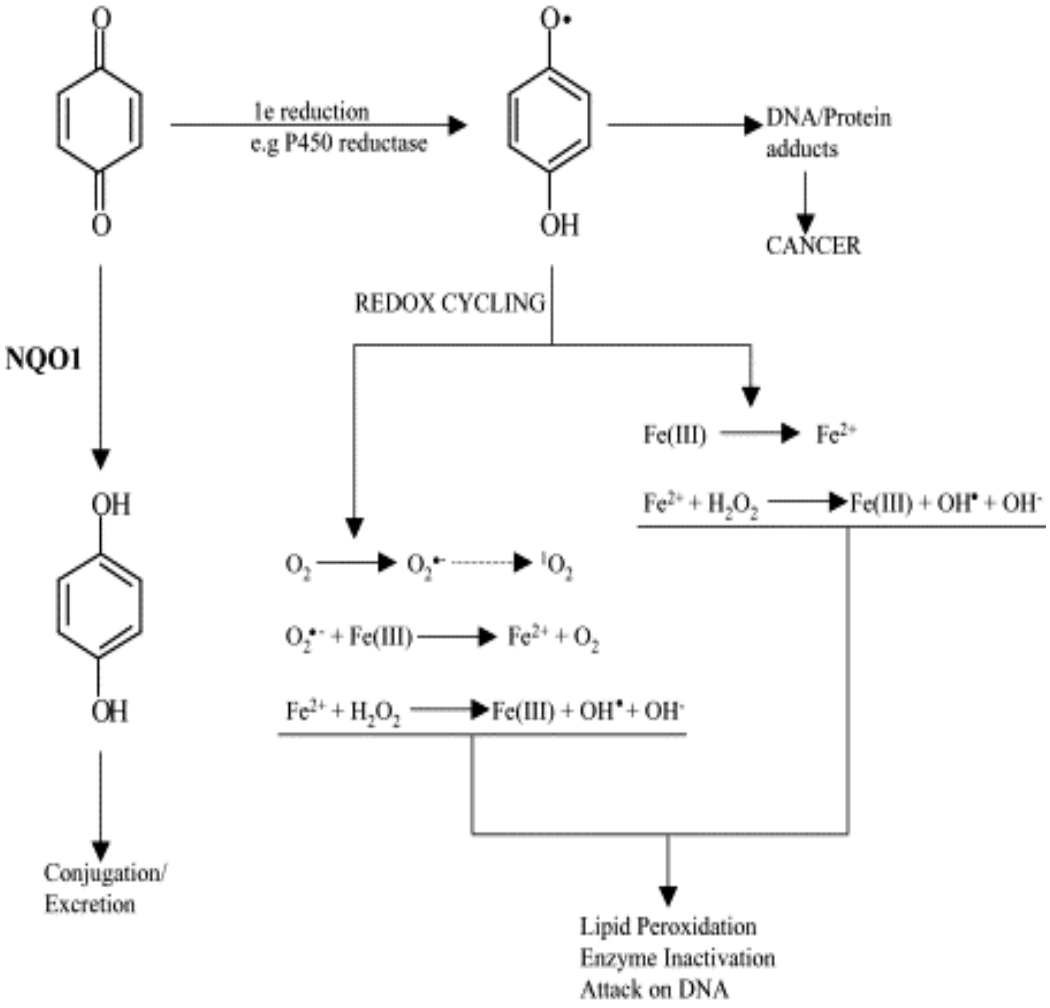

Figure 1.5. Consequences of quinone metabolism. Quinones will induce *NQO1* gene expression through the Nrf2 signaling pathway. Once induced, the NQO1 will start detoxifying these quinones to the corresponding hydroquinone. Due to their electrophilicity, semiquinones are capable of direct reaction with cellular macromolecules including protein and DNA, and this may ultimately lead to neoplasia. Redox-cycling may also occur; the unpaired electron from the semiquinone can be used to reduce Fe^{3+} to Fe^{2+} which in turn drives the Fenton reaction leading to hydroxyl radical production. Alternatively, a semiquinone may reduce molecular oxygen, which can lead to generation of singlet oxygen and superoxide-driven Fenton reactions. (Nioi and Hayes, 2004).

1.2.2.2. GSTA1

Based on the amino acid sequence identity, seven classes of cytosolic GSTs have been identified in mammalian species and designated GSTA (Alpha), M (Mu), O (Omega), P (Pi), S (Sigma), T (Theta), and Z (Zeta) (Hayes *et al.*, 2005; McIlwain *et al.*, 2006). Generally GSTs, known as glutathione S-transferases, catalyze the conjugation reactions of several reactive intermediates with glutathione (GSH) and the subsequent excretion of these metabolites out of the body, thus protecting cells against various chemical stresses and carcinogenesis (Nguyen and Pickett, 1992); (Lamb and Franklin, 2002). All of these enzymes catalyze the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) and thus exhibit GSH peroxidase activity toward cumene hydroperoxide (Hayes *et al.*, 1989; Hayes *et al.*, 2005); however; the regulatory mechanisms controlling their expression are different.

As with NQO1, analysis of the 5'-flanking region of the *GSTA1* gene demonstrated the presence of two main regulatory regions that control the basal and inducible expression of *GSTA1*. A sequence identity similar to the XRE core sequence found in the 5'-flanking region of the *CYP1A1* was reported (Rushmore *et al.*, 1990; Rushmore and Pickett, 1990). The second regulatory element found in the *GSTA1 gene* showed no homology with the XRE sequence identity. It was known later as ARE, and is important for the basal expression of the GSTA1 (Rushmore *et al.*, 1990; Rushmore and Pickett, 1990). Chemical inducers of GST have been classified as monofunctional such as PAHs, HAHs, and phenolic antioxidants. However, a number of these agents are also capable of inducing

CYP1A, and therefore are classified as bifunctional inducers. These observations strongly suggest that two distinct regulatory proteins participate in the transcriptional activation of *GST* genes. Previous studies have suggested that bifunctional inducers activate the AhR-XRE pathway, resulting in the transcription of both phase I and II genes (Hayes *et al.*, 2005; Giudice and Montella, 2006). Moreover, bifunctional inducers could first be metabolized by the induced phase I enzymes, thus liberating inducers of phase II enzymes via the Nrf2-ARE pathway (Nioi and Hayes, 2004; Hayes *et al.*, 2005; Giudice and Montella, 2006).

Significant evidence has supported the idea that the expression level of GST is an important factor in determining the sensitivity of cells to a broad spectrum of toxic and carcinogenic chemicals. Variations of GST expression have been shown to influence susceptibility to human diseases linked to oxidative stress, such as carcinogenesis (Sheweita and Tilmisany, 2003; McIlwain *et al.*, 2006), asthma, and cardiovascular diseases (Korashy and El-Kadi, 2006a). In this context, meta-analysis studies suggest that a deficiency of GSTT1 and GSTM genotypes increases the risk of carcinogenesis (Katoh *et al.*, 1998; Sheweita and Tilmisany, 2003; Boccia *et al.*, 2006), whereas overexpression of GSTs protects against various chemical carcinogens (Hayes *et al.*, 1989).

1.2.2.3. UGT1A6

According to the amino acid sequences identities, two families of UGTs, namely UGT1 and UGT2, have been characterized (Mackenzie *et al.*, 1997). The UGT1 family is composed of one subfamily, UGT1A, whereas the UGT2 family has two subfamilies, UGT2A and 2B (Mackenzie *et al.*, 1997; Meech and Mackenzie, 1997). UGTs catalyze the glucuronidation and conjugation of a wide range of endogenous substances, such as serotonin, xenobiotics, such as the drug paracetamol, planar phenols, aryl amines, and phenolic metabolites of B[a]P. UGT1A6 is mainly expressed in the liver; however low-to-moderate levels of expression have also been demonstrated in extrahepatic tissues, such as intestine, kidney, testis, and brain (Brands *et al.*, 2000; Shelby and Klaassen, 2006). The expression of UGT1A6 has been influenced by many factors, such as genetic tissue-specific, and environmental factors (Munzel *et al.*, 2003). With regard to the induction of *UGT1A6* gene expression, a previous study by Shelby and coworkers has shown that the induction is mediated through different signaling pathways, such as those involving the AhR-XRE, Nrf2-ARE, pregnane X receptor (PXR), and constitutive androstane receptor (CAR) (Shelby and Klaassen, 2006).

1.2.2.4. ALDH3

ALDH3 is a cytosolic enzyme which belongs to a family of NADP-dependent enzymes that catalyzes the oxidation of toxic endogenous and exogenous aliphatic and aromatic aldehydes to carboxylic acids (Harrington *et*

al., 1987). Tissue distribution studies demonstrated hepatic and extrahepatic expression of ALDH3. ALDH3 is not constitutively expressed in the normal liver, but it is significantly induced in response to PAHs (Takimoto *et al.*, 1992). However, high constitutive expression levels were reported in the lungs, stomach, urinary tract, skin, and cornea.

The induction of ALDH3 transcription in rat hepatoma H4IIE (Takimoto *et al.*, 1992) and Hepa 1c1c7 (Vasiliou *et al.*, 1992) cells in response to PAHs and HAHs is mediated through an AhR-XRE-dependent mechanism. However, constitutive expression of Aldh3 mRNA is elevated in untreated mutant Hepa 1c1c7 cells lacking a functional Cyp1a1 enzyme; and normal expression is restored by introduction of a functional murine Cyp1a1 (Vasiliou *et al.*, 1992). These studies suggest that TCDD-mediated induction of ALDH3 is distinct from the CYP1Al/CYP1A2 metabolism-dependent repression of constitutive gene expression (Vasiliou *et al.*, 1992).

1.3. ACTIVATION OF THE AhR

1.3.1. Ligand-Dependent Activation of the AhR

In the bHLH/PAS superfamily, the AhR is the only protein that requires activation by a ligand. In the absence of ligand, the AhR exists primarily in the cytoplasm in combination with two HSP90s, co-chaperone p23, and XAP2 (Denison *et al.*, 2002). The HSP90-associated co-chaperone, p23, acts as a stimulatory factor of AhR activity (Beischlag *et al.*, 2008). The interaction between HSP90 and the AhR is mediated by p23 which in turn prevents the

spontaneous formation of a AhR-ARNT heterodimer in the absence of ligand (Kazlauskas *et al.*, 1999). Additionally, p23 protects the receptor from being degraded and facilitates the nuclear uptake of the AhR following ligand treatment (Cox and Miller, 2002). On the other hand, XAP2 is essential for attenuating the nuclear export of the unliganded AhR to the nucleus (Kazlauskas *et al.*, 2000).

Activation of the AhR usually starts through binding with its ligand, leading to a conformational change that exposes its N-terminal nuclear localization sequence (Denison *et al.*, 2011). Thereafter, the activated AhR dissociates from the cytoplasmic complex and translocates to the nucleus where it heterodimerizes with ARNT (Whitelaw *et al.*, 1994). This binding results in the displacement of HSP90 and other subunits from the AhR, leading to more interactions between AhR and ARNT and activation/transformation of the ligand-AhR-ARNT complex into a high affinity DNA-binding form (Denison *et al.*, 2011). The ligand/AhR/Arnt complex then acts as a transcription factor that binds to a specific DNA recognition sequence, GCGTG, within the XRE, located in the promoter region of a battery of genes termed AhR-regulated genes such as CYP1A1, leading to DNA bending, coactivator recruitment, chromatin and nucleosome disruption, increased promoter accessibility to transcription factors, and increased rates of gene transcription (Denison and Nagy, 2003; Nebert *et al.*, 2004; Denison *et al.*, 2011) (Fig. 1.2).

1.3.2. Ligand-Independent Activation of the AhR

Several pieces of evidence suggest that the AhR without exogenous ligand

is shuttling between the nucleus and cytosol. This was confirmed by using the nuclear export inhibitor, leptomycin B, or by mutation of the AhR nuclear export sequence (NES) which resulted in nuclear accumulation of AhR in the absence of exogenous ligand (Richter *et al.*, 2001). However, binding to ligands increases the rate of nuclear import of the AhR but does not eliminate its nuclear export (Richter *et al.*, 2001). These studies suggest that the AhR travels between the nucleus and the cytosol in the absence of exogenous ligand, and hence activation of the AhR could be a ligand-independent process.

Numerous postulations have been reported to explain this observation. For example, there are several endogenous compounds that can act as AhR activators. These compounds, such as arachidonic acid metabolites, have been suggested to be elaborated and induce CYP1A1 when cells were exposed to hydrodynamic shearing stress (Mufti and Shuler, 1996). In addition, the formation of tryptophan metabolites from UV and visible light exposure can activate the AhR and induce CYP1A1 (Helferich and Denison, 1991; Ma, 2011). Another statement has been introduced to explain the ligand-independent activation of the AhR, suggesting that AhR can be affected by several other signaling pathways. In agreement with this hypothesis, it was previously reported that protein kinase C (PKC) is involved in the activation of AhR in a relatively different way than 3MC-mediated activation (Delescluse *et al.*, 2000).

On the other hand, omeprazole was able to induce CYP1A1 in rat and human hepatocytes in an AhR ligand-independent manner, however; it did not show any binding ability to the AhR (Backlund and Ingelman-Sundberg, 2004).

In this case, other mechanisms were reported to explain the effect of omeprazole, including its effect on protein tyrosine kinases, which differs from the effect of the high affinity AhR ligands such as TCDD (Delescluse *et al.*, 2000; Backlund and Ingelman-Sundberg, 2005).

1.3.3. Classical AhR Ligands

The classical ligands for the AhR include classes of naturally occurring, highly toxic and persistent environmental carcinogenic aromatic hydrocarbons, PAHs and HAHs. The most extensively studied members of the PAHs are 3-methylcholanthrene (3MC), β-naphthoflavone (βNF), and B[a]P, whereas dioxin-related compounds, such as TCDD, represent the prototypes of HAHs (Denison and Nagy, 2003). These classical AhR ligands share structural, functional, and physicochemical features in that they are planar, aromatic, and hydrophobic in nature (Denison and Nagy, 2003). PAHs and HAHs have been classified as bifunctional inducers, i.e. they induce both phase I and II xenobiotic metabolizing enzymes, and monofunctional inducers, i.e. induce phase II only (Prochaska and Talalay, 1988).

In this situation, two models have been suggested where non-metabolizable inducers, such as TCDD, act directly via the AhR to induce both phase I and II enzymes, while metabolizable inducers, such as B[a]P, act by inducing phase I enzymes specifically to yield metabolites resembling monofunctional inducers (Prochaska and Talalay, 1988). Furthermore, it has been demonstrated that 3MC-adduct formation, catalyzed by CYP1A2, mediates the

sustained CYP1A1 induction by covalently binding to the AhR (Moorthy *et al.*, 2007). Binding affinity of ligands to the AhR is critically important for their AhR-mediated effects, in which the stronger the binding affinity the greater the toxicity. For example, TCDD is the prototypical ligand and the most potent AhR activator and CYPlAl inducer ever known. Being resistant to metabolic breakdown, TCDD and other HAHs are more stable and produce sustained effects in the pM to nM range, whereas PAHs are considered metabolically more labile and therefore possess lower AhR affinity and CYPlAl induction, in the nM to µM range (Denison and Nagy, 2003). In this regard, it has been demonstrated that the toxicity of planar PAHs is extremely sensitive to both the number and position of halogen substituents. For example, TCDD toxicity was drastically decreased by adding non-lateral chlorines or removing chlorines from its structure, suggesting that AhR binding to ligands is sensitive to the chlorination pattern (Mhin *et al.*, 2002).

1.3.4. Non-Classical AhR Ligands

A large number of newly identified AhR ligands whose structures and physiochemical properties differ significantly from those of PAHs and HAHs have been reported (Seidel *et al.*, 2000; Gharavi and El-Kadi, 2005). The observations that the AhR is activated in the absence of exogenous ligand strongly support the existence of endogenous AhR ligands. For example, it has been shown that hypoxia induces CYP1A1 in rat lungs and liver *in vivo;* this induction coincided with the formation of endogenous AhR ligand. Furthermore,

it has been demonstrated that physiological compound(s), such as bilirubin and billiverdin contained in serum induce *CYP1A1*gene expression (Guigal *et al.*, 2000; Guigal *et al.*, 2001).

Recently, a variety of endogenous compounds has been identified *in vitro* as AhR ligands and activators of the AhR-regulated genes. Among these ligands are bilirubin (Sinal and Bend, 1997), biliverdin (Phelan *et al.*, 1998), lipoxin (Schaldach *et al.*, 1999), indoles such as tryptophan (Heath-Pagliuso *et al.*, 1998), and hemoglobin (Anwar-Mohamed and El-Kadi, 2010; Amara *et al.*, 2012b). However, the ability of these compounds to bind to and activate AhR *in vivo* remains to be confirmed. Although the majority of these non-classical AhR ligands are weak CYP1A1 inducers, this list has expanded to include a number of widely prescribed drugs such as omeprazole (Lemaire *et al.*, 2004), primaquine (Werlinder *et al.*, 2001), sulindac (Ciolino *et al.*, 2006), ketoconazole, and itraconazole (Korashy *et al.*, 2007).

1.3.5. Negative Regulation of the AhR and its Regulated Genes

It was previously thought that negative regulatory elements (NREs) and cognate repressor proteins, which are present in the promoter regions of several AhR-regulated genes, negatively modulate the expression of these genes (Boucher *et al.*, 1993; Boucher *et al.*, 1995). Studies in human and rat cells have identified an NRE in the *CYP1A1* gene promoter that appears to negatively modulate its transcriptional activity by down-regulating a heterologous promoter/enhancer involving specific nuclear protein binding (Jorgensen and Autrup, 1995; Walsh *et*

al., 1996). This was concluded from the fact that mutations in the cognate repressor protein inhibited DNA-protein binding, resulting in a 2- to 3-fold increase in the maximal CYP1A1 inducibility in response to the AhR ligand (Boucher *et al.*, 1995; Piechocki and Hines, 1998).

Likewise, superinducibility of CYP1A1 mRNA by TCDD in Hepa 1c1c7 and human breast cancer MCF10A cells treated with the protein synthesis inhibitor, cycloheximide (CHX), supports the existence and involvement of negative regulatory proteins in the regulation of *CYP1A1* gene expression (Joiakim *et al.*, 2004). In this regard, several studies have identified nuclear transcription proteins, such as OCT-1 and NF-Y (Boucher *et al.*, 1993; Boucher *et al.*, 1995; Sterling and Bresnick, 1996), that constitutively bind to the NREs of the *CYP1A1* gene and hence influence the relative TCDD-induced activity in human hepatoma HepG2, but not human breast cancer MCF7 cells (Jorgensen and Autrup, 1995). Furthermore, the interaction of the AhR-ARNT complex with corepressor transcriptional proteins such as silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) decreased the XRE binding affinity, which was demonstrated by the gel electrophoretic mobility shift assay (EMSA) (Nguyen *et al.*, 1999; Pollenz, 2002).

1.3.6. Physiological and Toxicological Consequences of Activation of the AhR

Previous investigations on AhR regulation and expression suggest that the AhR possesses xenobiotic-independent functions. Despite AhR being implicated

in several disorders of environmental etiology including atherosclerosis, chloracne, immunosuppression, thymic atrophy, and malignancies (Shimada and Fujii-Kuriyama, 2004; Marlowe and Puga, 2005), it has numerous physiological functions, such as cell proliferation, apoptosis, and embryogenesis (Marlowe and Puga, 2005). The availability of knockout mice allowed scientists to test the specific functions of particular genes and to observe the processes that these particular genes could regulate. Early pathological studies using knockout mice have shown that elimination of ARNT expression using a null allele resulted in embryonic death, whereas elimination of AhR expression resulted in pathology of several organs, but not in death (Fernandez-Salguero *et al.*, 1995; Schmidt *et al.*, 1996; Maltepe *et al.*, 1997). This implies that the AhR-ARNT-mediated signaling pathway plays a vital role in several organ systems. In contrast, conflicting data on the role of the AhR in cell cycle progression has been reported in a cell type-dependent manner. For example, it has been shown that AhR inhibited the growth of MCF7 cells, but promoted HepG2 cell proliferation (Abdelrahim *et al.*, 2003). In addition, it has been demonstrated that the AhR is involved in the regulation of normal liver growth (Schmidt *et al.*, 1996) and development of prostate, thymus, and ovaries (Hundeiker *et al.*, 1999), and is necessary for normal developmental closure of the ductus venous (Walisser *et al.*, 2005). It has been also proposed that the AhR plays a physiological role in the reproductive system, since AhR-null female mice showed difficulties in maintaining normal pregnancy (Abbott *et al.*, 1999). Moreover, the AhR has been involved in the development of the

mammary gland; however ligand-dependent activation of the AhR suppresses this process independently of CYP1A1 or CYP1B1 induction (Hushka *et al.,* 1998).

1.4. MECHANISMS INVOLVED IN THE MODULATION OF AhR-REGULATED GENES

1.4.1. Transcriptional Mechanism

A transcriptional mechanism is mainly involved in the induction of AhR-regulated genes. This induction occurs through the activation of several transcription factors that bind to specific DNA sequences to initiate gene transcription. Inhibition of their mRNA and protein synthesis by using actinomycin D (Act-D) and cycloheximide (CHX), respectively, suggests that a transcriptional regulatory mechanism is involved in the induction of CYP1A1 (Ramana and Kohli, 1998). In addition, recent studies have demonstrated that the superinduction of the *Cyp1a1* gene by CHX or MG-132, a 26S proteasome inhibitor, is a transcriptional mechanism and reflects a change in the synthesis, rather than stabilization, of Cyp1a1 mRNA (Ma and Baldwin, 2000; Ma and Baldwin, 2002; Joiakim *et al.*, 2004). On the other hand, ARE-dependent transcriptional activation of *Nqo1* and *Gsta1* genes requires the activation of a CHX-sensitive transcription factor, Nrf2 (Rushmore and Kong, 2002; Xu *et al.*, 2005). In this context, it has been reported that treatment of Hepa lclc7 cells with CHX inhibited the newly synthesized Nqol mRNA but did not affect the existing mRNA levels (Eickelmann *et al.,* 1995; Lamb and Franklin, 2002; Ma *et al.*, 2004).

1.4.2. Post-Transcriptional Mechanisms

It is well known that steady state mRNA levels reflect a balance between the rate of mRNA synthesis and the rate of mRNA degradation. Therefore, altering the rate of mRNA degradation will influence its steady state concentration and determine how quickly it can be expressed (Lekas *et al.*, 2000). Interestingly, mRNA decay is controlled mainly by the exonucleases that catalyze mRNA, but protected by a specific terminal structure poly (A) tail at the 3' end (Meyer *et al.*, 2004). In addition, other studies have demonstrated that blocking translation has been shown to stabilize mRNA (Meyer *et al.*, 2004). The stability of mRNA of CYPs is usually reflected by their mRNA half-lives ($t_{1/2}$). A previous study on HepG2 cells investigating the decay of CYP1Al, CYP1A2, and CYP1B1 mRNAs after treatment with TCDD showed that CYP1A2 and CYP1B1are long-lived CYPs with an approximate $t_{1/2}$ values of greater than 24 h (Lekas *et al.*, 2000). In contrast, CYPlAl mRNA decays quickly, with a $t_{1/2}$ of approximately 2.4 h in HepG2 cells. The rapid decay of CYPlAl was associated with a rapid loss in poly (A) tail length, suggesting that deadenylation is the first step in the decay pathway (Lekas *et al.*, 2000).

Post-transcriptional regulatory modulation of the AhR-regulated genes is still less well understood. Lee and Safe have demonstrated that inhibition of CYP1A1 mRNA expression in T47D cells in response to resveratrol, a polyphenolic plant extract, is attributed to increased rate of CYP1A1 mRNA degradation (Lee and Safe, 2001). In addition, the adrenal steroid hormone dehydroepiandrosterone decreased CYP1A1 mRNA stability in MCF7 cells by

increasing its degradation rate (Ciolino and Yeh, 1999). The degradation of NQOl mRNA has not been elucidated; however, a few studies have examined the half-life of NQOl mRNA transcripts. It has been shown that NQOl mRNA decay is regulated through the AU rich region in its untranslated 3' end (Gingerich *et al.*, 2004). This region is targeted by AU binding proteins (AUBPs), which in turn bind to the mRNA transcripts and govern their stability (Gingerich *et al.*, 2004). Activation of AUBPs may occur through different mechanisms and the most studied ones by far are the phosphorylation and ubiquitination processes that occur subcellularly in response to different extracellular and intracellular signals (Gingerich *et al.*, 2004). Studies examining the half-life of NQOl mRNA in different species have found NQOl transcripts are long lived with t ½ values of more than 17 h (Korashy and El-Kadi, 2006b; Elbekai and El-Kadi, 2008).

1.4.3. Translational and Post-translational Mechanisms

Post-translational modification can be defined as any functional modification of a translated protein (Han and Martinage, 1992). The majority of these modifications occur after the release of the polypeptide from the ribosome after being translated from its original mRNA transcripts (Han and Martinage, 1992). The post-translational modification of amino acids alters protein functionality by attaching to it other biochemical functional groups such as acetate, phosphate, and various lipids and carbohydrates. Protein phosphorylation is a common mechanism for regulating its function, i.e. activating or inactivating the protein (Aguiar *et al.*, 2005).

1.4.3.1. Phosphorylation

Protein phosphorylation is a reversible cellular process that involves transfer of phosphate from adenosine triphosphate (ATP) to a specific protein by protein kinases and phosphatases (Han and Martinage, 1992). Several studies suggested that the AhR undergoes changes through phosphorylation. For example, treatment of Hepa 1c1c7 cells with c-AMP resulted in activation and translocation of the AhR to the nucleus in a different way than that observed with TCDD (Oesch-Bartlomowicz *et al.*, 2005). Moreover, phosphatase inhibitors, such as ortho-vanadate, reduced the CYP1A1/2-dependent formation of mutagenic metabolites of aromatic amines and aromatic amides in rat liver hepatocytes and rat liver homogenates (Oesch-Bartlomowicz *et al.*, 1997).

1.4.3.2. 26S Proteosomal Degradation

Ubiquitin-proteasomal degradation is a two-step process that involves ubiquitination of the protein prior to its degradation by the proteasome (Roos-Mattjus and Sistonen, 2004). Proteins designated for degradation are covalently tagged by ubiquitin, a small 76 amino acid polypeptide, and this process is called ubiquitination (Roos-Mattjus and Sistonen, 2004). After being ubiquitinated, the tagged protein will be recognized by the proteasome, where the ubiquitin is recycled and the target protein is degraded (Roos-Mattjus and Sistonen, 2004). As its name suggests, ubiquitin is a highly conserved protein. It was first isolated in the 1970s by Goldstein, and was found to be ubiquitously expressed in all tissues and organisms (Goldstein, 1974; Goldstein *et al.*, 1975).

The 26S proteasome is a multi-enzyme protein complex that has plays an essential role in protein degradation inside the cells. The complex has critical functions in several vital cellular processes inside the cell such as cell cycling, differentiation and apoptosis (Weissman, 2001). It is a very large (2500 kDa), dynamic holoenzyme that consists of at least 32 different subunits in two copies (Glickman and Ciechanover, 2002; Cohen *et al.*, 2008). Proteasomes are present in the nucleus and cytoplasm of almost all organisms. Intriguingly, treatment of Hepa lclc7 cells with TCDD shortens the AhR $t_{1/2}$ from 28 h to 3 h (Ma *et al.*, 2000; Fujii-Kuriyama and Mimura, 2005). The down-regulation of AhR in response to its ligands is attributed to the ubiquitination of AhR protein prior to its degradation through the 26S proteasome pathway (Eltom *et al.*, 1999). This is supported by the observations that inhibition of the 26S proteasome pathway in Hepa 1c1c7 cells, using MG-132, increased the AhR and ARNT protein levels and hence enhanced the induction of *Cyp1a1* gene expression (Davarinos and Pollenz, 1999).

1.4.3.3. Cellular Heme and Heme Oxygenase

Enzymatic activity levels of CYPs could be enhanced or suppressed by the cellular heme contents. Several studies have shown that the mechanism of CYP monooxygenase induction is attributed to the enhancement of δ-aminolevulinate synthase, a rate-limiting step in the biosynthesis of heme (Lavrovsky *et al.*, 1993). Furthermore, modulation of the expression of heme oxygenase- 1 (HO-1), a rate-limiting step in heme degradation, has been shown to alter cellular heme content

and hence the enzymatic activity (Kikuchi *et al.*, 2005). Therefore, it seems that the balance between these two pathways could determine the level of CYP enzyme activity.

Heme oxygenase was discovered in 1968 when Tenhunen and colleagues described the mechanism for catabolism of heme (Tenhunen *et al.*, 1968). Subsequently, three distinct isoforms of the enzyme were described. Two of these are constitutively activated (HO-2 and HO-3) and one is an inducible form (HO-1) (Maines *et al.*, 1986; McCoubrey *et al.*, 1997). HO-1, is 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 free radicals (Marilena, 1997; Kikuchi *et al.*, 2005). It is the major isoform, which is ubiquitously expressed in a wide range of mammalian tissues (Lavrovsky *et al.*, 1993; Kikuchi *et al.*, 2005). Expression of HO-1 is induced by oxidative stress stimuli, such as hypoxia, inflammation, heavy metals, and hydrogen peroxide. Furthermore, 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 CYPs which are also endoplasmic reticulum-bound enzymes.

1.4.4. Oxidative Stress

The term oxidative stress was defined firstly by Helmut Sies as the ‘imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage’ (Sies, 1997). Therefore, oxidative stress, refers to a serious

imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses (Brigelius-Flohe, 2009). With regard to the AhR-mediated toxicity, it has been shown to be mediated through the oxidative stress, characterized by an increase in cellular oxidation state to produce an oxidative stress response (Dalton *et al.*, 2002). Therefore, increased production of ROS and activation of several redox-sensitive transcription factors, such as Nrf2, NF-κB and AP-1 are considered to directly regulate the expression of AhR-regulated genes.

1.4.4.1. ROS

One of the mechanisms by which CYP1A1 induction leads to toxicity is the production of ROS. It has been shown that during CYP catalytic cycles, monoxygenase enzymes release free radicals, such as hydroxyl radical ($HO^{\cdot}$) Subsequently, this causes oxidation of several biological macromolecules, such as DNA and proteins. This is supported by the observations that TCDD and B[a]P cause oxidative stress in various tissues (Barouki and Morel, 2001).

In addition, it has been shown that AhR ligands increase ROS production in Hepa 1c1c7 cells through an AhR-dependent mechanism. This was accompanied by a decrease in the Cyp1a1 catalytic activity but not mRNA or protein expressions (Elbekai *et al.*, 2004). The AhR ligand-mediated decrease in Cyp1a1 activity was reversed by the antioxidant *N*-acetylcysteine, suggesting a role for ROS in the modulation of AhR-regulated genes (Elbekai *et al.*, 2004). Mechanistically, oxidative stress plays a critical role in the CYP1Al auto-

regulatory loop in which generation of ROS as a result of induction of CYP1Al activity activates the NF-κB signaling pathway that suppresses AhR activation and hence suppression of its gene promoter (Barouki and Morel, 2001). With regard to oxidative stress inducers, heavy metals are well known to cause oxidative stress and subsequently produce ROS. Among these metals is Hg, and it has been shown that in an *in vivo* and *in vitro* models, the exposure to Hg can cause oxidative stress in biological systems (Clarkson, 1972; Farina *et al.*, 2003; Aschner and Syversen, 2005; Crespo-Lopez *et al.*, 2007; Augusti *et al.*, 2008; Grotto *et al.*, 2009), with generation of ROS, glutathione (GSH) depletion, and decrease of sulphydryl groups (–SH) of proteins (Shenker *et al.*, 2002), which can lead to pathological processes (Halliwell *et al.*, 1992).

1.4.4.2. Redox-Sensitive Transcription Factors

Although there is a wide range of toxic responses to PAHs and HAHs from cell proliferation to carcinogenesis, some of which are not directly AhR-dependent, several reports have suggested that the AhR is likely to interact with other transcription factors to cause such diverse effects (Tian *et al.*, 2002). Despite the fact that some PAHs elicit unique signal transduction pathways, it has been shown that most of these ligands could trigger other common signaling pathways in the cells. In this context more than 20 redox-sensitive transcription factors have been identified and characterized previously (Lyakhovich *et al.*, 2006). These transcription factors are responsible for changes in the redox status of the cell in response to stimulants. Among these factors,

Nrf2, NF-κB, and AP-1, have been shown to influence the expression of several genes that alter the activity of numerous metabolic processes.

1.4.4.2.1. Nrf2

Nrf2 is a redox sensitive member of the capʿn' collar basic leucine zipper family of transcription factors (Itoh *et al.*, 1997). Through an XRE-independent process, recent studies have shown that gene expression of phase II metabolizing enzymes is regulated by a labile protein transcriptional factor, Nrf2. These studies have labeled Nrf2 as the central transcription factor involved in the regulation and expression of many antioxidants and detoxifying phase II enzymes, such as NQO1 and GSTA1, against oxidative damage. This was supported by using the Nrf2-null mice, in which it has been shown that inducible, but not constitutive, *Nqo1* and *Gstp* gene expressions were abolished, whereas in AhR- and Nrf2-double knockout mice, both constitutive and inducible expressions of *Nqo1* and *Gstp* genes were completely inhibited (Noda *et al.*, 2003; Zhu *et al.*, 2005). These results not only support the notion that AhR- and Nrf2- mediated pathways could play an integral role in the regulation of *Nqo1* and *Gsta1*genes, but also suggest the existence of cross-talk between these pathways. This conclusion was supported by the observations of Ma and Marchand who showed that *Nrf2* gene expression is directly regulated through AhR activation and *NQO1* gene expression is controlled by CYPlAl activity (Ma *et al.*, 2004; Marchand *et al.*, 2004).

Several pieces of evidence support a direct link between AhR and Nrf2. First, *Nrf2* is a target gene for the AhR, in which three functional XRE and two ARE have been identified in the mouse, rat, and human *Nrf2* promoter (Miao *et al.*, 2005; Kohle and Bock, 2007). This is supported by observations that TCDD increased Nrf2 protein levels in a time-dependent manner (Miao *et al.*, 2005) and that studies using Nrf2-deficient cells revealed that the induction of NQO1 by TCDD requires functional Nrf2 (Ma *et al.*, 2004). Second, Nrf2 can be activated indirectly by Cyplal-generated highly reactive electrophiles that mediate the induction of phase II enzymes such as NQO1 (Marchand *et al.*, 2004; Miao *et al.*, 2005). Third, a direct interaction between ARE-XRE and Nrf2-ARE signaling pathways has been characterized. Sequence analysis of the enhancer region of mouse *Nqo1* showed that the putative ARE and XRE sequences are located close to each other, which may suggest a possible functional overlap between their mediated signaling pathways (Ma *et al.*, 2004). Several scenarios have been proposed, where ARE and XRE function as a composite response element to which both AhR and Nrf2 bind and mediate the induction of NQOl by TCDD. In addition, AhR and Nrf2 may interact with each other directly or through an adaptor protein; such interactions are required for induction of NQOl by TCDD.

1.4.4.2.2. NF-κB

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.,* 1999; Tian *et al.,* 2002). Among these dimers, NF-κB1/RelA, which refers to an NF-κB complex, is a transcriptionally active complex that was first identified as a transcriptional enhancer of the gene expression of immunoglobulin k light chain in B cells (Sen and Baltimore, 1986), and hence the term NF-κB.

Regulation of NF-κB is a complicated process, in which a wide range of stimuli, such as bacterial lipopolysaccharide (LPS), viruses, oxidative stress, and ultraviolet light trigger NF-κB activation through different mechanisms. For example, although most of these stimuli trigger the NF-κB signaling pathway by activating the IκK pathway, signal cross-talk between NF-κB and other transcription factors or MAPKs has been reported (Wullaert *et al.*, 2006). In this context, several studies have shown that NF-κB participates in many of the physiological as well as toxicological responses mediated by the PAHs and HAHs. Co-immunoprecipitation 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 the 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 1clc7 cells in which activation of NF-κB suppressed the expression of Cyp1a1 at the transcription level (Ke *et al.,* 2001). With regard to the CYPs, Zordoky and coworkers have suggested three mechanisms by which NF-κB can regulate CYP expression and activity. First, NF-κB can directly regulate the expression of

CYP1A1, CYP2B1/2, CYP2C11, CYP2D5, CYP2E1, CYP3A7, and CYP27B1 through binding to the promoter region of these genes. Second, NF-κB indirectly regulates the transcription of CYP genes through mutual repression with some nuclear receptors that are involved in CYP regulation such as AhR, constitutive androstane receptor, glucocorticoid receptor, pregnane X receptor, retinoid X receptor, peroxisome proliferator-activated receptor, farnesoid X receptor and liver X receptor (CAR, GR, PXR, RXR, PPAR, FXR, and LXR). Finally, NF-κB can regulate CYP activity at the post-transcriptional level by inducing heme oxygenase-1 or by affecting the CYP protein stability (Zordoky and El-Kadi, 2009).

To date the reaction between AhR and NF-κB is not clear, i.e. whether such interaction occurs at the cytoplasmic or nuclear levels; however, several studies suggested that the interaction of NF-κB and AhR occurs primarily in the cytoplasm since ARNT was not found to dimerize with RelA in a ligand-dependent manner (Tian *et al.*, 1999). Moreover, it has been reported that inactivated AhR and NF-κB in the cytoplasm are kept away by being sequestered by their inhibitory proteins, HSP90 and 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). Other studies suggested PKA- and PKC-dependent mechanisms (Zhong *et al.*, 1997; Tian *et al.*, 2002).

1.4.4.2.3. AP-1

AP-1 is a transcription factor that belongs to a family of basic domain/leucine zipper (bZIP) proteins 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). In addition, AP-1 controls the expression of a number of genes, such as those encoding collagenase, stromelysin, and cytokines through coupling to its binding site (Shen *et al.*, 2005). AP-1 is a heterodimeric complex of leucine-zipper proteins, c-Jun and c-Fos. Upon activation by a large number of stimuli, including proinflammatory cytokines, oxidative stress, and tumor promoters, AP-1 binds to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) responsive element (TRE) sequences within the promoter regions of several target genes (Shen *et al.*, 2005).

AP-1 activity has been shown to be regulated by mitogen-activated protein kinase (MAPK) signaling pathways such as c-Jun N-terminal kinase (JNK), extracellular-signal regulated kinase (ERK), and p38 MAPK (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). It has been reported previously that there is a well-established link between the AP-1 signaling pathway and the expression of AhR-regulated genes (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 showed 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, these results and the observation that AhR antagonists attenuate the TCDD-induced inhibition of AP-1 binding in CH12.LX cells (Suh *et al.*, 2002) strongly suggest a coordination between AhR and AP-1 signaling pathways. In contrast, treatment of Hepa 1c1c7 cells with TCDD or BaP 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 enzyme activity in HepG2 cells in response to 3MC is mediated through activation of AP-1 DNA binding (Quattrochi *et al.*, 1998).

In addition to CYP, the involvement of AP-1 signaling pathways in the induction of *NQO1* gene expression has been previously reported. Several studies showed that induction of the *NQO1*gene in human colon adrenocarcinoma HT29 (Yao *et al.*, 1997) and Hepa lclc7 (Park *et al.*, 2004) cells by the chemoprotective compounds mitomycin C and baicalin is AP-1-dependent. Furthermore, it has been demonstrated that the constitutive expression of *NQO1* in human non-small cell lung cancer is regulated through the AP-1 signaling pathway (Kepa and Ross, 1999).

1.5. Heavy metals

Historically, heavy metals are considered the oldest toxic substances known to humans. Lead (Pb^{2+}) is probably the oldest metal, having been used in 2000 B.C (Valko *et al.*, 2005). In addition, arsenic (A^{3+}) and mercury (Hg^{2+}) have been used early for the decoration of Egyptian tombs (Valko *et al.*, 2005). Heavy metals are defined as the metallic elements that are capable of forming polyvalent cations and possess a molecular size of more than 50 Da (Foulkes, 2000). Based on physiological and toxicological effects, heavy metals are classified into four classes (Foulkes, 2000). Class A represents heavy metals, such as iron, that play essential physiological functions such as mediation of enzyme activities. Class B contains metals for which no biological role has been established, and which at low-range concentrations exhibit little or no toxicity, such as strontium, lanthanum, and others. A third Class, C, includes zinc, nickel and copper which are essential metals for living systems; however, they are considered very toxic at relatively high concentrations. Class D includes heavy metals that are very toxic at very low concentrations and have no biological functions, such as arsenite, mercury, lead and cadmium (Foulkes, 1990; Foulkes, 2000).

Heavy metals are highly toxic non-essential elements in that they are neither created nor biodegradable and usually originate from natural and industrial resources (Barbier *et al.*, 2005). The available information suggests that heavy metals in the environment, even in trace amounts, can cause serious problems to all organisms including humans (Beyersmann, 2002). The frequent

persistent occurrence and accumulation of heavy metals in the environment and their potential exposure to humans, from numerous sources including contaminated air, water, soil and food, cause them to be ranked highly as most hazardous and toxic substances in the environment by the Agency for Toxic Substances and Diseases Registry (ASTDR) (ATSDR, 2011) and the Canadian Environmental Protection Act Registry (CEPA) (CEPA, 2012). Among all heavy metals, Hg^{2+} ranked the highest in these lists.

1.5.1. Mercury (Hg)

Hg is a metal that occurs naturally in the environment and is widely used in the foundry, mining, and manufacturing industries. In addition, Hg is a component in a number of electrical instruments and medical products such as thermometers, thermostats, dental amalgams, switches, and batteries (Gochfeld, 2003). Among the metals, Hg is unique in that it is found in the environment in several physical and chemical forms. At room temperature, elemental (or metallic) Hg exists as a liquid (Zalups, 2000). As a result of its high vapor pressure, this form of Hg is released into the environment as Hg vapor. Hg vapor is colorless and odorless. The higher the temperature, the more vapors will be released from liquid metallic Hg. Some people who have breathed mercury vapors report a metallic taste in their mouths. Metallic mercury has been found at 714 hazardous waste sites nationwide (ATSDR, 2011). There are three different oxidation states of Hg namely Hg^0, which exists in metallic form as vapor, and Hg^+ or Hg^{2+}, which can form stable organic compounds such as methylmercury

(Ercal *et al.*, 2001; Tezel *et al.*, 2001). In this regard, Hg usually enters water as a product of industrial pollution, where it is methylated by bacteria to form methylmercury, which can then be taken up by fish and ultimately by humans (Crinnion, 2000). Generally, the route and efficiency of exposure depends mainly on the oxidation state of Hg (Ercal *et al.*, 2001; Tezel *et al.*, 2001).

Inorganic mercury compounds occur when mercury combines with elements such as chlorine, sulfur, or oxygen. These mercury compounds are also called mercury salts. Most inorganic mercury compounds are white powders or crystals, except for mercuric sulfide (also known as cinnabar) which is red and turns black after exposure to light (ATSDR, 2011). Primarily through the action of micro-organisms, inorganic mercury can be combined with carbon to form organic mercury compounds, of which methylmercury is the most abundant (also known as monomethylmercury) (Risher *et al.*, 2002). In surface waters, it is rapidly accumulated by aquatic organisms, where it biomagnifies as it ascends the food chain (Risher *et al.*, 2002). There is a potentially large number of organic mercury compounds including phenylmercury and dimethylmercury. In the past, phenylmercury was used in some commercial products; however, dimethylmercury is used in small amounts as a reference standard for some chemical tests. http://www.chm.bris.ac.uk/motm/dimethylmercury/dmmh.htm. Dimethylmercury is the only organic mercury compound that has been identified at hazardous waste sites. It was only found in extremely small amounts at two hazardous waste sites nationwide, but it is very harmful to human and animals (ATSDR, 2011). Like the inorganic mercury compounds, both methylmercury

and phenylmercury exist as "salts" (for example, methylmercuric chloride or phenylmercuric acetate). When pure, most forms of methylmercury and phenylmercury are white crystalline solids. Dimethylmercury, however, is a colorless liquid.

1.5.2. Hg Toxicity

Mercury occurs in different chemical and physical forms. Therefore, the toxicity of mercury is thus very complex, and depends not only on the form of mercury and the route of entry, but also on dosage, frequency, and age at exposure (Pichichero *et al.,* 2002). In addition, mercury is a widespread environmental toxicant and pollutant which induces severe alterations in body tissues and causes a wide range of adverse health effects (Bhan and Sarkar, 2005). Both humans and animals are exposed to various chemical forms of mercury in the environment. These include elemental mercury vapor (Hg^0), inorganic mercurous (Hg^{1+}) and mercuric (Hg^{2+}) mercury, and the organic mercury compounds (Zahir *et al.*, 2005). Because mercury is ubiquitous in the environment, humans, plants, and animals are all unable to avoid exposure to some form of mercury (Holmes *et al.*, 2009). Humans are exposed to all forms of mercury through accidents, environmental pollution, food contamination, dental care, preventive medical practices, industrial and agricultural operations, and occupational operations (Bhan and Sarkar, 2005). The major sources of chronic low-level mercury exposure are dental amalgams and fish consumption. Mercury enters water as a natural process of off-gassing from the earth's crust and also

through industrial pollution (Dopp *et al.*, 2004). Algae and bacteria methylate the mercury entering the waterways. Methylmercury then makes its way through the food chain into fish, shellfish, and eventually into humans (Sanfeliu *et al.*, 2003).

All forms of mercury cause toxic effects in a number of tissues and organs, depending on the chemical form of mercury, the level of exposure, the duration of exposure, and the route of exposure. The kidneys are the primary target organ where inorganic mercury is taken up, accumulated, and expresses toxicity (Clarkson, 1972; Zalups, 2000; ATSDR, 2011). It is well known that inorganic mercury causes severe kidney damage after acute and chronic exposure (Zalups and Diamond, 1987b; Zalups and Diamond, 1987a; Sharma *et al.*, 2005). In this regard, mercury has been recognized as a nephrotoxic agent for several centuries. The fact that renal disorder could be produced by mercurial salts used in the treatment of syphilis has been known since the 16th century.

In 1860 there was a case of a woman who had been acquitted by English courts of murdering her husband with white precipitate (79 percent mercury). The consensus of medical opinion at that time held that mercury was not a poison (Rodin and Crowson, 1962). To dispute this, Pavy, investigated the effect of orally administered the same white precipitate on dogs, rabbits and mice he found gross renal alterations consisting of enlarged, roughly speckled kidneys with marked striation of the cortex (Pavy, 1860). In addition, organic mercuric compounds are also nephrotoxic but to a lesser degree than inorganic mercurous or mercuric compounds. Systemic distributions of organic mercury are more

diffuse than inorganic forms, and they affect other target organs, including hematopoietic and neural tissues (ATSDR, 2011).

Organic mercury can produce a variety of neurologic effects through all routes of exposure. Other isolated instances of alkyl mercury poisoning have been reported (Engleson and Herner, 1952; Cinca *et al.*, 1980), and epidemic poisonings in Japan and Iraq demonstrated conclusively the neurotoxicity of these compounds. The first reported widespread outbreak of neurologic disorders associated with methylmercury involved the ingestion of contaminated fish in the Minamata area of Japan (Kutsuna, 1968; Takizawa, 1970). The neurologic syndrome observed in this poisoning incident was characterized by a long list of symptoms, including paresthesia (prickling, tingling sensation in the extremities) impaired peripheral vision, hearing, taste, and smell, slurred speech, unsteadiness of gait and limbs, muscle weakness, irritability, memory loss, depression and sleeping difficulties (Kutsuna, 1968; Tsubaki, 1986). Elevated concentrations of methylmercury were observed in the hair and brains of victims.

Epidemics of neurologic disorders similar to those described above were reported in Iraq in 1956 and 1960 (Jalili and Abbasi, 1961; Bakir *et al.*, 1973), as the result of eating flour made from seed grain treated with ethylmercury *p*-toluene sulfonanilide. Effects observed in affected individuals included inability to walk, cerebellar ataxia, speech difficulties, paraplegia, spasticity, abnormal reflexes, restriction of visual fields or blindness, tremors, paresthesia, insomnia, confusion, hallucinations, excitement, and loss of consciousness. In another incident following the ingestion of contaminated bread prepared from wheat and

other cereals treated with a methylmercury fungicide in 1971-72, more than 6000 patients required hospitalization and more than 500 deaths occurred, mainly due to central nervous system failure. Attempts to correlate symptoms with an estimate of methylmercury intake in the 1971-72 epidemic (based on average levels found in grain and self-reported estimates of number of loaves ingested) indicated that no effects were observed in persons consuming a total of 3.6 mg Hg/kg at ages 5-9 years, 2.8 mg Hg/kg at ages 10-14 years, or 1.7 mg Hg/kg at ages 15 years and above (consumption values reflect total intake and not daily intake) (Al-Mufti *et al.*, 1976).

1.5.3. Effect of Hg on Aryl Hydrocarbon Receptor Regulated-Genes

Previous studies have focused primarily on the toxicology of alkyl mercurials in laboratory animals and on the central nervous system, with some investigations involving early *in vivo* biochemical effects of methylmercury in other tissues (Miyakawa and Deshimaru, 1969; Verity *et al.*, 1975). Nevertheless, few early studies have demonstrated the effect of mercury on the hepatic microsomal enzymes, the UDP glucuronyltransferases and the mixed-function oxidases (Lucier *et al.*, 1972). Salts of methylmercury, when administered to rats, decreased the activity of mixed function oxidase, whereas mercuric acetate administration was reported to result in an increase in p-nitroanisole demethylation (Lucier *et al.*, 1972; Wagstaff, 1973). In 1978, Aitio and his colleagues proved that mercury is a potent inhibitor of mixed function oxidation, whereas the effect on cytochrome c reduction was less pronounced. Recently, our

laboratory and others have published results showing that mercury differentially alters the expression of constitutive and inducible cytochrome P450s 1A1 (Cyp1a1), (Cyp1a2) and (Cyp1b1) at different signaling pathway levels in murine hepatoma Hepa 1c1c7, human hepatoma HepG2, and human hepatocyte cultures (Vakharia *et al.*, 2001a; Korashy and El-Kadi, 2005; Amara *et al.*, 2010).

Moreover, mercury was able to differentially alter the expression of phase II drug metabolizing enzymes such NADP(H):quinone oxidoreductase1(Nqo1), and glutathione S-transferase a1 subunit (Gsta1), as well as heme oxygenase-1 (HO-1) *in vitro* in human hepatoma HepG2 and isolated mice hepatocytes, and *in vivo* in C57Bl/6J mice (Korashy and El-Kadi, 2006b; Amara *et al.*, 2012a; Amara *et al.*, 2012b).

1.5.4. Hg Toxicokinetics

Absorption of metallic mercury is high (approximately 70–80%) for inhaled vapor, and negligible for oral exposure to liquid metallic mercury. Absorption of inorganic mercuric salts is dependent upon the form and test conditions, which may range from 2 to 38%. Oral absorption of organic mercury is nearly complete, but respiratory absorption data are lacking, particularly for the alkyl mercurials (ATSDR, 2011). The distribution data for metallic, inorganic, and organic mercury are consistent in identifying the kidney as the organ with the highest mercury bioaccumulation (Clarkson, 1972; Rice, 1989; Zalups, 2000; Clarkson, 2002). Compared to inorganic mercury, metallic mercury has a high lipophilicity that makes it transferred readily through the placenta and blood-brain

barrier. The oxidation of metallic mercury to an inorganic divalent cation in the brain can result in retention in the brain. Although inorganic mercury compounds can reach most organs, their low lipophilicity reduces their ability to penetrate barriers to and accumulate in the brain and fetus. Methylmercury has a similar distribution to that of metallic mercury. However, a relatively large amount of mercury can accumulate in the brain and fetus (compared to inorganic mercury) because of its ability to penetrate the blood-brain and placental barriers and its conversion in the brain and fetus to the inorganic divalent cation (Rice, 1989; ATSDR, 2011).

Metallic mercury can be oxidized primarily in the red blood cells to inorganic divalent mercury by the hydrogen peroxide-catalase pathway, which is present in most tissues (Halbach and Clarkson, 1978; Hursh *et al.*, 1988). Evidence from animal studies suggests the liver as an additional site of oxidation (ATSDR, 2011). In 1978, it was believed that the rate of metallic mercury oxidation is dependent on: (1) the concentration of catalase in the tissue; (2) endogenous production of hydrogen peroxide; and (3) availability of mercury vapor at the oxidation site (Magos *et al.*, 1978). Furthermore, hydrogen peroxide production is probably a rate-determining step because in another *in vivo* study Nielsen-Kudsk has found that stimulation of hydrogen peroxide production in red cells increased the uptake of mercury vapors in red blood cells (Nielsen-Kudsk, 1973). In addition to the red cells, the oxidation of metallic mercury may also occur in the brain, in both adult and fetal liver (Magos *et al.*, 1978), lungs (Hursh *et al.*, 1980), and probably all other tissues to some degree (Clarkson, 1989).

Unlike oxidation in red cells, the rate-limiting step in *in vitro* oxidation in the liver is dependent on the rate of mercury delivery to the catalase enzyme (Magos *et al.*, 1978). Despite of the oxidation process, unoxidized parts of metallic mercury can still reach the brain because the oxidation of metallic mercury is a slow process compared the circulation time from the lungs to the brain (Magos, 1967). In 1972 another study suggested that mercury oxidation also occurs in the placenta and fetus (Yang *et al.*, 1972).

Once absorbed the inorganic divalent cation can, in turn, be reduced to metallic mercury. The mercurous ion is unstable in the presence of sulfhydryl groups and undergoes disproportionation into one atom of metallic mercury and one ion of mercuric mercury (ATSDR, 2011). The oxidation-reduction cycle occurring between metallic and inorganic mercury has been evidenced by *in vivo* studies. In this regard, rats and mice that were exposed to mercuric chloride exhaled metallic mercury vapor (Clarkson and Rothstein, 1964; Dunn *et al.*, 1981a; Dunn *et al.*, 1981b).

As for metallic mercury, organic mercury can also be converted to inorganic divalent mercury. However, the extent of conversion is less than that for metallic mercury (Dunn and Clarkson, 1980). The conversion of organic mercury such as methylmercury or phenylmercury into divalent inorganic mercury can probably occur soon after absorption, and also feeding into the oxidation-reduction pathway. Several investigators have reported high levels of inorganic mercury in tissues (Magos and Butler, 1972; WHO, 1990) and feces after methylmercury exposure (Turner, 1975). The degradation of methylmercury into

inorganic mercury has been supported experimentally in that rat liver microsomes can degrade methylmercury into inorganic mercury. The final product of inorganic mercury from degradation of methylmercury is in parallel with hydroxyl radical production (Suda and Hirayama, 1992). The promotion and inhibition of hydroxyl radical formation and the hydroxyl radical scavenger, affects inorganic mercury production. These results suggest that hydroxyl radicals produced from microsomes may play a predominant role in alkyl mercury degradation (Fig 1.6).

It is well known that the NADPH-cytochrome P-450 reductase is responsible for hydroxyl radical production in liver microsomes. Furthermore, alkyl mercury degradation varies in proportion to the enzyme activities and hydroxyl radical production. Therefore, these results imply that hydroxyl radicals produced by cytochrome P-450 reductase might be the primary reactive species that induces alkyl mercury degradation. In vitro studies using a peroxidase hydrogen peroxide-halide system indicated that besides the hydroxyl radical, hypochlorous acid (HOCl) scavengers are also capable of degrading methylmercury (Suda and Takahashi, 1992). A small amount of an oral dose of methylmercuric chloride can also be converted into inorganic mercury in the intestinal flora (Nakamura *et al.*, 1977; Rowland *et al.*, 1980). However, inorganic mercury is poorly absorbed across the intestinal wall and, therefore, most of it is excreted.

Following exposure to metallic mercury, the elimination of mercury can occur via the urine, feces, and expired air. Following exposure to inorganic

mercury (mercuric), mercury is eliminated in the urine and feces. The urine and feces are the main excretory pathways of metallic and inorganic mercury in humans, with a body burden half-life of approximately 1–2 months (Clarkson, 1989). With regard to the organic mercury, the fecal (biliary) pathway is the predominant excretory route for methylmercury, with less than one-third of the total mercury excretion occurring through the urine, following oral and inhalation exposure (Norseth and Clarkson, 1970). In humans, nearly all of the total mercury in the feces after organic mercury administration is in the inorganic form (Fig 1.6). The conversion of methylmercury to inorganic mercury is a major step that is dependent on the duration of exposure and/or the duration after cessation of exposure (ATSDR, 2011).

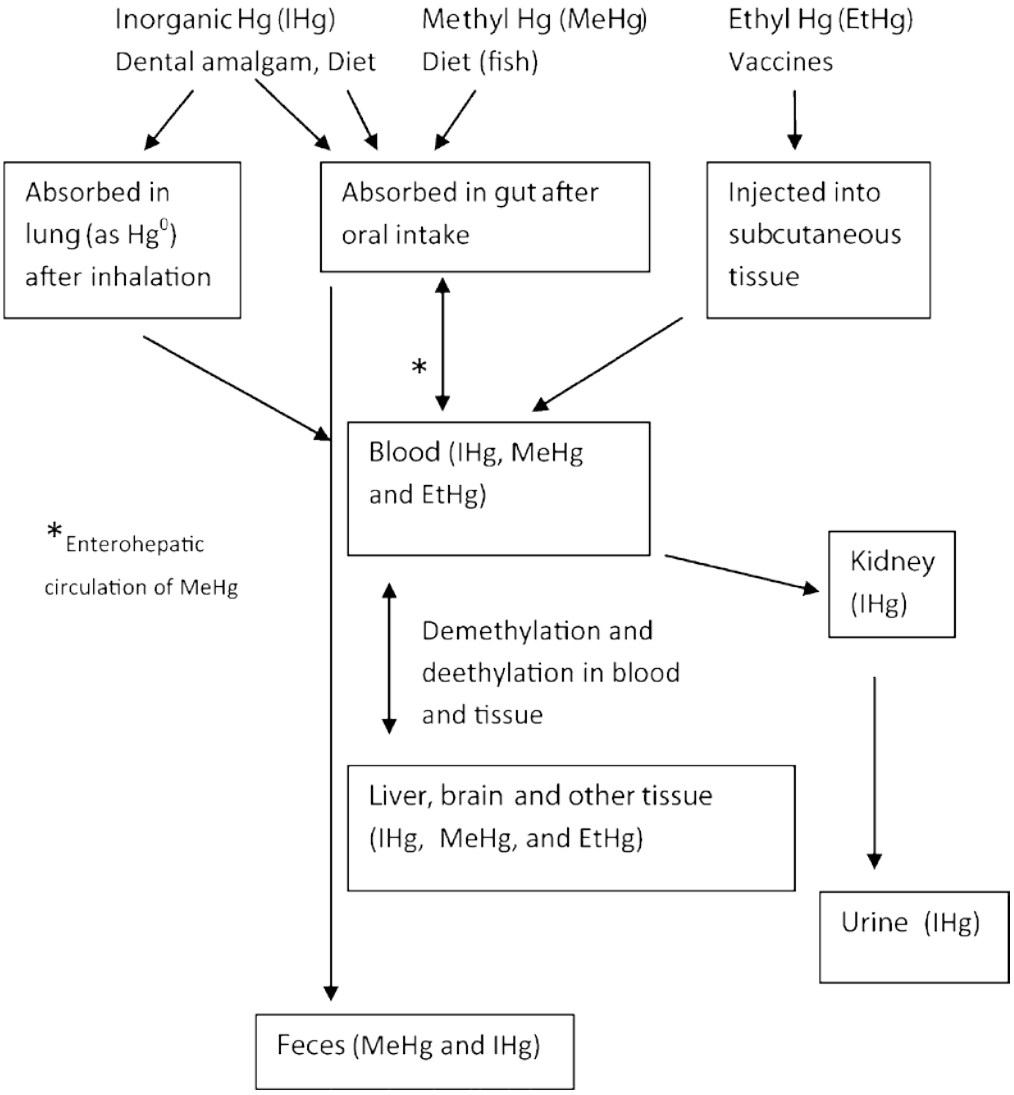

Fig. 1.6. Simplified outline of the sources and metabolism of different mercury species. (Barregard *et al.*, 2011).

1.6. RATIONALE, HYPOTHESES AND OBJECTIVES

1.6.1. Rationale

It well known that classical AhR ligands typified by HAHs and PAHs produce a wide array of toxic effects. Most of the toxic effects produced by these hazardous contaminants have been shown to be mediated by the AhR, a cytosolic receptor to which these contaminants bind. Upon binding to the AhR, these ligands induce the transcription of CYPs responsible for their metabolism into toxic intermediates. These toxic intermediates then act as second messengers for the induction of phase II drug metabolizing enzymes. Therefore, the toxicity of these AhR ligands cannot be assessed by measuring CYP induction alone, because it has been shown that these AhR ligands will also induce the phase II AhR regulated genes as a counterproductive mechanism to this process.

Many sources of environmental exposure to AhR ligands involve heavy metal co-exposure. Heavy metals and AhR ligands are common contaminants of hazardous waste sites and co-released from sources such as fossil fuel combustion and municipal waste incineration and as components of tobacco. Several studies have reported that significant traces of metals were detected in the air of several cities in Italy, Pakistan, and the Czech Republic (Sram *et al.*, 1996; Monarca *et al.*, 1997), in surface soils in Greece (Stalikas *et al.*, 1997), in Sweden, and in fish from the Sheboygan River in Wisconsin (Kaminsky, 2006). In Canada, studies examined mercury levels in hair samples taken from 361 and 228 children living in three regions in Nunavut and Northern Quebec, a region called Nunavik, respectively showed that the mean hair Hg concentration of Inuit children from

Nunavut was 1.43 μg/g of hair (Tian *et al.*, 2011), which was about half of that found in children from Nunavik (Northern Quebec) with a mean of 2.7 μg/g of hair (Despres *et al.*, 2005). Furthermore, results from the Baffin region, have showen that the mean Hg concentration was 2.09 μg/g of hair. These levels are 10–20 times higher than children from the U.S. with a mean of 0.22μg/g of hair (McDowell *et al.*, 2004).

Several previous studies have addressed heavy metals as modifiers of xenobiotic metabolizing enzymes, suggesting a possible role of trace metals in altering the mutagenicity and carcinogenicity of PAHs and HAHs. Among these metals, Hg is ranked highly among the most hazardous and toxic substances in the environment (ATSDR, 2011; CEPA, 2012). However, very little information is available on the effects of Hg on the regulation and expression of AhR-regulated genes. Initial reports on the effects of Hg on CYP activity demonstrated that Hg decreased hepatic CYPs in both phenobarbital- and 3MC-treated mice (Abbas-Ali, 1980). Our laboratory has also demonstrated previously the effect of Hg on Cyp1a1 expression in murine hepatoma Hepa 1c1c7 cells (Korashy and El-Kadi, 2005). In addition, Hg has been shown to enhance the activity of NQO1 and catecholamine-O-methyl-transferase in human placenta (Boadi *et al.*, 1991) and NQO1 and Gsta1 in murine hepatoma Hepa 1c1c7 cells (Korashy and El-Kadi, 2006b). Therefore, it was of great importance to evaluate the combined toxic effects, in particular the AhR-driven carcinogenicity and mutagenicity of these AhR ligands typified by TCDD and Hg, a common co-

contaminant of TCDD in an *in vitro* and *in vivo* model using human hepatoma HepG2 cells, isolated mice primary hepatocytes and C57Bl/6J mice, respectively.

1.6.2. Hypotheses

Based on a literature review, we proposed the following hypotheses:

1. Co-exposure to Hg^{2+} and the AhR ligand, TCDD, disrupts the expression of phase I and phase II AhR-regulated genes, such as Cyp1a1, Cyp1a2 and Cyp1b1 and Nqo1 and Gsta1 respectively, in a tissue-, time-, and gene-specific manner in vivo
2. Hg^{2+} influences the activity of the AhR ligand, TCDD, by altering the expression of AhR-regulated genes through AhR-XRE and Nrf2-ARE signaling pathways
3. HO-1 and Nrf2 are involved in the modulation of AhR-regulated genes by Hg^{2+}
4. MeHg alters CYP1A1 expression in a manner different to that of Hg^{2+}

1.6.3. Objectives

The specific objectives of this study are: 1) to determine the possible effects of Hg 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 Hg^{2+} on both the basal and TCDD- or Sulforaphane (SUL)-mediated induction of NQO1, as bifunctional and monofunctional inducers in human hepatoma HepG2 cells, and to determine the

molecular mechanisms involved in this modulation, 3) to examine the effect of Hg^{2+} *in vitro* and *in vivo* using mice liver and isolated mice primary hepatocytes and to determine the effect of Hg^{2+} on AhR-regulated genes in extrahepatic tissues: kidney, lung and heart, and 4) to investigate whether similar effects to those of Hg^{2+} will be observed upon exposure to MeHg on CYP1A1 expression gene and investigating the molecular mechanisms involved in this alteration in human hepatoma HepG2 cells.

The importance and the significance of this research project stems from its great impact on increasing our knowledge about the effects of Hg on the AhR-regulated genes. This will allow us to understand and identify the mechanistic steps and signaling pathways likely to be involved in the modulation of xenobiotic metabolizing enzymes by Hg, which will help in improving the treatment strategies for mutagenicity mediated by AhR ligands.

CHAPTER 2- MATERIALS AND METHODS

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Amara, I.E., Anwar-Mohamed, A., Abdelhamid, G., El-Kadi, A.O., 2013a. Mercury modulates the cytochrome P450 1a1, 1a2 and 1b1 in C57BL/6J mice: in vivo and in vitro studies. Toxicology and applied pharmacology 266, 419-429.

Amara, I.E., Anwar-Mohamed, A., El-Kadi, A.O., 2010. Mercury modulates the CYP1A1 at transcriptional and posttranslational levels in human hepatoma HepG2 cells. Toxicology letters 199, 225-233.

Amara, I.E., Anwar-Mohamed, A., El-Kadi, A.O., 2013b. Posttranslational mechanisms modulating the expression of the cytochrome P450 1A1 gene by methylmercury in HepG2 cells: A role of heme oxygenase-1. Toxicology letters.

Amara, I.E., El-Kadi, A.O., 2011. Transcriptional modulation of the NAD(P)H:quinone oxidoreductase 1 by mercury in human hepatoma HepG2 cells. Free radical biology & medicine 51, 1675-1685.

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, mercuric chloride ($HgCl_2$), 2,6-dichlorophenolindophenol (DCPIP), dicoumarol, isothiocyanate sulforaphane (SUL) methyl mercury chloride ($MeHgCl_2$), β-glucuronidase, arylsulfatase, chlorpromazine HCl, collagenase, collagen from rat tail, and hemoglobin (Hb), were purchased from Sigma Chemical Co. (St. Louis, MO). Tin mesoporphyrin (SnMP), was 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 (San Diego, CA). High-Capacity cDNA Reverse Transcription Kit, SYBR® Green PCR Master Mix, human Hmox1 (HO-1) validated 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). CYP1A1/1A2 mouse polyclonal primary antibody, heme oxygenase-1 (HO-1) goat polyclonal primary antibody glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-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 (ab34173) was purchased from Abcam (Cambridge, MA, USA). Anti-mouse IgG peroxidase secondary antibody was purchased from R&D Systems, Inc. (Minneapolis, MN). Nrf2 mouse monoclonal primary antibody (MAB3925) and anti-mouse IgG peroxidase secondary antibody were purchased from R&D Systems (Minneapolis, MN, USA). Human Nrf2 validated siRNA and primers were purchased from Integrated DNA Technologies (Coralville, IA, USA) pRL-CMV plasmid and dual luciferase assay reagents were obtained from Promega (Madison, WI, USA). All other chemicals 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 mice 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 the 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 cytochrome P450–dependent 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

The 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. 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 C57BL/6J mice and plated onto different well sizes (12-, 24- and 96-wells) of 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 125 μl, respectively of Type I

rat-tail collagen (50 μg/ml acetic acid, 0.02 N) 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, 50 ng/ml dexamethasone phosphate, 100 IU/ml penicillin G, 10 μg/ml streptomycin, and 25 ng/mL amphotericin B. The plastic culture plates were incubated at 37°C in a cell culture incubator with 95% O_2: 5% CO_2. Viability was assessed before and after the incubation period by the trypan blue (0.2%) exclusion method, and in both instances the viability was over 90%.

2.2.3. Animal Model

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

2.2.4. Isolation of Primary Hepatocytes

Three solutions were utilized for the isolation of 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 filtered through a 22μm membrane before use.

Mouse hepatocytes were isolated by a two-step collagenase perfusion method as described previously (Seglen, 1976). Mice 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. 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 of solution C. The capsule was stripped away from one side of the liver, and the cells were detached by brushing the liver with a plastic comb and filtered through cotton gauze. The suspension of cells was incubated in a shaker water bath at 37°C for 5 min with 95% O_2: 5% CO_2, filtered through a (70 μm) cell strainer and divided into two aliquots which were placed on ice. Once the temperature of 4°C was reached, the cells were centrifuged at 100 × *g* for 2 min, the supernatant was aspirated and the sediment was resuspended in DMEM and re-centrifuged at 100 × *g* for 2 min, an operation that was repeated twice. The

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

2.2.5. Chemical Treatment

Human hepatoma HepG2 cells and isolated mouse hepatocytes were treated in serum free medium with various concentrations of Hg^{2+} (2.5 - 10 μM) and MeHg (1.25 - 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 Hb (0.5 - 2 μM) (Burgstahler and Nathanson, 1995). TCDD, SUL and SnMP were dissolved in dimethylsulfoxide (DMSO) and maintained in DMSO at −20 °C until use. Hg^{2+}, MeHg and hemin (10 mM stocks) were prepared freshly in double de-ionized water. 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 Hg^{2+} (dissolved in saline) at 2.5 mg/kg, in the absence and presence of 15 μg/kg TCDD (dissolved in corn oil) injected i.p. The mice were divided into 4 groups. The first group (*n*=12) of control mice received saline (0.4 mL) plus corn oil (0.4 mL). The second group (n=12) consisted of mice which received Hg^{2+} dissolved in saline (0.4 mL) plus corn oil (0.4 mL). The third group (n=12) comprised mice which received TCDD dissolved in corn oil (0.4 mL) plus saline (0.4 mL). The fourth group (n=12) received Hg^{2+} dissolved in saline (0.4 mL) plus TCDD dissolved in corn oil (0.4

mL). After a single injection of Hg^{2+} with and without TCDD, the animals were euthanized via cervical dislocation at 6 h (*n*=6) and 24 h (*n*=6). Liver, kidney, lung and heart 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. All animals were allowed free access to food and water throughout the treatment period.

2.2.7. Measurement of Cell Viability

The effect of Hg^{2+} and MeHg on cell viability was determined using the MTT assay as described previously (Anwar-Mohamed and El-Kadi, 2009). 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 mouse 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 Hg^{2+} (2.5-50 μM) and MeHg (1.25-20 μM) in the absence and presence of 1 nM TCDD. After 24 h incubation, the medium was removed and replaced with cell culture medium containing 1.2 mM MTT dissolved in phosphate buffered saline (PBS) (pH 7.4). After 2 h of incubation, the crystals formed were dissolved in isopropanol. The intensity of the color in each well was measured at a wavelength of 550 nm using the Bio-Tek EL 312e microplate reader (Bio-Tek Instruments, Winooski, VT).

2.2.8. RNA Extraction and cDNA Synthesis

Total RNA from the frozen tissues or cells treated for 6 h, 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. Thereafter, cell lysate was collected in a 1.5-mL Eppendorf tubes 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 Eppendorf 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 using 75% ethanol in diethyl pyrocarbonate (DEPC)-treated water. The tubes were vortexed lightly 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 supernatant removed by aspiration 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 to ensure that the pellet was completely dissolved. 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 analysis 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 to 85°C and kept these for 5 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 by 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. Melting curve (dissociation stage) was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product.

2.2.10. 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 ΔC_T of control (vehicle treated animals at 6 or 24 h time points) from the ΔC_T of the corresponding treatment groups. The values and ranges given in different figures were determined as follows: $2^{-\Delta\ (\Delta CT)}$ with $\Delta\Delta C_T$ + S.E. and $\Delta\Delta C_T$ – S.E., where S.E. is the standard error of the mean of the Δ (ΔC_T) value.

Table 2.1. Primers and siRNA sequences used for real-time PCR reactions

Gene	Forward primer	Reverse primer
Human *CYP1A1*	5'-CTATCTGGGCTGTGGGCAA-3'	5'-CTGGCTCAAGCACAACTTGG-3'
Human *NQO1*	5'-CGCAGACCTTGTGATATTCCAG-3'	5'-CGTTTCTTCCATCCTTCCAGG-3'
Human *HO-1*	5'-ATGGCCTCCCTGTACCACATC-3'	5'-TGTTGCGCTCAATCTCCTCCT-3'
Human *AhR*	5'-CCCTTGGAAATTCATTGCCA-3'	5'-GGAGAGGTGCTTCATATGTCGTC-3'
Human *Nrf2*	5'-AACCACCCTGAAAGCACAGC-3'	5'-TGAAATGCCGGAGTCAGAATC-3'
Human *Actin*	5'-CTGGCACCCAGGACAATG-3'	5'-GCCGATCCACACGGAGTA-3'
Murine *Cyp1a1*	5'-GGTTAACCATGACCGGGAACT-3'	5'-TGCCCAAACCAAAGAGAG TGA-3'
Murine *Cyp1a2*	5'-TGGAGCTGGCTTTGACACAG-3'	5'-CGTTAGGCCATGTCACAAGTA GC-3'
Murine *Cyp1b1*	5'-AATGAGGAGTTCGGGCGCACA-3'	5'-GGCGTGTGGAATGGTGAC AGG-3'
Murine *Nqo1*	5'-GGAAGCTGCAGACCTGGTGA-3'	5'-CCTTTCAGAATGGCTGGCA-3'
Murine *Gsta1*	5'-CCCCTTTCCCTCTGCTGAAG-3'	5'-TGCAGCTTCACTGAATCTTGA AAG-3'
Murine *HO-1*	5'-GTGATGGAGCGTCCACAGC-3'	5'-TGGTGGCCTCCTTCAAGG-3'
Murine *Actin*	5'-TATTGGCAACGAGCGGTTCC-3'	5'-GGCATAGAGGTCTTTACGGAT GTC-3'
Nrf2 siRNA	5′-GUAAGAAGCCAGAUGUUAAdUdU-3′	3′-dUdUCAUUCUUCGGUCUACAATT-5′
HO-1 siRNA	5'-CAAUGCAGUAUUUUUGUtt-3'	5'-AACAAAAAUACUGCAUUUGag-3'

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 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 x 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 potassium chloride [1.15% (w/v)]. Consequently, they were cut into pieces and homogenized separately in cold sucrose solution (1 g of tissue in 5 ml of 0.25 M sucrose). After homogenizing, the tissues were separated by differential ultracentrifugation. The final pellet was reconstituted in cold sucrose and supernatant containing cytosol, 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 magnesium chloride, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 µl/ml of protease inhibitor cocktail. The cell homogenates were obtained by incubating the cell lysates on ice for 1 h, with intermittent vortexing every 10 min, followed by centrifugation at 12,000 × *g* for 10 min at 4 °C. Briefly 50 µg of CYP1A1 and 5 µg of NQO1 of cell homogenates 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 1X sample buffer, boiled for 5 min, separated by 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. Protein blots were blocked for 24 h at 4 °C in blocking buffer containing 5% skim milk powder, 2% bovine serum albumin and 0.05% (v/v) Tween-20 in tris-buffered saline solution (TBS; 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris–base). After blocking, the blots were incubated with the following antibodies: primary polyclonal mouse CYP1A1, primary polyclone rabbit NQO1 primary monoclonal mouse Nrf2, primary polyclonal mouse Cyp1a1/1a2, primary polyclonal rabbit Cyp1b1, primary polyclonal goat Nqo1, primary polyclonal goat Gsta1, primary polyclone rabbit HO-1, and primary polyclonal rabbit actin, or primary polyclonal goat Gapdh for 2 h at room temperature. Incubation with a

peroxidase-conjugated goat anti-rabbit IgG secondary antibody for Nqo1, Cyp1b1, HO-1, Nrf2 and actin or goat anti-mouse IgG secondary antibody for Cyp1a1/1a2, or rabbit anti-goat IgG secondary antibody for Gsta1 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 Enzyme Activity in Cells

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

2.2.15. 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 magnesium chloride hexahydrate 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 proteins.

2.2.16. 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.

2.2.17. 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 *et al.*, 1974). Briefly, 20 µg of cytosolic or microsomal proteins 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.

2.2.18. Transient Transfection and Luciferase Assay

Human hepatoma HepG2 cells and isolated mouse hepatocytes were plated in 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).

2.2.19. HO-1 and Nrf2 siRNA Transfection in Human Hepatoma HepG2 Cells

HepG2 cells were plated in 24-well cell culture plates. Each well of cells was transfected with HO-1 and Nrf2 siRNA at concentrations of 20 and 150 nM, respectively, using INTERFERin reagent according to manufacturer's instructions (Polyplus). HO-1 siRNA sequences were sense: CAA AUG CAG UAU UUU UGU Utt, and antisense: AAC AAA AAU ACU GCA UUU Gag. Nrf2 siRNA sequences were sense, 5′-GUAAGAAGCCAGAUGUUAAdUdU-3′, and antisense, 3′-dUdUCAUUCUUCGGUCUACAATT-5′. Transfection efficiency was determined using real-time PCR to detect HO-1 and Nrf2 mRNA post-transfection at 6, 12, and 24 h. Therefore, cells were treated 6 h post-transfection with TCDD in the absence and presence of Hg^{2+} (10 μM) and MeHg (5 μM) for 6 h to determine HO-1, CYP1A1, Nrf2 and NQO1 mRNA levels, or 24 h to determine CYP1A1 and NQO1 catalytic activity levels.

2.2.20. Determination of mRNA Half-lives

The half-lives of CYP1A1 and NQO1 mRNA were analyzed by an actinomycin D (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 Hg^{2+} (10 µM). Total RNA was extracted at 0, 1, 3, 6, 12 and 24 h after incubation with Hg^{2+}. The fold change in the level of *CYP1A1* and *NQO1* (target genes) between treated and untreated cells, corrected by the level of ß-actin, was determined using the following equation: Fold change = $2^{-\Delta\ (\Delta Ct)}$, where $\Delta Ct = Ct_{(target)} - Ct_{(ß\text{-}actin)}$ and $\Delta(\Delta Ct) = \Delta Ct_{(treated)} - \Delta Ct_{(untreated)}$.

2.2.21. Determination of Protein Half-lives

The half-lives of CYP1A1 and NQO1 proteins were analyzed by the cycloheximide (CHX)-chase assay. Cells were pre-treated with 1 nM TCDD for 24 h. Cells were then washed and incubated with 10 µg/ml CHX, to inhibit further protein synthesis, immediately before treatment with Hg^{2+}. Cells were collected in lysis buffer containing 50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 µl/ml of protease inhibitor cocktail, at 0, 1,3, 6, 12, 24, 36 and 48 h after incubation with the metal. Cellular protein was determined using the method of Lowry (Lowry *et al.*, 1951). CYP1A1 and NQO1 proteins were measured by Western blotting. The intensity of bands was quantified, relative to the signals obtained for GAPDH protein, using ImageJ software. The protein half-life values were determined from semilog plots of integrated densities versus time.

2.2.22. Preparation of Nuclear Extract and Cytosolic Protein

Nuclear and cytosolic protein extracts from HepG2 cells were prepared according to a previously described procedure (Andrews and Faller, 1991) with slight modifications. Briefly, HepG2 cells grown on 100-mm Petri dishes were treated for 6 h with vehicle or 10 μM Hg^{2+}. Thereafter, cells were washed twice with cold PBS, pelleted, and suspended in cold buffer A [10 mM Hepes–KOH, 1.5 mm $MgCl_2$, 10mM KCl, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)], pH 7.9, at 4 °C. After 15 min on ice, the cells were centrifuged at 6500 Xg; the supernatant, cytosolic fraction was removed into new microcentrifuge tubes and the pellets were suspended again in high-salt-concentration cold buffer C (20 mM Hepes–KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM PMSF) to extract nuclear proteins. The cells were then incubated on ice with vigorous agitation every 5 min for 30 min followed by centrifugation for 10 min at 12,000 g at 4 °C, and the supernatant (nuclear extract) was removed into new microcentrifuge tubes. The cytosolic and nuclear extracts were stored at −80 °C until further use.

2.2.23. Measurement 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.

2.2.24. 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 the Dunnett test was carried out to assess statistical significance between treatment groups compared to TCDD. For mRNA and protein half-lives, statistical significance was assessed using two way ANOVA followed by the Dunnett test. The differences were considered significant when $P < 0.05$.

CHAPTER 3- RESULTS

A version of this chapter has been published in:

Amara, I.E., Anwar-Mohamed, A., Abdelhamid, G., El-Kadi, A.O., 2012. Effect of mercury on aryl hydrocarbon receptor-regulated genes in the extrahepatic tissues of C57BL/6 mice. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 50, 2325-2334.

Amara, I.E., Anwar-Mohamed, A., Abdelhamid, G., El-Kadi, A.O., 2013a. Mercury modulates the cytochrome P450 1a1, 1a2 and 1b1 in C57BL/6J mice: in vivo and in vitro studies. Toxicology and applied pharmacology 266, 419-429.

Amara, I.E., Anwar-Mohamed, A., El-Kadi, A.O., 2010. Mercury modulates the CYP1A1 at transcriptional and posttranslational levels in human hepatoma HepG2 cells. Toxicology letters 199, 225-233.

Amara, I.E., Anwar-Mohamed, A., El-Kadi, A.O., 2013b. Posttranslational mechanisms modulating the expression of the cytochrome P450 1A1 gene by methylmercury in HepG2 cells: A role of heme oxygenase-1. Toxicology letters.

Amara, I.E., El-Kadi, A.O., 2011. Transcriptional modulation of the NAD(P)H:quinone oxidoreductase 1 by mercury in human hepatoma HepG2 cells. Free radical biology & medicine 51, 1675-1685.

3.1. Mercury Modulates CYP1A1 at Transcriptional and Post-translational Levels in Human Hepatoma HepG2 Cells

3.1.1. Effect of co-exposure to Hg^{2+} and TCDD on cell viability

To determine the non-toxic concentrations of Hg^{2+} to be utilized in the current study, HepG2 cells were exposed for 24 h with increasing concentrations of Hg^{2+} (2.5 – 50 µM) in the absence and presence of 1 nM TCDD. Thereafter cytotoxicity was assessed using MTT assay. Figure 3.1 shows that Hg^{2+} at concentrations of 2.5 – 25 µM in the presence and absence of 1 nM TCDD did not affect cell viability. Therefore, all subsequent studies were conducted using the concentrations of 2.5 – 10 µM.

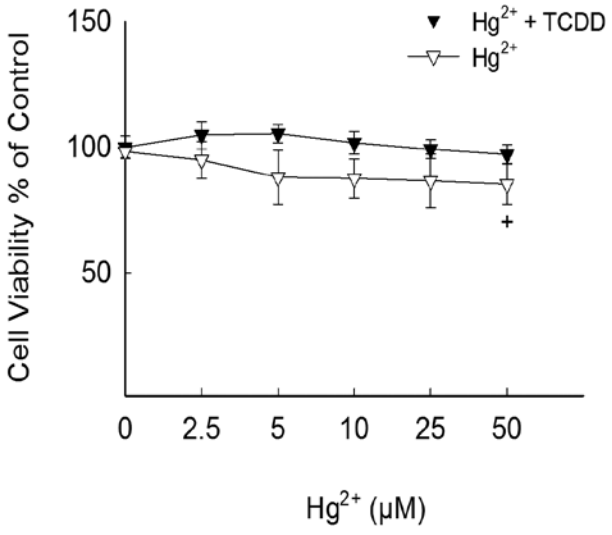

Figure 3.1. Effect of Hg^{2+} on cell viability. HepG2 cells were treated for 24 h with Hg^{2+} (2.5 - 50 μM) in the absence and presence of 1 nM TCDD. Cell cytotoxicity was determined using the MTT assay. Data are expressed as percentage of untreated control (which is set at 100%) ± SE (n = 8). (+) $P < 0.05$, compared to control (concentration = 0 μM).

3.1.2. Concentration-Dependent Effect of Co-exposure to Hg^{2+} and TCDD on Inducible CYP1A1 mRNA

To examine the effect of co-exposure to Hg^{2+} and TCDD on CYP1A1 mRNA, HepG2 cells were treated with various concentrations of Hg^{2+} (2.5-10 μM) in the presence of 1 nM TCDD (Fig. 3.2). Thereafter, CYP1A1 mRNA was assessed using real-time PCR. Our results show that Hg^{2+} alone did not affect the CYP1A1 mRNA (data not shown). On the other hand, TCDD alone caused a significant increase of CYP1A1 mRNA, by 43-fold, that was non-significantly inhibited by Hg^{2+} at the concentrations of 2.5 and 5 μM, while significant inhibition took place at the highest concentration tested, 10 μM. Ten μM Hg^{2+} significantly decreased the TCDD-mediated induction of CYP1A1 mRNA to 16-fold compared to TCDD alone (Fig. 3.2).

3.1.3. Concentration-Dependent Effect of Co-exposure to Hg^{2+} and TCDD on Inducible CYP1A1 Protein and Catalytic Activity

To investigate whether the observed inhibition of the TCDD-mediated induction of CYP1A1 mRNA by Hg^{2+} is further translated to the protein and catalytic activity levels, HepG2 cells were treated for 24 h with increasing concentrations of Hg^{2+} (2.5-10 μM) in the presence of 1 nM TCDD. Figure 3.3A shows that TCDD alone caused a 24-fold increase of CYP1A1 protein level. In agreement with the CYP1A1 mRNA results, Hg^{2+} at the concentration of 10 μM significantly decreased the TCDD-mediated induction of CYP1A1 protein to ~14-fold compared to TCDD alone. In addition, TCDD alone significantly increased

CYP1A1 activity by approximately 11-fold. However, Hg^{2+} decreased the TCDD-mediated induction of CYP1A1 catalytic activity level in a concentration-dependent manner to 7.5-, 5- and 2-fold compared to control at Hg^{2+} concentrations of 2.5, 5, and 10, μM, respectively (Fig. 3.3B).

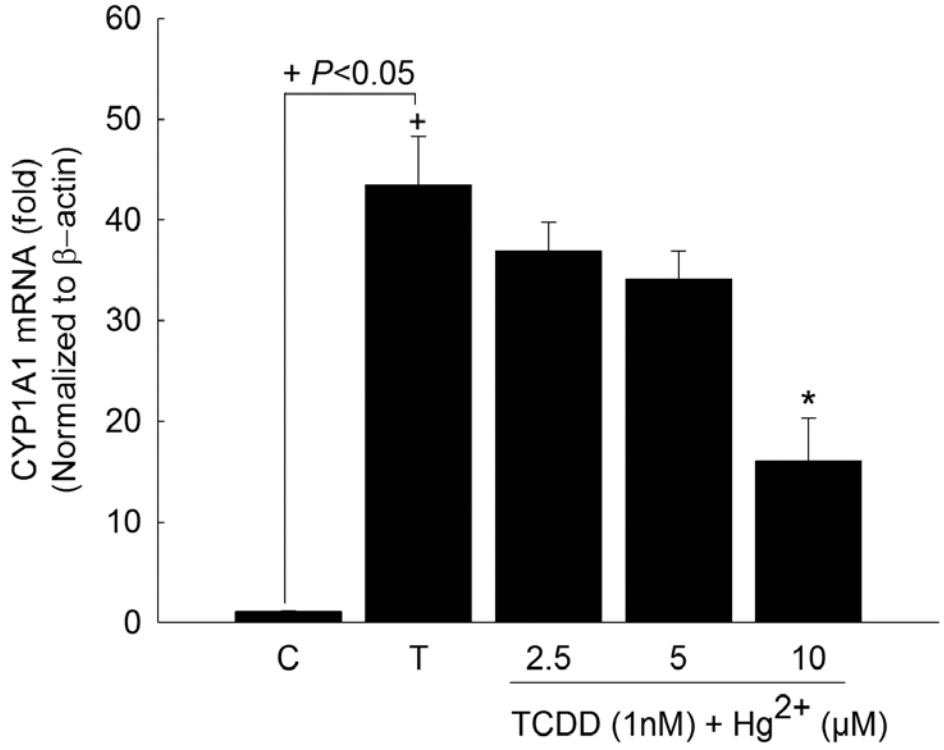

Figure 3.2. Effect of Hg^{2+} on CYP1A1 mRNA using real-time PCR. HepG2 cells were treated with increasing concentrations of Hg^{2+} in the presence of 1 nM TCDD for 6 h. First-strand cDNA was synthesized from total RNA (1 μg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described in the Materials and Methods section. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments ± SE (n = 6). (+) $P < 0.05$, compared to control (C) (concentration = 0 μM); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

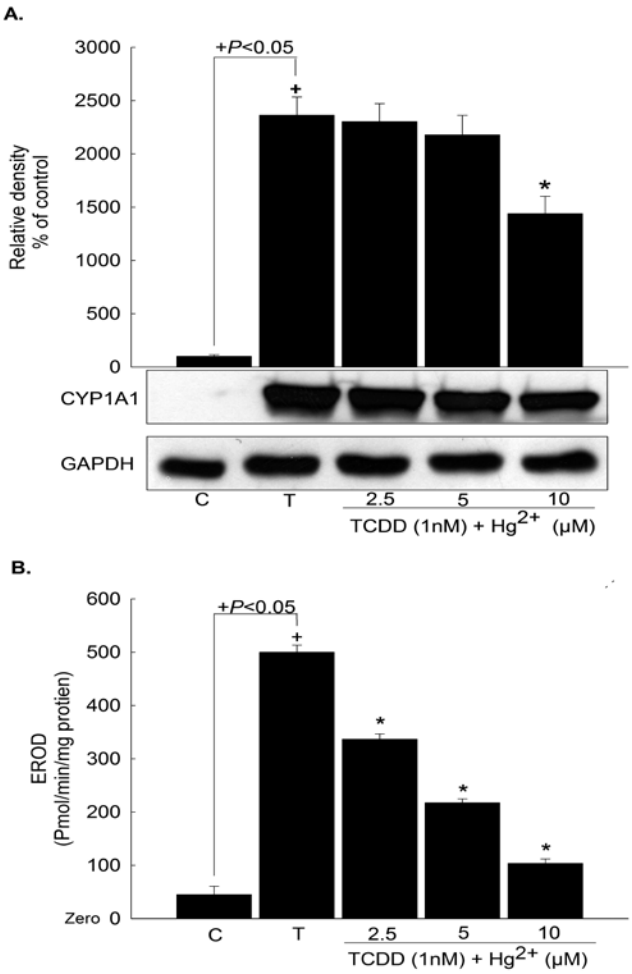

Figure 3.3. Effect of Hg^{2+} on CYP1A1 protein and EROD activity levels. HepG2 cells were treated for 24 h with increasing concentrations of Hg^{2+} in the presence of 1 nM TCDD. **A**, Protein (50 µg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C and then incubated with a primary CYP1A1 antibody for 24 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 GAPDH signals, which was used as loading control. One of three representative experiments is shown. **B**, EROD activity was measured in intact living cells treated with increasing concentrations of Hg^{2+}, in the absence and presence of 1 nM TCDD for 24 h. CYP1A1 activity was measured using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SE (n = 6). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

3.1.4. Transcriptional Inhibition of CYP1A1 gene by Hg^{2+}

In order to understand whether or not the inhibitory effect of Hg^{2+} on TCDD-mediated induction of CYP1A1 mRNA is actually due to a transcriptional mechanism, HepG2 cells were transiently transfected with the XRE-driven luciferase reporter gene. Luciferase activity results showed that 10 µM Hg^{2+} alone inhibited the constitutive expression of the luciferase activity to 0.7-fold compared to the control (Fig. 3.4). On the other hand, 1 nM TCDD alone caused a significant increase in the luciferase activity that reached 60-fold compared to the control. Interestingly, co-treatment with Hg^{2+} and TCDD significantly decreased the TCDD-mediated induction of luciferase activity to 34-fold compared to TCDD alone (Fig. 3.4).

3.1.5. Post-transcriptional Modification of CYP1A1 mRNA by Hg^{2+}

Although a transcriptional mechanism is involved in the Hg^{2+} mediated down-regulation of the TCDD-mediated induction of CYP1A1 mRNA levels, there was still a possibility that a post-transcriptional mechanism might be involved. 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 Hg^{2+} decreases CYP1A1 mRNA via decreasing its stability, a decrease in half-life would be expected to take place. To examine the effect of Hg^{2+} on the CYP1A1 mRNA stability, we performed the Act-D chase experiment on intact viable HepG2 cells. Figure 3.5 shows that CYP1A1 mRNA decayed with a half-life of 5.57 $\pm$ 0.99 h. Furthermore, Hg^{2+} did not significantly alter the CYP1A1 mRNA half-life, which was 7.14 $\pm$ 0.95 h (Fig. 3.5).

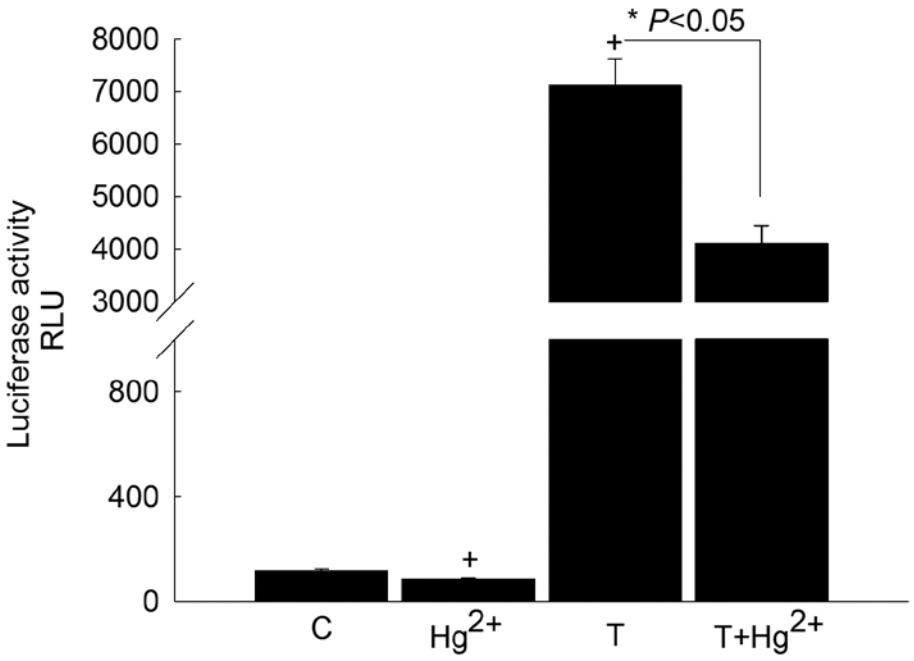

Figure 3.4. Effect of Hg^{2+} on luciferase activity. HepG2 cells were transiently transfected with the XRE-luciferase transporter plasmid pGudLuc 6.1. Cells were treated with vehicle, TCDD (1 nM), Hg^{2+} (10 μM), and TCDD (1 nM) + Hg^{2+} (10 μM) for 24 h. Cells were lysed and luciferase activity was measured according to the 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.

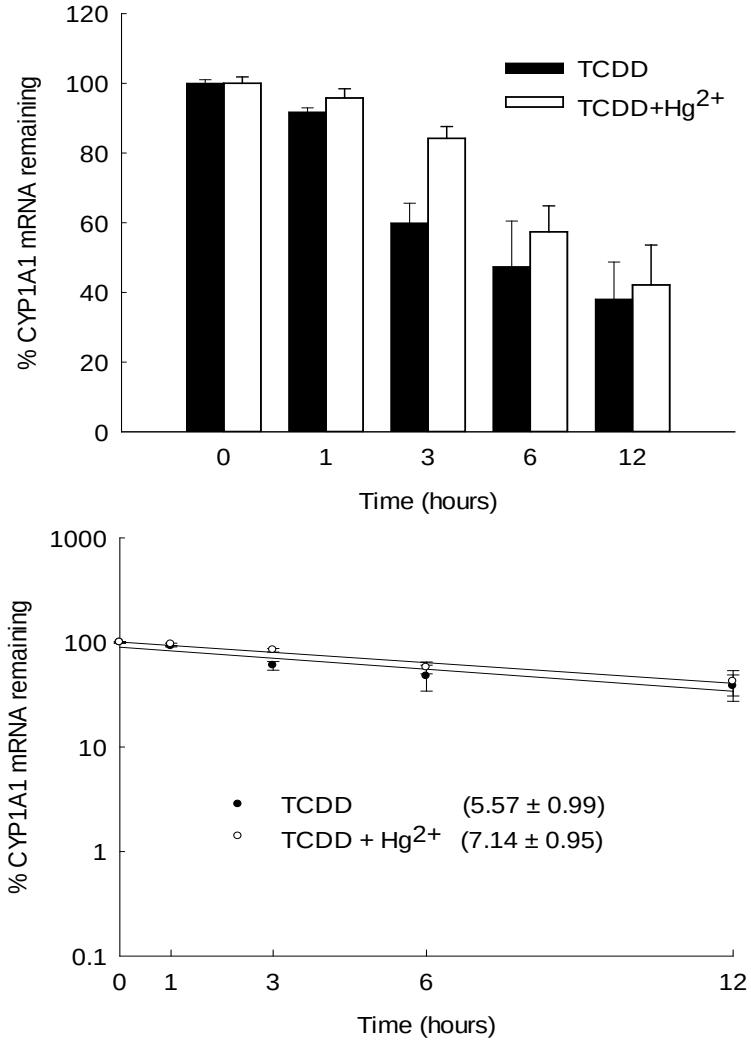

Figure 3.5. Effect of Hg^{2+} on CYP1A1 mRNA half-life using real-time PCR. HepG2 cells were grown to 90% confluence in six-well cell culture plates, and then treated with 1 nM TCDD for 12 h. The cells were then washed and incubated in fresh media containing 10 μM Hg^{2+} plus 5μg/ml Act-D, a RNA synthesis inhibitor. First-strand cDNA was synthesized from total RNA (1 μg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using an ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. mRNA decay curves were analyzed individually, and the half-life was estimated from the slope of a straight line fitted by linear regression analysis ($r2 > 0.81$) to a semilog plot of mRNA quantity, expressed as a percent of treatment at time = 0 h (maximum, 100%) level, versus time. The half-lives obtained from six independent experiments were then used to calculate the mean half-life (mean ± SE, n = 6).

3.1.6. Post-translational Modification of CYP1A1 Protein by Hg^{2+}

The fact that Hg^{2+} inhibited the TCDD-mediated induction of CYP1A1 catalytic activity much more than that observed at the mRNA level raised the question of whether Hg^{2+} could modify CYP1A1 protein stability. Therefore, we measured the effect of Hg^{2+} on CYP1A1 protein half-life using CHX-chase experiment. Figure 3.6 shows that CYP1A1 protein induced by TCDD degraded with a half-life of 10.78 $\pm$ 0.61 h. Interestingly, Hg^{2+} significantly decreased the stability of CYP1A1 protein, which degraded with a half-life of 4.75 $\pm$ 0.81 h (Fig. 3.6).

3.1.7. Effect of Co-exposure to Hg^{2+} and TCDD on HO-1 mRNA

The inverse relation between HO-1 expression and CYP1A1 activity directed us to probe the role of Hg^{2+} in inhibiting the TCDD-mediated induction of CYP1A1 at the catalytic activity level. Therefore, we examined the effect of Hg^{2+} on HO-1 mRNA, a rate-limiting enzyme of heme degradation. For this purpose, HepG2 cells were treated with increasing concentrations of Hg^{2+} (2.5–10 µM) in the presence of 1 nM TCDD. Thereafter, HO-1 mRNA was measured using real-time PCR. Figure 3.7 shows that TCDD alone did not alter HO-1 mRNA levels. On the other hand, co-exposure to TCDD and Hg^{2+} significantly increased the HO-1 mRNA level by 4.5-, 8-, and, 22-fold with concentrations of 2.5, 5, and 10 µM, respectively. Thus, HO-1 might be participating in the Hg^{2+}-mediated decrease in the TCDD-mediated induction of CYP1A1 at the catalytic activity (Fig. 3.7).

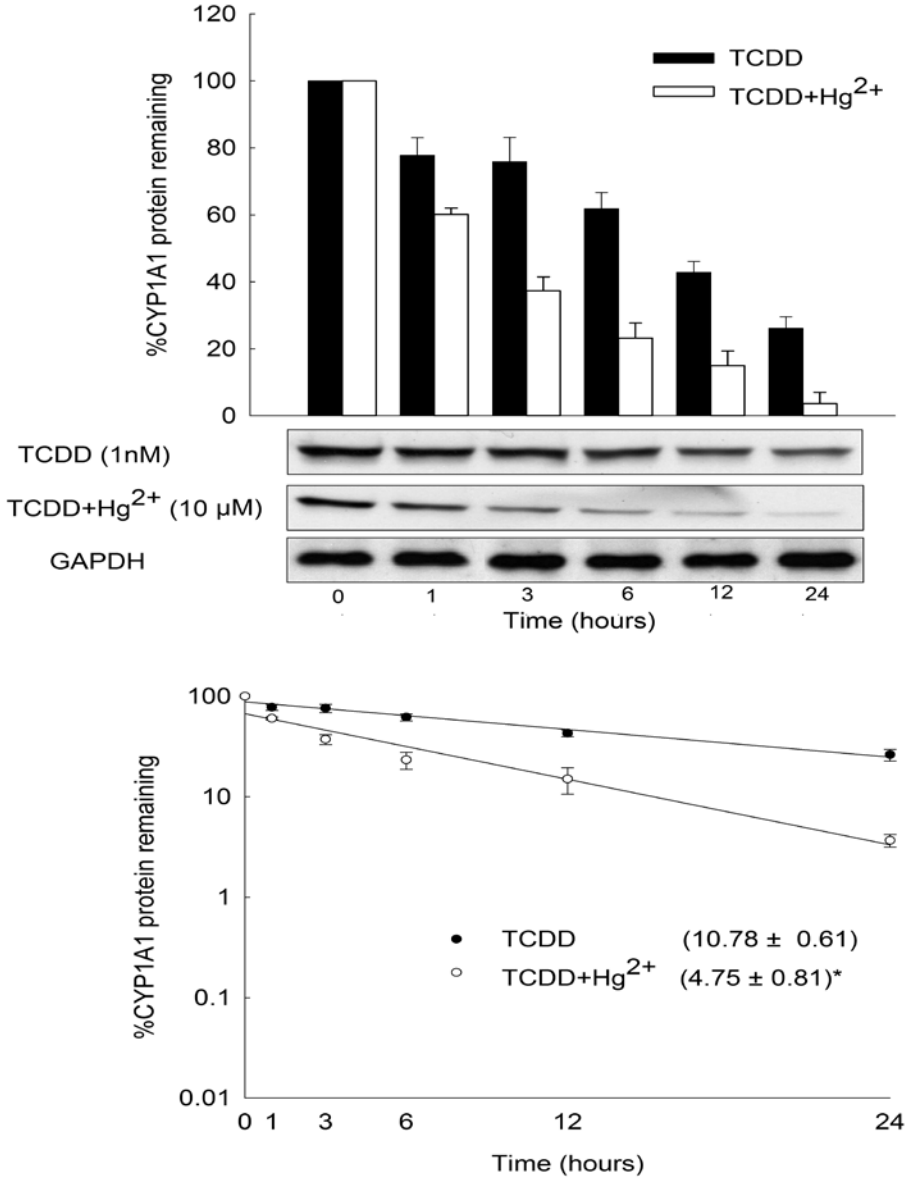

Figure 3.6. Effect of Hg^{2+} on the CYP1A1 protein half-life. HepG2 cells were grown to 90% confluence in six-well cell culture plates. Thereafter, the cells were treated with 1 nM TCDD for 24 h. Cells were washed and incubated in fresh media containing 10 µM Hg^{2+} plus 10 µg/ml CHX, a protein translation inhibitor. Total cellular protein was extracted at the designated time points after the addition of CHX. Protein (50 µg) was separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The intensities of CYP1A1 protein bands were normalized to GAPDH signals, which were used as loading controls. All protein decay curves were analyzed individually. The half-life was estimated from the slope of a straight line fitted by linear regression analysis to a semilog plot of protein amount, expressed as a percentage 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 ± S.E., n = 3). (*) $p < 0.05$ compared with TCDD.

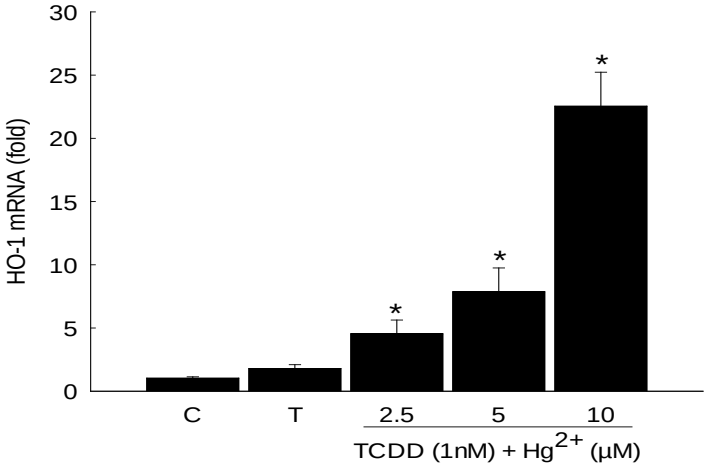

Figure 3.7. Effect of Hg^{2+} on HO-1 mRNA. HepG2 cells were treated for 6 h with increasing concentrations of Hg^{2+} in the presence of 1 nM TCDD. First-strand cDNA was synthesized from total RNA (1 µg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments ± SE (n = 6). (*) $P < 0.05$, compared with the respective TCDD (T) treatment.

3.1.8. Effect of SnMP as a Competitive Inhibitor of HO-1 on the Post-translational modification of CYP1A1 Catalytic Activity by Hg^{2+}

To confirm the role of HO-1 in Hg^{2+}-mediated decrease in the TCDD-mediated induction of CYP1A1 catalytic activity, we examined the effect of an HO-1 inhibitor, SnMP, on the decrease of CYP1A1 catalytic activity-mediated by Hg^{2+}. For this purpose, HepG2 cells were co-exposed to 10 μM Hg^{2+} and 1 nM TCDD in the presence and absence of 5 μM SnMP. SnMP alone, or in the presence of TCDD or Hg^{2+} plus TCDD, did not affect CYP1A1 mRNA levels for all treatments, thus eliminating the possibility that SnMP reverses the inhibitory effect of Hg^{2+} on CYP1A1 catalytic activity through affecting its mRNA levels (Fig. 3.8A). Similarly, SnMP alone or in the presence of TCDD did not alter the CYP1A1 catalytic activity. TCDD alone increased the CYP1A1 catalytic activity by ~11-fold. On the other hand, Hg^{2+} at the concentration of 10 μM decreased the TCDD-mediated induction of CYP1A1 catalytic activity to ~5-fold compared to control (Fig. 3.8B). Intriguingly, SnMP partially reversed the Hg^{2+}-mediated decrease of CYP1A1 activity to reach ~9-fold compared to the control. In spite of being successful in partially reversing the Hg^{2+}-mediated decrease of CYP1A1 activity through inhibiting HO-1, SnMP was unable to completely restore the CYP1A1 activity.

3.1.9. Effect of Exogenous Heme on Hg^{2+}-mediated decrease of CYP1A1 Catalytic Activity

In an attempt to examine whether the presence of external heme could

restore the Hg^{2+}-mediated decrease in CYP1A1 activity, HepG2 cells were co-exposed to 10 μM Hg^{2+} 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. Interestingly, the addition of hemin partially restored the Hg^{2+}-mediated decrease of CYP1A1 activity by 2- fold (Fig. 3.9).

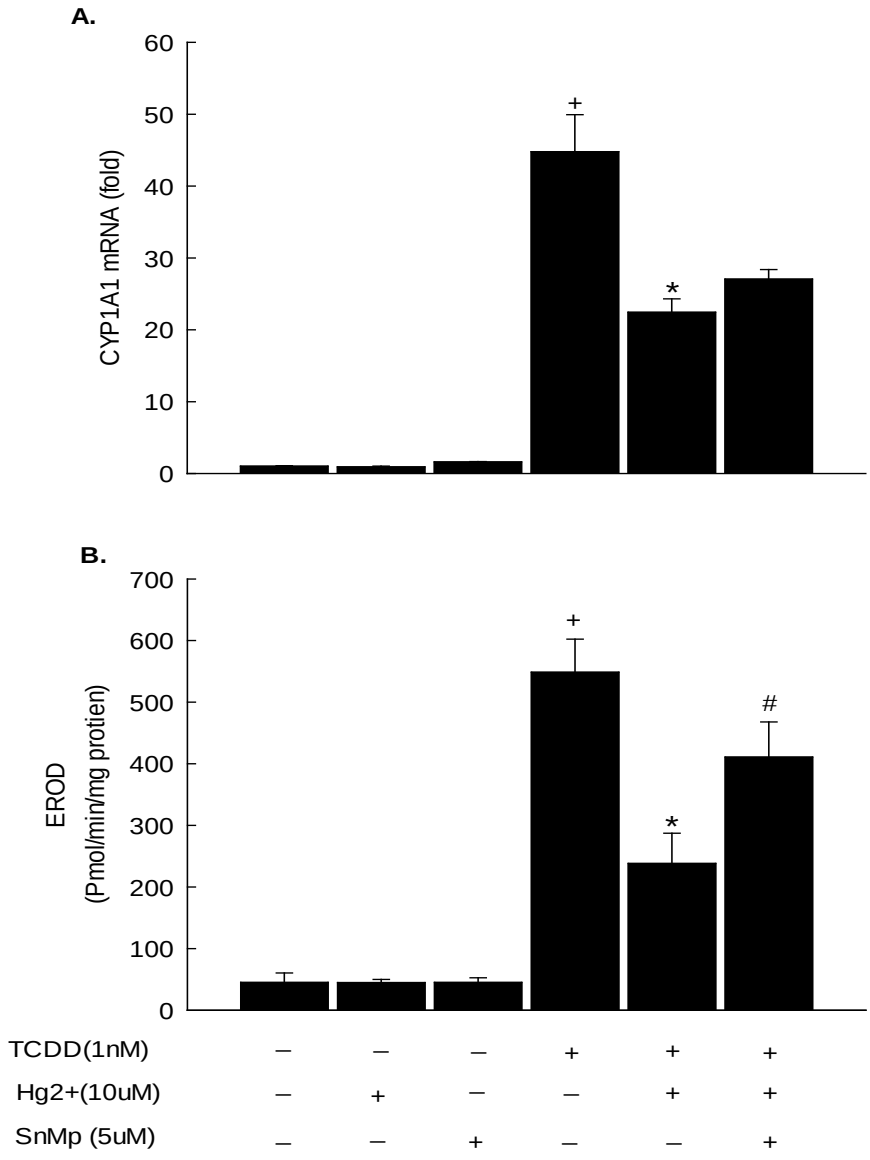

Figure 3.8. Effect of SnMP on CYP1A1 mRNA and catalytic activity levels in the presence of Hg^{2+}. HepG2 cells were treated with 10 μM of Hg^{2+} and 1 nM TCDD in the presence and absence of 5 μM SnMP for 6 h for CYP1A1 mRNA, and for 24 h for CYP1A1 catalytic activity. **A**, First-strand cDNA was synthesized 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 six independent experiments ± SE ($n = 6$). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment. **B,** CYP1A1 activity was measured using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SE ($n = 8$). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment; (#) $P < 0.05$, compared to respective Hg^{2+} + TCDD treatment.

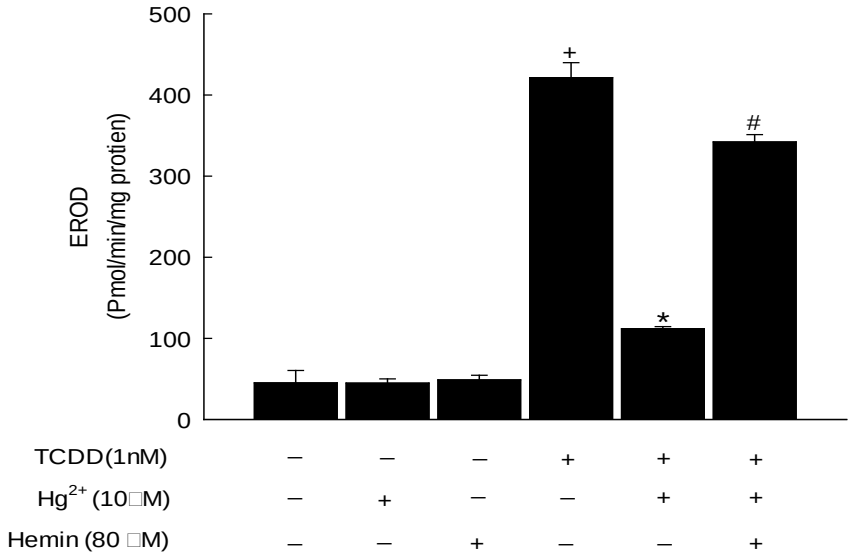

Figure 3.9. Effect of supplementing external heme on Hg^{2+}-mediated decrease of CYP1A1 activity. HepG2 cells were treated with 10 μM Hg^{2+} and 1 nM TCDD in the presence and absence of 80 μM hemin for 24 h. CYP1A1 activity was measured using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SE ($n = 8$). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment; (#) $P < 0.05$, compared to respective Hg^{2+} + TCDD treatment.

3.1.10. Effect of HO-1 siRNA on Hg^{2+}-mediated Inhibition of CYP1A1 Catalytic Activity

Despite using selective pharmacological inhibitors such as SnMP and heme precursors like hemin to inhibit HO-1 activity, it was of importance to confirm our hypothesis that the Hg^{2+}-mediated increase in HO-1 is in part responsible for the down-regulation of CYP1A1 at the catalytic activity level. Therefore, we took a genetic approach to confirm whether or not HO-1 is involved in the Hg^{2+}-mediated decrease in the TCDD-mediated induction of CYP1A1 catalytic activity. For this purpose, HepG2 cells were transfected with human HO-1 siRNA for 6 h, and then the cells were treated with 10 μM Hg^{2+} 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. 3.10A). On the other hand, Hg^{2+} was able to increase HO-1 mRNA levels, in the absence and presence of TCDD, to reach 25-fold compared to the control. When the cells were transfected with HO-1 siRNA, and then treated with Hg^{2+} in the presence or absence of TCDD, there was a statistically significant decrease in HO-1 mRNA to reach ~4.5-fold compared to control (Fig. 3.10A). 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 3.10B shows that CYP1A1 mRNA levels were not altered by the HO-1 siRNA. Thus, the observed effects on the CYP1A1 catalytic activity levels are solely through knocking down HO-1.

Looking at CYP1A1 catalytic activity, Hg^{2+} alone or in the presence of HO-1 siRNA did not affect CYP1A1 catalytic activity (Fig. 3.10C). TCDD alone

increased the CYP1A1 catalytic activity by 21-fold, whereas Hg^{2+} significantly decreased the TCDD-mediated induction of CYP1A1 catalytic activity to reach 6-fold compared to control. Interestingly, when HepG2 cells were transfected with HO-1 siRNA and then co-exposed to Hg^{2+} and TCDD, Hg^{2+} decreased the TCDD-induced catalytic activity to reach 17-fold, compared to the control, and was unable to maintain the same inhibitory effect on CYP1A1 catalytic activity when compared to non-transfected cells (Fig. 3.10C).

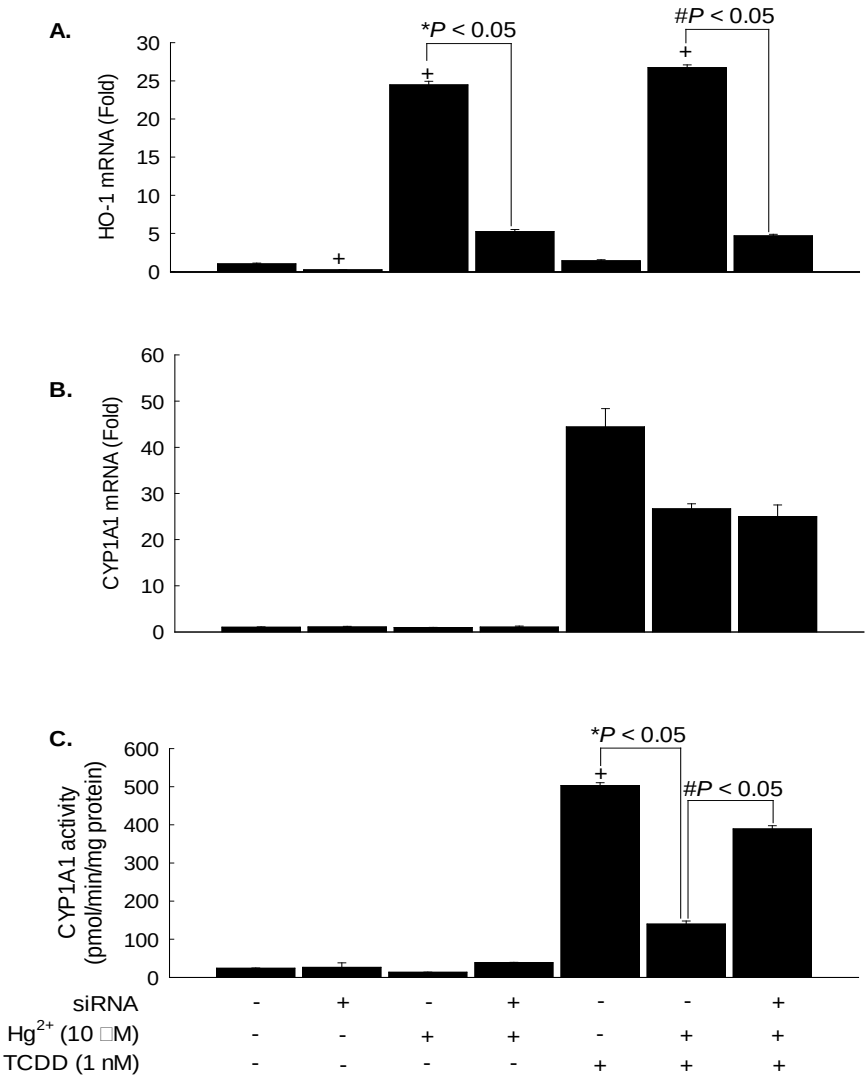

Figure 3.10. Effect of HO-1 siRNA on Hg^{2+}-mediated induction of HO-1 mRNA, and Hg^{2+}-mediated inhibition of CYP1A1 mRNA and catalytic activity. HepG2 cells were transiently transfected with 20 nM HO-1 siRNA (siRNA) for 6 h. Thereafter cells were treated with vehicle, TCDD (1 nM), Hg^{2+} (10 μM), TCDD (1 nM) + Hg^{2+} (10 μM) for 6 h for HO-1 and CYP1A1 mRNA or 24 h for CYP1A1 protein. **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 six independent experiments ± SE (n = 6). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective Hg^{2+} treatment; (#) $P < 0.05$, compared to respective Hg^{2+} + TCDD treatment. **C,** 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 Hg^{2+} + TCDD treatment.

3.2. Transcriptional Modulation of NAD(P)H: Quinone Oxidoreductase-1 by Mercury in Human Hepatoma HepG2 Cells

3.2.1. Time-Dependent Effect of Hg^{2+} on NQO1 mRNA

To better understand the kinetics of NQO1 mRNA in response to the exposure to Hg^{2+}, the time-dependent effect was determined at various time points (0, 1, 3, 6, 12, and 24 h) following the incubation of HepG2 cells with 10 μM Hg^{2+} (Fig. 3.11). Thereafter, NQO1 mRNA was assessed using real-time PCR. Our results clearly demonstrated that Hg^{2+} significantly increased the NQO1 mRNA transcripts in a time-dependent manner. Hg^{2+} treatment caused an induction of the NQO1 by 12%, 33%, 127 % at 1, 3, and 6 h respectively, compared to the control, followed by a 54% and 118 % drop of the maximal mRNA levels at 12 and 24 h, respectively.

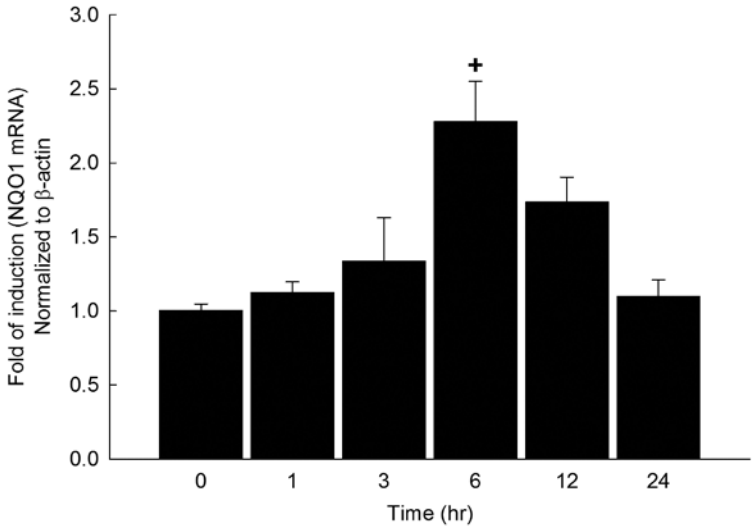

Figure 3.11. Time-dependent effect of Hg^{2+} on NQO1 mRNA. HepG2 cells were treated with 10 μM Hg^{2+} for the time point indicated. First-strand cDNA was synthesized from total RNA (1.5 μg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using an ABI 7500 real-time PCR system as described under "Materials and methods." Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (+) $P < 0.05$, compared to control (zero time).

3.2.2. Concentration-Dependent Effect of Hg^{2+} on the Constitutive and TCDD-Inducible Expression of NQO1 mRNA

To examine the ability of Hg^{2+} to modulate NQO1 gene expression, HepG2 cells were treated with various concentrations of Hg^{2+} in the absence and presence of 1 nM TCDD. Thereafter, NQO1 mRNA was assessed using real-time PCR. The concentrations of Hg^{2+} used hereafter were chosen after determining the ability of a wide range of concentrations to modulate the NQO1 gene expression without significantly affecting cell viability (Anwar-Mohamed and El-Kadi, 2008; Amara *et al.*, 2010). Initially, Hg^{2+} at the concentrations of 2.5, 5, and 10 µM caused a concentration-dependent increase in NQO1 mRNA levels by 148%, 186%, and 217%, respectively. Moreover, TCDD alone caused a 229% increase in NQO1 mRNA levels that was further potentiated by Hg^{2+} in a dose-dependent manner, starting at the lowest concentration tested which is 2.5 µM (264%), and 361% with the middle dose (5 µM), and reaching the maximum induction at the concentration of 10 µM (386%), compared to the control (Fig. 3.12A).

3.2.3. Concentration-Dependent Effect of Hg^{2+} on the Constitutive and TCDD-Inducible of NQO1 Protein and Catalytic Activity Levels

To investigate whether the observed induction of constitutive and inducible NQO1 mRNA by Hg^{2+} is further translated to the protein and catalytic activity levels, HepG2 cells were treated for 24 h with increasing concentrations of Hg^{2+} in the absence and presence of 1 nM TCDD. In agreement with the NQO1 mRNA results, Hg^{2+} alone significantly increased the constitutive NQO1 protein and

catalytic activity in a dose-dependent manner (2.5, 5, and 10 μM) by 157%, 198% and 236%, respectively, for the protein levels and 128%, 164% and 203%, respectively, for the activity levels. Furthermore, TCDD alone caused 214% and 213% increases in NQO1 protein and catalytic activity levels, respectively. Hg^{2+} at the concentration of 2.5 μM failed to potentiate the TCDD-mediated induction of NQO1. On the other hand, Hg^{2+} at the concentrations of 5 and 10 μM significantly potentiated the TCDD-mediated induction of NQO1 protein and catalytic activity levels by 284% and 373%, respectively, at the protein level, and by 217% and 295%, respectively, at the catalytic activity level (Figs. 3.12B and C).

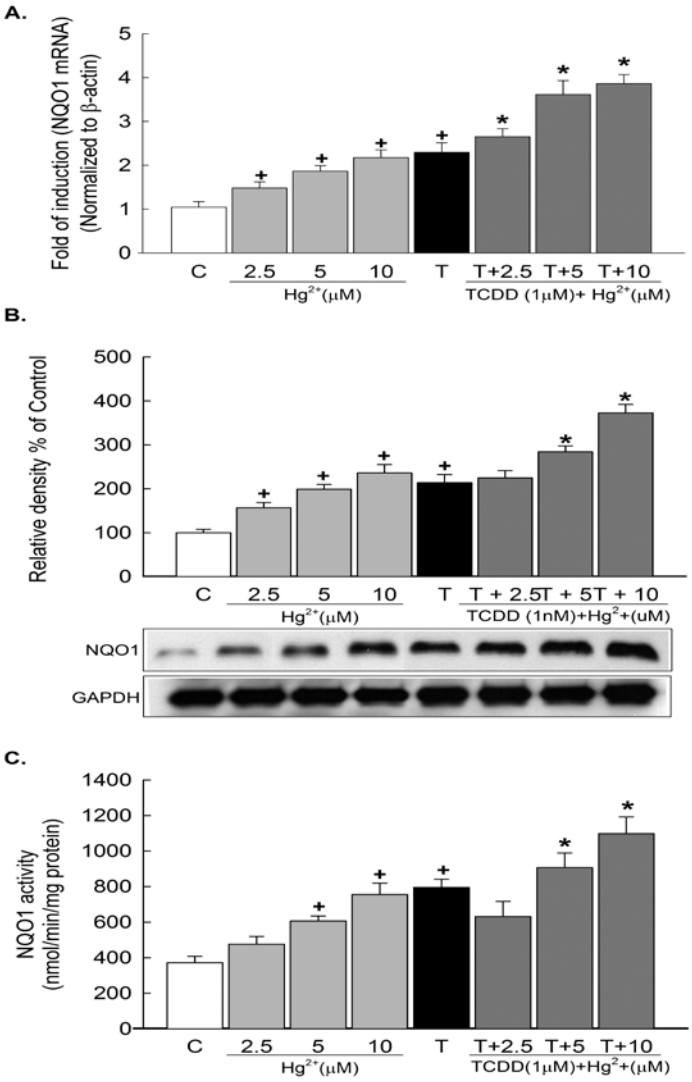

Figure 3.12. Concentration-dependent effect of Hg^{2+} on the constitutive and TCDD-mediated induction of NQO1 at mRNA, protein, and catalytic activity levels in HepG2 cells. HepG2 cells were treated with increasing concentrations of Hg^{2+} in the presence and absence of 1 nM TCDD for 6 h for mRNA or 24 h for protein and catalytic activity. **A**, First-strand cDNA was synthesized from total RNA (1.5 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. (+) $P < 0.05$, compared to control (C) (concentration = 0 μM); (*) $P < 0.05$, compared to respective TCDD (T) treatment. **B**, Protein (5 μg) was separated on a 10% SDS-PAGE. NQO1 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**, 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.

3.2.4 Concentration-Dependent Effect of Hg^{2+} on the Constitutive and SUL-mediated Induction of NQO1 mRNA, Protein and Catalytic Activity Levels

The fact that Hg^{2+} induced the NQO1 gene expression at constitutive and TCDD-induced levels raised the question whether Hg^{2+} will behave similarly in the presence of SUL, which induces NQO1 mainly through the Nrf2 pathway (Venugopal and Jaiswal, 1996). For this purpose, HepG2 cells were treated with various concentrations of Hg^{2+} in the absence and presence of 5 μM SUL. If Hg^{2+} exerts its effect solely through the AhR pathway we expected to see a limited effect of Hg^{2+} on the SUL-mediated induction of NQO1. Our results showed that SUL alone significantly increased NQO1 mRNA levels by 213% that was non-significantly potentiated by the lowest dose of Hg^{2+} tested 2.5 μM while the significant potentiation took place with Hg^{2+} at the concentrations of 5 and 10 μM by 318% and 393%, respectively compared to the control (Fig.3.13A). In addition, this induction was further translated to the protein and catalytic activity levels, in which SUL alone showed a significant induction of NQO1 protein and catalytic activity levels by 288% and 199%, respectively (Figs. 3.13B and C). On the other hand, Hg^{2+} at the concentration of 5 and 10 μM significantly potentiated the SUL-mediated induction of NQO1 at the protein and activity levels by 341% and 392% at the protein levels and 213% and 248%, respectively at the activity levels, respectively (Figs. 3.13B and C).

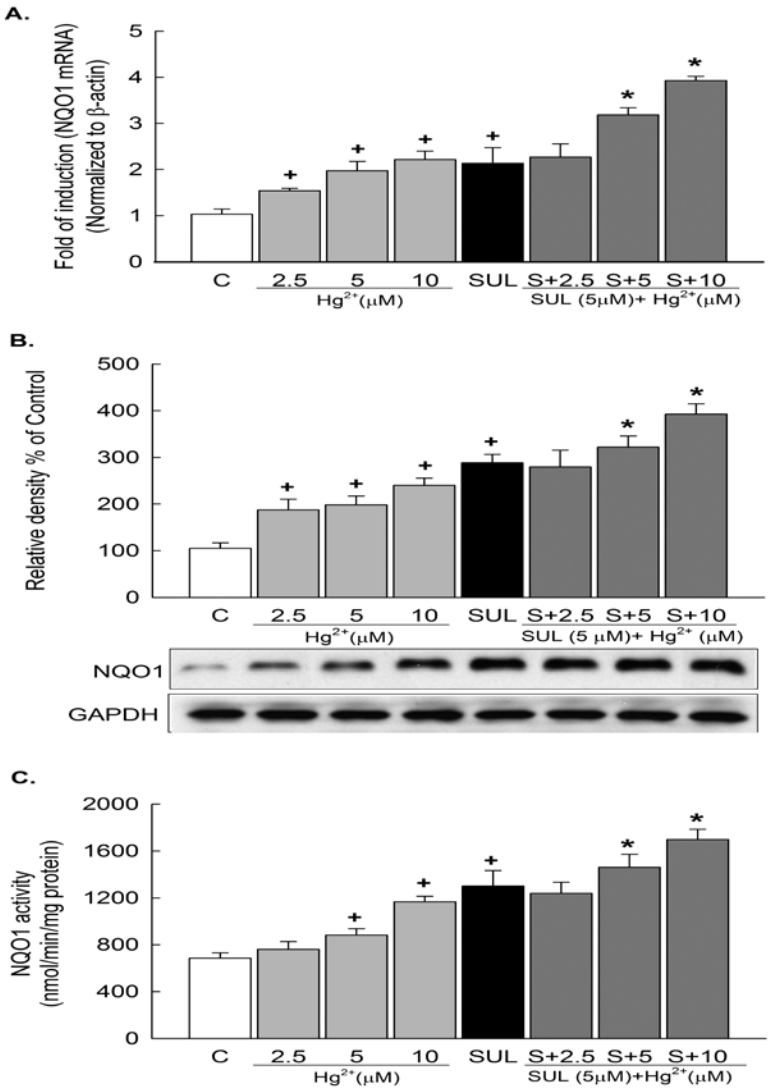

Figure 3.13. Concentration-dependent effect of Hg^{2+} on the constitutive and SUL-mediated induction of NQO1 at mRNA, protein, and catalytic activity levels in HepG2 cells. HepG2 cells were treated with increasing concentrations of Hg^{2+} in the presence and absence of 5 μM SUL for 6 h for mRNA or 24 h for protein and catalytic activity. **A**, 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. (+) $P < 0.05$, compared to control (C) (concentration = 0 μM); (*) $P < 0.05$, compared to respective SUL treatment. **B**, Protein (5 μg) was separated on a 10% SDS-PAGE. NQO1 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**, 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 SUL treatment.

3.2.5 Transcriptional Induction of the NQO1 gene by Hg^{2+}

To determine if the observed effect of co-exposure to Hg^{2+} and TCDD on NQO1 is occurring through an ARE-dependent mechanism, HepG2 cells were transiently transfected with the ARE-driven luciferase reporter gene. Luciferase activity results showed that 10 μM Hg^{2+} alone significantly increased luciferase activity by 193% (Fig. 3.14). TCDD (1 nM) and SUL (5 μM) were capable of causing a significant induction of the luciferase activity by 205% and 189%, respectively compared to the control. On the other hand, Hg^{2+} (10 μM) potentiated the TCDD- and SUL-mediated induction of luciferase activity by 53% and 56%, respectively compared to TCDD and SUL alone, (Fig. 3.14).

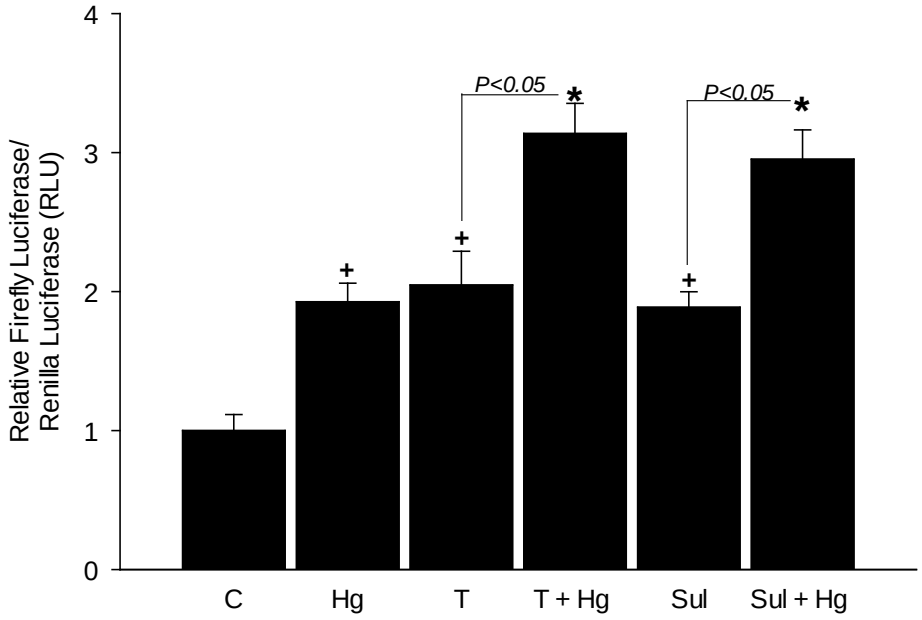

Figure 3.14. Effect of Hg^{2+} on ARE-dependent luciferase activity. HepG2 cells were transiently co-transfected with the reporter plasmid PGL3-ARE and the renilla luciferase pRL-CMV vector. Cells were treated with vehicle, Hg^{2+} (10 μM), TCDD (1 nM), TCDD (1 nM) + Hg^{2+} (10 μM), SUL (5 μM), or SUL (5 μM) + Hg^{2+} (10 μM) for 24 h. Thereafter, cells were lysed, and luciferase activity was measured according to the manufacturer's instruction. Luciferase activity is reported as relative light units (RLU). Values are presented as mean ± SE (n=3). (+) $P < 0.05$, compared with control (C); (*) $P < 0.05$, compared with the respective TCDD (T) or SUL treatment.

3.2.6 Effect of Hg^{2+} on the Levels of Nrf2

In the current study, Hg^{2+} increased NQO1 mRNA at the constitutive and the inducible levels. Therefore, it was of interest to examine the time-dependent effects of Hg^{2+} on the Nrf2 mRNA and protein levels. Our results demonstrated that Hg^{2+} did not affect the gene expression of Nrf2 at 2, 4, and 6 h (Fig. 3.15A). On the other hand, Hg^{2+} increased Nrf2 protein levels at 6, 12, and 24 h by 104%, 113% and 114% respectively, compared to the control (Fig. 3.15B). Thus, the induction of NQO1 gene expression by Hg^{2+} could be attributed to the stabilization of Nrf2 protein. In an attempt to investigate whether or not Hg^{2+} increased NQO1 gene expression was in fact due to increasing its nuclear accumulation as a consequence to Nrf2 stabilization, we measured the Nrf2 protein levels in the nuclear and cytosolic protein extracts of HepG2 cells treated with Hg^{2+} for 6 h. Our results demonstrated that Hg^{2+} increased the nuclear accumulation of Nrf2 protein by 45%, compared to control (Fig. 3.15C). On the contrary, Hg^{2+} decreased the cytosolic Nrf2 protein level by 53%, compared to control (Fig. 3.15C). Thus, the induction of NQO1 gene expression by Hg^{2+} is due to increasing the stability and nuclear accumulation of Nrf2 protein via increasing its shuttling from the cytosol to the nucleus.

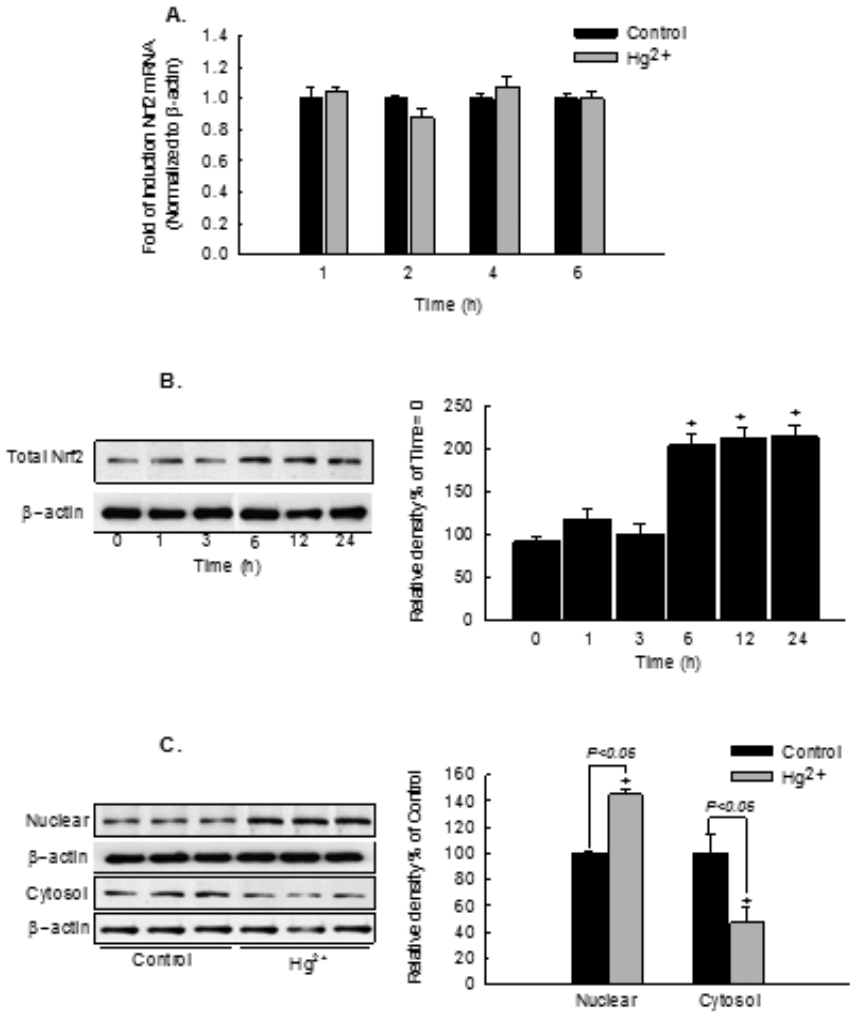

Figure 3.15. Time-dependent effect of Hg^{2+} on Nrf2 mRNA (A), time-dependent effect of Hg^{2+} on total Nrf2 protein level (B), and effect of Hg^{2+} on Nrf2 cellular distribution. A, HepG2 cells were treated with 10 μM Hg^{2+} for 2, 4, and 6 h. First-strand cDNA was synthesized from total RNA (1.5 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. **B,** HepG2 cells were treated with 10 μM Hg^{2+} for different time points. Total cell lysate (25 μg) was separated on a 10% SDS-PAGE. Nrf2 protein was detected using the enhanced chemiluminescence method. One of three representative experiments is shown ± SE (n = 3). (+) $P < 0.05$, compared to control (Time = 0 h). **C,** HepG2 cells were treated with 10 μM Hg^{2+} for 6 h. Nuclear or cytosolic protein (25 μg) was separated on a 10% SDS-PAGE. Nrf2 protein was detected using the enhanced chemiluminescence method. One of three representative experiments is shown ± SE (n = 3). (+) $P < 0.05$, compared to control (C).

3.2.7 Post-transcriptional Modification of NQO1 mRNA by Hg^{2+}

The level of mRNA expression is a function of the transcription and elimination rates through processing or degradation. Therefore, we examined the effect of Hg^{2+} on the stability of human NQO1 mRNA transcripts, using an Act-D chase experiment. Our results showed that NQO1 mRNA is a long-lived mRNA with a half-life of 26.98 ± 2.73 h (Fig. 3.16). On the other hand, co-exposure to Hg^{2+} did not alter the NQO1 mRNA half-life, indicating that the induction of NQO1 mRNA transcripts in response to Hg^{2+} was not due to any increase in its stability.

3.2.8 Post-translational Modification of NQO1 Protein by Hg^{2+}

Although a translational mechanism is involved in the Hg^{2+}-mediated potentiation of the TCDD-mediated induction of NQO1 protein levels, there was still a possibility that a post-translational mechanism might be involved. Therefore, the effect of Hg^{2+} on NQO1 protein half-life was determined using an CHX chase experiment. Figure 3.17 shows that NQO1 protein induced by TCDD degraded with a half-life of 45.33 ± 3.15 h. On the other hand, Hg^{2+} did not alter the NQO1 protein half-life compared with TCDD alone, indicating that the increase in NQO1 protein level in response to Hg^{2+} was not due to any increase in its stability (Fig. 3.17).

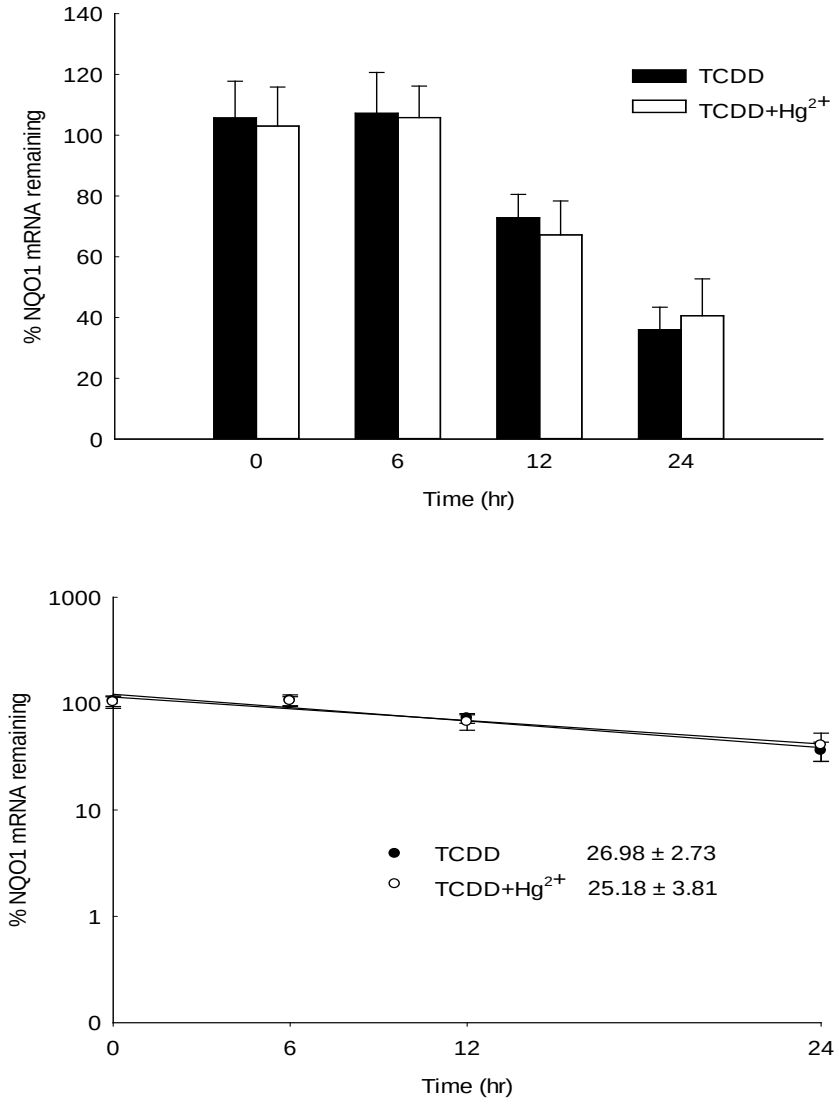

Figure 3.16. Effect of Hg^{2+} on NQO1 mRNA half-life. HepG2 cells were grown to 90% confluence in six-well cell culture plates and were treated with 1 nM TCDD for 6 h. The cells were then washed three times and incubated in fresh media containing 10 µM Hg^{2+} plus 5 µg/ml Act-D, a RNA synthesis inhibitor. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using an ABI 7500 real-time PCR system as described under Materials and Methods. mRNA decay curves were analyzed individually, and the half-life was estimated from the slope of a straight line fitted by linear regression analysis to a semilog plot of mRNA amount, expressed as a percentage 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).

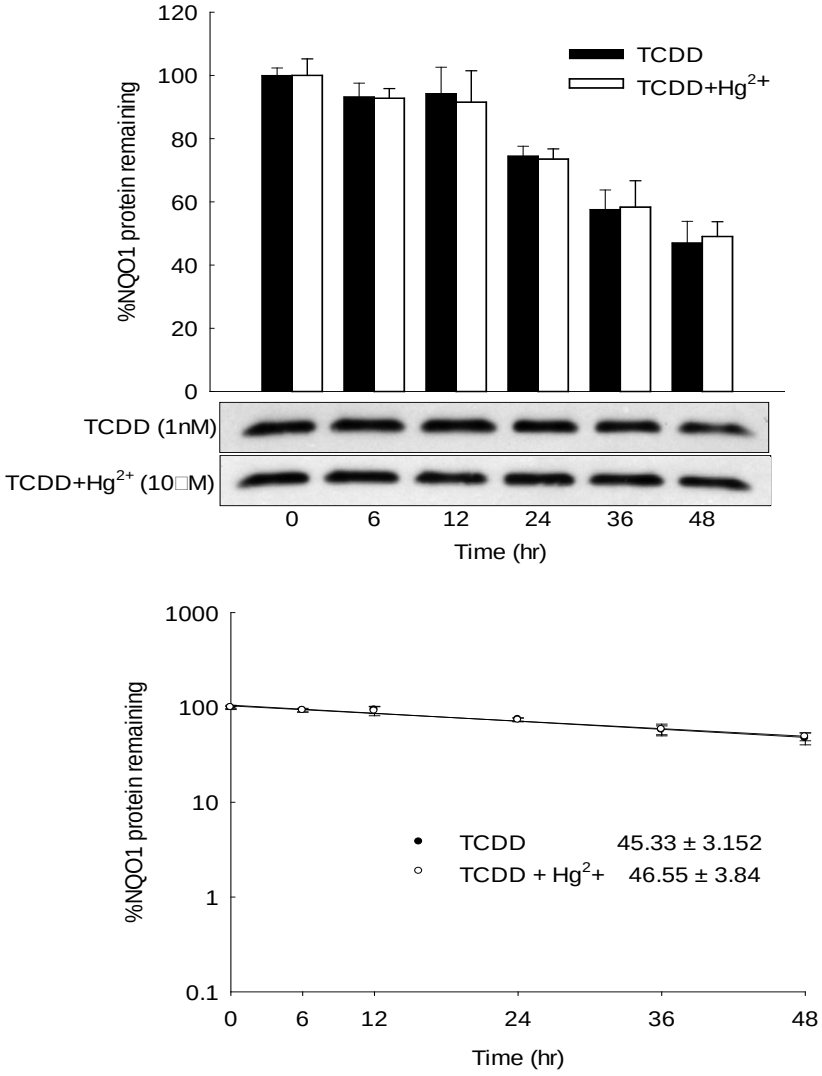

Figure 3.17. Effect of Hg^{2+} on the NQO1 protein half-life. HepG2 cells were grown to 90% confluence in six-well cell culture plates. Thereafter, the cells were treated with 1 nM TCDD for 24 h. Cells were washed and incubated in fresh media containing 10 µM Hg^{2+} plus 10 µg/ml CHX, a protein translation inhibitor. Protein was extracted at the designated time points after the addition of CHX. Protein (5 µg) was separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The intensities of NQO1 protein bands were normalized to GAPDH signals, which were used as loading controls. All protein decay curves were analyzed individually. The half-life was estimated from the slope of a straight line fitted by linear regression analysis to a semilog plot of protein amount, expressed as a percentage 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).

3.2.9 Effects of Nrf2 siRNA on the Hg^{2+}-mediated Induction of NQO1 mRNA and Catalytic Activity Levels

To test the selectivity of the siRNA for Nrf2, we determined the Nrf2 mRNA levels in cells transfected with siRNA for Nrf2. Fig. 3.18A shows that Nrf2 mRNA level was decreased by 67% compared to the control. To further confirm that the knockdown of Nrf2 is in fact causing a decrease in NQO1, HepG2 cells were transfected with human Nrf2 siRNA or Silencer negative control siRNA for 6 h, and then the cells were treated with 10 μM Hg^{2+} in the presence and absence of 1 nM TCDD. Our results showed that Nrf2 siRNA significantly decreased NQO1 mRNA by 63% compared to control (Fig. 3.18B). When the cells were transfected with Nrf2 siRNA and then treated with Hg^{2+} (10 μM) in the absence or presence of 1nM TCDD, there was a statistically significant decrease of the NQO1 mRNA induced by Hg^{2+} to 78% and 66%, respectively (Fig. 3.18B). Furthermore, the Silencer select negative control siRNA did not affect the inducible level of NQO1 mRNA by Hg^{2+}, eliminating the possibility that the inhibitory effects of Nrf2 siRNA might have been due to any toxicity. Looking at the NQO1 protein levels, cells transfected with Nrf2 siRNA encountered a significant decrease of constitutive expression of NQO1 by 45% compared to the control. Moreover, the knockdown of Nrf2 caused a significant decrease in the Hg^{2+}-mediated increase of the NQO1 catalytic activity in the absence and presence of TCDD by 43% and 54%, respectively (Fig. 3.18C).

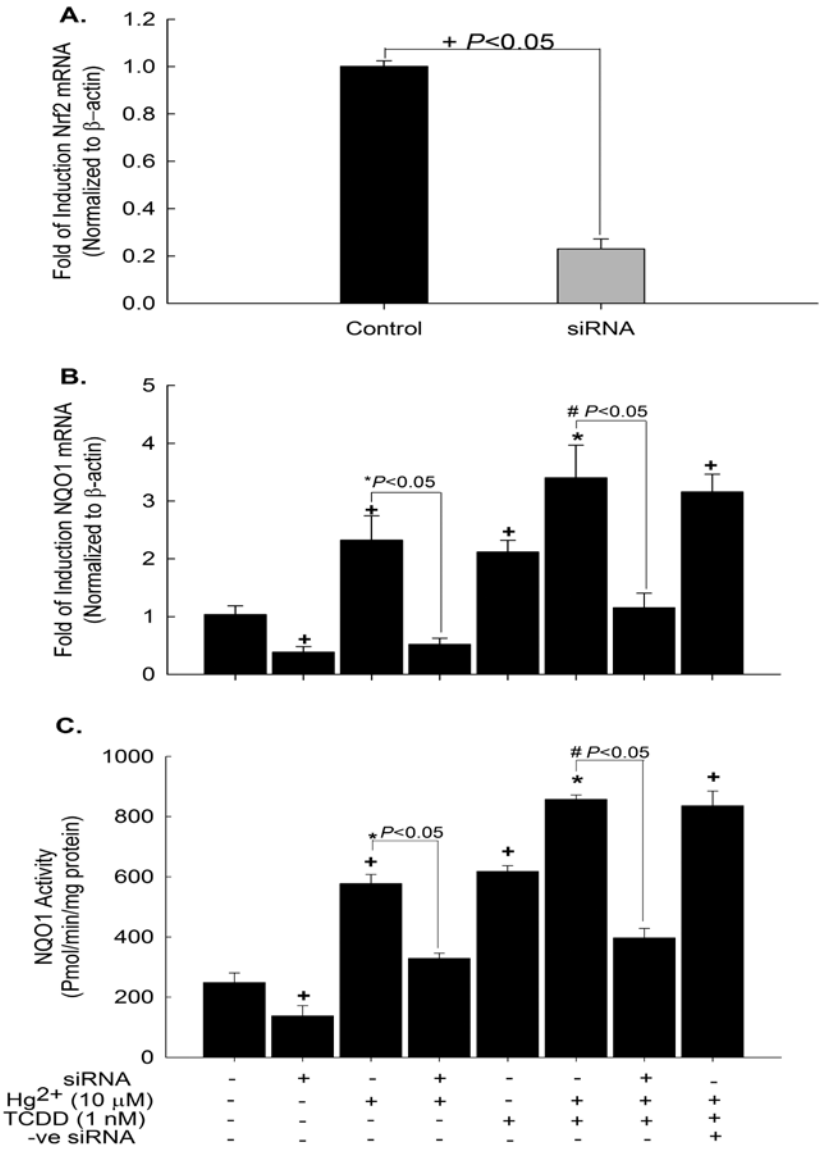

Figure 3.18. Effect of Nrf2 siRNA on Nrf2 mRNA and on Hg^{2+}-mediated induction of NQO1 mRNA and catalytic activity levels. HepG2 cells were transiently transfected with 150 nM Nrf2 siRNA for 6 h. Thereafter, cells were treated with vehicle, TCDD (1 nM), Hg^{2+} (10μM), TCDD (1 nM) + Hg^{2+} (10μM) for 6 h for Nrf2 and NQO1 mRNA or 24 h for NQO1 catalytic activity. **A,** and **B,** 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 Section 2. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments ± SE (n = 6). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective Hg^{2+} treatment; (#) $P < 0.05$, compared to respective Hg^{2+} +TCDD treatment. **C,** NQO1 enzyme activity was determined spectrophotometrically using DCPIP as 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 Hg2+ +TCDD treatment.

3.2.10 Effects of Nrf2 siRNA on AhR and CYP1A1 mRNA Levels

The fact that NQO1 mRNA and catalytic activity levels were decreased in Nrf2 siRNA transfected cells to 90 % compared to cells treated either with Hg^{2+} or TCDD prompted us to investigate the effect of Nrf2 siRNA on the mRNA of AhR and its downstream regulated gene, CYP1A1. For this purpose, HepG2 cells were transfected with human Nrf2 siRNA for 6 h, and then the cells were treated with 10 μM Hg^{2+} in the presence and absence of 1 nM TCDD. Our results showed that Nrf2 siRNA significantly decreased AhR mRNA levels by 41% compared to control (Fig. 3.19A). In addition, TCDD-induced CYP1A1 mRNA was significantly decreased in the cells transfected with Nrf2 siRNA by 54% compared to non-transfected cells treated with TCDD alone (Fig. 3.19B).

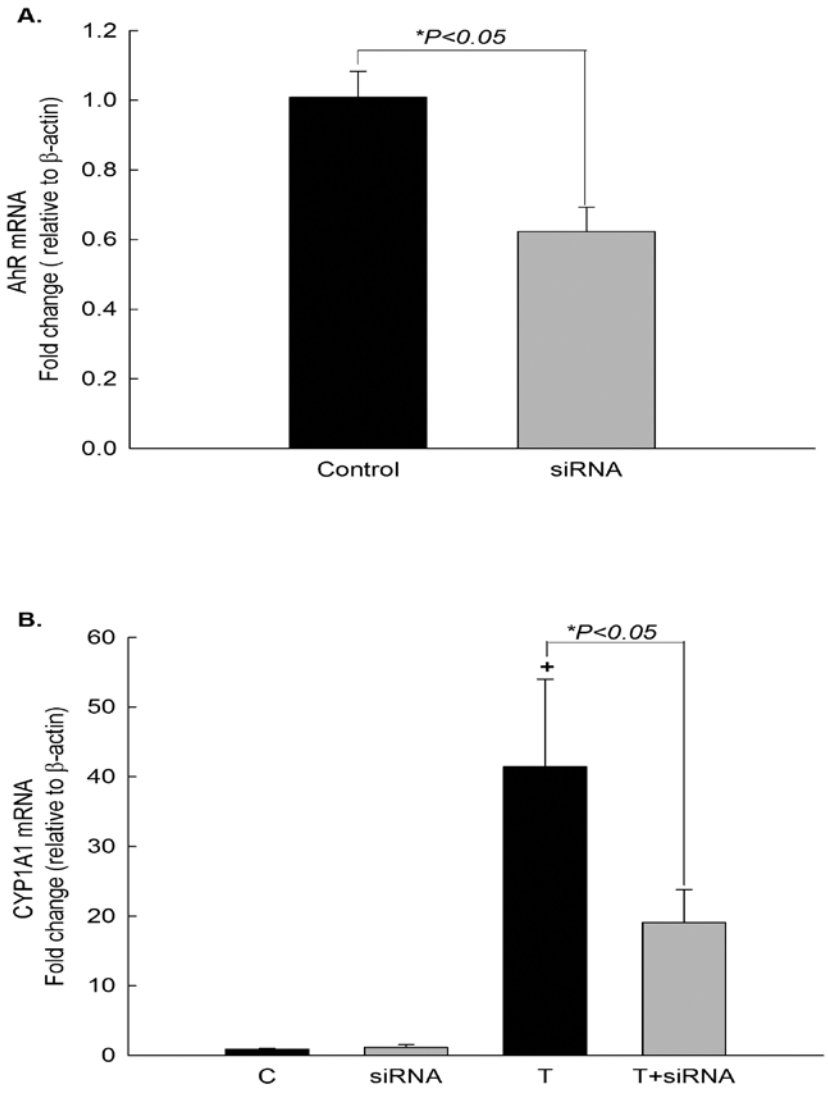

Figure 3.19. Effect of Nrf2 siRNA on AhR and CYP1A1 mRNA levels. HepG2 cells were transiently transfected with 150 nM Nrf2 siRNA (siRNA) for 6 h. Thereafter cells were treated with vehicle, TCDD (1 nM), for 6 h for AhR and CYP1A1 mRNA. **A,** and **B,** First-strand cDNA was synthesized from total RNA (1.5 μg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Section 2. Duplicate reactions were performed for each experiment, and the values presented are the means of four independent experiments ± SE (n = 4). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment.

3.3. Mercury Modulates the Cytochrome P450 1a1, 1a2 and 1b1 in C57Bl/6 Mice: in vivo and in vitro Studies

3.3.1. Effect of Co-exposure to Hg^{2+} and TCDD on Cyp1a1, Cyp1a2 and Cyp1b1 mRNA Levels in the Liver

At 6 and 24 h, Hg^{2+} alone failed to significantly affect Cyp1a1, Cyp1a2, or Cyp1b1 mRNA levels in the liver (Fig. 3.20A, 20B, and 20C). On the other hand, TCDD alone significantly induced Cyp1a1, Cyp1a2 and Cyp1b1 mRNA levels in the liver at 6 h by 12400-, 11.7- and 6-fold, respectively, compared to the control (Fig. 3.20A, 20B, and 20C). At 24 h TCDD alone was able to significantly induce Cyp1a1, Cyp1a2, and Cyp1b1 mRNA levels in the liver by 5600-, 15-, and 220-fold, respectively, compared to the control (Fig. 3.20A, 20B, and 20C). When animals were co-exposed to Hg^{2+} and TCDD, Hg^{2+} at 6 h significantly inhibited the TCDD-mediated induction of Cyp1a1 and Cyp1a2 mRNA levels in the liver by –17.7-and – 6.6-fold, respectively, compared to TCDD alone, while there was no effect of this co-exposure on Cyp1b1 mRNA levels (Fig. 3.20A, 20B, and 20C). On the contrary, Hg^{2+} at 24 h significantly potentiated the TCDD-mediated induction of Cyp1a1, Cyp1a2 and Cyp1b1 mRNA levels by 1.4-, 1.4- and 2.7-fold, compared to TCDD alone (Fig. 3.20A, 20B, and 20C).

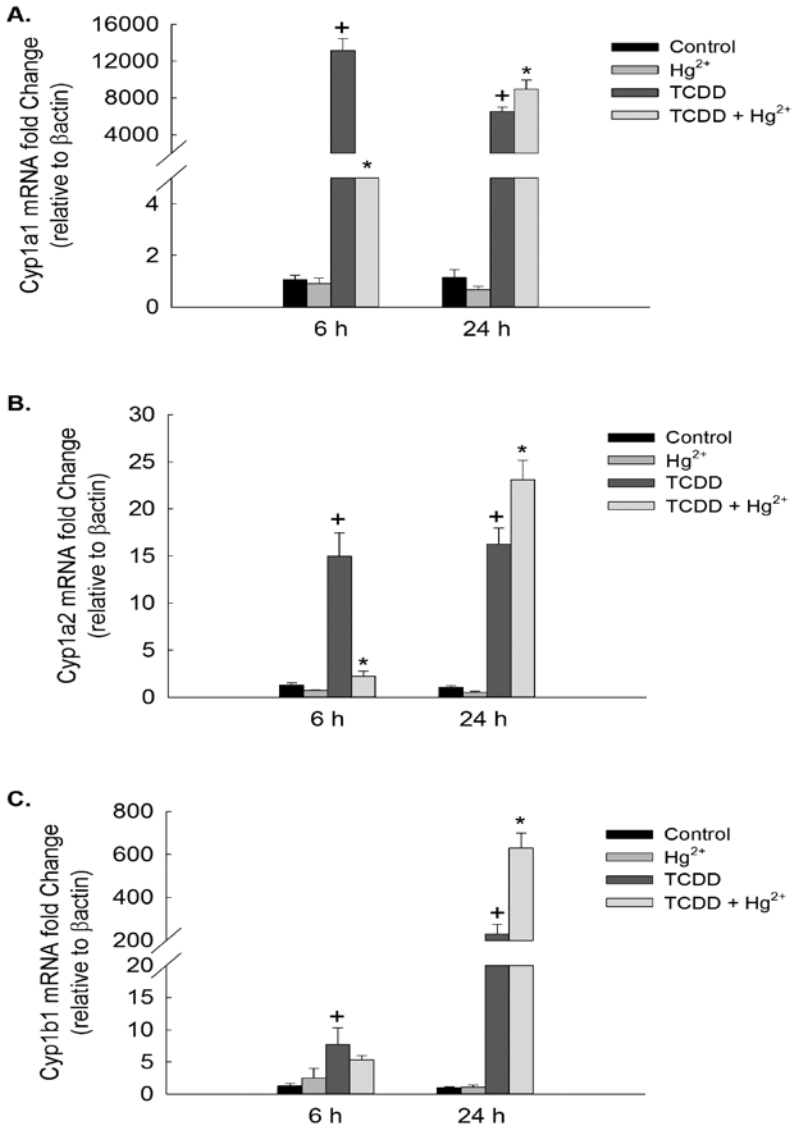

Figure 3.20. Effect of co-exposure to Hg^{2+} and TCDD on liver Cyp1a1, Cyp1a2, and Cyp1b1 mRNA in C57BL/6J mice. Animals were injected i.p. with 2.5 mg/kg Hg^{2+} in the absence and presence of 15 µg/kg TCDD for 6 h and 24 h. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from liver and the expression of Cyp1a1, Cyp1a2, and Cyp1b1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. (+) $P < 0.05$, compared to control (untreated animals); (*) $P < 0.05$, compared to respective TCDD treatment.

3.3.2. Effect of Co-exposure to Hg^{2+} and TCDD on Cyp1a, and Cyp1b1 Protein and Catalytic Activity Levels in the Liver

Our results showed that Hg^{2+} alone significantly increased Cyp1b1 protein expression levels in the liver by 1.8-fold, while it did not affect the Cyp1a protein levels (Fig. 3.21A). As expected, TCDD alone significantly induced Cyp1a and Cyp1b1 protein expression levels in the liver by 31- and 6-fold, respectively, compared to the control (Fig. 3.21A). When animals were co-exposed to Hg^{2+} and TCDD, Hg^{2+} significantly potentiated the TCDD-mediated induction of Cyp1a protein expression levels in the liver by 2.5- and 2.3-fold, respectively, compared to TCDD alone (Fig. 3.21A).

At the catalytic activity levels, Hg^{2+} alone did not significantly affect EROD or MROD activity in the liver (Fig. 3.21B and 21C). TCDD alone significantly induced EROD and MROD activities in the liver by 8- and 6-fold, respectively, compared to the control (Fig.3.21B and 21C). However, when animals were co-exposed to Hg^{2+} and TCDD, Hg^{2+} significantly potentiated the TCDD-mediated induction of EROD and MROD activities in the liver by 2- and 2.4-fold respectively, compared to TCDD alone (Fig. 3.21B and 21C).

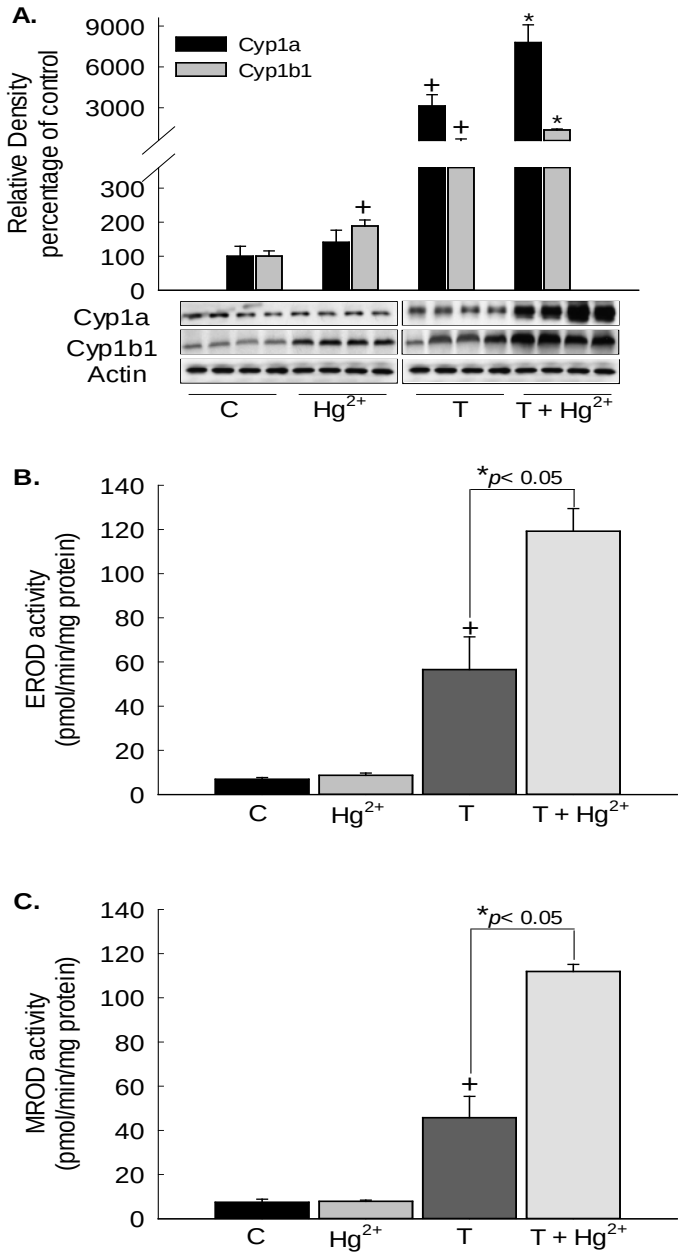

Figure 3.21. Effect of co-exposure to Hg^{2+} and TCDD on liver Cyp1a and Cyp1b1 protein expression levels, and EROD and MROD activities in C57BL/6J mice. (A) Liver microsomal proteins were isolated after 24 h of treatment. 30 µg of microsomal proteins were separated on a 10% SDS-PAGE. Proteins were detected using the enhanced chemiluminescence method. The graph represents the relative amount of protein normalized to actin signals (mean ± SEM, n = 6), and the results are expressed as percentage of the control values taken as 100%. **(B)** and **(C)** EROD and MROD activities were measured using 7-ethoxyresorufin and 7-methoxyresorufin as substrates, respectively. The reaction was started by the addition of 1 mM NADPH and lasted for 5 min for EROD and 10 min for MROD. The reaction was terminated by the addition of ice cold acetonitrile. Values are presented as mean ± SEM (n = 6). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment.

3.3.3. Effect of Co-exposure to Hg^{2+} and TCDD on HO-1 mRNA Levels in the Liver of C57Bl/6J mice

At 6 h Hg^{2+} and TCDD were able to significantly induce HO-1 mRNA levels in the liver by 13-and 4.6-fold respectively, compared to the control, while at 24 h they did not affect its mRNA levels (Fig.3.22). When animals were co-exposed to Hg^{2+} and TCDD, TCDD at 6 h significantly potentiated the Hg^{2+}-mediated induction of HO-1 mRNA levels in the liver by 7-fold, compared to Hg^{2+} alone (Fig.3.22), while at 24 h it did not significantly affect the Hg^{2+}-mediated induction of HO-1 mRNA levels in the liver compared to Hg^{2+} alone (Fig.3.22).

3.3.4. Effect of Co-exposure to Hg^{2+} and TCDD on Cell Viability

To determine the non-toxic concentrations of Hg^{2+} to be utilized in the current study, isolated hepatocytes from C57Bl/6 mice were exposed for 24 h to increasing concentrations of Hg^{2+} (2.5 – 50 µM) in the absence and presence of 1 nM TCDD; thereafter cytotoxicity was assessed using the MTT assay. Figure 3.23 shows that Hg^{2+} at concentrations of 2.5 – 10 µM in the presence and absence of 1 nM TCDD did not affect cell viability (Fig.3.23). Therefore, all subsequent studies were conducted using the concentrations of 2.5 – 10 µM.

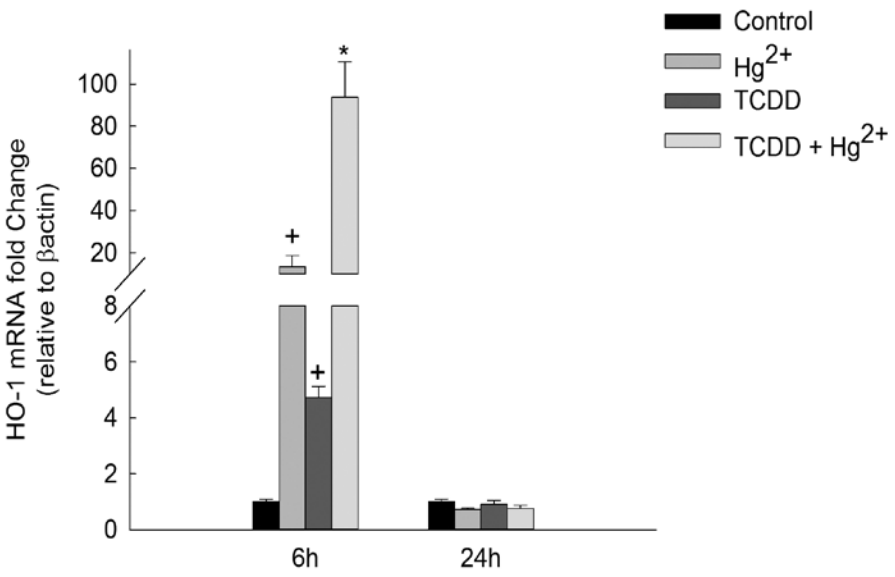

Figure 3.22. Effect of co-exposure to Hg^{2+} and TCDD on liver HO-1 mRNA in C57BL/6J mice. Animals were injected i.p. with 2.5 mg/kg Hg^{2+} in the absence and presence of 15 µg/kg TCDD for 6 h and 24 h. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from liver and the expression of HO-1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. (+) $P < 0.05$, compared to control (untreated animals); (*) $P < 0.05$, compared to respective TCDD treatment.

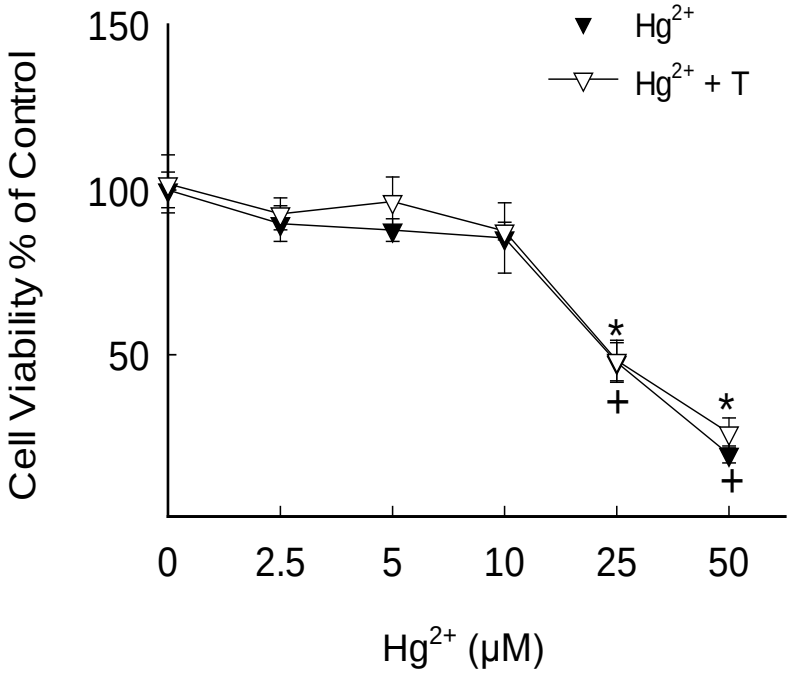

Figure 3.23. Effect of Hg^{2+} on cell viability. Isolated hepatocytes were treated for 24 h with Hg^{2+} (0, 2.5, 5, 10, 25, and 50 μM) in the absence and presence of 1 nM TCDD. Cell cytotoxicity was determined using the MTT assay. Data are expressed as percentage of untreated control (which is set at 100%) ± SE (n = 8). (+) $P < 0.05$, compared to control (concentration = 0 μM); (*) $P < 0.05$, compared to respective TCDD treatment.

3.3.5. Concentration- and Time- Dependent Effect of Co-exposure to Hg^{2+} and TCDD on Cyp1a1 mRNA in Hepatocytes

To examine the effect of co-exposure to Hg^{2+} and TCDD on Cyp1a1 mRNA, isolated mouse hepatocytes were treated with various concentrations of Hg^{2+} (2.5–10 μM) in the presence of 1nM TCDD (Fig. 3.24A). Hg^{2+} alone did not significantly affect the CYP1A1 mRNA (data not shown). On the contrary, TCDD alone caused 308-fold increase in Cyp1a1 mRNA levels that was inhibited in a dose-dependent manner by Hg^{2+}. Initially, Hg^{2+} at the concentration of 2.5 μM caused a significant decrease in TCDD-mediated induction of Cyp1a1 mRNA levels by 1.3-fold. The maximum inhibition took place at the highest concentration tested (10 μM), which caused a decrease in the TCDD-mediated induction of Cyp1a1 mRNA levels by 3.7-fold (Fig. 3.24A).

To better understand the kinetics of Cyp1a1 mRNA in response to the co-exposure to Hg^{2+} and TCDD, the time-dependent effect was determined at various time points up to 24 h after treatment of isolated mouse hepatocytes with 1nM TCDD in the absence and presence of 10 μM Hg^{2+}. Figure 3.24B shows that TCDD-induced Cyp1a1 mRNA in a time-dependent manner. TCDD treatment caused a maximal induction of the Cyp1a1 mRNA by 412-fold at 12 h, compared to 0 h. However, a 22.6-fold of induction occurred as early as 3 h (Fig. 3.24B). In contrast, Hg^{2+} significantly decreased in the Cyp1a1 mRNA levels at 6 h by 1.7-fold, compared to TCDD alone. Similarly, Hg^{2+} significantly decreased the TCDD-mediated induction of Cyp1a1 mRNA levels at 12 h, and 24 h, by 1.6-, 2.7-fold, respectively, compared to TCDD alone (Fig. 3.24B).

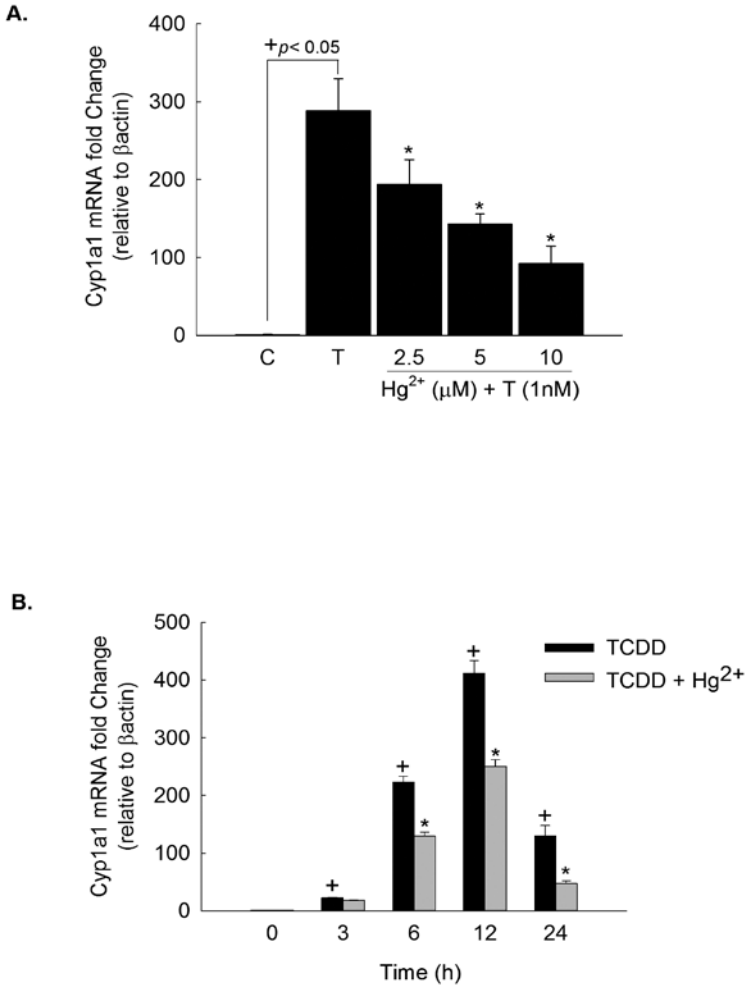

Figure 3.24. Concentration- and time-dependent effect of Hg^{2+} on Cyp1a1 mRNA in isolated mouse hepatocytes. Hepatocytes were treated with increasing concentrations of Hg^{2+} in the presence of 1 nM TCDD for 6 h for concentration dependent **(A)** or for different time points for time-dependence **(B)**. First-strand cDNA was synthesized from total RNA (1 μg) extracted from isolated mouse hepatocytes. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. (+) $P < 0.05$, compared to control (C) (concentration = 0 μM or time = 0 h); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

3.3.6. Concentration-Dependent Effect of Co-exposure to Hg^{2+} and TCDD on Cyp1a Protein and Catalytic Activity Levels in Hepatocytes

To examine whether the observed inhibition of the TCDD-mediated induction of Cyp1a mRNA by Hg^{2+} is further translated to the protein and activity levels, isolated mouse hepatocytes were treated for 24 h with increasing concentrations of Hg^{2+} (2.5–10 μM) in the presence of 1 nM TCDD. Hg^{2+} alone did not significantly affect the CYP1A1 protein and catalytic activity levels (data not shown). Figure 3.25A and 25B show that TCDD alone caused 3.8- and 15-fold increases in Cyp1a protein and catalytic activity, respectively. Of interest, Hg^{2+} decreased the TCDD-mediated induction of Cyp1a protein and catalytic activity levels in a dose-dependent manner. This inhibitory effect of Hg^{2+} on the Cyp1a protein and catalytic activity levels is in concordance with the observed effect on the mRNA levels, in which the initial significant inhibition took place at 2.5μM Hg^{2+}, and inhibition reached maximum at 10 μM (Fig. 3.25A and 25B).

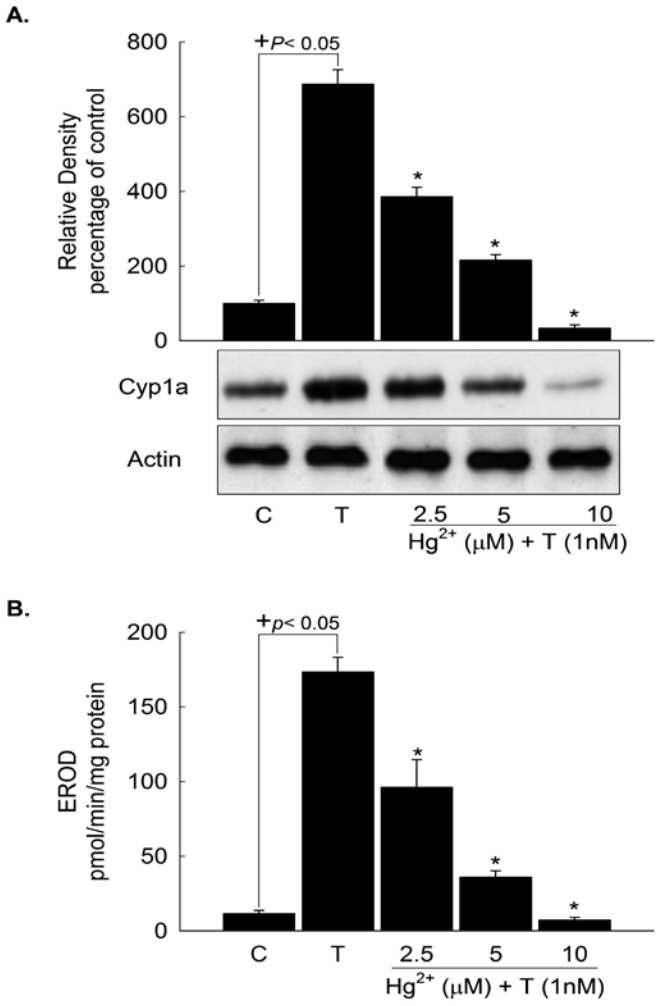

Figure 3.25. Effect of Hg^{2+} on Cyp1a protein and catalytic activity in isolated mouse hepatocytes. Hepatocytes were treated with increasing concentrations of Hg^{2+} in the presence of 1 nM TCDD for 24 h for protein and catalytic activity. **(A)** Protein (20 µg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C and then incubated with a primary Cyp1a antibody for 24 h at 4°C, followed by 1 h incubation with secondary antibody at room temperature. Cyp1a protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to actin signals, which was used as loading control. One of three representative experiments is shown. **(B)** Cyp1a1 activity was measured in intact living cells treated with increasing concentrations of Hg^{2+}, in the absence and presence of 1 nM TCDD for 24 h. Cyp1a1 activity was measured using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SE (n = 6). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

3.3.7. Transcriptional and Post-translational Inhibition of Cyp1a by Hg^{2+}

In order to study the effect of Hg^{2+} on the AhR-dependent transcriptional activation, isolated mouse hepatocytes were transiently transfected with the XRE-driven luciferase reporter gene. Luciferase activity results showed that 10 µM Hg^{2+} alone did not affect the constitutive expression of the luciferase activity (Fig. 3.26). On the other hand, 1nM TCDD alone caused a significant increase of luciferase activity by 12.75-fold as compared to the control (Fig. 3.26). Interestingly, co-treatment with Hg^{2+} and TCDD significantly decreased the TCDD-mediated induction of luciferase activity by 1.8-fold (Fig. 3.26).

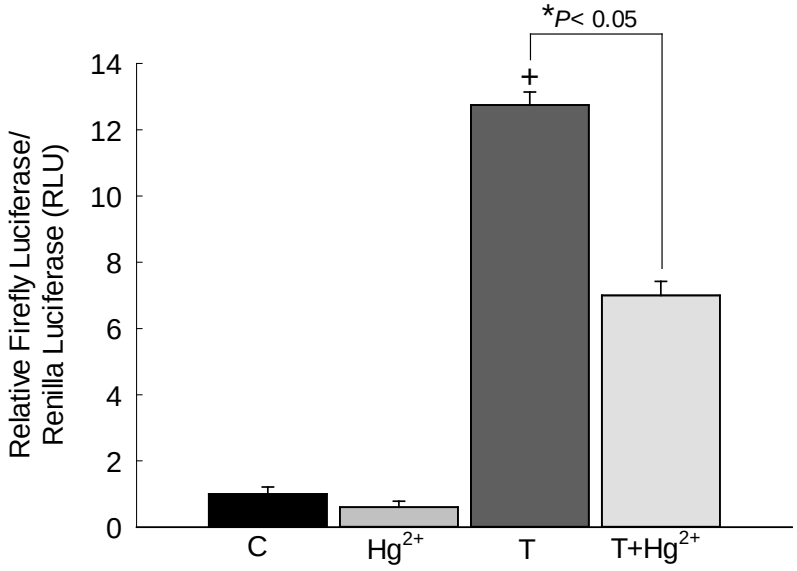

Figure 3.26. Effect of Hg^{2+} on luciferase activity in isolated mouse hepatocytes. Hepatocytes were transiently transfected with the XRE-luciferase transporter plasmid pGudLuc1.1 and renilla luciferase plasmid pRL-CMV plasmid. Cells were treated with vehicle, Hg^{2+} (10 μM), TCDD (1 nM), or TCDD (1 nM) + Hg^{2+} (10 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. Values are presented as mean ± SE (n = 6). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

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

The fact that Hg^{2+} inhibited the TCDD-mediated induction of Cyp1a1 at the catalytic activity level more than inhibiting its mRNA or protein levels prompted us to investigate the possible effect of Hg^{2+} on HO-1 mRNA levels. Our results showed that TCDD alone did not significantly affect HO-1 mRNA levels. In contrast, Hg^{2+} in the presence of 1nM TCDD was able to increase HO-1 mRNA in a dose-dependent manner by 12-, 24-, and 36-fold, respectively, compared to TCDD respective treatment (Fig. 3.27A).

To confirm the role of Hg^{2+}-induced HO-1 in decreasing the TCDD-mediated induction of Cyp1a1 catalytic activity, we examined the effect of HO-1 inhibitor, SnMP, on the Cyp1a1 catalytic activity that was inhibited by Hg^{2+}. For this purpose isolated mouse hepatocytes were co-exposed to 10 µM Hg^{2+} and 1 nM TCDD in the presence and absence of 5 µM SnMP. SnMP alone or in the presence of TCDD did not alter the Cyp1a1 catalytic activity. TCDD alone increased the Cyp1a1 catalytic activity by 14-fold. On the other hand, Hg^{2+} at the concentration of 10 µM decreased the TCDD-mediated induction of Cyp1a1 catalytic activity by 9-fold, compared to TCDD treatment (Fig. 3.27B). Intriguingly, SnMP partially reversed the Hg^{2+}-mediated decrease of Cyp1a1 activity to reach 8-fold compared to control. In spite of being successful in partially reversing the Hg^{2+}-mediated decrease of Cyp1a1 activity through inhibiting HO-1, SnMP was unable to completely restore the Cyp1a1 activity.

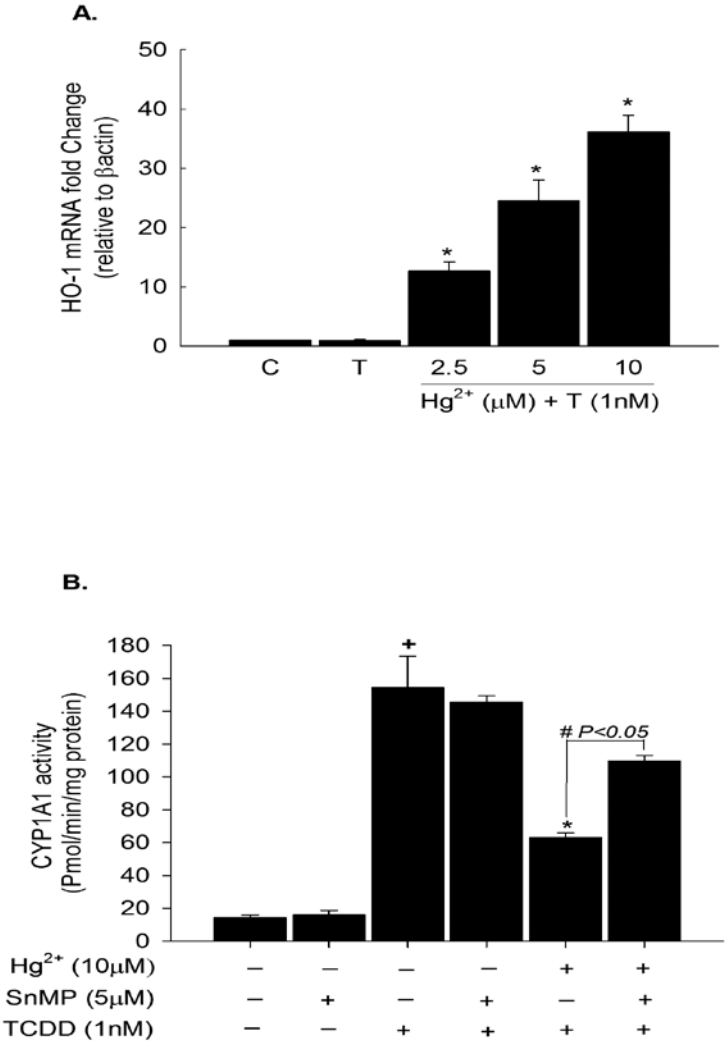

Figure 3.27. Effect of Hg^{2+} on HO-1 mRNA and Effect of SnMP on Cyp1a1 catalytic activity levels. (A) Hepatocytes were treated with increasing concentrations of Hg^{2+} in the presence of 1 nM TCDD for 6 h. First-strand cDNA was synthesized from total RNA (1 μg) extracted from hepatocytes. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. (+) $P < 0.05$, compared to control (C) (concentration = 0 μM); (*) $P < 0.05$, compared to respective TCDD (T) treatment. **(B)** Hepatocytes were treated with 10 μM of Hg^{2+} and 1 nM TCDD in the presence and absence of 5 μM SnMP for 24 h for Cyp1a1 catalytic activity. Cyp1a1 activity was measured using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SE ($n = 8$). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment; (#) $P < 0.05$, compared to respective Hg^{2+} +TCDD treatment.

3.3.9. The Effect of Hg^{2+} on Serum Hb Levels *in vivo* and Effect of Hb on XRE-Luciferase Activity *in vitro*

The discrepancy between the effects of Hg^{2+} on the TCDD-mediated induction of Cyp1a1 mRNA, protein, and catalytic activity levels *in vivo* and those observed at the *in vitro* level prompted us to examine the role of an endogenous mediator that could have influenced the effect of Hg^{2+} on the TCDD-mediated induction of Cyp1a1 *in vivo* but not *in vitro*. For this purpose, we measured serum Hb levels in C57BL/6J mice treated with Hg^{2+} in the absence and presence of TCDD. Our results demonstrated that Hg^{2+} increased serum Hb levels by 2-fold, compared to the control (Fig. 3.28A). In contrast, TCDD alone did not significantly affect serum Hb levels. Interestingly, when animals were co-exposed to Hg^{2+} and TCDD, Hg^{2+} increased serum Hb levels by 2.5-fold, compared to TCDD alone (Fig. 3.28A).

The effects of Hg^{2+} on serum Hb levels further motivated us to examine its effect alone on the XRE-driven luciferase activity. For this purpose, isolated mouse hepatocytes were treated with 1 μM Hb in the absence and presence of 1nM TCDD. Our results demonstrated that Hb alone was able to significantly induce luciferase activity by 3.7-fold, compared to the control (Fig. 3.28B). TCDD alone significantly induced luciferase activity by 46.6-fold, compared to control. Interestingly, when isolated mouse hepatocytes were co-exposed to Hb and TCDD, Hb significantly potentiated the TCDD-mediated induction of luciferase activity by 1.27-fold, compared to TCDD alone (Fig. 3.28B).

In order to correlate the *in vivo* effects with those observed at the *in vitro* levels we tried to mimic the *in vivo* situation using an *in vitro* model. For this purpose, isolated mouse hepatocytes were treated with TCDD for 2 hours in the absence and presence of Hg^{2+}. Thereafter, the treatment medium for cells receiving Hg^{2+} was replaced with another medium containing 1 μM Hb with TCDD. The reason behind replacing the medium with new treatment medium is that we were unable to detect any Hg^{2+} in the serum of animals who received Hg^{2+} treatment for 24 h using the slow poke reactor (data not shown). In addition, we needed to remove Hg^{2+} from the medium to rule out any direct effect of Hg^{2+}. Our results showed that when cells were treated with Hg^{2+} and then treated with Hb there was no significant effect of Hb on the XRE-driven luciferase activity. Importantly, when cells were treated with Hg^{2+} and then co-exposed to TCDD and Hb, there was a significant potentiation to the XRE-driven luciferase activity by 1.98-fold, compared to Hg^{2+} in the presence of TCDD treatment (Fig. 3.28C).

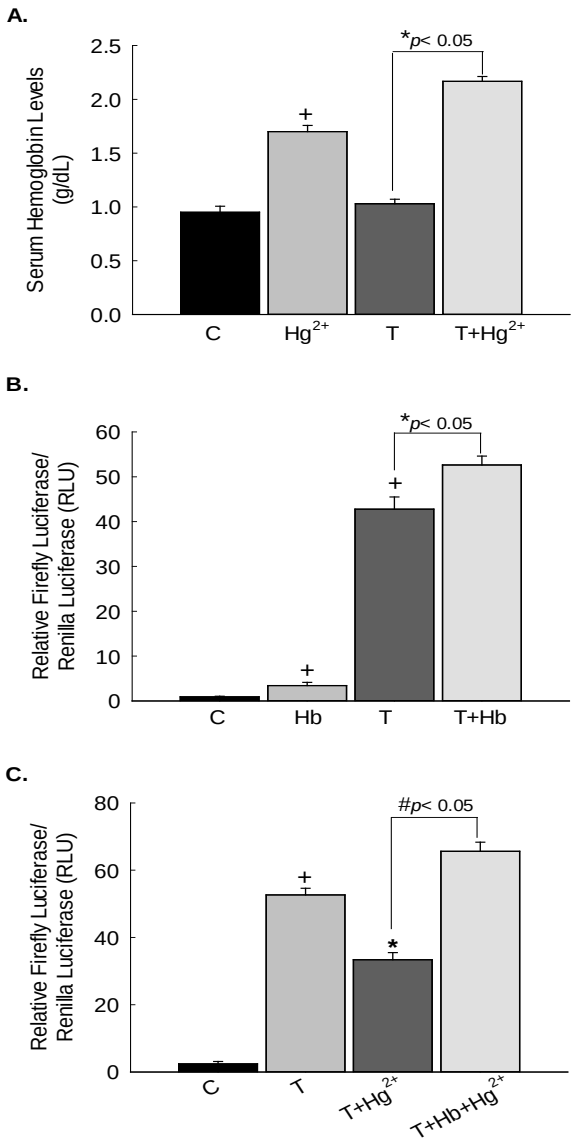

Figure 3.28. Effect of Hg^{2+} on serum Hb levels in vivo and the effect of Hb on luciferase activity in isolated mouse hepatocytes. (A) Animals were injected i.p. with 2.5 mg/kg Hg^{2+} in the absence and presence of 15 µg/kg TCDD for 24 h. Total blood was collected and centrifuged to isolate serum. Serum Hb levels were measured as previously described under Materials and Methods section. **(B)** Hepatocytes were transiently transfected with the XRE-luciferase transporter plasmid pGudLuc1.1 and renilla luciferase plasmid pRL-CMV plasmid. Cells were treated with Hb (1 µM), TCDD (1 nM), or TCDD (1 nM) + Hb (1 µM) for 24 h. Cells were lysed and luciferase activity was measured according to manufacturer's instructions. Luciferase activity is reported relative to renilla activity. Values are presented as mean ± SE ($n = 6$). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment. **(C)** Hepatocytes were transiently transfected with the XRE-luciferase transporter plasmid pGudLuc1.1 and renilla luciferase plasmid pRL-CMV plasmid. Cells were treated with vehicle, Hg^{2+} (10 µM), TCDD (1 nM), or TCDD (1 nM) + Hg^{2+} (10 µM) for 2 h. Cells were then washed and cells that were treated with Hg^{2+} or TCDD + Hg^{2+} were further incubated with Hb for additional 24 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.

3.3.10. Effect of Hb on the Cyp1a1 protein and catalytic activity levels

Western blot analysis was carried out to examine whether the obtained transcriptional activation of XRE by Hb is further translated to the protein level. Figure 3.29A shows that Hb caused a significant concentration-dependent induction of the Cyp1a1 protein by 2.5-, 3-, and 4.5-fold when incubated for 24 h with Hb concentrations of 0.5, 1, and 2 μM, respectively. Expectedly, TCDD significantly induced the Cyp1a1 protein by 9-fold (Fig. 3.29A). To determine whether the effect of Hb on Cyp1a1 protein is further translated to Cyp1a1 catalytic activity, isolated mouse hepatocytes were incubated with increasing concentrations of Hb (0.5, 1 and 2 μM) for 24 h. Thereafter, Cyp1a1 catalytic activity was determined using EROD assay. Our results showed that Hb significantly induced Cyp1a1 catalytic activity in a concentration-dependent manner by 1.7-, 2.3-, and 3-fold with Hb concentrations of 0.5, 1, and 2 μM, respectively (Fig. 3.29B). Moreover, the positive control, TCDD, significantly induced Cyp1a1 catalytic activity by 14-fold (Fig. 3.29B).

To examine the role of AhR in the induction of Cyp1a1 catalytic activity by Hb, isolated mouse hepatocytes were pre-incubated with the AhR antagonist, resveratrol, (20 μM) for 2 h before the treatment with Hb or TCDD for additional 24 h. Our results showed that resveratrol significantly reduced the induction of Cyp1a1 at catalytic activity mediated either by Hb or TCDD (Fig. 3.29B).

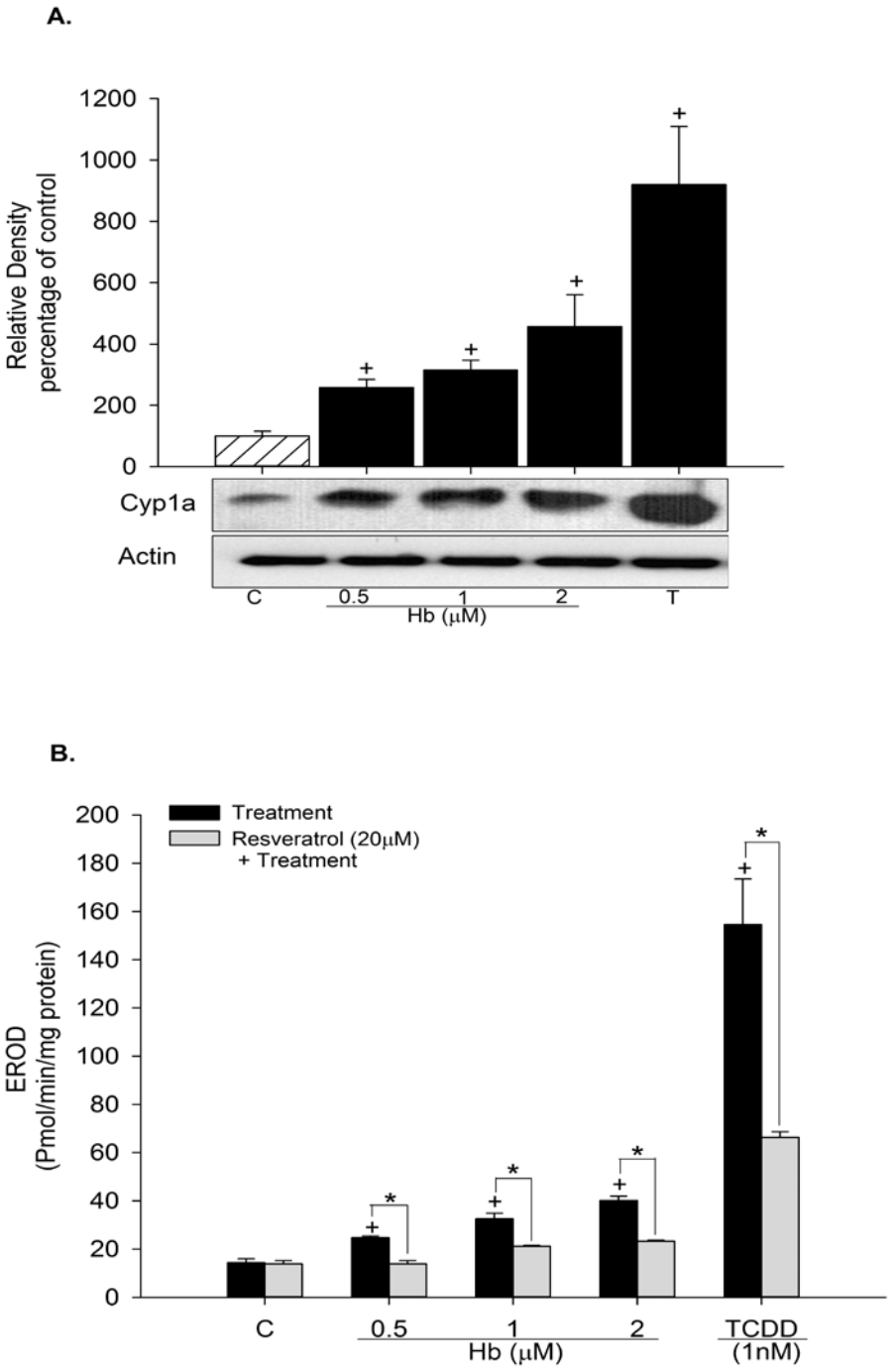

Figure 3.29. Effect of Hb on Cyp1a1 protein and catalytic activity in isolated mouse hepatocytes. Cells were treated with increasing concentrations of Hb (0.5–2 μM) or TCDD (1 nM) as a positive control for 24 h. **(A)** Cells were then harvested and Cyp1a1 protein was determined using Western blot analysis. The graph represents the average optical density (± S.E.M.) of bands from three different experiments as a percent of control. **(B)** Cyp1a1 activity was determined using Cyp1a1-dependent EROD assay. To investigate the role of AhR in the induction of Cyp1a1 by Hb, cells were incubated with resveratrol (20 μM) for 2 h prior to the treatment with Hb for an additional 24 h. Values represent mean activity ± S.E.M. (n = 8). (+) $P < 0.05$ compared to control,(C); (*) $P < 0.05$ compared to the treatment.

3.4. Effect of Mercury on Aryl Hydrocarbon Receptor-Regulated Genes in the Extrahepatic Tissues of C57Bl/6 Mice.

3.4.1. Expression of Cyp1a1, Cyp1a2, Cyp1b1, Nqo1, Gsta1 and HO-1 mRNA in the Kidney, Lung and Heart of C57Bl/6J Mice

To examine the constitutive expression of various AhR-regulated genes in the kidney, lung, and heart tissues of male C57Bl/6 mice, total RNA was isolated from different tissues, and steady-state mRNA for all tested genes was 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. 3.30A**,** 30B and 30C). Furthermore, Cyp1a2 was the lowest expressed gene in the three examined tissues and was considered as a calibrator.

In the kidney, Nqo1 was the most highly expressed gene, about 7000-fold higher than the calibrator (Fig. 3.30A). Cyp1a1 and HO-1 were also highly expressed genes at 233- and 1970-fold higher than the calibrator, respectively (Fig. 3.30A). Cyp1b1 and Gsta1 were moderately expressed genes at 58- and 12.5-fold higher than the calibrator, respectively (Fig. 3.30A). In contrast to the kidney, lung Nqo1 was a low expressed gene at 2.1-fold higher than the calibrator. However, Cyp1a1 and HO-1 were the most highly expressed genes at 1060- and 162-fold higher than the calibrator, respectively (Fig. 3.30B). Lung Cyp1b1 and Gsta1 were low to moderate expressed genes at 1.4- and 3-fold higher than the calibrator, respectively (Fig. 3.30B). Similar to the kidney, heart Nqo1 was a highly expressed gene at 6300-fold higher than the calibrator (Fig. 3.30C). Furthermore, Cyp1a1 and HO-1 were also highly expressed genes at 1900- and 632-fold higher than the calibrator, respectively (Fig. 3.30C). Cyp1b1

and Gsta1 were moderately expressed genes at 100- and 64-fold higher than the calibrator, respectively (Fig. 3.30C).

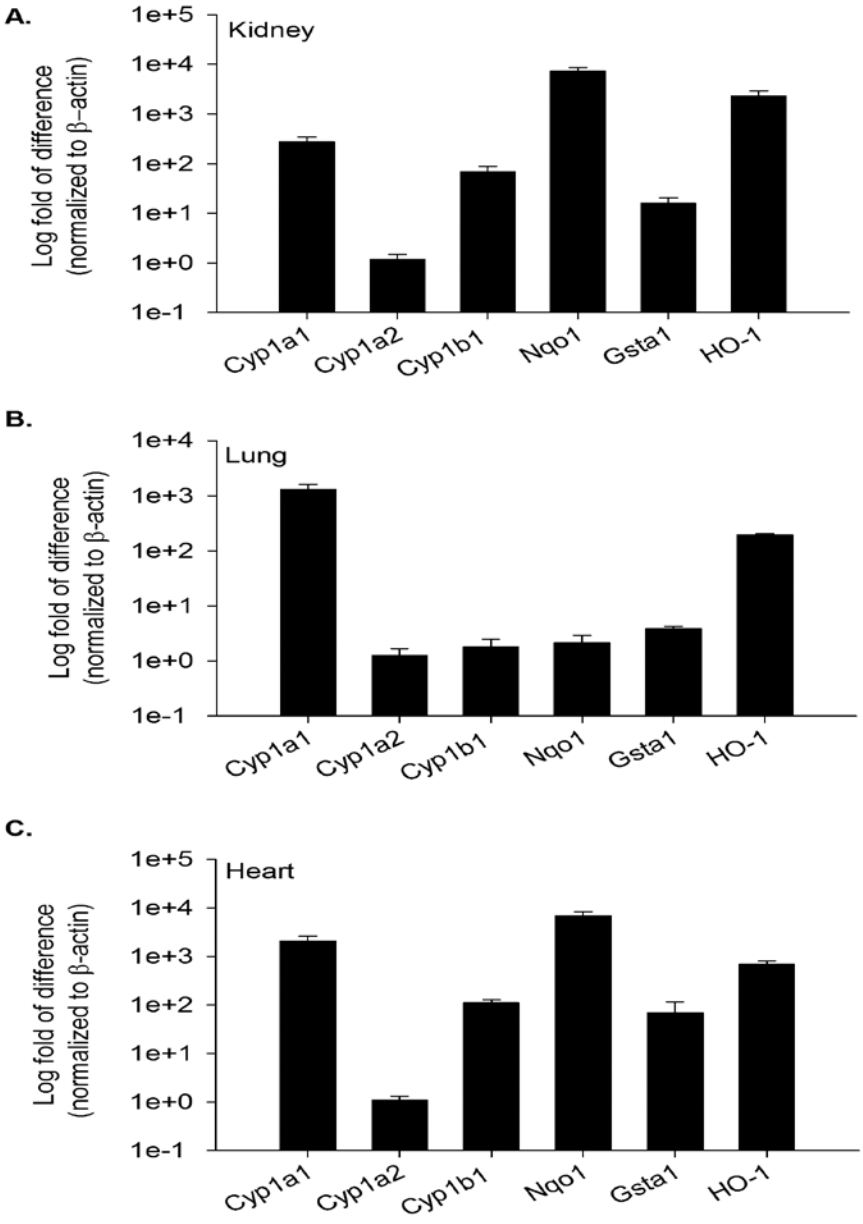

Figure 3.30. Constitutive expression of AhR-regulated and HO-1 genes in the kidney, lung, and heart. Total RNA was isolated from extrahepatic tissues 6 h and 24 h after treatment, and the relative expression of these genes in the kidney (**A**), lung (**B**), and heart (**C**) were determined by reverse 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 were calculated as mean ± S.E.M. (n=6).

3.4.2. Effect of Co-exposure to Hg^{2+} and TCDD on Cyp1a1, Cyp1a2 and Cyp1b1 mRNA in the Kidney, Lung and Heart of C57Bl/6J Mice

At 6 h, Hg^{2+} significantly increased the Cyp1a1 mRNA levels in both lung and heart but not in the kidney by 2- and 3.5-fold, respectively, compared to the control (Fig. 3.31A). However, at 24 h no change was observed in the expression of this gene in all tested tissues (Fig. 3.31B). TCDD alone significantly increased Cyp1a1 mRNA at 6 h in kidney, lung, and heart by 958-, 205-and 366- fold, respectively, and at 24 h by 271-, 56-, and 3.5-fold, respectively (Fig. 3.31A and 31B). When animals were co-exposed to Hg^{2+} and TCDD, Hg^{2+} at 6 h significantly inhibited the TCDD-mediated induction of Cyp1a1 mRNA levels in the kidney by -10-fold while it did not significantly affect its levels in the lung or heart (Fig. 3.31A). In contrast, at 24 h Hg^{2+} increased the TCDD-mediated induction of Cyp1a1 in the kidney, lung, and heart by 1.8-, 1.7- and 1.5-fold, respectively, compared to TCDD alone (Fig. 3.31B).

At 6 h, Hg^{2+} alone was able to significantly increase the Cyp1a2 mRNA levels in the kidney and heart by 15.6-, and 10-fold, respectively, whereas, it decreased its level in the lung by 25-fold (Fig. 3.32A). However, at 24 h Hg^{2+} alone failed to significantly affect the Cyp1a2 mRNA levels in all tested tissues (Fig. 3.32B). TCDD alone significantly induced the Cyp1a2 mRNA levels at 6 h in the kidney, lung, and heart by 36-, 1.7-, and 35-fold, respectively and at 24 h by 11.2-, 1.8- and 17.1-fold, respectively (Fig. 3.32A and 32B). When animals were co-exposed to Hg^{2+} and TCDD, Hg^{2+} at 6 h did not significantly affect the TCDD-mediated induction of Cyp1a2 mRNA levels in either kidney or heart while it

significantly decreased its level in the lung by 11.1-fold (Fig. 3.32A). On the other hand, at 24 h Hg^{2+} significantly potentiated the TCDD-mediated induction of Cyp1a2 mRNA levels in both kidney and lung by 2.9- and 1.9-fold, respectively, while it decreased its level in the heart by 3.3-fold compared to TCDD alone (Fig. 3.32B).

At 6 h, Hg^{2+} significantly increased the Cyp1b1 mRNA levels in the kidney and heart by 19.2- and 15-fold, respectively (Fig. 3.33A). At 24 h Hg^{2+} did not significantly affect the Cyp1b1 mRNA levels in all the tested tissues (Fig. 3.33B). At 6 h TCDD alone was able to significantly induce the Cyp1b1 mRNA levels in the kidney, lung, and heart by 30-, 59- and 33-fold, respectively and by 6.5-, 144- and 7.6-fold at 24 h, respectively (Fig. 3.33A and 33B). When animals were co-exposed to Hg^{2+} and TCDD, Hg^{2+} at 6 h, significantly decreased the TCDD-mediated induction of Cyp1b1 mRNA levels in the kidney by 2-fold compared to TCDD alone; however, it did not significantly affect Cyp1b1 mRNA levels in either the lung or the heart (Fig. 3.33A). Interestingly, at 24 h Hg^{2+} significantly potentiated the TCDD-mediated induction of Cyp1b1 mRNA levels in the kidney and lung by 4- and 1.4-fold, respectively compared to TCDD alone, while it did not affect its level in the heart (Fig. 3.33B).

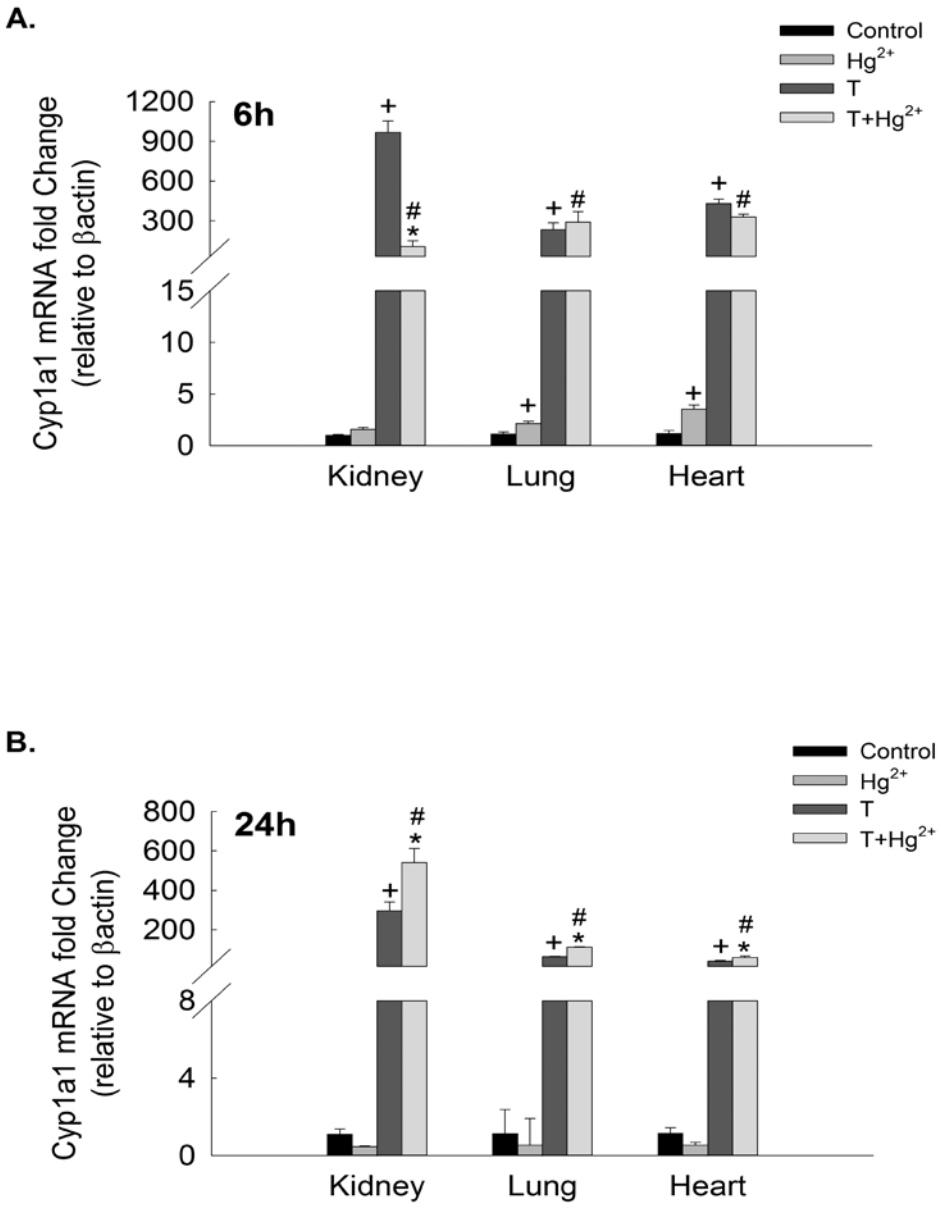

Figure 3.31. Effect of co-exposure to Hg^{2+} and TCDD on Cyp1a1 mRNA in the kidney, lung, and heart of C57Bl/6 mice. Animals were injected i.p. with 2.5 mg/kg Hg^{2+} in the absence and presence of 15 µg/kg TCDD for 6 h (A) and 24 h (B). First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from kidney, lung and heart, 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. Results were calculated as mean ± S.E.M. (n=6). (+) $P < 0.05$, compared to control (untreated animals); (*) $P < 0.05$, compared to respective TCDD treatment; (#) $P < 0.05$, compared to Hg^{2+} treated animals.

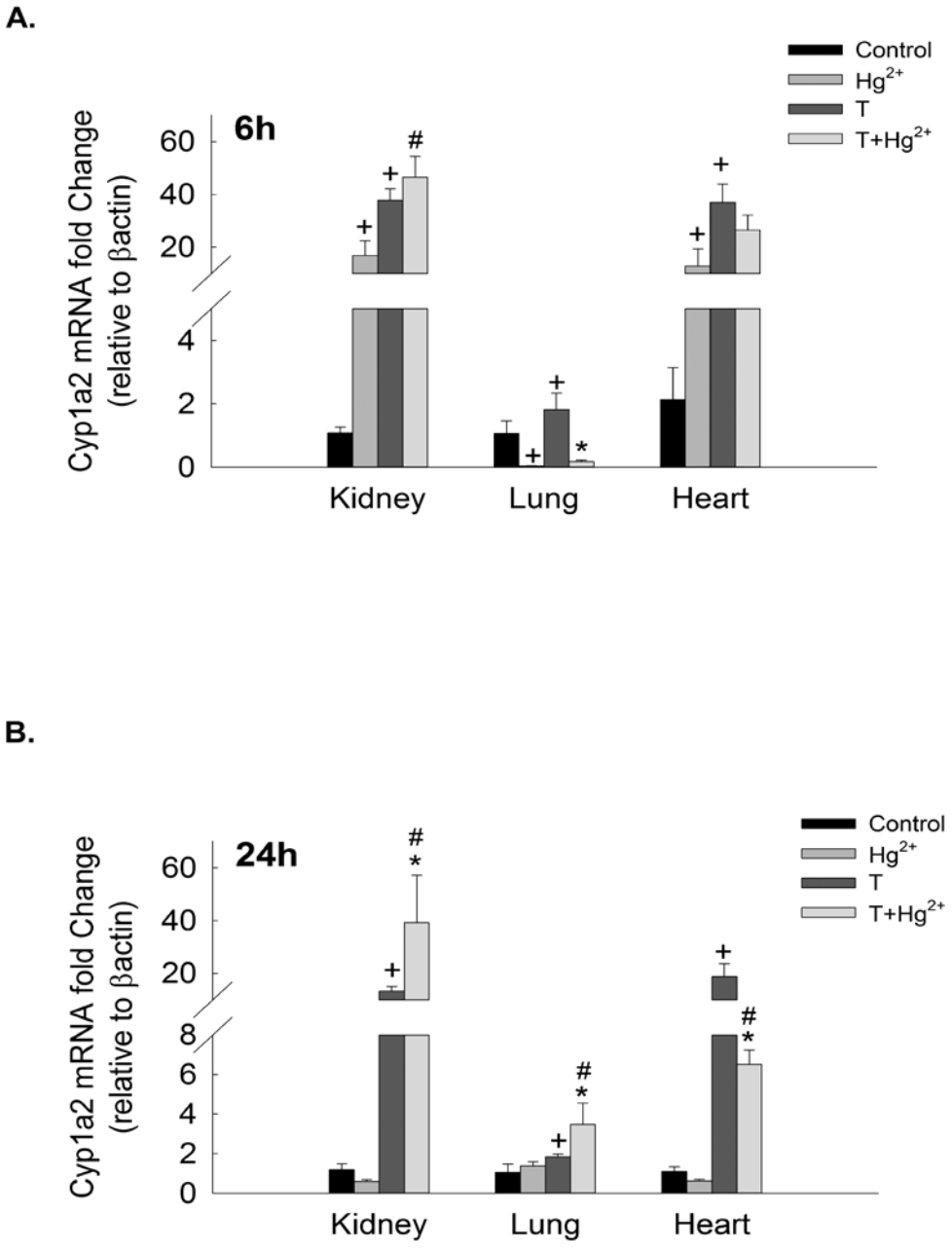

Figure 3.32. Effect of co-exposure to Hg^{2+} and TCDD on Cyp1a2 mRNA in the kidney, lung, and heart of C57Bl/6 mice. Animals were injected i.p. with 2.5 mg/kg Hg^{2+} in the absence and presence of 15 µg/kg TCDD for 6 h (A) and 24 h (B). Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. Results were calculated as mean ± S.E.M. (n=6). (+) $P < 0.05$, compared to control (untreated animals); (*) $P < 0.05$, compared to respective TCDD treatment; (#) $P < 0.05$, compared to Hg^{2+} treated animals.

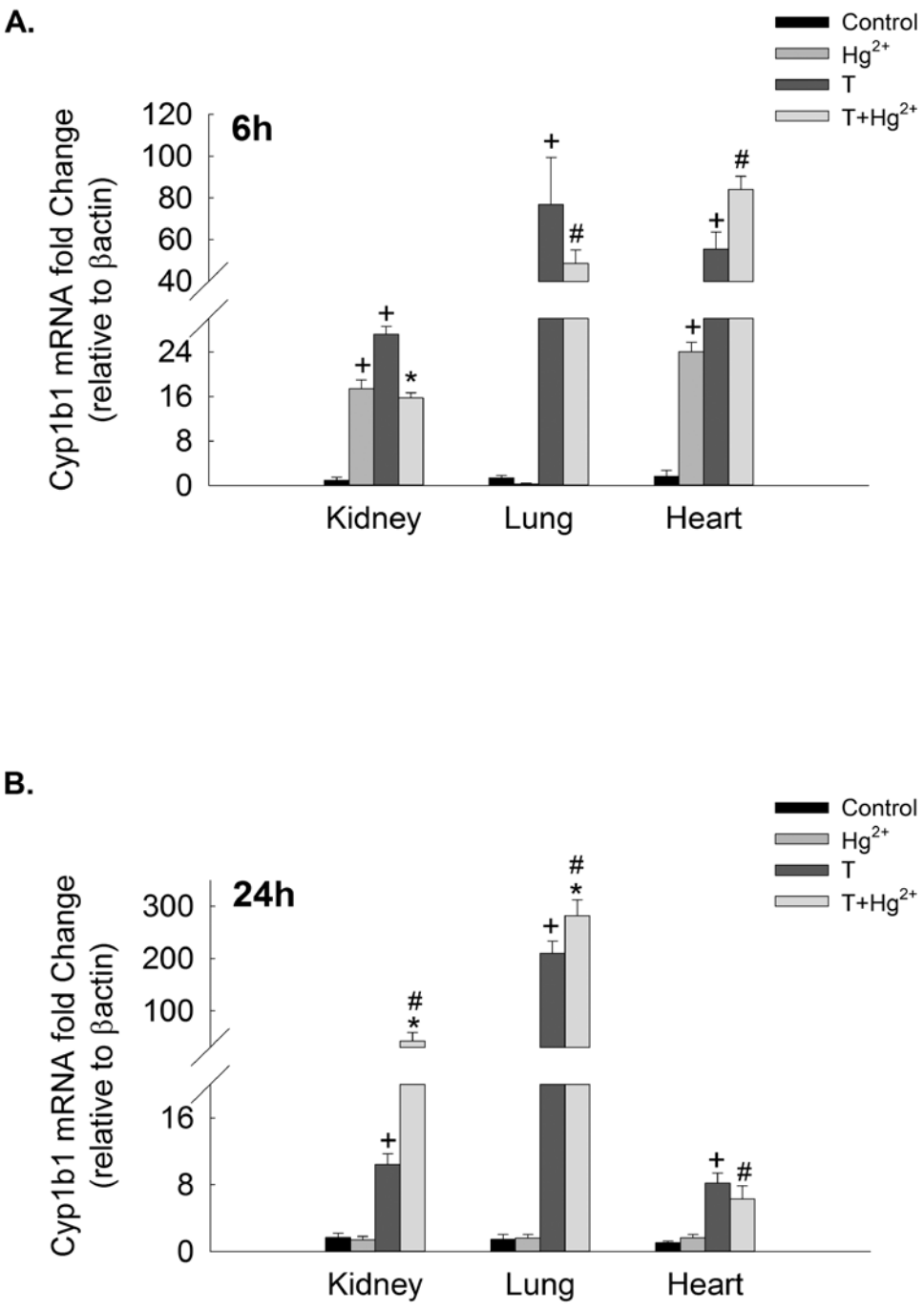

Figure 3.33. Effect of co-exposure to Hg^{2+} and TCDD on Cyp1b1 mRNA in the kidney, lung, and heart of C57Bl/6 mice. Animals were injected i.p. with 2.5 mg/kg Hg^{2+} in the absence and presence of 15 µg/kg TCDD for 6 h (A) and 24 h (B). Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. Results were calculated as mean ± S.E.M. (n=6). (+) $P < 0.05$, compared to control (untreated animals); (*) $P < 0.05$, compared to respective TCDD treatment; (#) $P < 0.05$, compared to Hg^{2+} treated animals.

3.4.3. Effect of Co-exposure to Hg^{2+} and TCDD on Cyp1a and Cyp1b1 Protein Expression and EROD and MROD Catalytic Activity Levels in the Kidney and Lung of C57Bl/6J Mice

Our results showed that Hg^{2+} alone significantly induced kidney Cyp1a and Cyp1b1 protein expression levels by 1.5- and 2.2-fold, respectively, compared to the control (Fig. 3.34A and 34B). However, in the lung Hg^{2+} significantly induced Cyp1b1 by 5.7-fold, whereas Cyp1a protein was not significantly affected (Fig. 3.34A and 34B). TCDD alone significantly induced the kidney and lung Cyp1a protein expression levels by 5.7- and 11-fold, respectively, and the kidney and lung Cyp1b1 protein expression levels by 2.8- and 6.8-fold, respectively, compared to the control (Fig. 3.34A and 34B). In contrast, Hg^{2+} significantly inhibited the TCDD-mediated induction of Cyp1a and Cyp1b1 protein levels in the kidney by -1.6- and -2-fold, respectively, compared to TCDD alone (Fig. 3.34A and 34B). On the other hand, Hg^{2+} significantly potentiated the TCDD-mediated induction of Cyp1a and Cyp1b1 proteins levels in the lung by 1.4- and 1.5-fold, respectively, compared to TCDD alone (Fig. 3.34A and 3.34B). The limitation of minimal protein quantities that could be extracted from heart samples hindered us from measuring protein expression and catalytic activities of these genes in this organ.

At the catalytic activity levels, Hg^{2+} alone significantly induced the kidney EROD and MROD catalytic activity by 1.5-and 2-fold, respectively (Fig. 3.35A and 35B). On the other hand, Hg^{2+} significantly inhibited the lung EROD activity by 1.6-fold. Interestingly, lung MROD activity was not affected by Hg^{2+}

treatment (Fig. 3.35A and 35B). TCDD alone significantly induced kidney EROD and MROD catalytic activity by 11.6- and 13-fold, respectively, and lung EROD and MROD catalytic activity by 10.4- and 3.3-fold, respectively, compared to the control (Fig. 3.35A and 35B). When animals were co-exposed to Hg^{2+} and TCDD, Hg^{2+} significantly inhibited the TCDD-mediated induction of kidney EROD and MROD activity by 1.4- and 1.6-fold. However; this inhibition did not reach statistical significant for lung EROD and MROD activity compared to TCDD alone (Fig. 3.35A and 35B).

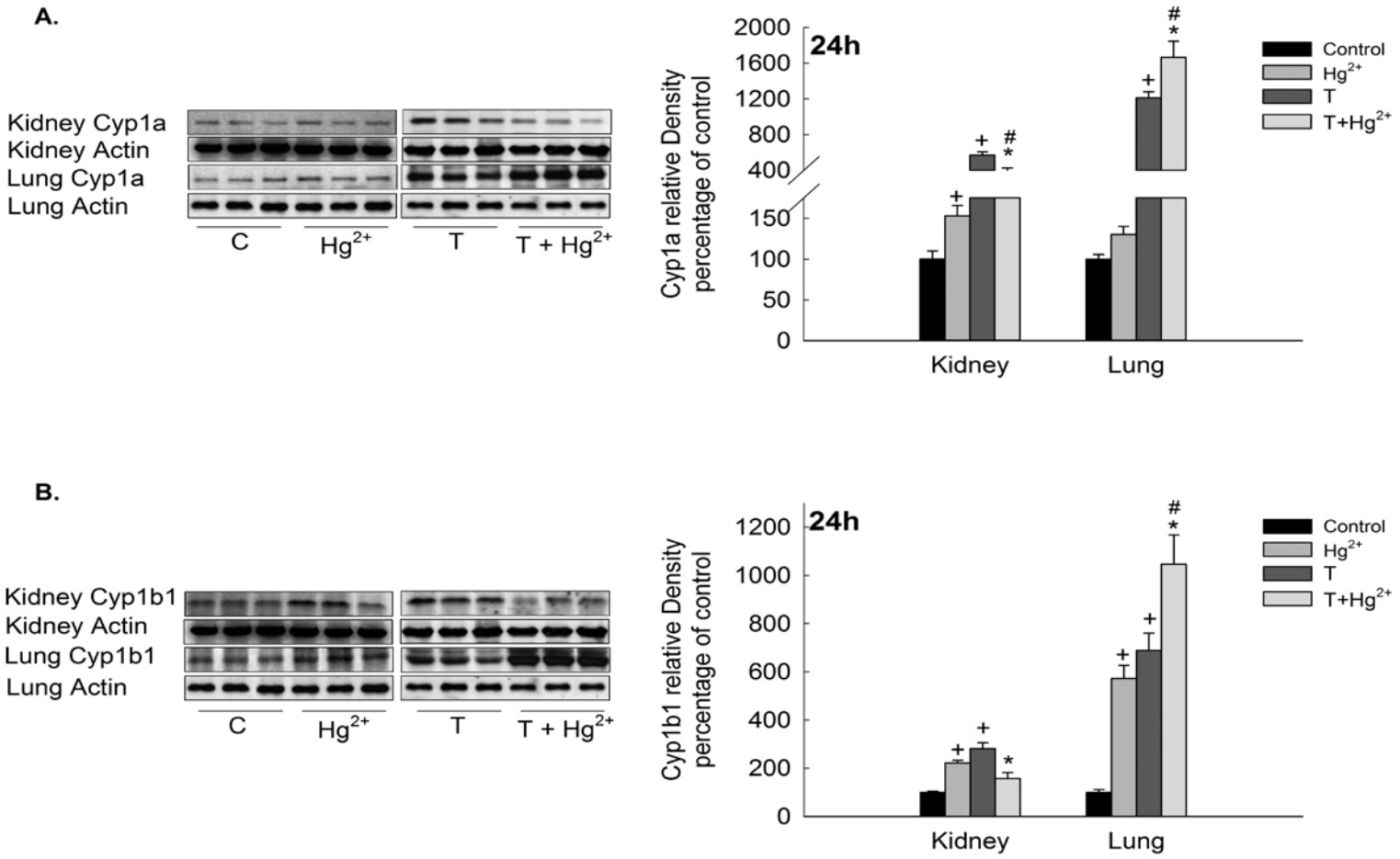

Figure 3.34. Effect of co-exposure to Hg^{2+} and TCDD on Cyp1a and Cyp1b1 protein expression levels in the kidney and lung of C57Bl/6 mice. Kidney and lung microsomal proteins were isolated after 24 h of treatment. 30 µg microsomal proteins for Cyp1a and Cyp1b1 were separated on a 10% SDS-PAGE. Proteins were detected using the enhanced chemiluminescence method. The graph represents the relative amount of protein normalized to β-actin signals, and the results are expressed as percentage of the control values taken as 100%. Results were calculated as mean ± S.E.M. (n=6). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment; (#) $P < 0.05$, compared to Hg^{2+} treated animals.

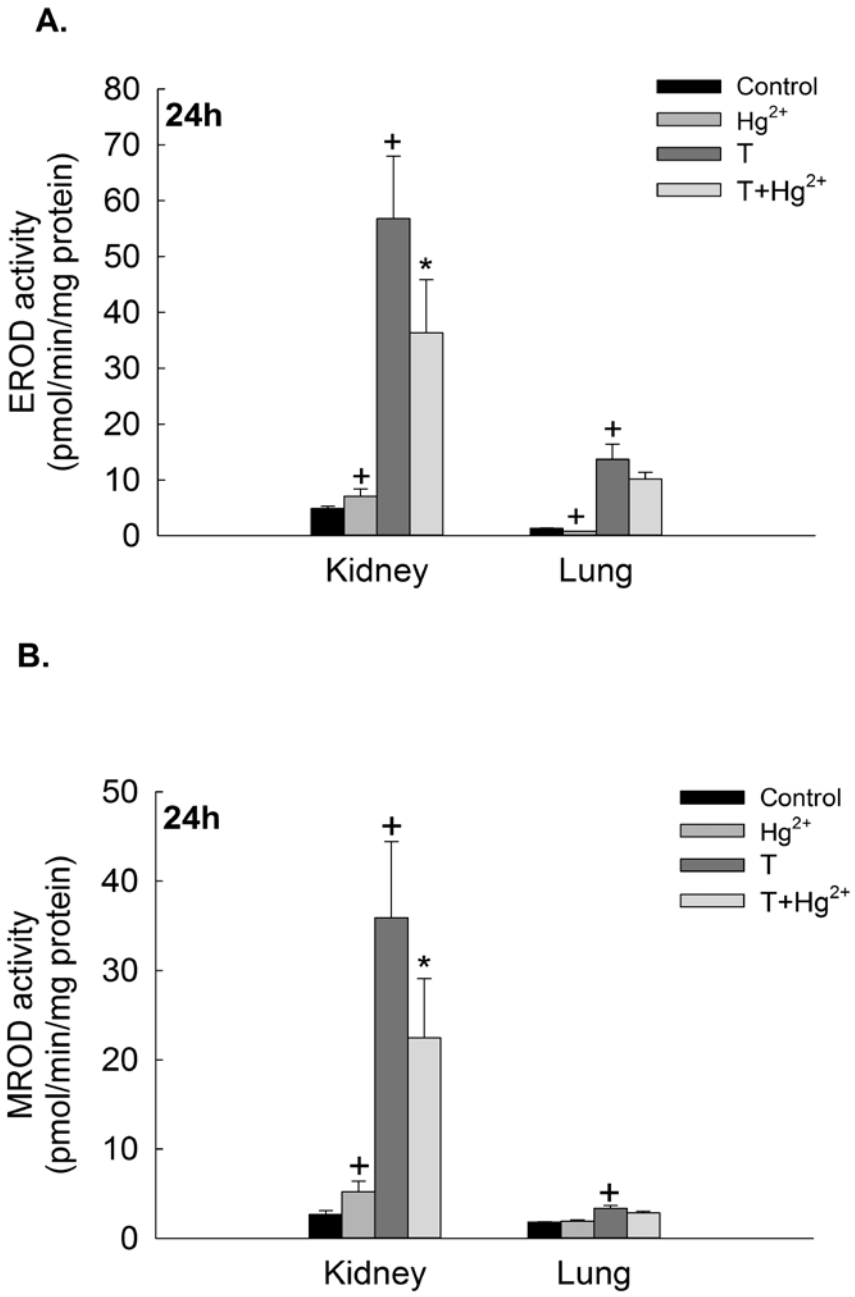

Figure 3.35. Effect of co-exposure to Hg^{2+} and TCDD on EROD and MROD catalytic activities levels in the kidney and lung of C57Bl/6 mice. Kidney and lung microsomal protein was isolated after 24 h of treatment. EROD and MROD activities were measured using 7-ethoxyresorufin, and 7-methoxyresorufin as 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. Results were calculated as mean ± S.E.M. (n=6). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment.

3.4.4. Effect of Co-exposure to Hg^{2+} and TCDD on Nqo1 and Gsta1 mRNA in the Kidney, Lung and Heart of C57B/6J Mice

The fact that Hg^{2+} was capable of altering kidney, lung and heart phase I AhR-regulated genes prompted us to investigate its possible effect on their phase II AhR-regulated genes which are not only regulated by AhR but are also regulated by the redox sensitive transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2). At 6 h Hg^{2+} alone significantly induced Nqo1 mRNA levels in the kidney, lung, and heart by 2.8-, 1.8- and 1.4-fold, respectively, compared to the control. However, at 24 h it was able to significantly induce the Nqo1 mRNA levels by 8- and 2-fold, respectively, in the kidney and heart but not in the lung, compared to the control (Fig. 3.36A and 36B). TCDD alone significantly induced the Nqo1 mRNA levels in the kidney, lung, and heart by 2-, 6.7-, and 2-fold at 6 h, and by 2.8-, 6.4-, and 3.5-fold at 24 h, respectively, compared to the control (Fig. 3.36A and 36B). When animals were co-exposed to Hg^{2+} and TCDD, at 24 h Hg^{2+} significantly potentiated the TCDD-mediated induction of Nqo1 mRNA levels in the kidney, lung, and heart by 6-, 2.3-, and 1.7-fold, respectively, compared to TCDD alone (Fig.3.36A and 36B). However, this potentiation did not reach statistical significance at 6 h in all tested tissues (Fig. 3.36A).

Hg^{2+} alone significantly induced the Gsta1 mRNA levels in both the kidney and heart by 446- and 2.4-fold at 6 h, and by 2417- and 8.4-fold at 24 h, respectively, compared to control (Fig. 3.37A and 37B). In addition, TCDD alone significantly induced the Gsta1 mRNA levels in the kidney and heart by 7- and

2.3-fold at 6 h, and by 3.2- and 12.4-fold at 24 h, respectively, compared to the control (Fig. 3.37A and 37B). On the other hand, TCDD did not significantly affect Gsta1 mRNA levels in the lung at 6 or 24 h. Upon co-exposure to Hg^{2+} and TCDD, at 6 h Hg^{2+} was able to significantly potentiate the TCDD-mediated induction of Gsta1 mRNA levels in the kidney and lung by 37- and 3.3-fold, respectively, but not in the heart, compared to TCDD alone (Fig. 3.37A). Interestingly, Hg^{2+} significantly potentiated the TCDD-mediated induction of Gsta1 mRNA in the kidney, lung, and heart by 1100-, 1.5- and 2-fold at 24 h, respectively, compared to TCDD alone (Fig. 3.37B).

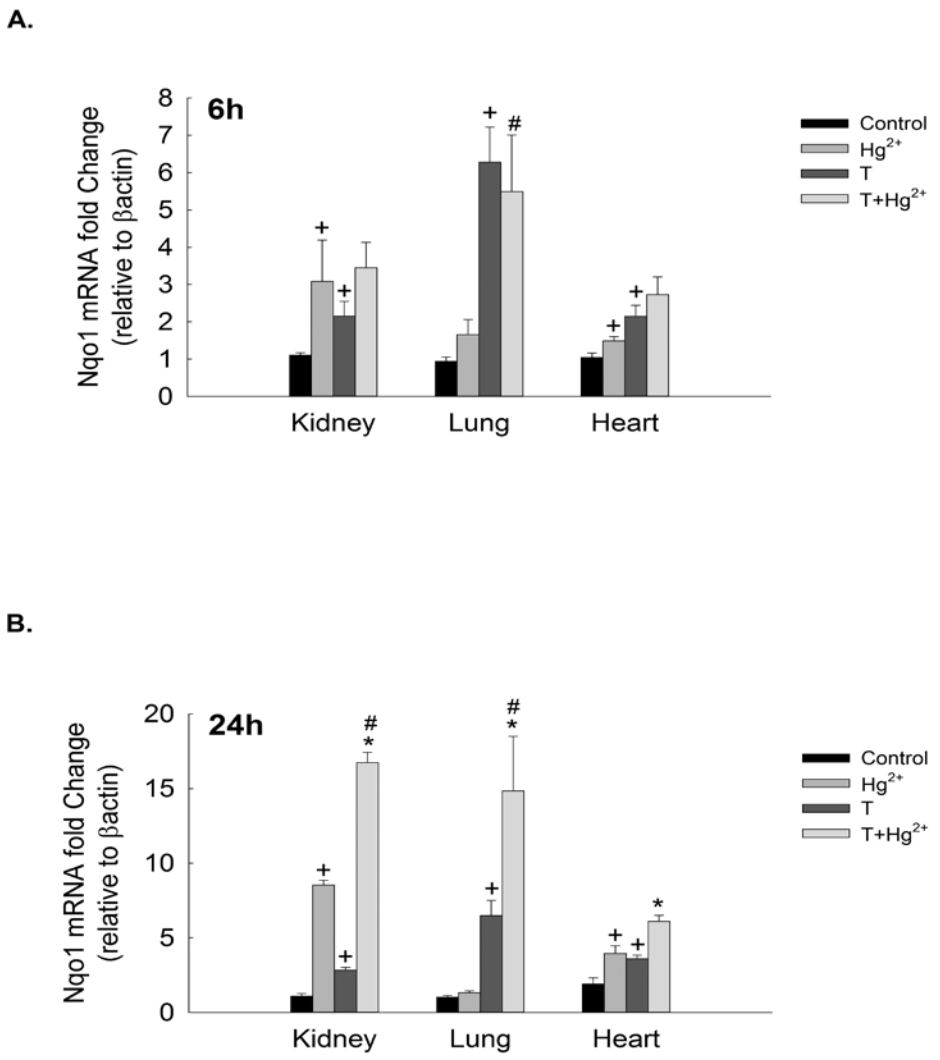

Figure 3.36. Effect of co-exposure to Hg^{2+} and TCDD on Nqo1 mRNA in the kidney, lung, and heart of C57Bl/6 mice. Animals were injected i.p. with 2.5 mg/kg Hg^{2+} in the absence and presence of 15 µg/kg TCDD for 6 h (A) and 24 h (B). First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from kidney, lung and heart, 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. Results were calculated as mean ± S.E.M. (n=6). (+) $P < 0.05$, compared to control (untreated animals); (*) $P < 0.05$, compared to respective TCDD treatment; (#) $P < 0.05$, compared to Hg^{2+} treated animals.

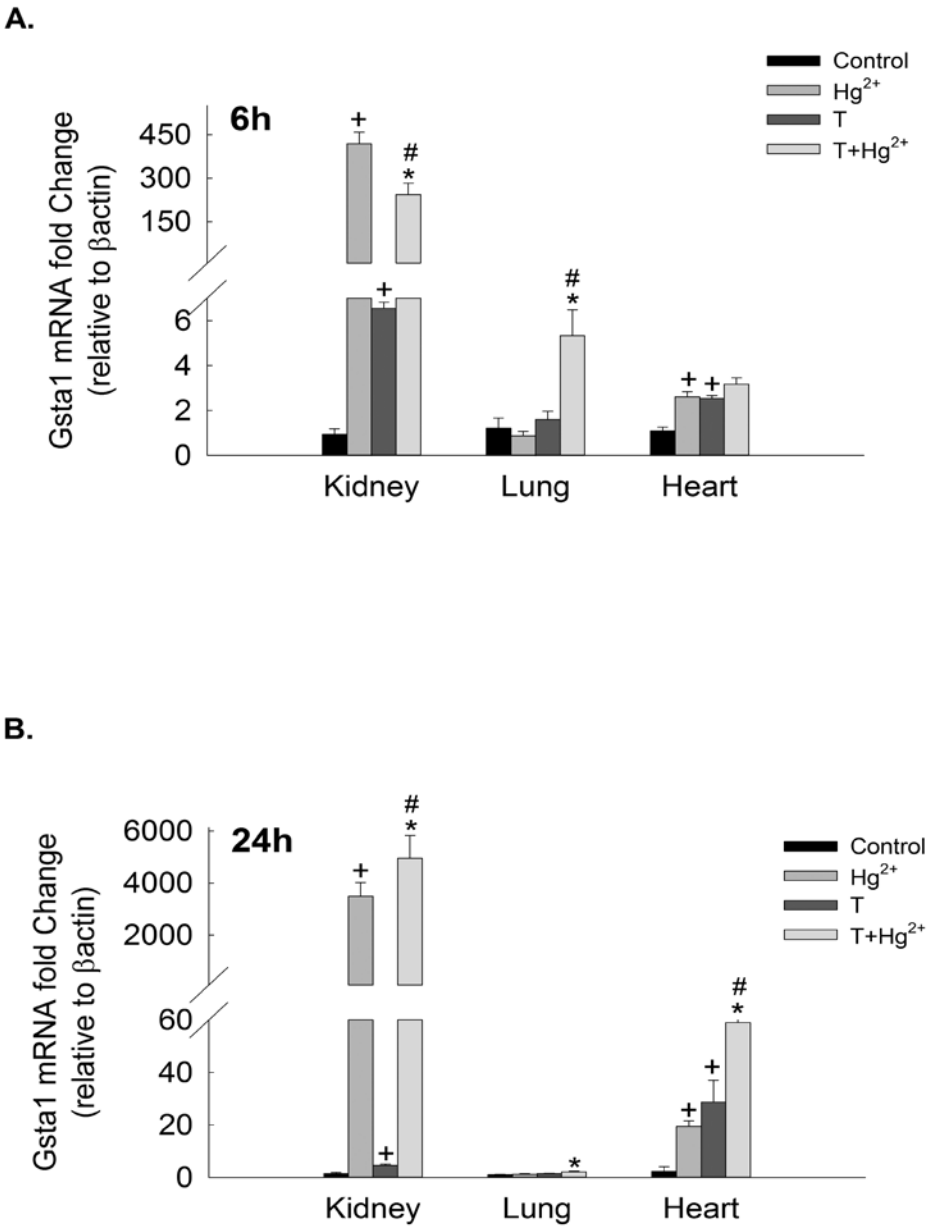

Figure 3.37. Effect of co-exposure to Hg^{2+} and TCDD on Gsta1 mRNA in the kidney, lung, and heart of C57Bl/6 mice. Animals were injected i.p. with 2.5 mg/kg Hg^{2+} in the absence and presence of 15 µg/kg TCDD for 6h (A) and 24 h (B). First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from kidney, lung and heart, 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. Results were calculated as mean ± S.E.M. (n=6). (+) $P < 0.05$, compared to control (untreated animals); (*) $P < 0.05$, compared to respective TCDD treatment; (#) $P < 0.05$, compared to Hg^{2+} treated animals.

3.4.5. Effect of Co-exposure to Hg^{2+} and TCDD on Nqo1 and Gsta1 Protein Expression and Catalytic Activity Levels in the Kidney and Lung of C57Bl/6 Mice

Hg^{2+} alone significantly induced Nqo1 protein level in the kidney and lung by 1.9- and 2.4-fold, respectively, and Gsta1 protein level in the kidney by 15.5-fold, while it did not significantly affect the lung Gsta1 protein, compared to the control (Fig. 3.38B). TCDD alone significantly induced Nqo1 and Gsta1 in the kidney by 1.6- and 3.6-fold, respectively, and in the lung by 2.5- and 1.7-fold, respectively, compared to control (Fig. 3.38A and 38B). Upon co-exposure to Hg^{2+} and TCDD, Hg^{2+} significantly potentiated the TCDD-mediated induction of Nqo1 protein in both the kidney and lung by 1.5- and 1.4-fold, respectively, compared to TCDD alone (Fig. 3.38A). Similarly, Hg^{2+} significantly potentiated the TCDD-mediated induction of Gsta1 in the kidney and lung by 3.5- and 1.3-fold, respectively, compared to TCDD alone (Fig. 3.38B).

At the catalytic activity levels, Hg^{2+} alone significantly induced the kidney Nqo1 and Gsta1 activities by 3.3- and 12.6-fold, respectively, compared to the control, while it did not significantly affect these activities in the lung (Fig. 3.39A and 39B). TCDD alone significantly induced Gsta1 activity, but not Nqo1 activity, in the kidney and the lung by 9.5- and 1.8-fold, respectively (Fig. 3.39A and 39B). When animals were co-exposed to Hg^{2+} and TCDD, Hg^{2+} significantly potentiated the TCDD-mediated induction of Nqo1 and Gsta1 activities in the kidney by 3.5- and 1.5- and by 1.6- and 1.05-fold in the lung, respectively, compared to TCDD alone (Fig. 3.39A and 39B).

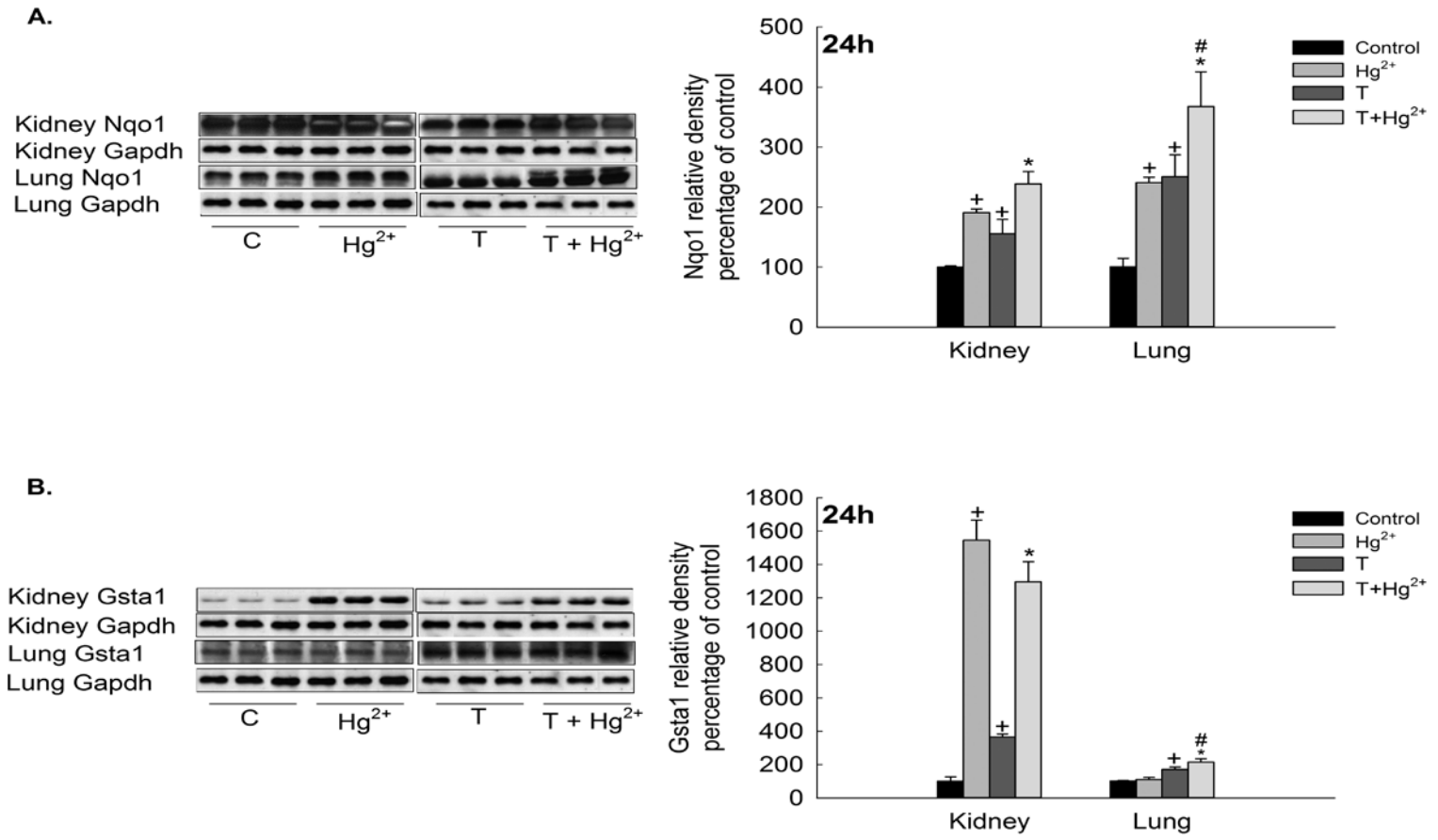

Figure 3.38. Effect of co-exposure to Hg^{2+} and TCDD on Nqo1 and Gsta1 protein expression levels in the kidney and lung of C57Bl/6 mice. Kidney and lung cytosolic proteins were isolated after 24 h of treatment. 5 or 30 µg cytosolic proteins for Nqo1 and Gsta1, respectively were separated on a 10% SDS-PAGE. Proteins were detected using the enhanced chemiluminescence method. The graph represents the relative amount of protein normalized to Gapdh signals, and the results are expressed as percentage of the control values taken as 100%. Results were calculated as mean ± S.E.M. (n=6). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment; (#) $P < 0.05$, compared to Hg^{2+} treated animals.

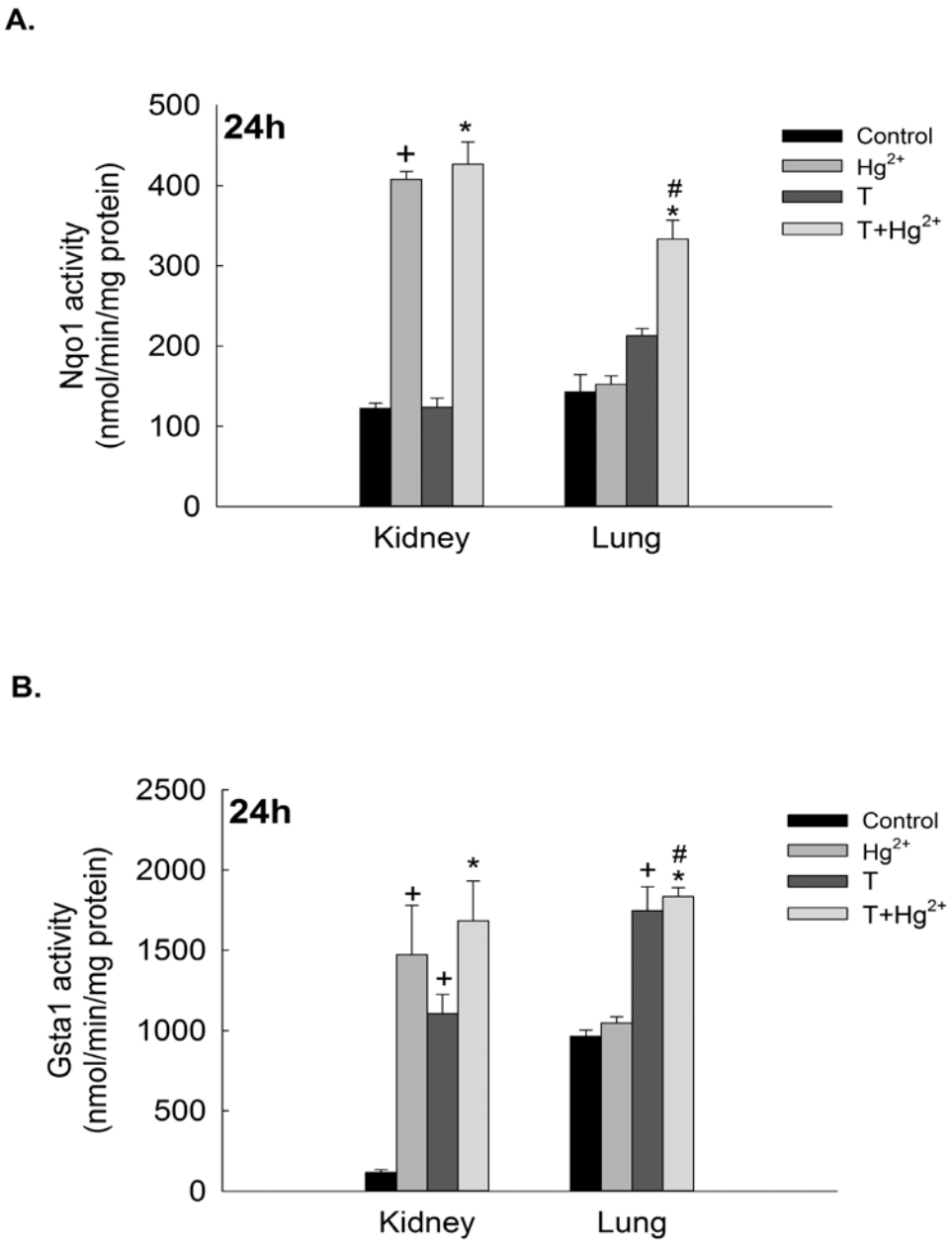

Figure 3.39. Effect of co-exposure to Hg^{2+} and TCDD on Nqo1 and Gsta1 catalytic activities levels in the kidney and lung of C57Bl/6 mice. Kidney and lung cytosolic protein was isolated after 24 h of treatment. Nqo1 enzyme activity was determined spectrophotometrically using DCPIP as substrate, and dicoumarol a specific Nqo1 inhibitor. Gsta1 activity was determined spectrophotometrically using CDNB. Results were calculated as mean ± S.E.M. (n=6). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective TCDD treatment; (#) $P < 0.05$, compared to Hg^{2+} treated animals.

3.4.6. Effect of Co-exposure to Hg^{2+} and TCDD on HO-1 mRNA in the Kidney, Lung and Heart and Protein Levels in the Kidney and Lung of C57Bl/6 Mice

We have previously demonstrated that Hg^{2+}-mediated induction of HO-1 mRNA levels coincided with a significant decrease in CYP1A1 activity, possibly through degrading its heme content (Amara *et al.*, 2010). Furthermore, inhibition of HO-1, addition of heme precursor, or knockdown of HO-1 with siRNA partially restored the TCDD-mediated induction of CYP1A1 catalytic activity. Therefore, it was of importance for us to determine if Hg^{2+} will induce HO-1 or not at 6 h and 24 h, and whether or not this effect will correlate with the effect of Hg^{2+} on P450 activities. Our results demonstrated that Hg^{2+} alone was able to induce HO-1 mRNA levels in the kidney, lung, and heart at 6 h by 209-, 5.4- and 14.6-fold, respectively, compared to control (Fig. 3.40A). Importantly, Hg^{2+} alone at 24 h did not significantly affect HO-1 mRNA levels in the kidney, lung, or heart (Fig. 3.40 B). TCDD alone at 6 h did not alter HO-1 in the kidney, lung, or heart, while it significantly induced HO-1 mRNA levels at 24 h by 1.9-fold in the kidney only (Fig. 3.40B). When animals were co-exposed to Hg^{2+} and TCDD at 6 h, Hg^{2+} significantly potentiated the TCDD-mediated induction of HO-1 mRNA levels in the kidney, lung, and heart by 200-, 7.4-, and 8.5-fold, respectively, compared to TCDD alone (Fig. 3.40A). At 24 h, however, HO-1 mRNA levels were significantly potentiated only in the kidney by 1.5-fold compared to TCDD alone (Fig. 3.40B).

To investigate whether the effect of Hg^{2+} on HO-1 mRNA is further

translated to the protein level, we examined the effect of Hg^{2+} on HO-1 protein levels at 6 and 24 h. Our results showed that Hg^{2+} at 6 h significantly induced HO-1 protein expression levels in the kidney and lung by 2.1- and 2-fold, respectively, compared to the control (Fig. 3.40C). TCDD alone was able to significantly decrease the HO-1 protein by -2.5-fold in both the kidney and lung, compared to the control. Upon co-exposure to Hg^{2+} and TCDD, at 6 h Hg^{2+} significantly increased the HO-1 protein level in the kidney and lung by 5.3- and 3.6-fold, respectively compared to TCDD treatment (Fig. 3.40C). However, Hg^{2+} with or without TCDD was not able to induce HO-1 protein levels in the kidney or lung at 24 h (data not shown).

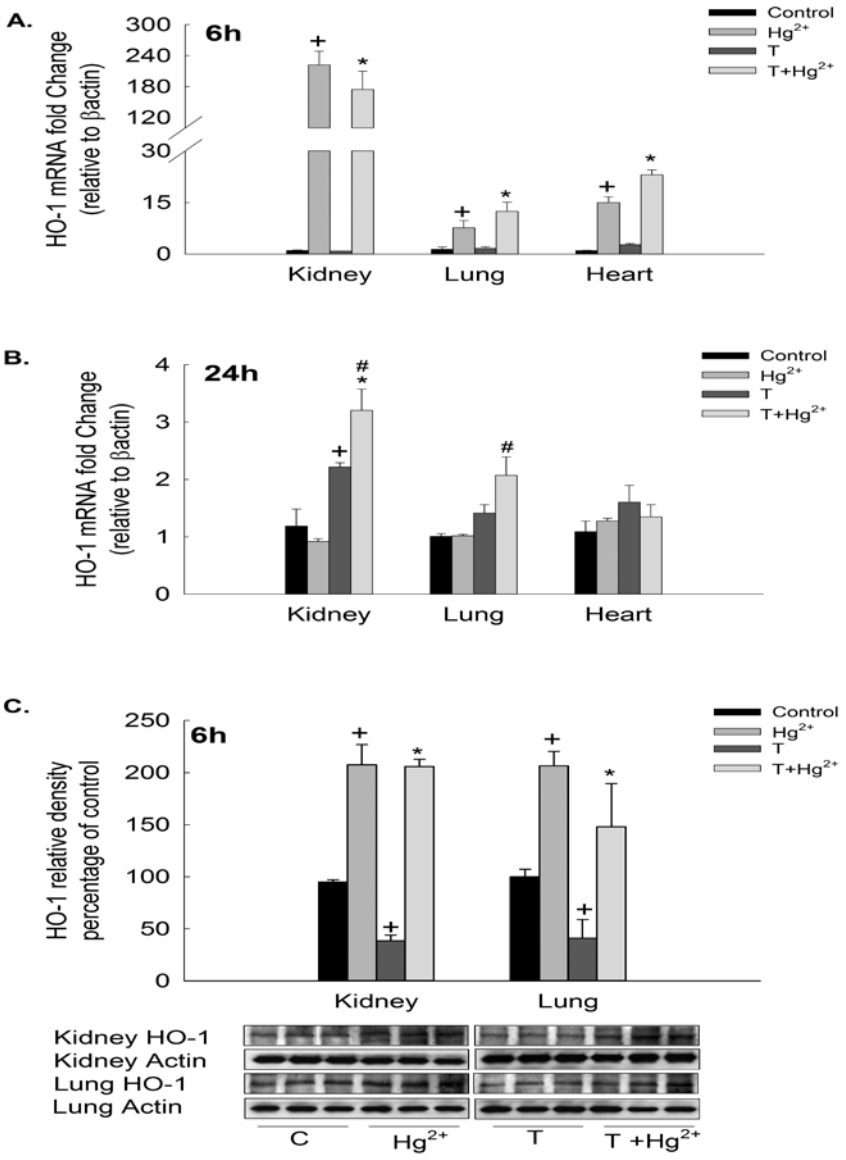

Figure 3.40. Effect of co-exposure to Hg^{2+} and TCDD on HO-1 mRNA in the kidney, lung, and heart of C57Bl/6 mice. Animals were injected i.p. with 2.5 mg/kg Hg^{2+} in the absence and presence of 15 µg/kg TCDD for 6 h (A) and 24 h (B). First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from kidney, lung and heart, 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. (C). Kidney and lung microsomal proteins were isolated after 24 h of treatment. 40 µg proteins for HO-1 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, and the results are expressed as percentage of the control values taken as 100%. Results were calculated as mean ± S.E.M. (n=6). (+) $P < 0.05$, compared to control (untreated animals); (*) $P < 0.05$, compared to respective TCDD treatment; (#) $P < 0.05$, compared to Hg^{2+} treated animals.

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

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

To determine the non-toxic concentrations of MeHg to be utilized in the current study, HepG2 cells were exposed for 24 h with increasing concentrations of MeHg (1.25–20 µM) in the absence and presence of 1 nM TCDD; thereafter cytotoxicity was assessed using the MTT assay. Fig.3.41 shows that MeHg at concentrations of 1.25–5 µM in the presence and absence of 1 nM TCDD did not affect cell viability. However, the highest concentrations tested (10-20 µM), significantly reduced the cell viability by 35 and 90 %, respectively (Fig. 3.41). Therefore, all subsequent studies were conducted using the concentrations of 1.25–5 µM.

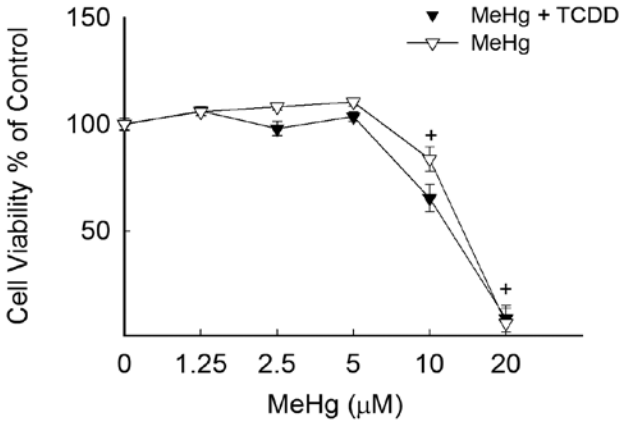

Figure 3.41. Effect of MeHg on cell viability. HepG2 cells were treated for 24 h with MeHg (0–20 μM) in the absence and presence of 1 nM TCDD. Cell cytotoxicity was determined using the MTT assay. Data are expressed as percentage of untreated control (which is set at 100%) ± SE (n = 8). (+) $P < 0.05$, compared to control (concentration= 0 μM); (*) $P < 0.05$, compared to respective TCDD treatment.

3.5.2. Concentration-dependent effect of MeHg and TCDD on inducible CYP1A1 mRNA

To examine the effect of co-exposure to MeHg and TCDD on CYP1A1 mRNA, HepG2 cells were treated with various concentrations of MeHg (1.25–5 μM) in the presence of 1 nM TCDD (Fig. 3.42). Thereafter, CYP1A1 mRNA was assessed using real-time PCR. Our results show that Hg^{2+} alone did not significantly affect the CYP1A1 mRNA (data not shown). On the other hand, TCDD alone caused a significant increase of CYP1A1 mRNA by 45-fold that was non-significantly affected by MeHg at all concentrations tested (Fig. 3.42).

3.5.3. Concentration-dependent effect of MeHg and TCDD on CYP1A1 protein and catalytic activity

HepG2 cells were treated for 24 h with increasing concentrations of MeHg (1.25–5 μM) in the presence of 1 nM TCDD. Hg^{2+} alone did not significantly affect the CYP1A1 protein and activity levels (data not shown). Figure 3.43A shows that TCDD alone caused a 24-fold increase in CYP1A1 protein level. In agreement with the CYP1A1 mRNA results, MeHg did not affect the TCDD-mediated induction of CYP1A1 protein expression. On the other hand, TCDD alone significantly induced the CYP1A1 catalytic activity by 16.5-fold, which was significantly decreased by MeHg in a concentration-dependent manner to 59, 84 and 94 % with concentrations of 1.25, 2.5, and 5, μM, respectively, compared to TCDD treatment (Fig. 3.43B).

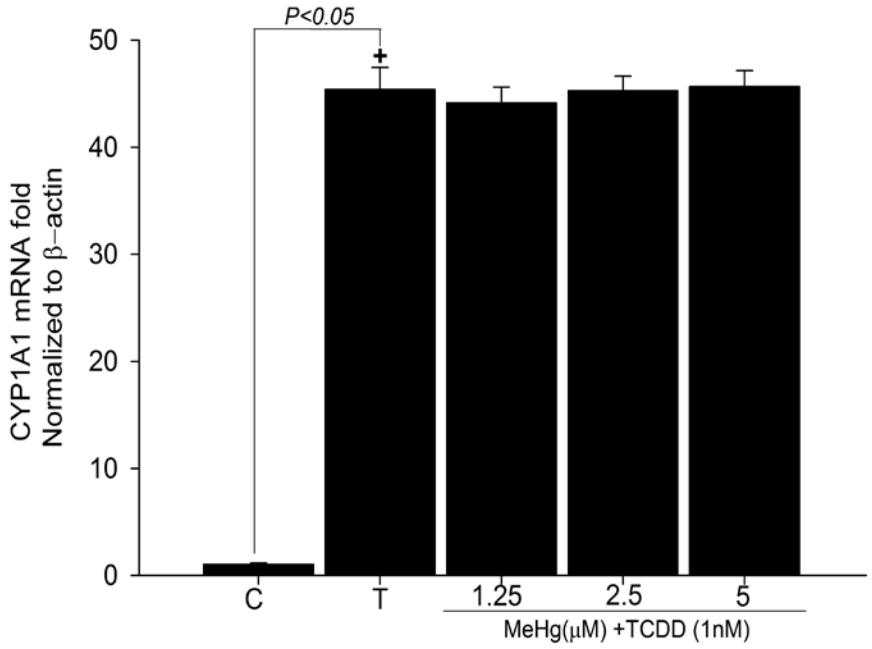

Figure 3.42. Effect of MeHg on CYP1A1 mRNA using real-time PCR. HepG2 cells were treated with increasing concentrations of MeHg in the presence of 1 nM TCDD for 6 h. First-strand cDNA was synthesized from total RNA (1 µg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Section 2. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments ± SE (n = 6). (+) $P < 0.05$, compared to control (C) (concentration = 0 µM).

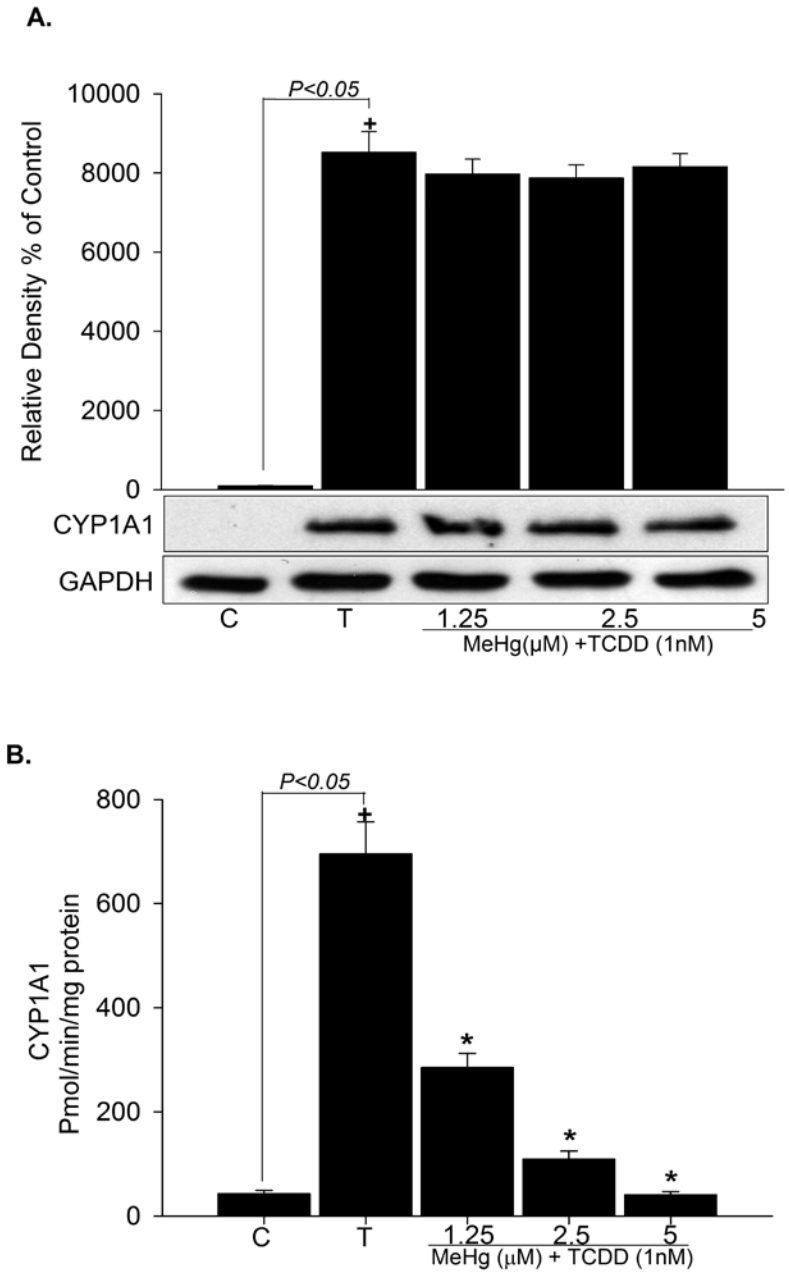

Figure 3.43. Effect of MeHg on CYP1A1 protein and EROD activity. HepG2 cells were treated for 24 h with increasing concentrations of MeHg in the presence of 1 nM TCDD. (A) Protein (50 µg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4 ◦C and then incubated with a primary CYP1A1 antibody for 2 h at room temperature, 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 GAPDH signals, which was used as loading control. One of the three representative experiments is shown. (B) EROD activity was measured in intact living cells treated with increasing concentrations of MeHg, 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.

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

HepG2 cells were plated onto 12-well cell culture plates. The XRE-driven luciferase reporter gene and the Renilla luciferase pRL-CMV vector, used for normalization, were cotransfected into HepG2 cells. Luciferase activity results showed that 5 μM MeHg alone did not affect the constitutive expression of the luciferase activity compared to control (Fig. 3.44). On the other hand, 1 nM TCDD alone caused a significant increase in the luciferase activity by 62-fold compared to control. Expectedly, co-treatment with MeHg and TCDD did not affect the TCDD-mediated induction of luciferase activity compared to TCDD alone (Fig. 3.44).

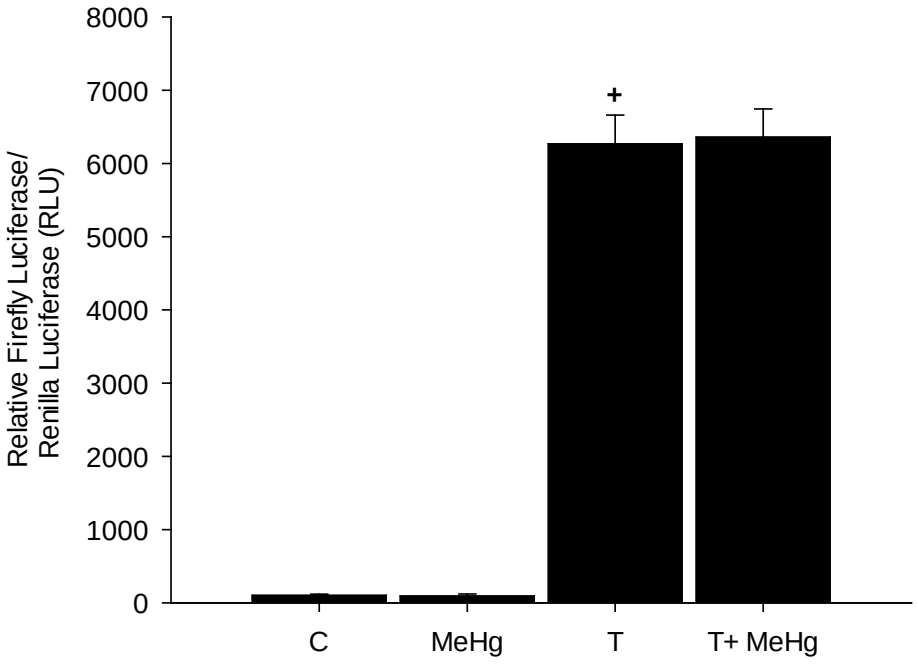

Figure 3.44. Effect of MeHg on luciferase activity. HepG2 cells were transiently transfected with the XRE-luciferase transporter plasmid pGudLuc 6.1 and the Renilla luciferase pRL-CMV vector. Cells were treated with vehicle, TCDD (1 nM), MeHg (5 µM), or TCDD (1 nM) + MeHg (5 µM) for 24 h. Cells were lysed and luciferase activity was measured according to manufacturer's instruction. Luciferase activity is reported as relative light unit. Values are presented as mean ± SE (n = 6). (+) $P < 0.05$, compared to control (C).

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

To examine the possible direct inhibitory effect of MeHg on CYP1A1 catalytic activity, HepG2 cells were treated for 24 h with 1 nM TCDD. Thereafter, cells were incubated with increasing concentrations of MeHg for 2 h, and the CYP1A1 catalytic activity levels were determined in intact living cells using EROD assay. Our results showed that TCDD alone significantly increased CYP1A1 catalytic activity, by 17-fold. In contrast, MeHg did not cause a direct inhibitory effect on the TCDD-mediated induction of CYP1A1 catalytic activity levels (Fig. 3.45A).

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

To investigate whether the MeHg-mediated decrease in CYP1A1 activity is a result of decreased intracellular NADPH, HepG2 cells were treated for 24 h with 5 μM MeHg with or without 1 nM TCDD and the CYP1A1 activity was assessed in total cellular lysates in the presence of NADPH. Figure 3.45B shows that TCDD alone caused an 18-fold increase in CYP1A1 catalytic activity. On the other hand, MeHg decreased the TCDD-mediated induction of CYP1A1 catalytic activity by 90 % in total cell lysate supplemented with NADPH (Fig. 3.45B). This inhibitory effect of MeHg on the CYP1A1 catalytic activity level in total cell lysate was similar to that observed at the catalytic activity levels in intact cells.

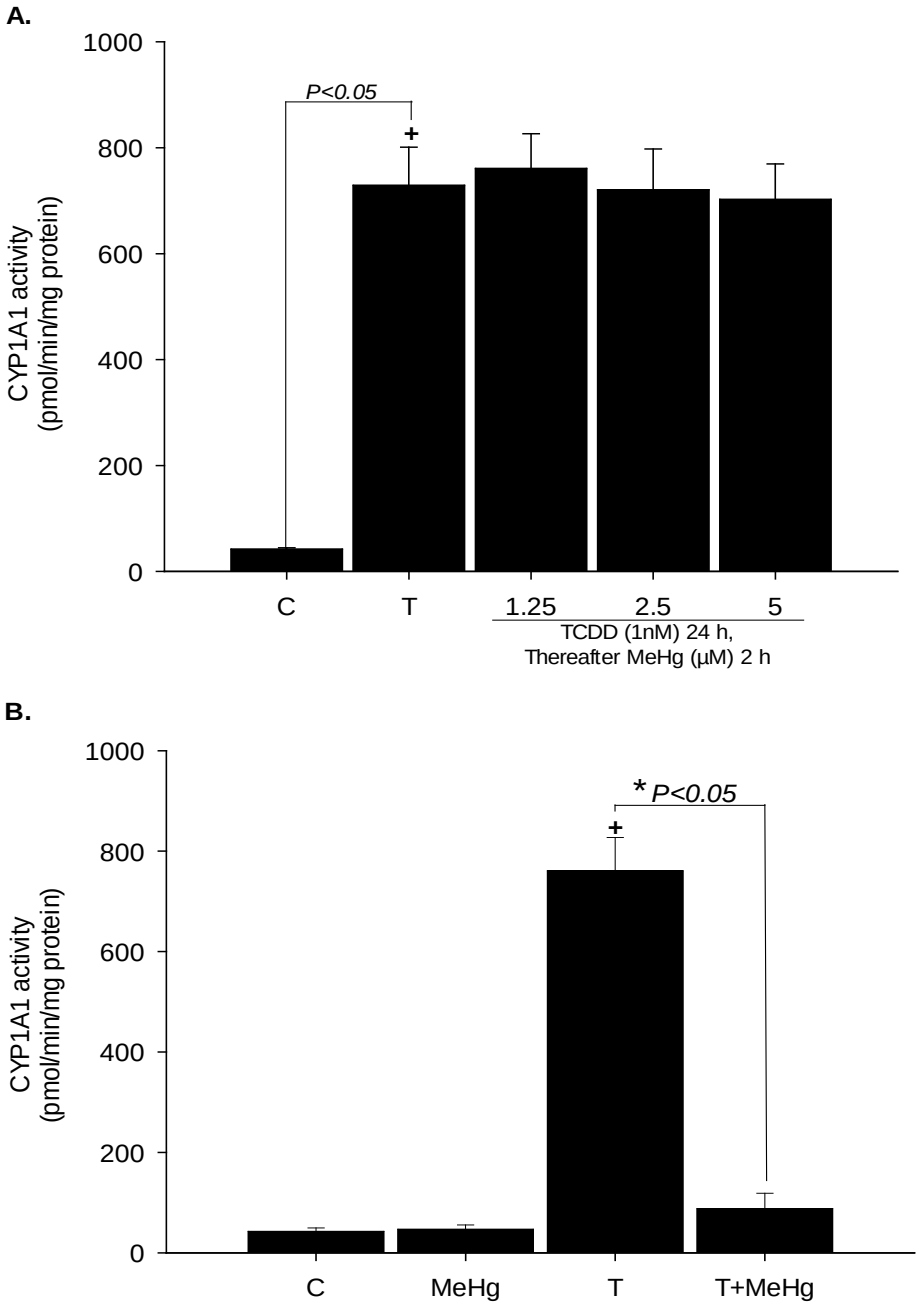

Figure 3.45. Direct effect of MeHg on inducible CYP1A1 activity and effect of supplementing NADPH on MeHg-mediated decrease in CYP1A1 activity. (A) HepG2 cells were treated for 24 h with 1 nM TCDD and then cells were incubated with 5 μM MeHg for additional 2 h. Thereafter, CYP1A1 activity was measured using 7-ethoxyresorufin as a substrate. (B) HepG2 cells were treated with 5 μM MeHg 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 supplemented with NADPH using 7-ethoxyresorufin as a substrate. Values are presented as means ± SE (n=8). + *P < 0.05*, compared to control (C); * *P < 0.05*, compared to respective TCDD (T) treatment.

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

The inverse relationship between HO-1 expression and CYP1A1 activity directed us to probe the role of MeHg in inhibiting the TCDD-mediated induction of CYP1A1 at the catalytic activity level. Therefore, we examined the effect of MeHg on HO-1 mRNA, a rate limiting enzyme of heme degradation. For this purpose, HepG2 cells were treated with increasing concentrations of MeHg (1.25-5 μM) in the presence of 1 nM TCDD. Thereafter, HO-1 mRNA was measured using real-time PCR. Our findings show that TCDD alone did not alter the HO-1 mRNA level, whereas, co-exposure to TCDD and MeHg significantly increased the HO-1 mRNA level by 1.2-, 1.6-, and 4-fold with concentrations of 1.25, 2.5 and 5 μM, respectively. Thus, HO-1 might be participating in the MeHg-mediated decrease of the TCDD-mediated induction of CYP1A1 at the catalytic activity levels (Fig. 3.46A).

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

The fact that MeHg inhibited the TCDD-mediated induction of CYP1A1 at the catalytic activity level but not at the mRNA or protein levels prompted us to investigate the possible role of HO-1 in this inhibitory effect. For this purpose HepG2 cells were co-exposed to 5 μM MeHg and 1 nM TCDD in the presence and absence of 5 μM SnMP. Our results showed that, SnMP alone caused no effect on the CYP1A1 catalytic activity. Similarly, the TCDD-mediated induction of CYP1A1 catalytic activity was not affected by SnMP treatment. On the other

hand, MeHg at the concentration of 5 μM decreased the TCDD-mediated induction of CYP1A1 catalytic activity. Intriguingly, SnMP totally reversed the MeHg-mediated decrease in CYP1A1 activity. Upon treatment of the cells with SnMP in the presence of both MeHg and TCDD, there was a complete restoration of the MeHg-mediated down regulation of CYP1A1 catalytic activity induced by TCDD (Fig. 3.46B).

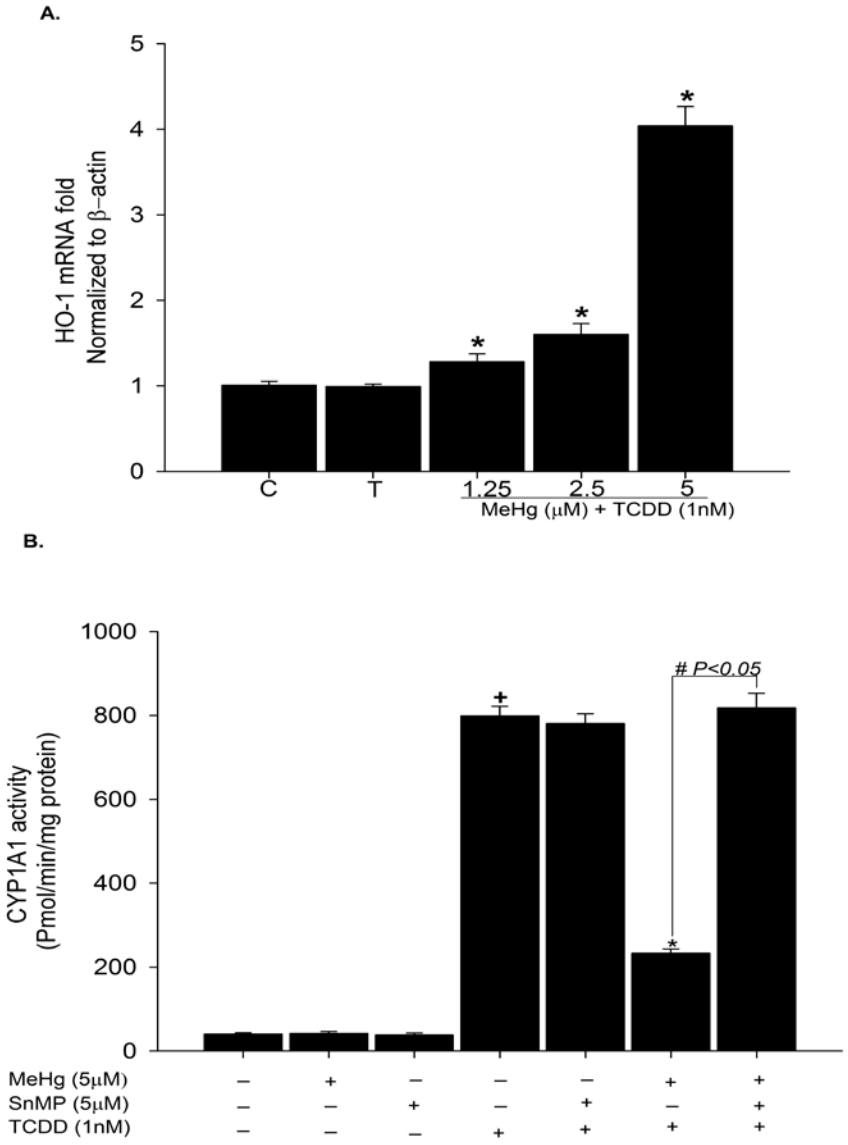

Figure 3.46. Effect of co-exposure to MeHg and TCDD on HO-1 mRNA, and the effect of SnMP on the posttranslational modification of CYP1A1 catalytic activity by MeHg. (A) HepG2 cells were treated for 6 h with increasing concentrations of MeHg in the presence of 1 nM TCDD. First-strand cDNA was synthesized 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 Section 2. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments ± SE (n = 6). (+) *P < 0.05,* compared with control (C); (*) *P < 0.05*, compared with the respective TCDD (T) treatment. (B) HepG2 cells were treated with 5 µM of MeHg and 1 nM TCDD in the presence and absence of 5 µM SnMP for 24 h for CYP1A1 catalytic activity. CYP1A1 activity was measured using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SE (n = 8). (+) *P < 0.05*, compared to control; (*) *P < 0.05*, compared to respective TCDD treatment; (#) *P < 0.05*, compared to respective MeHg +TCDD treatment.

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

Despite using selective pharmacological inhibitors such as SnMP to inhibit HO-1 activity; it was of importance to confirm our hypothesis that the MeHg-mediated increase in HO-1 is responsible for the down-regulation of CYP1A1 at the catalytic activity level. Therefore, we took a genetic approach to confirm whether or not HO-1 is involved in the MeHg-mediated decrease of the TCDD-mediated induction of CYP1A1 catalytic activity. Thereafter, HepG2 cells were transfected with human HO-1 siRNA for 6 h, and then the cells were treated with 5 μM MeHg in the presence and absence of 1 nM TCDD. Our results showed that HO-1 siRNA significantly decreased HO-1 mRNA by 80 % as compared to control (Fig. 3.47A). On the other hand, MeHg was able to increase HO-1 mRNA levels, in the absence and presence of TCDD, to reach 24-fold compared to control. When the cells were transfected with HO-1 siRNA, and then treated with MeHg alone or in the presence of TCDD there was a statistically significant decrease in HO-1 mRNA by 79 and 83 % respectively, (Fig. 3.47A). Furthermore, the Silencer select negative control siRNA did not affect the inducible level of HO-1 mRNA by MeHg, eliminating the possibility that the inhibitory effects of HO-1 siRNA might have been due to any toxicity.

Looking at CYP1A1 catalytic activity, MeHg alone or in the presence of HO-1 siRNA did not affect CYP1A1 catalytic activity (Fig. 3.47B). TCDD alone increased the CYP1A1 catalytic activity by 21-fold, whereas MeHg significantly decreased the TCDD-mediated induction of CYP1A1 catalytic activity by 82 %

compared to TCDD treatment. Interestingly, when HepG2 cells were transfected with HO-1 siRNA and then co-exposed to MeHg and TCDD, MeHg was unable to maintain the inhibitory effect on CYP1A1 catalytic activity when compared to non-transfected cells (Fig. 3.47B).

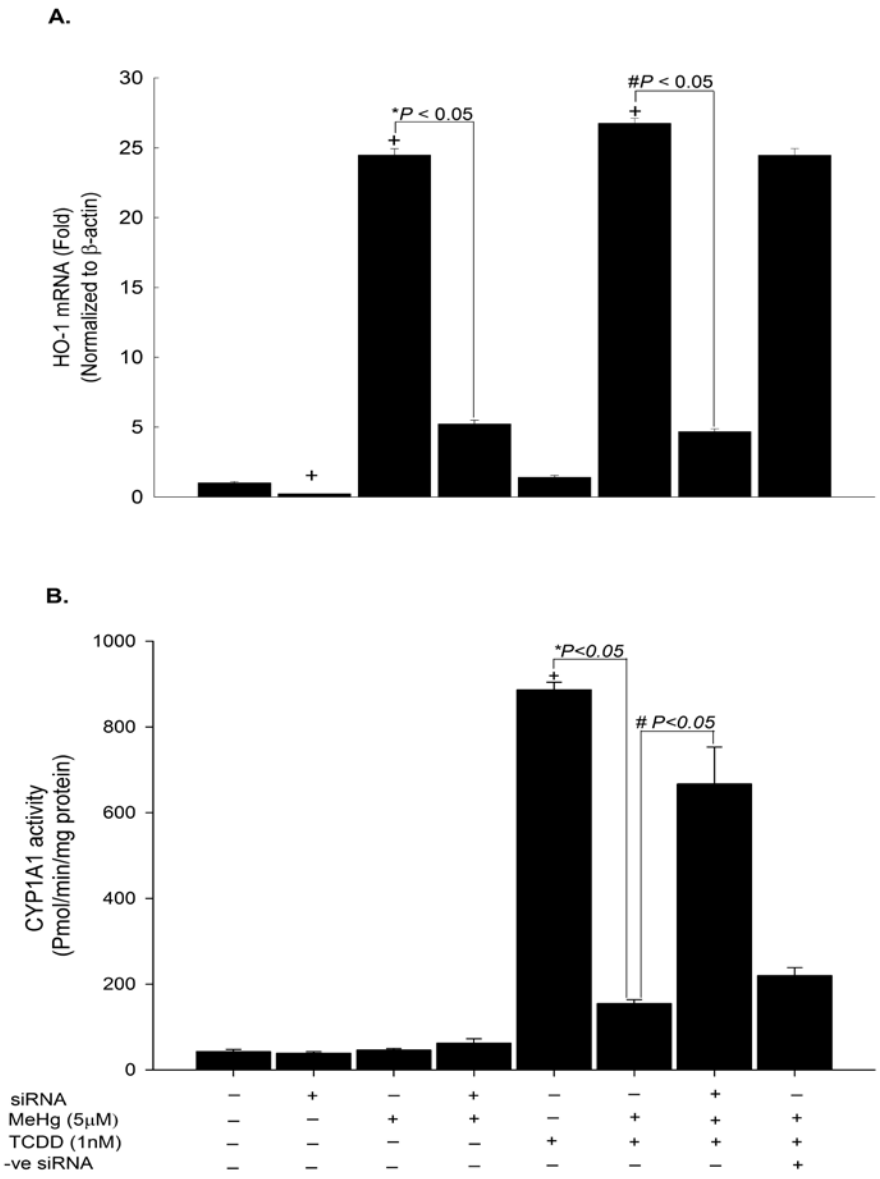

Figure 3.47. Effect of HO-1 siRNA on MeHg-mediated induction of HO-1 mRNA, and MeHg-mediated inhibition of CYP1A1 catalytic activity. HepG2 cells were transiently transfected with 20nM HO-1 siRNA (siRNA) for 6 h, thereafter cells were treated with vehicle, TCDD (1 nM), MeHg (5 μM), TCDD (1 nM) +MeHg (5 μM) for 6 h for HO-1 and 24 h for CYP1A1 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 Section 2. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments ± SE (n = 6). (+) $P < 0.05$, compared to control; (*) $P < 0.05$, compared to respective MeHg treatment; (#) $P < 0.05$, compared to respective MeHg +TCDD treatment. (B) 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 MeHg +TCDD treatment.

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

To investigate whether the inhibitory effect of MeHg is specific for CYP1A1 or other AhR-regulated genes are also affected, the effect of MeHg on the expression of NADP(H):quinone oxidoreductase 1 (NQO1), an AhR-regulated gene, was examined. For this purpose, HepG2 cells were treated with various concentrations of MeHg in the absence and presence of 1 nM TCDD. Initially, MeHg at 1.25, 2.5, and 5, μM caused a concentration-dependent increase in NQO1 mRNA levels by 1.6-, 2-, and 2.4-fold, respectively. Moreover, TCDD alone caused a 2.3-fold increase in NQO1 mRNA level that was further potentiated by MeHg in a dose-dependent manner, starting at the lowest concentration tested, which was 1.25 μM (14.5 %), and reaching 54 % with the middle dose (2.5 μM) and the maximum induction of 87 % at the concentration of 5 μM, compared to the control (Fig. 3.48A). In agreement, with the NQO1 mRNA results, MeHg alone significantly increased the constitutive NQO1 protein in a dose-dependent manner (1.25, 2.5, and 5 μM) by 1.5-, 2.7-, and 3.7-fold, respectively. Furthermore, TCDD alone caused a 3.5-fold increase in NQO1 protein, which was not significantly potentiated by MeHg at the concentration of 1.25 μM, whereas MeHg at the concentrations of 2.5 and 5 μM was able to significantly potentiate the TCDD-mediated induction of NQO1 protein levels by 48 and 71 % compared to TCDD treatment (Fig. 3.48B).

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

In order to examine whether the induction of HO-1 mRNA by MeHg is an ARE-dependent pathway, HepG2 cells were transiently transfected with the ARE-driven luciferase reporter gene. Luciferase activity results showed that MeHg alone significantly induced the ARE luciferase activity by 2-fold, compared to the control (Fig. 3.48C). Expectedly, TCDD alone significantly induced ARE luciferase activity by 2.1-fold compared to the control (Fig. 3.48C). Furthermore, co-treatment with 5 μM of MeHg significantly potentiated the TCDD-mediated induction of ARE luciferase activity by 62 % (Fig. 3.48C).

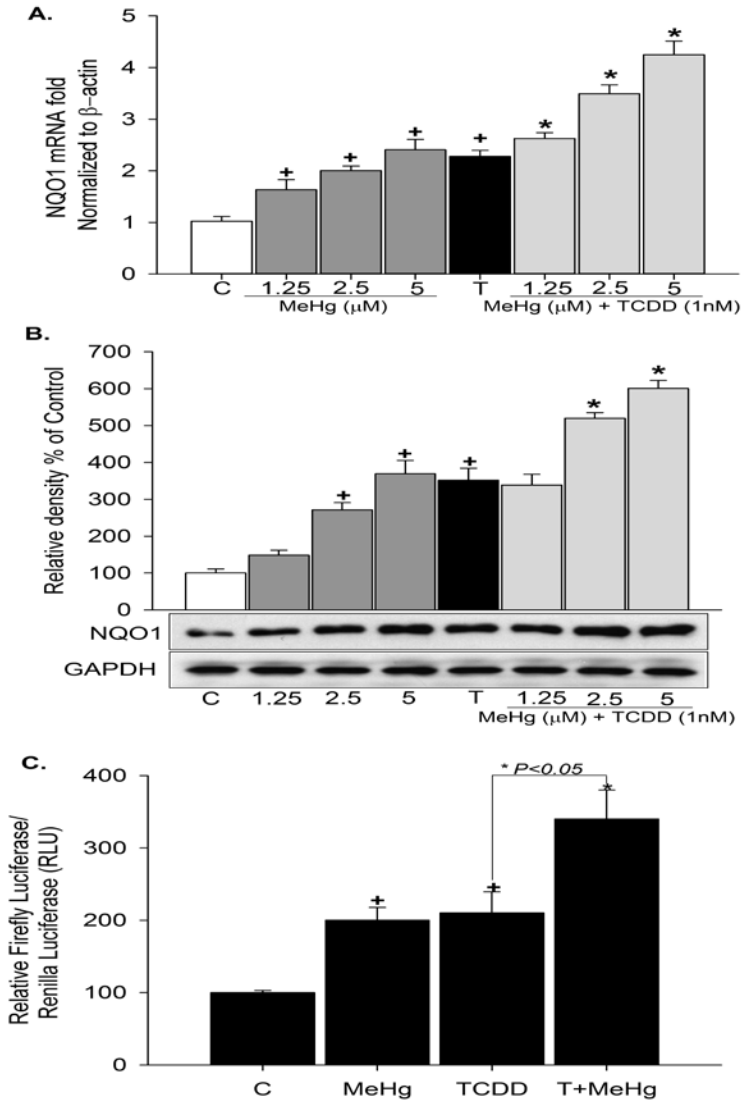

Figure 3.48. Effect of MeHg on the constitutive and TCDD-mediated induction of NQO1 mRNA, protein, and ARE-dependent luciferase activity. HepG2 cells were treated with increasing concentrations of MeHg in the presence and absence of 1 nM TCDD for 6 h for mRNA or 24 h for protein levels. (A) cDNA fragments were amplified and quantitated using the ABI 7500 real-time PCR system. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (B) Protein (5 μg) was separated by 10% SDS–PAGE. NQO1 protein was detected using the enhanced chemiluminescence method. The intensity of the bands was normalized to the GAPDH signal, which was used as loading control. One of three representative experiments is shown. + $P < 0.05$, compared to control (C); * $P < 0.05$, compared to respective TCDD (T) treatment. (C) HepG2 cells were transiently cotransfected with the reporter plasmid PGL3-ARE and the Renilla luciferase pRL-CMV vector. Cells were treated with vehicle, MeHg (5 μM), TCDD (1 nM), and TCDD (1 nM) + MeHg (5 μM), for 24 h. Thereafter, cells were lysed, and luciferase activity was measured according to the manufacturer's instructions. Luciferase activity is reported as relative light units (RLU). Values are presented as means ± SE (n=6). + $P < 0.05$, compared with control (C); * $P < 0.05$, compared with the respective TCDD (T) treatment.

CHAPTER 4- DISCUSSION

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Amara, I.E., Anwar-Mohamed, A., Abdelhamid, G., El-Kadi, A.O., 2013a. Mercury modulates the cytochrome P450 1a1, 1a2 and 1b1 in C57BL/6J mice: in vivo and in vitro studies. Toxicology and applied pharmacology 266, 419-429.

Amara, I.E., Anwar-Mohamed, A., El-Kadi, A.O., 2010. Mercury modulates the CYP1A1 at transcriptional and posttranslational levels in human hepatoma HepG2 cells. Toxicology letters 199, 225-233.

Amara, I.E., Anwar-Mohamed, A., El-Kadi, A.O., 2013b. Posttranslational mechanisms modulating the expression of the cytochrome P450 1A1 gene by methylmercury in HepG2 cells: A role of heme oxygenase-1. Toxicology letters.

Amara, I.E., El-Kadi, A.O., 2011. Transcriptional modulation of the NAD(P)H:quinone oxidoreductase 1 by mercury in human hepatoma HepG2 cells. Free radical biology & medicine 51, 1675-1685.

4.1. GENERAL DISCUSSION

Co-contamination by heavy metals, such as mercury (Hg), and halogenated aromatic hydrocarbons (HAHs) such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), is a common environmental problem with multiple biological consequences. However, the majority of published studies on AhR ligand toxicities have been conducted individually, yet human exposures are usually to mixtures of these ligands and metals such as Hg^{2+}. Nevertheless, there have been relatively few studies of the combined effects of Hg and AhR ligands on AhR-regulated genes. Our findings in this study were important as they showed the potential interactions between Hg and TCDD on the regulation of AhR-regulated genes, and hence the toxicity and carcinogenicity of TCDD in *in vitro* and *in vivo* situations. In addition, the current study explores the molecular mechanisms involved in these alterations.

Among heavy metals, Hg which is a highly toxic non-essential element in that it is neither created nor biodegradable (Barbier *et al.*, 2005). Due to industrialization and changes in the environment during the twentieth century, humans and animals are exposed to numerous chemical forms of Hg, including elemental mercury vapor (Hg^0), inorganic mercurous (Hg^+) and mercuric (Hg^{2+}) compounds, and organic mercuric ($R\text{-}Hg^+$ or R-Hg-R; where R represents any organic ligand) compounds (Fitzgerald and Clarkson, 1991). Inasmuch as mercury is ubiquitous in the environment, it is nearly impossible for most humans to avoid exposure to some form of mercury on a regular basis. Both AhR ligands and Hg are ranked high on the list of the most hazardous xenobiotics in the

environment, as reported by the Agency for Toxic Substances and Diseases Registry and the Canadian Environmental Protection Act (ATSDR, 2011; CEPA, 2012). Yet, the effects of Hg on the modulation of AhR-regulated genes and the molecular mechanisms involved still warrant further investigations.

The concentrations of Hg utilized in this work were selected based on our cell viability test (Fig. 3.1, 23 and 41) and previous studies on human hepatoma HepG2 cells and isolated mouse hepatocytes (Vakharia *et al.*, 2001b; Korashy and El-Kadi, 2008a). Furthermore, the concentrations of Hg^{2+} and TCDD utilized in the *in vivo* study were selected based on previous *in vivo* studies that used the same and different mouse strains (Mehra and Kanwar, 1980; Tanaka-Kagawa *et al.*, 1998; Hu *et al.*, 1999; Uno *et al.*, 2008; Wong *et al.*, 2010). The reasons for choosing these single doses in an *in vivo* model that because administration of repeated doses of Hg^{2+} leads to a considerable fall in total protein content in different tissues previously studied, with the maximum decrease (39.6 %) being observed in the liver (Mehra and Kanwar, 1980). In addition, the reported LD50 values of mercury for various rodent species including rats, mice and guinea pigs range are 29.9 mg/kg, 57.6 mg/kg, and 21 mg/kg, respectively (RTECS, 1985-1986).

The low concentrations of Hg used in the current study are within the estimated human plasma and tissue levels in individuals without known exposure (Gerhardsson *et al.*, 1988; Tezel *et al.*, 2001). Taken together, chronic human exposure to these long half-life toxic metals in addition to the high risk of accumulation in the body tissues (Snow *et al.*, 1989) make the concentrations

used in the present study of high relevancy to the corresponding human plasma and tissue levels, and calculated exposure levels in the environment (ATSDR, 2011; CEPA, 2012). The 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). Furthermore, the isolated mouse hepatocytes were used in this study because it is well established previously that these cells which are isolated from C57BL/6 strain (the prototypical AhR-responsive mouse), possess a high-affinity receptor (Lubet *et al.*, 1984; Chang *et al.*, 1993; Okino *et al.*, 1993; Ema *et al.*, 1994).

4.1.1. Mercury Modulates CYP1A1 at Transcriptional and Post-translational Levels in Human Hepatoma HepG2 Cells

Although environmental co-exposures involve both AhR ligands, typified by TCDD, and heavy metals, typified by Hg^{2+}, the current methods for assessing the potential toxicological consequences are often assuming the additivity of responses. However, this might not be the case, because there is still a possibility of antagonistic or synergistic effects. In the current study we examined the potential effect of Hg^{2+} on the TCDD-mediated induction of the *CYP1A1* gene in human hepatoma HepG2 cells. *CYP1A1* gene expression involves the 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). Initially, our results showed that Hg^{2+} significantly inhibited the TCDD-mediated induction of CYP1A1 at the mRNA, and protein levels at the highest concentration tested which is 10 μM, whereas at the catalytic activity level, Hg^{2+} caused a concentration-dependent inhibition of CYP1A1 (Fig.3.2 and 3.3A). These results are consistent with previously reported observations in HepG2 cells and isolated human hepatocytes showing that Hg^{2+} significantly reduced CYP1A1 induction mediated by benzo[a]pyrene derivatives (Vakharia *et al.*, 2001b). In addition, these results are in agreement with our previous observations in human hepatoma HepG2 cells, where Hg^{2+} at 5 and 10 μM significantly inhibited the induction of the TCDD-mediated induction of CYP1A1 at both protein and catalytic activity levels (Korashy and El-Kadi, 2008a). In contrast, previous data from our laboratory have shown that Hg^{2+} up-regulates *Cyp1a1* gene expression and causes further potentiation of the TCDD-mediated induction of Cyp1a1 mRNA and protein levels in murine hepatoma Hepa 1c1c7 cells (Korashy and El-Kadi, 2004; Korashy and El-Kadi, 2005).

The controversy between the effect of Hg^{2+} 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 with 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; 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 is unaffected (Suzuki and Nohara, 2007).

At the transcriptional level, we demonstrated that Hg^{2+} alone or in the presence of TCDD was able to significantly decrease AhR-dependent luciferase reporter gene expression (Fig. 3.4). Thus, the down-regulation of *CYP1A1* gene expression by Hg^{2+} was mediated through an AhR-dependant mechanism. Moreover, the ability of Hg^{2+} to decrease the *CYP1A1* gene expression through a transcriptional mechanism prompted further investigation. Therefore, we examined the effect of Hg^{2+} on the CYP1A1 mRNA stability, using Act-D-chase experiment. Our results showed that Hg^{2+} was unable to significantly alter CYP1A1 mRNA half-life (Fig. 3.5), implying that Hg^{2+} is mediating its effect through a transcriptional mechanism to inhibit the *CYP1A1* gene expression.

The concentration-dependent decrease of CYP1A1 activity by Hg^{2+} could be attributed, at least in part, to a post-translational mechanism. Therefore, we examined the effect of Hg^{2+} on the TCDD-mediated induction of CYP1A1 protein half-life using CHX-chase experiments. Our results showed that Hg^{2+} significantly increased CYP1A1 protein degradation rate (Fig. 3.6), implying the

presence of a post-translational down-regulation of CYP1A1 by Hg^{2+}. In contrast to this finding, we have previously shown that Hg^{2+} increased the TCDD-mediated induction of Cyp1a1 protein stability in Hepa 1c1c7 cells (Korashy and El-Kadi, 2005). These results suggest that the effect of Hg^{2+} on CYP1A1 half-life is species-specific.

In this study, we showed that Hg^{2+} significantly increased the HO-1 mRNA level at all concentrations tested, implying the possibility that Hg^{2+} might have decreased the CYP1A1 activity through degrading its heme group via HO-1. 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 free radicals (Marilena, 1997). HO-1 anchors to the endoplasmic reticulum membrane via a stretch of hydrophobic residues at the C-terminus (Schuller *et al.*, 1998). Thus, it is expected to interact with CYP450s which are also endoplasmic reticulum-bound enzymes. The results of the current study are in agreement with our previous published data in which Hg^{2+} was shown to be able to significantly induce HO-1 mRNA expression in Hepa 1c1c7 cells (Korashy and El-Kadi, 2005). The fact that Hg^{2+} induces HO-1 with a consequent decrease in the heme pool could result in the failure to form a functioning CYP1A1 protein. Moreover, the apoprotein would be more susceptible to proteasomal degradation (Anwar-Mohamed *et al.*, 2009).

The role of HO-1 in the down-regulation of CYP1A1 at the catalytic activity level was supported by series of pieces of evidence. Firstly, SnMP was able to partially prevent the decrease in TCDD-mediated induction of CYP1A1

activity by Hg^{2+}. The observed effect of SnMP on the Hg^{2+}-mediated decrease of CYP1A1 catalytic activity was solely through competitively inhibiting HO-1 protein and not through altering CYP1A1 mRNA. In addition, it was previously demonstrated that SnMP is incapable of producing any effect on HO-1 mRNA and CYP1A1 mRNA and catalytic activity (Anwar-Mohamed and El-Kadi, 2010). Secondly, we dissected out the role of HO-1 in the Hg^{2+}-mediated decrease of CYP1A1 catalytic activity using hemin, a precursor of heme. If Hg^{2+} decreases the TCDD-mediated induction of CYP1A1 activity through degrading its heme, then supplying heme will restore the TCDD-mediated induction of CYP1A1 activity. Interestingly, our results demonstrated that supplying external heme partially prevented the decrease in TCDD-mediated induction of CYP1A1 activity by Hg^{2+}.

Despite using a specific HO-1 inhibitor, SnMP, and heme precursor, hemin, we wanted to confirm the role of HO-1 in the Hg^{2+}-mediated decrease of CYP1A1 catalytic activity using a genetic approach. Our results showed that HO-1 mRNA was successfully knocked-down using HO-1 siRNA in HepG2 cells. Interestingly, Hg^{2+}-mediated decrease in CYP1A1 catalytic activity was partially reversed upon transfection with HO-1 siRNA. To the best of our knowledge this is the first study examining the role of HO-1 in the Hg^{2+}-mediated decrease of CYP1A1 catalytic activity using HO-1 siRNA. Studies from our laboratory and others have shown that in HepG2 cells, HO-1 knock-down partially reverses arsenite-mediated decrease of the TCDD and benzo-k-fluoranthene-induced CYP1A1 catalytic activity levels (Bessette *et al.*, 2009; Anwar-Mohamed and El-

Kadi, 2010) .

In conclusion, data presented herein demonstrated for the first time that Hg^{2+} down-regulates the expression of CYP1A1 at transcriptional and posttranslational levels in HepG2 cells. In addition, Hg^{2+}–mediated induction of HO-1 and CYP1A1 protein degradation are partially involved in the modulation of CYP1A1 at the post-translational level.

4.1.2. Transcriptional Modulation of NAD (P) H: Quinone Oxidoreductase-1 by Mercury in Human Hepatoma HepG2 Cells

NQO1 is an important regulator of intracellular redox status, by virtue of its ability to maintain antioxidants in their active forms. Hence, NQO1 protects against cellular oxidative stress and PAH-induced carcinogenicity (Long *et al.*, 2000). Furthermore, the tight regulation of this enzyme is critical for cellular protection against oxidative stress, macromolecule damage, and subsequent mutagenesis and carcinogenesis. Data from our laboratory and others showed that heavy metals including Hg^{2+} are capable of modifying the basal and inducible NQO1 through different stages of its regulatory pathway.

In the current study we hypothesize that Hg^{2+} up-regulates the constitutive and inducible NQO1 gene expression through enhancing the Nrf2 signaling pathway. Hence the main objective of the current study was to determine the potential effect of Hg^{2+} on both the basal and TCDD- or SUL-mediated induction of NQO1, as bifunctional and monofunctional inducers, respectively. Our results showed that Hg^{2+}caused a rapid time-dependent of induction of NQO1

mRNA (Fig. 3.11), which is in agreement with previous work reported in murine hepatoma Hepa 1c1c7 cells (Korashy and El-Kadi, 2006b). Consequently, Hg^{2+} was capable of causing significant induction of the basal and TCDD- and SUL-mediated induction of NQO1 at mRNA, protein, and catalytic activity levels in HepG2 cells in a concentration-dependent manner. This finding is in agreement with previous work in that Hg^{2+} induced the constitutive and inducible Nqo1 at mRNA, protein and catalytic activity in Hepa 1c1c7 cells (Korashy and El-Kadi, 2004).

The transcriptional induction of NQO1 by Hg^{2+} was supported by a series of pieces of evidences. First, Hg^{2+} was able to induce the constitutive and TCDD- and SUL-mediated induction of NQO1 mRNA in a dose-dependent manner. Second, Hg^{2+} was able to increase the basal and TCDD- and SUL-induced ARE-luciferase activity. These results are consistent with our previous study in which Hg^{2+} modulates the Nqo1 gene expression at the transcriptional level in Hepa 1c1c7 cells (Korashy and El-Kadi, 2006b). Although a transcriptional mechanism is involved in the Hg^{2+}-mediated up-regulation of the basal and inducible NQO1 mRNA levels, there was still a possibility that a post-transcriptional mechanism might be involved. If Hg^{2+} increases NQO1 mRNA via increasing its stability, an increase in half-life would be expected to take place. In this regard, Act-D chase experiment was carried out to determine the effect of Hg^{2+} on the NQO1 mRNA stability. Our results showed that Hg^{2+} did not significantly alter the NQO1 mRNA half-life, ruling out the role of a post-transcriptional mechanism. The sustained elevation of basal and TCDD and SUL-mediated induction of NQO1

mRNA, protein and catalytic activity prompted further investigation to examine whether Hg^{2+} could modify the stability of NQO1 protein. In this context, the stability of NQO1 protein did not appear to be significantly altered by Hg^{2+} up to 48h. The result of this experiment indicates a lack of a post-translational regulation of the NQO1 by Hg^{2+}.

To address whether Hg^{2+} exerts its effect through enhancing the transcription of Nrf2, we examined the effect of Hg^{2+} on the Nrf2 mRNA. Our results demonstrated that Hg^{2+} did not affect the mRNA of Nrf2, further confirming that the induction of NQO1 gene expression by Hg^{2+} was not due to any increase in the expression of Nrf2. Although the transcription of Nrf2 was not affected by Hg^{2+} treatment, it was still important to see if Hg^{2+} would stabilize the already existing Nrf2 protein. Our results demonstrated that Hg^{2+} increases Nrf2 stability over the time course of 24 h. In agreement with our findings, a previous study reported that Hg^{2+} increased the Nrf2 protein levels in human monocytes at 6 h (Wataha *et al.*, 2008). To further testify whether the stabilization effect of Hg^{2+} on Nrf2 protein is reflected in its distribution we measured Nrf2 protein in cytosolic and nuclear fractions of HepG2 cells at 6 h. Although there was a limitation of using loading control, such as actin in nuclear extract, few pieces of evidence have supported that (Bettinger *et al.*, 2004). Our results demonstrated that Hg^{2+} increased Nrf2 protein nuclear accumulation which was accompanied by a decrease in its cytosolic protein levels. Thus, these results confirm that the induction of NQO1 by Hg^{2+} is mediated through activation of Nrf2. In this regard, it has been previously demonstrated that NQO1 inducing agents through the Nrf2

pathway dissociate Nrf2 from Keap1, causing stabilization and nuclear accumulation of Nrf2 (Nioi *et al.*, 2003; Nioi and Hayes, 2004). In addition to Nrf2, we have previously reported that activation of activator protein-1 (AP-1), and/or nuclear factor-κB (NF-κB) signaling pathways are also involved in the modulation of NQO1 gene expression by Hg^{2+} in Hepa 1c1c7 cells (Korashy and El-Kadi, 2008b). Therefore, there is a possibility that AP-1 and/or NF-κB may directly or indirectly be involved in the modulation of NQO1 expression by Hg^{2+} in human hepatoma HepG2 cells as well.

Interestingly, it has been shown that ARE-dependent transcriptional activation of the NQO1 gene requires the activation of Nrf2 that would consequently bind to ARE and subsequently activate NQO1 transcription (Rushmore and Kong, 2002; Ma *et al.*, 2004; Miao *et al.*, 2005; Xu *et al.*, 2005). Nrf2 is a basic leucine zipper transcriptional protein that is degraded via the 26s proteasomal pathway (Itoh *et al.*, 1997). In the current study our results showed that transfecting HapG2 cells with Nrf2 siRNA significantly decreased the constitutive and inducible NQO1 gene expressions. Consistent with our results, previous findings were observed in Nrf2-knokout mice embryonic fibroblast cells (Chen and Kunsch, 2004; Jaiswal, 2004; Ma *et al.*, 2004). In addition, transfecting HepG2 cells with siRNA for Nrf2 significantly decreased the Hg^{2+}-mediated induction of NQO1 mRNA and catalytic activity by approximately 90%. These results demonstrate that the induction of NQO1 by Hg^{2+} is mainly due to activating the Nrf2/ARE signaling pathway.

Since the NQO1 is also regulated by the AhR, it was of importance to

investigate whether the decreased expression of NQO1 in the Nrf2 siRNA transfected cells could be due to a decrease in the expression of the AhR as a transcriptional factor controlling it. For this purpose, we examined the effect of Nrf2 siRNA on the constitutive expression of AhR mRNA levels. Our results showed that in HepG2 cells transfected with Nrf2 siRNA the AhR mRNA levels were lower than in non-transfected cells. These findings are in agreement with our recent report in that the AhR and Cyp1a1 mRNA levels were lower in the Nrf2 knockout mice compared to the wild type mice (Anwar-Mohamed *et al.*, 2012). Moreover, previous studies have shown that Nrf2 regulates the transcription of AhR through an ARE available in its enhancer region (Shin *et al.*, 2007; Yeager *et al.*, 2009). From these previous studies we can summarize that the cross-talk between Nrf2 and AhR is evident by the presence of XRE element in the Nrf2 promotor (Miao *et al.*, 2005) and the presence of ARE element in the AhR promotor (Shin *et al.*, 2007). Therefore, our results demonstrated that the AhR level is dependent on the expression of Nrf2.

Nrf2 siRNA does not only influence phase II drug metabolizing enzymes but it also affects phase I drug metabolizing enzymes, typified by CYP1A1. Our findings have shown that the TCDD-mediated induction of CYP1A1 mRNA is significantly decreased in cells transfected with Nrf2 siRNA compared to non-transfected cells. Thus, the effect of Nrf2 knockout on the AhR at the mRNA level has a consequent effect on its downstream target CYP1A1.

Despite the fact that Hg^{2+} increases NQO1 expression through activating Nrf2, which is considered an important cellular defense mechanism, it still

remains undeniably a carcinogenic agent. The reason behind this claim is that the induction of NQO1 expression via Nrf2 by Hg^{2+} is a necessity to protect the cells from Hg^{2+}-mediated toxic effects. In this context, it has been recently demonstrated that activation of Nrf2 by isothiocyanates causes a decrease in Hg^{2+} accumulation and cytotoxicity during exposure of mice to methylmercury (MeHg). An explanation offered to explain this phenomenon is that isothiocyanates activate Nrf2, resulting in up-regulation of glutamyl cysteine ligase (GCL), GSTs and multi-drug resistance proteins (MRPs) which are responsible for conjugation of MeHg with GSH and excretion of the MeHg-GSH adduct into the extracellular space. As such, increased Nrf2 activation was associated with a reduction in the cellular and organ levels of mercury and substantial suppression of MeHg-induced cytotoxicity and intoxication in both primary hepatocytes and wild-type but not in Nrf2-dificient mice (Toyama *et al.*, 2011). This was in agreement with previous work which reported that Nrf2 deletion significantly enhances MeHg accumulation and cytotoxicity in primary mouse hepatocytes (Toyama *et al.*, 2011). Thus, it is apparent that activation of Nrf2 in response to Hg^{2+} is a defense mechanism by which the cells respond to protect against Hg^{2+}-induced cytotoxicity.

In conclusion, the present study demonstrated that Hg^{2+} up-regulates human NQO1 primarily through a transcriptional mechanism. Although Hg^{2+} is considered a toxic metal, we have demonstrated that Hg^{2+} may have cytoprotective properties by increasing the expression of NQO1, which is considered a carcinogenesis-preventing enzyme. Furthermore, Nrf2 is involved in

the regulation of NQO1 by Hg^{2+}.

4.1.3. Mercury Modulates Cytochromes P450 1a1, 1a2 and 1b1 in C57Bl/6 Mice: in vivo and in vitro Studies

The current study provides the first evidence that Hg^{2+} differentially modulates the constitutive and TCDD-inducible Cyp1a and Cyp1b1expression and activity in C57BL/6J mouse liver and isolated mouse hepatocytes. Our results showed that Hg^{2+} alone did not affect liver Cyp1a1, Cyp1a2, or Cyp1b1 at the mRNA, protein or catalytic activity levels, with the exception of Cyp1b1 protein expression levels. Importantly, Hg^{2+} inhibited the TCDD-mediated induction of liver Cyp1a1 and Cyp1a2 mRNA levels at 6 h while significantly potentiating the TCDD-mediated induction of liver Cyp1a1, Cyp1a2 and Cyp1b1 mRNA levels at 24 h. Interestingly, Hg^{2+} potentiated the TCDD-mediated induction of liver Cyp1a1, Cyp1a2, and Cyp1b1 protein expression levels with a concomitant potentiation in the EROD and MROD catalytic activity levels.

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

release of other physiological modulators such as bilirubin or billiverdin which are known AhR ligands (Denison and Nagy, 2003).

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

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

genetic manipulations in Hepa 1c1c7 cells which could have affected the AhR regulatory pathway.

The transcriptional regulation of *Cyp1a1* gene expression by Hg^{2+} was also investigated. In this context, we have demonstrated that Hg^{2+} in the presence of TCDD was able to significantly decrease the AhR-dependent luciferase reporter gene expression (Fig. 3.4). Thus, the down-regulation of *Cyp1a1* gene expression by Hg^{2+} was mediated through an AhR-dependent mechanism. The ability of Hg^{2+} to inhibit Cyp1a1 at the activity level more than that observed effect on the mRNA or protein expression levels in isolated mouse hepatocytes raised the question whether there is a post-translational modification that might have occurred to the Cyp1a1 protein. Evidence from our laboratory and others suggests a role of HO-1 in the Hg^{2+}-mediated decrease in Cyp1a1 catalytic activity levels in Hepa 1c1c7 cells and HepG2 cells (Korashy and El-Kadi, 2005; Amara *et al.*, 2010).

In this study, we have shown that Hg^{2+} significantly increased the HO-1 mRNA level at all concentrations tested, implying the possibility that Hg^{2+} might have decreased Cyp1a1 activity through degrading its heme content via HO-1. HO-1, an enzyme of 32 kDa, catalyzes the oxidative conversion of heme into billiverdin, which serves an important role in protecting cells from oxidative damage, such as 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 results of the current

study are in agreement with our previous published data in which Hg^{2+} was shown to be able to significantly induce HO-1 mRNA expression in Hepa 1c1c7 and HepG2 cells (Korashy and El-Kadi, 2005; Amara *et al.*, 2010). The fact that Hg^{2+} induces HO-1 with a consequent decrease in the heme pool could result in the failure to form a functioning Cyp1a1 protein. Moreover, the apoprotein would be more susceptible to proteasomal degradation (Anwar-Mohamed *et al.*, 2009). However, this might have not occurred at the *in vivo* level due to the fact that HO-1 was not persistently elevated, and thus its effect could not be seen at the activity level.

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

An important difference between our current *in vivo* and *in vitro* studies with regard to the effect of Hg^{2+} on the TCDD-mediated induction of Cyp1a1 mRNA, protein, and catalytic activity is the distribution of Hg^{2+} which might have contributed to the differential expression between both *in vitro,* which is expressed as the direct effect of the metal and *in vivo* which is applying an

indirect effect (Sundberg *et al.*, 1998). As such, it has been demonstrated previously that metallic mercury is preferentially deposited in the kidneys and lungs over other vital organs in mice (Kim *et al.*, 1995). However, at the *in vitro* level, Hg^{2+} was persistently present with the cells throughout the treatment time-course. Thus, the effect we observe *in vitro* is the direct effect of Hg^{2+} which would be the opposite case in the *in vivo* situation.

It is well established that Hg^{2+} has higher binding capacity with thiol groups such as in glutathione (GSH) which plays an important role in Hg^{2+} metabolism (Clarkson, 1997; Custodio *et al.*, 2005). Whereas human hemoglobin has six free thiol groups, one in each of the α-chains and two in each of the β-chains, hemoglobin of other vertebrates may have several numbers of thiol functions (Lawn *et al.*, 1980; Wilson *et al.*, 1980). Rats for example, have ten reactive thiol functions per hemoglobin, three in each α-chain and two in the β-chain (Chua *et al.*, 1975; Radosavljevic and Crkvenjakov, 1989). A previous study showed that about 80% of mercury can be recovered in red blood cells (RBC) (Trumpler *et al.*, 2009). Since hemoglobin is the most highly abundant constituent of RBC besides water, a reaction between mercury and hemoglobin is expected. In this context, binding between mercury and hemoglobin has been proven (Janzen *et al.*, 2011). Furthermore, Hg^{2+} even at low concentrations, was able to make an adduct formation with hemoglobin which in turn lead to increase plasma hemoglobin levels (Janzen *et al.*, 2011).

The fact that Hg^{2+} possess as higher capacity for binding to thiol groups such as in hemoglobin, which in turn leads to increased Hb levels in the plasma,

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

In this context, mechanistic experiments have been carried out to explore whether the effect of Hb on Cyp1a1 luciferase activity is occurring through the AhR signaling pathway. Firstly, we tested the effect of Hb on Cyp1a1 protein expression using isolated mouse hepatocytes. Our results showed that Hb significantly induces the Cyp1a1 protein in a dose-dependent manner. Secondly we investigated its effect on Cyp1a1 catalytic activity levels in the absence and presence of a known AhR antagonist; resveratrol (a competitive inhibitor of AhR-

ligands) in isolated mouse hepatocytes. Our results showed that Hb was able to induce Cyp1a1 catalytic activity levels in a concentration-dependent manner. On the other hand, resveratrol significantly inhibited the Hb-mediated induction of Cyp1a1 activity, suggesting the involvement of an AhR-dependent mechanism. In agreement with our findings, it would be reasonable to postulate that this effect could not be due to the direct effect of Hb but rather its metabolic products heme, bilirubin, and biliverdin which are previously known AhR ligands (Sinal and Bend, 1997; Phelan *et al.*, 1998).

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

4.1.4. Effect of Mercury on Aryl Hydrocarbon Receptor-Regulated Genes in the Extrahepatic Tissues of C57Bl/6 Mice.

In the current study we have demonstrated that Hg^{2+} modulates the constitutive and TCDD-inducible AhR-regulated genes in a time-, tissue-, and AhR-regulated enzyme genes manner. The results of the current study have been summarized in Table 2. Our findings showed that Cyp1a1, Cyp1a2, Cyp1b1,

Nqo1, Gsta1 and HO-1 are constitutively expressed in all the examined tissues. Moreover, Nqo1 and HO-1 were the highest constitutively expressed genes, followed by Cyp1a1, Cyp1b1, Gsta1 and lastly Cyp1a2 in the kidney of C57Bl/6 mice. In the lung, 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 the current study.

In the current study Hg^{2+} significantly increased Cyp1a1 mRNA levels in the lung and heart but not in the kidney of C57Bl/6 mice. Furthermore, Hg^{2+} significantly induced Cyp1a2 and Cyp1b1 mRNA levels in the kidney and heart of C57Bl/6 mice while it significantly inhibited its mRNA levels in the lung. At the protein and catalytic activity levels, Hg^{2+} significantly increased the protein expression levels of Cyp1a and Cyp1b1 in the kidney with a subsequent increase in EROD and MROD activities. In addition Hg^{2+} significantly induced Cyp1b1 but not Cyp1a in the lung with a subsequent inhibition of EROD without affecting MROD activities.

Our results, despite the discrepancies across organs, is in agreement with

previously published work from our laboratory and others in which it was shown that Hg^{2+} alone was able to induce Cyp1a1 mRNA, protein and catalytic activity, in a concentration- and time-dependent manner in mouse cell lines derived from liver and shark rectal gland cells (Ke *et al.*, 2002; Korashy and El-Kadi, 2004). An important difference between our current study and the previous *in vitro* studies is the tissue distribution of Hg^{2+} which might have contributed to the differential expression across organs (Sundberg *et al.*, 1998). As such, it has been demonstrated previously that metallic mercury is preferentially deposited in the kidneys and lungs over other vital organs in mice (Kim *et al.*, 1995).

In all tested tissues TCDD alone significantly increased Cyp1a1, Cyp1a2, and Cyp1b1 mRNA, protein, as well as EROD and MROD catalytic activities. Despite these significant inductions, TCDD did not increase the expression of these genes at the mRNA, protein, or catalytic activity levels to the same extent across tissues or within a single tissue. An important factor that might have contributed to these differences across tissues is the expression level of AhR co-regulatory proteins such as co-activators and co-repressors in different tissues (Nishimura *et al.*, 2003; Nishimura and Naito, 2006). Of interest, the differences in fold of induction between these genes in a single tissue could be also explained by the presence of different quantities and qualities of XRE promoters in each of these genes, which is undeniably another important factor.

When animals were co-exposed to Hg^{2+} and TCDD at 6 h, Hg^{2+} significantly inhibited Cyp1a1 and Cyp1b1 mRNA levels in the kidney and Cyp1a2 mRNA level in the lung while there was no significant effect in the heart.

On the contrary, at 24 h Hg^{2+} further potentiated kidney and lung Cyp1a1, Cyp1a2, and Cyp1b1, and heart Cyp1a1 mRNA levels, while heart Cyp1a2 mRNA levels were significantly inhibited. In the kidney, Hg^{2+} significantly inhibited TCDD-mediated induction of Cyp1a and Cyp1b1 protein levels with a subsequent inhibition in the EROD and MROD catalytic activities. Lastly, in the lung, Hg^{2+} significantly potentiated the TCDD-mediated induction of Cyp1a and Cyp1b1 protein expression levels while it did not affect EROD and MROD catalytic activities.

To the best of our knowledge, this is the first study to examine the effect of co-exposure to Hg^{2+} and TCDD on the AhR-regulated P450s in extrahepatic tissues. The differences between the results of mRNA expression obtained at 6 h and 24 h of treatment could be due to the long half-life of mercury which is 155 h (Sundberg *et al.*, 1998). Thus, we hypothesize that the effect observed at 6 h is the direct effect of Hg^{2+}, while, the effect at 24 h is an indirect effect that could have arisen due to other endogenous mediators.

Unlike the *in vitro* studies where it was proven that Hg^{2+}-induced HO-1 plays a crucial role in decreasing TCDD-mediated induction of Cyp1a1 catalytic activity through degrading its heme content (Amara *et al.*, 2010), in the current study the effect of HO-1 on EROD and MROD catalytic activities was not observed. Another explanation offered to explain this discrepancy is that it has been previously demonstrated that Hg^{2+} induces HO-1 through an activator protein-1 (AP-1) binding site present in its promoter (Alam *et al.*, 1995). Another explanation for the observed discrepancy could be the time-dependent factor of

inducible HO-1. In this context, previous studies have postulated that HO-1 mRNA and protein half-lives are 1.6 and 8 h, respectively (Panchenko *et al.*, 2000; Lam *et al.*, 2005). These previous findings are consistent with our HO-1 mRNA and protein results in which we have demonstrated that HO-1 mRNA and protein expression could be only detected at 6 h but not at 24 h (Panchenko *et al.*, 2000; Lam *et al.*, 2005).

With regard to AhR-regulated phase II enzymes, Hg^{2+} or TCDD alone significantly induced Nqo1 and Gsta1 mRNA, protein, and catalytic activity levels. Importantly, the co-exposure to Hg^{2+} and TCDD significantly potentiated the TCDD-mediated induction of Gsta1 mRNA at both time points tested, and Nqo1 mRNA at 24 h only. The regulation of Nqo1 and Gsta1 involves, in addition to the AhR-XRE, the antioxidant response element (ARE). In the current study it is not clear if Hg^{2+} potentiated the TCDD-mediated effects or TCDD potentiated the Hg^{2+}-mediated effects on the phase II AhR-regulated genes. However, according to the literature, TCDD is considered a bi-functional inducer as it induces phase I and phase II AhR-regulated genes (Kohle and Bock, 2006), and a mono-functional inducer as it induces these genes mainly through activating AhR (Kohle and Bock, 2006). In contrast, Hg^{2+} is well known to be an oxidative stress inducer (Korashy and El-Kadi, 2008c). With this in mind, it is thus expected that Hg^{2+} would significantly induce phase II AhR-regulated genes through activating the redox sensitive transcription factor, Nrf2. Our recent finding indicates that Hg^{2+} induces Nqo1 through the Nrf2-ARE signaling pathway in an *in vitro* model (Amara and El-Kadi, 2011). Nonetheless, it is still not clear whether or not a

similar mechanism would occur at the *in vivo* level.

In conclusion, the present study demonstrates for the first time that Hg^{2+}modulates the constitutive and TCDD-induced AhR-regulated genes in a time-, tissue-, and molecular level-dependent manner. Furthermore, the effect on one of these genes could not be generalized to other genes despite the fact that it is an AhR-regulated gene, as there are multiple factors that could act separately or concomitantly to cause differential effects.

Table 4.1.

Summary of the effects of Hg^{2+} and TCDD on the expression of AhR-regulated genes in kidney, lung and heart

Treatment	Gene	Tissues Kidney		Lung		Heart	
		6h	24h	6h	24h	6h	24h
Hg^{2+}	Cyp1a1	↔ mRNA	↔ mRNA, ↑ protein, ↑ activity	↑ mRNA	↔ mRNA, ↔ protein, ↓ activity	↑ mRNA	↔ mRNA
	Cyp1a2	↑ mRNA	↔ mRNA, ↑ protein, ↑ activity	↓ mRNA	↔ mRNA, ↔ protein, ↔ activity	↑ mRNA	↔mRNA
	Cyp1b1	↑ mRNA	↔ mRNA, ↑ protein, ↑ activity	↔ mRNA	↔ mRNA, ↑ protein, ↔ activity	↑ mRNA	↔mRNA
	Nqo1	↑mRNA	↑ mRNA, ↑ protein, ↑ activity	↔ mRNA	↔ mRNA, ↑ protein, ↔ activity	↑mRNA	↑mRNA
	Gst 1a	↑mRNA	↑ mRNA, ↑ protein, ↑ activity	↔ mRNA	↔ mRNA, ↔ protein, ↔ activity	↑mRNA	↑mRNA
	HO-1	↑ mRNA	↓ mRNA ↑ protein N/M	↑ mRNA	↔ mRNA ↑ protein N/M	↑ 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
	Cyp1b1	↑mRNA	↑ mRNA, ↑ protein, ↑ activity	↑mRNA	↑ mRNA, ↑ protein, ↑ activity	↑ mRNA	↑ mRNA
	Nqo1	↑mRNA	↑ mRNA, ↑ protein, ↔ activity	↑ mRNA	↑ mRNA, ↑ protein, ↔ activity	↑mRNA	↑ mRNA
	Gst 1a	↑mRNA	↑ mRNA, ↑ protein, ↑activity	↔ mRNA	↔ mRNA, ↑ protein, ↑activity	↑mRNA	↑ mRNA
	HO-1	↓ mRNA	↑ mRNA, ↓ protein, N/M	↔ mRNA	↔ mRNA, ↓ protein, N/M	↔ mRNA	↔ mRNA
Hg^{2+} +	Cyp1a1	↓ mRNA	↑ mRNA, ↓ protein, ↓ activity	↔ mRNA	↑ mRNA, ↑ protein, ↔ activity	↔ mRNA	↑ mRNA
TCDD	Cyp1a2	↔mRNA	↑ mRNA, ↓ protein, ↓ activity	↓ mRNA	↑ mRNA, ↑ protein, ↔ activity	↔ mRNA	↓ mRNA
	Cyp1b1	↓ mRNA	↑ mRNA, ↓ protein, ↓ activity	↔ mRNA	↑ mRNA, ↑ protein, ↔ activity	↔ mRNA	↔ mRNA
Versus	Nqo1	↔ mRNA	↑ mRNA, ↑ protein, ↑ activity	↔ mRNA	↑ mRNA, ↑ protein, ↑ activity	↔ mRNA	↑ mRNA
	Gst 1a	↑mRNA	↑ mRNA, ↑ protein, ↑ activity	↑ mRNA	↑ mRNA, ↑ protein, ↑ activity	↔ mRNA	↑ mRNA
TCDD	HO-1	↑ mRNA	↑ mRNA, ↑ protein, N/M	↑ mRNA	↔ mRNA, ↑ protein, N/M	↑ mRNA	↔ mRNA

(↑) increase; (↓) decrease; (↔) no change; (N/M) not measured

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

MeHg is a highly toxic non-essential environmental element that is neither created nor biodegradable (Barbier *et al.*, 2005). The concentrations of MeHg used in the present study were chosen after determining the ability of a wide range of concentrations to modulate the expression of CYP1A1 without significantly affecting HepG2 cell viability (Fig. 3.41). Moreover, the estimated human plasma and tissue concentrations of mercury in individuals without known exposure have been reported to be in the µM range (Gerhardsson *et al.*, 1988; Tezel *et al.*, 2001) and within the range that might be expected to be encountered in the environment (Vakharia *et al.*, 2001a; Vakharia *et al.*, 2001b; ATSDR, 2011; CEPA, 2012). Taken together, chronic human exposure to this long half-life toxic metal in addition to the high risk of accumulation in the body tissues, particularly the liver (Barbier *et al.*, 2005), makes the concentrations used in the present study of high relevancy to the corresponding human plasma and tissue levels and calculated exposure levels in the environment (ATSDR, 2011; CEPA, 2012).

The capacity of mercury to modulate the induction of CYP1A1expression by TCDD has been reported previously from our laboratory and others in murine Hepa 1c1c7 cells, human hepatoma HepG2 and human hepatocyte cultures, at the mRNA, protein, and catalytic activity levels (Vakharia *et al.*, 2001a; Korashy and El-Kadi, 2005; Amara *et al.*, 2010). However, our efforts along with others were mainly concerned with inorganic mercury, namely mercuric chloride (Hg^{2+}).

Therefore, it was of importance to investigate whether other forms of mercury, especially organic forms, such as MeHg, will exhibit an effect on CYP1A1 mRNA, protein, and catalytic activity levels. In this regard, the potential effect of MeHg on the induction of CYP1A1 gene expression by TCDD was assessed. Our results have shown that MeHg was not able to alter the TCDD-mediated induction of CYP1A1 at the mRNA or protein expression levels at any of the concentrations tested. In contrast to these results, our previous work using Hg^{2+} showed that Hg^{2+} was able to significantly inhibit the TCDD-mediated induction of CYP1A1 at the mRNA and protein levels in HepG2 cells at the highest concentration tested (10 µM) (Amara *et al.,* 2010). The discrepancy between previous and current work might be due to the concentration of mercury, where the effective concentration in previous study was 10 µM. However in the present study and based on our cell viability test, we were limited to using MeHg up to 5 µM only. In addition, our current results imply that each form of mercury have different effects on the TCDD-mediated induction of CYP1A1 gene expression. Despite these differences, at the catalytic activity levels MeHg significantly inhibited the TCDD-mediated induction of CYP1A1 catalytic activity in a dose-dependent manner, which is consistent with previously reported observations from our laboratory and others that Hg^{2+} significantly reduced CYP1A1 induction mediated by TCDD and benzo[a]pyrene derivatives in HepG2 cells and isolated human hepatocytes (Vakharia *et al.*, 2001a; Vakharia *et al.*, 2001b; Amara *et al.*, 2010). This inhibitory effect on the TCDD-mediated induction of CYP1A1 was not specific to mercury *per se*. As such, several other heavy metals such as lead

(Korashy and El-Kadi, 2012), arsenic (Anwar-Mohamed and El-Kadi, 2010) and vanadium (Abdelhamid *et al.*, 2010) exhibited similar inhibitory effects on the TCDD-mediated induction of CYP1A1 at the mRNA, protein, and catalytic activity levels in HepG2 cells. Although, MeHg did not affect the TCDD-mediated induction of CYP1A1 at the mRNA level, there was still a possibility that a transcriptional mechanism might be involved. Therefore, we examined the effect of MeHg on the XRE-driven luciferase activity in transiently transfected HepG2 cells with the XRE-driven luciferase reporter plasmid pGudLuc6.1. Our results showed that MeHg alone or in the presence of TCDD did not significantly affect the XRE-driven luciferase activity (Fig. 3.44).

Interestingly, the observation that MeHg-mediated inhibition of the TCDD-mediated induction of CYP1A1 occurred only at the catalytic activity levels suggested a possible involvement of a posttranslational mechanism(s). In this context, several posttranslational mechanisms, such as phosphorylation, proteasomal degradation, modulation of HO-1 gene expression, and the possible competition between MeHg and the CYP1A1 substrate 7-ethoxyresorufin have been reported previously in different cell lines (Werlinder *et al.*, 2001; Korashy and El-Kadi, 2005; Amara *et al.*, 2010). For this purpose, we performed further experiments as follows first; we examined the possible direct inhibitory effect of MeHg on CYP1A1 catalytic activity. Our results showed that MeHg did not directly affect the CYP1A1 activity, suggesting that a competitive inhibitory mechanism was not involved in the modulation of CYP1A1 activity by MeHg. Second; it has been previously reported that MeHg is able to decrease glucose-6-

phosphate dehydrogenase (G6PD) in yeast (Tsuzuki and Yamada, 1979). In addition, the deficiency in G6PD decreases the ability of the cells to generate NADPH (Scott *et al.*, 1991), which is required for CYP450 activity. Therefore, we examined whether supplementing cells with excess of NADPH would restore the TCDD-mediated induction of CYP1A1 activity that was decreased by MeHg. Our results showed that MeHg preserved its inhibitory effect on the TCDD-mediated induction of CYP1A1 activity despite supplementation of the enzymatic system with excessive NADPH. Thus, the effect of MeHg on the TCDD-mediated induction of CYP1A1 activity does not involve an effect on intracellular NADPH levels. Third; HO-1 gene expression, a rate-limiting enzyme in heme catabolism, has been shown to alter content of cellular heme, the prosthetic group of CYP450, and hence the enzymatic activity of heme containing enzymes (Kikuchi *et al.*, 2005). Interestingly, our results showed that MeHg-mediated inhibition of the TCDD-mediated induction of CYP1A1 activity was accompanied with a proportional increase in HO-1 mRNA levels. These results imply that MeHg might have decreased CYP1A1 activity through degrading its heme content via the induction of HO-1. Although total heme content was not measured in the current study, we have previously demonstrated in Hepa 1c1c7 cells that Hg^{2+} caused a significant decrease in heme availability, causing a subsequent reduction in Cyp1a1 activity, suggesting a direct correlation between HO-1 and Cyp1a1 (Korashy and El-Kadi, 2005).

In the current study, the role of HO-1 in the inhibition of CYP1A1 at the catalytic activity level is supported by series of pieces of evidences. First; the

inhibition of HO-1 activity by administration of SnMP, a specific inhibitor of HO-1, completely abolished the MeHg-mediated inhibition of the TCDD-mediated induction of CYP1A1 activity. In this regard, it has been previously shown that the effect of SnMP is solely occurring through competitive inhibition of HO-1 protein and not through altering CYP1A1 expression (Amara *et al.*, 2010; Anwar-Mohamed and El-Kadi, 2010). Second, knocking down of HO-1 using siRNA against HO-1 reversed the MeHg-mediated inhibition of TCDD-mediated induction of CYP1A1 activity. In agreement with our results, data from our laboratory and others have previously demonstrated that in HepG2 cells, knocking-down HO-1 partially reverses the effect of metals such as Hg^{2+} and As^{3+} on the TCDD-mediated induction of CYP1A1 catalytic activity (Bessette *et al.*, 2009; Amara *et al.*, 2010; Anwar-Mohamed and El-Kadi, 2010).

Lastly, we investigated whether the inhibitory effect of MeHg is specific for CYP1A1 or whether AhR-regulated genes such as NQO1 are also affected. Therefore, the effect of MeHg on the expression of NQO1 was examined. Our findings show that MeHg was able to significantly increase the constitutive and TCDD-inducible NQO1 mRNA and protein levels, which is consistent with our previous findings with Hg^{2+} (Amara and El-Kadi, 2011), confirming that the inhibitory effect of MeHg on the induction of CYP1A1 is specific. With the fact that *NQO1* along with *HO-1* are up-regulated in response to oxidative stress stimuli, such as hydrogen peroxide, through the activation of ARE in their promoter regions, we wanted to examine the effect of MeHg on the ARE-driven luciferase activity. Our results demonstrated that MeHg increases ARE-luciferase

activity. In this context, several studies have reported the capability of heavy metals, including Hg^{2+} (Stohs and Bagchi, 1995; Kaliman *et al.*, 2001), to induce HO-1. Furthermore, in mouse isolated hepatocytes it was shown that MeHg increases the nuclear accumulation of the transcription factor nuclear factor erythroid 2-like 2 (Nrf2) (Lamsa *et al.*, 2010) which is the transcription factor that binds to and activates the ARE initiating the transcription of NQO1 and HO-1. An explanation offered for this phenomenon is that metal-mediated increase in oxidative stress, including the production of reactive oxygen species (ROS) (Stohs and Bagchi, 1995; Kaliman *et al.*, 2001) and lipid peroxidation (Stohs and Bagchi, 1995; Ossola *et al.*, 1997) might be in part responsible for the nuclear accumulation of Nrf2 and the subsequent activation of the ARE (Inamdar *et al.*, 1996). Taken together, we suggest that induction of ROS by Hg^{2+} (Korashy and El-Kadi, 2005) triggers ARE activation and induction of NQO1 in addition to HO-1 which subsequently decreases CYP1A1 activity.

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

4.2. GENERAL CONCLUSION

In the current study we have demonstrated that Hg^{2+} significantly inhibited the TCDD-mediated induction of CYP1A1 expression at mRNA, protein and catalytic activity levels in human hepatoma HepG2 cells and

isolated mouse hepatocytes. However, it was able to induce the constitutive and inducible NQO1expression. Our data clearly showed that Hg^{2+} may decrease the carcinogenicity and muatgenicity of AhR ligands by decreasing the induction of *CYP1A1* while enhancing the induction of *NQO1* genes. Particularly, co-administration of AhR ligands with Hg^{2+} significantly potentiated NQO1 at the mRNA, protein and activity levels. However, it inhibited the TCDD-mediated induction of CYP1A1 mRNA, protein and catalytic activity levels. Furthermore, both forms of mercury (Hg^{2+} and MeHg) were able to significantly increase the expression of HO-1 which coincided with the decrease in phase I and the increase in phase II activities. HO-1, which is regulated by Nrf2 transcription factor, is partially involved in the Hg^{2+}-mediated down-regulation of CYP1A1 at the catalytic activity level, as treatment with HO-1 inhibitor, tin mesoporphyrin, or hemin (heme precursor) or transfecting the HepG2 cells with siRNA against HO-1 caused a partial restoration of the inhibition of TCDD-mediated induction of CYP1A1 catalytic activity caused by Hg^{2+}.

The current study provided the first evidence that Hg^{2+} modulates *CYP1A1* gene expression at the transcriptional and post-translational levels through an AhR- dependent pathway. At the transcriptional level, co-exposure to Hg^{2+} and TCDD significantly decreased the TCDD-mediated induction of AhR-dependent luciferase reporter gene expression. Moreover, the CYP1A1 mRNA and protein decay experiments showed that Hg^{2+} did not significantly affect the half-life of mRNA; however, it significantly decreased the degradation rate of its protein, implying a post-translational regulation of the

CYP1A1by Hg^{2+}. A post-translational mechanism and the role of HO-1 in Hg^{2+}-mediated decrease of CYP1A1 catalytic activity was also confirmed by treating the cells with an organic form of Hg such as MeHg.

On the other hand, Hg^{2+} induced the expression of *NQO1* genes at the transcriptional level. This was supported by a series of pieces of evidences. First, Hg^{2+} was able to induce the constitutive and TCDD- and SUL-mediated induction of NQO1 mRNA in a dose-dependent manner. Second, Hg^{2+} was able to increase the basal and TCDD- and SUL-induced ARE-luciferase activity. The role of Nrf2 in Hg-mediated increases in the expression of *NQO1* genes at the basal and inducible levels was supported by the capability of Hg^{2+} to increase the protein stability and consequently the nuclear accumulation of Nrf2 protein, while decreasing its cytosolic level. Thus the induction of *NQO1* gene expression by Hg^{2+} is due to increasing the stability and nuclear accumulation of Nrf2 protein via increasing its shuttling from the cytosol to the nucleus.

The current study also provides the first evidence that Hg^{2+} differentially modulates the constitutive and TCDD-inducible Cyp1a and Cyp1b1expression and activity in C57BL/6J mouse liver and isolated mouse hepatocytes. In that, Hg^{2+} inhibited the TCDD-mediated induction of liver Cyp1a1 and Cyp1a2 mRNA levels at 6 h while significantly potentiating the TCDD-mediated induction of liver Cyp1a1, Cyp1a2 and Cyp1b1 mRNA levels at 24 h. Moreover, Hg^{2+} potentiated the TCDD-mediated induction of liver Cyp1a1, Cyp1a2, and Cyp1b1 protein expression levels with a concomitant potentiation in the EROD and MROD catalytic activity levels. In this work, the discrepancy

was also between *in vivo* and *in vitro* studies with regard to the effect of Hg^{2+} on the TCDD-mediated induction of Cyp1a1 mRNA, protein, and catalytic activity, which might be due to the distribution of Hg^{2+} between both in vitro which is expressed the direct effect of the metal, and in vivo, which applies an indirect effect (Sundberg *et al.*, 1998). We also demonstrated that Hg^{2+} has the capability to increase Hb levels in the serum. Thus, the in vivo effect of Hg^{2+} on the TCDD-mediated induction of Cyp1a1 mRNA, protein, and catalytic activity levels at 24 h might be attributed to the direct effect of Hb on AhR signaling or due to an indirect effect through heme release or heme degradation by-products (billiverdin and bilirubin). Furthermore, our finding demonstrated that Hb significantly induces Cyp1a1 protein and catalytic activity in a dose-dependent manner.

Moreover, resveratrol (AhR antagonist) significantly inhibited the Hb-mediated induction of Cyp1a1 activity, suggesting the involvement of an AhR-dependent mechanism. The effect of Hg^{2+} indicates more complex regulation of Cyp1a1 at the *in vivo* level as there are several factors that can confound the *in vivo* results which are not present *in vitro*. In addition, caution should be taken when extrapolating *in vitro* data to an *in vivo* situation that warrants further investigation.

Finally, yet importantly, this study demonstrated that Hg^{2+} modulates the constitutive and TCDD-inducible AhR-regulated phase I and phase II genes in a time-, tissue-, and AhR-regulated enzyme-specific manner in kidney, lung and heart of C57Bl/6J mice. Furthermore, the effect on one of these genes could not

be generalized to other genes despite the fact that it is an AhR-regulated gene, as there are multiple factors that could act separately or concomitantly to cause differential effects.

4.3. FUTURE DIRECTIONS AND STUDIES

The results obtained from the current research raise several questions that need to addressed and in order to increase our understanding about the potential interactions between metals and AhR ligands. Therefore, further studies need to be conducted:

1. To characterize the cross-talk between AhR and Nrf2 transcription factor proteins in the modulation of AhR-regulated genes by Hg.
2. To examine the role of co-regulatory proteins such as co-activators and co-repressors in mediating the interactions between the AhR and Nrf2.
3. To determine the role of AP-1 and NF-κB signaling pathways in the cross-talk between AhR and Nrf2.
4. To identify the genes that are induced by Hg and mediate the cross-talk between the AhR and redox-sensitive transcription factors using global microarray analysis.
5. To examine the chronic exposure to Hg with and without AhR-ligands on the AhR-regulated phase I and phase II enzymes.

CHAPTER 5- REFERENCES

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