MODULATION OF ARYL HYDROCARBON RECEPTOR (AHR)-REGULATED CARCINOGEN ACTIVATING ENZYMES BY ORGANIC ARSENICALS: A POTENTIAL THERAPEUTIC TARGET

By

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ABSTRACT

Arsenic is a worldwide environmental pollutant that is associated with skin and several types of internal cancers, such as liver, lung, kidney, urinary bladder, and prostate cancers. Recent reports revealed that organic arsenic metabolites, such as trimethylarsine oxide (TMAO), dimethylarsinic acid (DMA(V)), and monomethylarsonous acid (MMA(III)) could activate the toxic and carcinogenic potential of arsenic. Therefore, the objectives of the current dissertation were to: 1) Investigate the effect of TMAO on the activation of hepatic aryl hydrocarbon receptor (AhR)-regulated genes, and to investigate the underlying mechanisms *in vitro*. 2) Examine the effect of TMAO on modulation of AhR-regulated genes *in vivo* in extrahepatic tissues. 3) Investigate the effect of DMA(V) on the alteration of AhR-regulated genes in the hepatic and extrahepatic tissues. 4) Examine the effects of MMA(III) as compared to its parent compound, As(III), on the expression of prototypical AhR-regulated gene, CYP1A1, *in vitro*. Our *in vivo* results demonstrated that TMAO increased carcinogen activating enzymes Cyp1a1, Cyp1a2, and Cyp1b1, in addition to Nqo1, Gsta1, and Ho-1 at the mRNA level. Upon co-exposure to TMAO and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), TMAO potentiated the TCDD-mediated induction of Cyp1a1, Cyp1b1, and Nqo1 mRNA levels. Western blotting revealed that TMAO increased Cyp1a1, Cyp1a2, Nqo1, Gsta, and Ho-1 protein levels, and potentiated the TCDD-mediated induction of Cyp1a1 and Cyp1b1 at the protein levels. In addition, TMAO significantly increased Cyp1a1, Cyp1a2, Nqo1, Gst, and Ho-1 activities, and significantly potentiated the TCDD-mediated induction of Cyp1a1 activity. At the *in vitro* level, TMAO induced Cyp1a1 and potentiated the TCDD-mediated induction of Cyp1a1 at mRNA, protein and activity levels. In addition, TMAO increased the nuclear localization of AhR and AhR-dependent XRE-driven

luciferase activity. With regard to the effect of TMAO on the extrahepatic tissues, TMAO increased Cyp1a1 and Cyp1b1 mRNA, protein, and activity in the lung. TMAO potentiated the TCDD-mediated induction of Cyp1a1 and Cyp1a2 mRNA, protein and activity in the lung. In the kidney, TMAO increased Cyp1b1 mRNA and protein. TMAO potentiated the TCDD-mediated induction of Cyp1a1 and Cyp1b1 mRNA, protein and activity. In the heart, TMAO potentiated the TCDD-mediated induction of Cyp1a1 and Cyp1b1 mRNA. Moreover, TMAO induced Nqo1 mRNA in the lung, kidney and heart, with subsequent increase in Nqo1 protein and activity in the lung. TMAO increased Gsta1 mRNA in the heart, and increased Gsta protein and activity in the lung and kidney. TMAO increased Nqo1 mRNA compared to TCDD in the kidney and heart, and potentiated the TCDD-mediated induction of Gsta protein and activity in the kidney. As for the DMA(V), our results demonstrated that it has no significant effect on Cyp1a mRNA and protein expression levels or catalytic activity in the liver. On the other hand, DMA(V) significantly potentiated the TCDD-mediated induction of Cyp1a mRNA and protein expression levels, with a subsequent potentiation of catalytic activity in the lung. Moreover, DMA(V) significantly inhibited the TCDD-mediated induction of Cyp1a mRNA and protein expression levels with subsequent inhibition of catalytic activity in the kidney. Regarding phase II AhR-regulated genes, DMA(V) had no significant effect on Nqo1 mRNA and protein expression levels or activity in the liver, lung, or kidney. With regard to MMA(III) as compared to As(III), our *in vitro* results showed that MMA(III) and As(III) decreased CYP1A1 mRNA, protein, and catalytic activity levels, and inhibited the TCDD-mediated induction of CYP1A1 mRNA, protein, and catalytic activity levels. MMA(III) and As(III) significantly inhibited XRE-driven luciferase activity and inhibited the TCDD-mediated induction of XRE-driven luciferase activity, in addition both compounds showed inhibition of nuclear accumulation of the AhR transcription factor. MMA(III) and As(III) had no

effect on CYP1A1 mRNA stability; however, MMA(III), but not As(III), decreased the protein stability of CYP1A1. As(III), but not MMA(III), induced HO-1 mRNA expression level. In addition, both MMA(III) and As(III) increased ROS production. In conclusion, the present work demonstrates for the first time that organic arsenic metabolites modulate phase I and phase II AhR-regulated genes in a tissue-, and enzyme-specific manner. This modulation could potentially participate in arsenic-induced toxicity and carcinogenicity. Our work opened new avenues for treatment of arsenic-induced carcinogenicity via using prodrugs that will be specifically activated inside tumor cells with CYP enzymes. The use of these rational therapies would decrease morbidity and mortality and hence diminish health care costs.

PREFACE

This thesis is an original work by Osama Elshenawy. All experimental procedures involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Sections 3.1 and 4.1 of this thesis have been published as O.H. Elshenawy and A.O.S. El-Kadi, " Modulation of aryl hydrocarbon receptor-regulated enzymes by trimethylarsine oxide in C57BL/6 mice: *In vivo* and *in vitro* studies," Toxicology Letters 2015;238(1):17-31. doi: 10.1016/j.toxlet.2015.06.1646. I was responsible for designing the research, conducting experiments, and analysis as well as the manuscript composition. A. O. El-Kadi was the supervisory author and was involved with concept formation and manuscript composition. Sections 3.2 and 4.2 of this thesis have been published as O.H. Elshenawy and A.O.S. El-Kadi, " Modulation of aryl hydrocarbon receptor regulated genes by acute administration of trimethylarsine oxide in the lung, kidney and heart of C57BL/6 mice," Xenobiotica: the fate of foreign compounds in biological systems 2015;45(10):930-43. doi: 10.3109/00498254.2015.1032385. I was responsible for designing the research, conducting experiments, and analysis as well as the manuscript composition. A. O. El-Kadi was the supervisory author and was involved with concept formation and manuscript composition. Sections 3.3 and 4.3 of this thesis have been published as O.H. Elshenawy, G. Abdelhamid, H.N. Althurwi, and A.O.S. El-Kadi, " Dimethylarsinic acid modulates the aryl hydrocarbon receptor-regulated genes in C57BL/6 mice: *in vivo* study," Xenobiotica: the fate of foreign compounds in biological systems 2017:1-11. doi: 10.1080/00498254.2017.1289423. I was responsible for designing the research, conducting experiments, and analysis as well as the manuscript composition. G. Abdelhamid, and H.N. Althurwi assisted with conducting experiments. G.

Abdelhamid assisted with analysis and contributed to manuscript edits. A. O. El-Kadi was the supervisory author and was involved with concept formation and manuscript composition. Sections 3.4 and 4.4 of this thesis have been published as O.H. Elshenawy, G. Abdelhamid, A.A. Soshilov, M.S. Denison, and A.O.S. El-Kadi, " Down-regulation of cytochrome P450 1A1 by monomethylarsonous acid in human HepG2 cells," Toxicology Letters 2017;270:34-50. doi: 10.1016/j.toxlet.2017.02.012. I was responsible for designing the research, conducting experiments and analysis as well as the manuscript composition. G. Abdelhamid, A.A. Soshilov, and M.S. Denison assisted with conducting experiments, and analysis. G. Abdelhamid, and M.S. Denison contributed to manuscript edits. A. O. El-Kadi was the supervisory author and was involved with concept formation and manuscript composition.

THIS WORK IS DEDICATED TO

MY BELOVED PARENTS AND FAMILY

WITH ALL LOVE AND SINCERITY

OSAMA

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2-OH-E^+	2-Hydroxyethidium
3MC:	3-Methylcholanthrene
8-OHdG:	8-Hydroxydeoxyguanosine
Act-D:	Actinomycin D
AhR:	Aryl Hydrocarbon Receptor
AhRC:	Aryl Hydrocarbon Receptor Complex
AIP:	AhR Interacting Protein
Aldh3:	Aldehyde Dehydrogenase 3 (cytosolic, class 3)
ANOVA:	Analysis of Variance
AP-1:	Activator Protein 1
AP-2:	Activator Protein 2
APL:	Acute Promyelocytic Leukemia
ARA9:	Ahr-Activated 9
ARE:	Antioxidant Response Element
ARNT:	Aryl Hydrocarbon Receptor Nuclear Translocator
As(III):	Arsenite
As(V):	Arsenate
AS3MT:	Arsenic Methyltransferase
ATCC:	American Type Culture Collection
ATO:	Arsenic Trioxide
ATP:	Adenosine Triphosphate

ATSDR:	The Agency For Toxic Substances And Disease Registry
BaA:	Benzo[A]Anthracene
BAL	British anti-Lewisite
BaP:	Benzo[a]Pyrene
BbF:	Benzo[b]Fluoranthene
BEAS-2B:	Human Bronchial Epithelial Cells
bHLH:	Protein Family of Basic Region Helix-Loop-Helix
BkF:	Benzo[k]Fluoranthene
BPDE:	Benzo[a]Pyrene-7,8-Diol-9,10-Epoxide
BVR	Biliverdin Reductase
bZIP:	Basic Leucine Zipper
cDNA:	Complementary DNA
CDNB:	1-Chloro-2,4-Dinitrobenzene
CHX:	Cycloheximide
CNC:	Cap'n'Collar
c-Rel:	NF-κB c-Rel subunit
CYP or Cyp:	Cytochrome P450
CYP1A1 or Cyp1a1:	Cytochrome P450 1A1
CYP1A2 or Cyp1a2:	Cytochrome P450 1A2
CYP1B1 or Cyp1b1:	Cytochrome P450 1B1
CYP2S1 or Cyp2s1:	Cytochrome P450 2S1
CYT19:	Arsenic Methyltransferase
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride

DAR:	Darinaparsin (S-Dimethylarsino-Glutathione)
DBahA:	Dibenzo[a,h]Anthracene
DCF	2′,7′-Dichlorofluorescein
DCFH-DA:	2′,7′-Dichlorodihydrofluorescein Diacetate
DCPIP:	2,6-Dichlorophenolindophenol
DHE:	Dihydroethidium
DMA(III):	Dimethylarsinous Acid
DMA(V):	Dimethylarsinic Acid
DMEM:	Dulbecco's Modified Eagle Medium
DMPS	Dimercaptopropanesulfonic Acid
DMSA	2,3-Dimercaptosuccinic Acid
DMSO:	Dimethyl sulfoxide
DRE:	Dioxin Response Element
EDTA:	Ethylenediaminetetraacetic Acid
EGTA:	Ethylene Glycol-bis(β-Aminoethyl Ether)-N,N,N',N'-Tetraacetic Acid
EPA:	Us Environmental Protection Agency
ERKs:	Extracellular signal regulated kinases
EROD:	7-Ethoxyresorufin O-deethylase Assay
ES:	Embryonic Stem Cells
FICZ:	6-Formylindolo[3,2-b]carbazole
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GSH:	Glutathione
GST:	Glutathione S-Transferase

GSTA1 or Gsta1:	Glutathione S-Transferase Alpha 1
h	Hours
H_2O_2:	Hydrogen Peroxide
HAH:	Halogenated Aromatic Hydrocarbons
HAP:	Hydroxyapatite Assay
Hb:	Hemoglobin
Hepa-1c1c7:	Murine Hepatoma Hepa-1c1c7
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HepG2:	Human hepatoma HepG2
HL	Hodgkin Lymphoma
HO-1 or Ho-1:	Heme Oxygenase 1
HSP90 or Hsp90:	Chaperone Protein Heat Shock Protein 90-kDa
HUVECs:	Human Umbilical Vein Endothelial Cells
i.p.:	Intraperitoneal
IARC:	International Agency for Research on Cancer
IκBs:	Inhibitory Proteins of κB
JAr:	Human Choriocarcinoma Cells
JNKs:	c-Jun NH_2 Terminal Kinases
Keap1:	Kelch-Like Ech-Associated Protein 1
LD50:	Median lethal dose
MAC:	Maximum Acceptable Concentration
MAPK:	Mitogen-activated protein kinases
MMA(III):	Monomethylarsonous Acid

MMA(V):	Monomethylarsonic Acid
MMC:	Mitomycin C
mRNA:	Messenger RNA
MROD:	7-Methoxyresorufin O-Demethylase Assay
MTT:	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NES:	Nuclear Export Signal
NF-κB:	Nuclear Factor-kappaB
NF-κB1:	NF-κB p50 and its precursor p105
NF-κB2:	NF-κB p52 and its precursor p100
NHL	Non-Hodgkin Lymphoma
NLS:	Nuclear Localization Signal or Sequence
NO:	Nitric Oxide
NQO1 or Nqo1:	NAD(P)H:Quinone Oxidoreductase 1
Nrf2:	Nuclear Factor Erythroid 2-Related Factor 2
NSCLCs:	Non-Small Cell Lung Carcinomas
$O_2^{\bullet -}$:	Superoxide Anion
ODC	Ornithine Decarboxylase
$^{\bullet}OH$:	Hydroxyl Radical
OSCC:	Oral Squamous Cell Carcinoma Cells
OVCAR-3/AsR:	Arsenic-Resistant Ovarian Cancer Cells, OVCAR-3 Subline
p23:	23-kDa Co-Chaperone Protein
p38:	p38 mitogen-activated protein kinases

p53(low)HBECs	p53-Knocked Down Human Bronchial Epithelial Cells
PAH:	Polycyclic Aromatic Hydrocarbons
PAS:	Per-ARNT-Sim Domain
PBMC:	Peripheral Blood Mononuclear Cells
PBS:	Phosphate-Buffered Saline
PCR:	Polymerase Chain Reaction
Per-ARNT-Sim:	Periodicity-Aryl Hydrocarbon Receptor Nuclear Translocator-Simple Minded
PKC:	Protein kinase C
PTCL	Peripheral T-Cell Lymphoma
Q-rich:	Glutamine-rich
RelA:	NF-κB p65 subunit
RelB:	NF-κB RelB subunit
ROS:	Reactive Oxygen Species
SAM:	S-Adenosylmethionine
SFM	Serum-Free Medium
SFN:	Sulforaphane
shRNA	Short Hairpin RNA
siRNA:	Small Interfering RNA
tBHQ:	Tert-Butylhydroquinone
TBS	Tris-Buffered Saline
TCDD:	2,3,7,8-Tetrachlorodibenzo-p-Dioxin
TCL	T-Cell Lymphoma

TMAO:	Trimethylarsine Oxide
TNF:	Tumor Necrosis Factor
UGT1A6 or Ugt1a6:	Uridine diphosphate glucuronosyltransferase 1A6
VSMCs:	Vascular Smooth Muscle Cells
WHO:	World Health Organization
WT-MEF:	Wild-Type Mouse Embryo Fibroblast
XAP2:	Hepatitis B Virus X-Associated Protein 2
XRE:	Xenobiotic Response Element
Znpp:	Zinc Protoporphyrin

1 CHAPTER ONE - INTRODUCTION

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Elshenawy, O.H., El-Kadi, A.O., 2015a. Modulation of aryl hydrocarbon receptor-regulated enzymes by trimethylarsine oxide in C57BL/6 mice: In vivo and in vitro studies. Toxicol. Lett. ***238****, 17-31.*

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1.1 Aryl hydrocarbon receptor (AhR)

The aryl hydrocarbon receptor (AhR) (also known as dioxin receptor) is a highly conserved, ligand-activated, basic region helix-loop-helix (bHLH) transcription factor. It is a member of the periodicity-aryl hydrocarbon receptor nuclear translocator-simple minded (Per-Arnt-Sim; PAS) protein family of chemosensors and developmental regulators. AhR has well-established functions during different stages of embryonic development, in physiologic homeostatic processes, in addition to cell proliferation and differentiation (Denison and Nagy, 2003; Bock and Kohle, 2006; Ko and Shin, 2012; Mohammadi-Bardbori *et al.*, 2015). AhR is a multifunctional molecular switch which plays a crucial role in mediating endobiotic and xenobiotic metabolism through activation of phase I and phase II xenobiotic-metabolizing enzymes in a wide range of species and tissues (Denison and Nagy, 2003; Bock and Kohle, 2006; Ko and Shin, 2012; Mohammadi-Bardbori *et al.*, 2015). The AhR pathway is modulated by endogenous ligands such as tryptophan metabolites, and exogenous ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) which is considered the prototypical AhR ligand (Bock and Kohle, 2006; Ko and Shin, 2012). Although AhR has different physiological functions, it has been recognized as the culprit for most toxic responses observed after exposure to dioxins and related compounds such as TCDD (Bock and Kohle, 2006; Ko and Shin, 2012). Of interest is that several AhR ligands are not only agonists of the AhR, but, with the exception of TCDD which is poorly metabolized, are also substrates for the induced phase I enzymes. This results in their conversion into genotoxic diol epoxide compounds which react with guanines in critical genes to form covalent adducts and potentially initiate tumorigenesis and other toxic responses (Spink *et al.*, 2002; Elbekai and El-Kadi, 2004; Hamouchene *et al.*, 2011).

1.1.1 Yin-yang role of AhR in cell cycle

Scientists have shown interest in understanding the role of the AhR in tumor development in the absence and presence of xenobiotics, in addition to the influence of cell phenotype on AhR-dependent tumor promotion (Marlowe and Puga, 2005; Yin *et al.*, 2016). Interestingly, the AhR was reported to have a yin-yang role in cell cycle regulation depending on the cell phenotype. This means that under some circumstances its activation is pro-proliferative, whereas under other circumstances its activation is anti-proliferative. This yin-yang activity of the AhR could play a crucial role in the abnormal proliferative and apoptotic responses associated with the carcinogenicity of several AhR ligands (Abdelrahim *et al.*, 2003; Marlowe and Puga, 2005; Mulero-Navarro *et al.*, 2006; Yin *et al.*, 2016). For example, a positive correlation has been reported between increased nuclear localization of AhR and the number of poorly differentiated cells in prostate tissue sections (Murray *et al.*, 2014; Richmond *et al.*, 2014). Increased levels of nuclear AhR are also associated with poor prognosis for patients with lung squamous cell carcinoma (Su *et al.*, 2013; Murray *et al.*, 2014). Similarly, constitutive activation of AhR induces gastric tumorigenesis and was found to be associated with adult T-cell human leukemia (ATL) (Mulero-Navarro *et al.*, 2006). On the other hand, the levels of AhR in breast cancer are inversely correlated with the histological grade of the tumor which could be a result of the AhR ability to antagonize estrogen receptor activity (Murray *et al.*, 2014). Constitutive AhR activation was also responsible for growth inhibition of Jurkat T cells and thymus involution in transgenic mice (Mulero-Navarro *et al.*, 2006). In addition, AhR activation by exogenous ligands was found to inhibit growth of human pancreatic and prostate cancer cells (Mulero-Navarro *et al.*, 2006).

Similarly, downregulation of AhR activity was observed in some hematological malignancies such as acute lymphoblastic leukemia (ALL) (Mulero-Navarro *et al.*, 2006).

1.1.2 Domain structure for the AhR

The AhR has several domains defined in its structure, and these domains are shown in Figure 1.1. First, the AhR contains a bHLH domain at the N-terminal region that functions in dimerization with Arnt and DNA response element, known as xenobiotic response element (XRE; dioxin response element; DRE). In addition, bHLH domain contains sequences important for both AhR nuclear localization (nuclear localization signal or sequence (NLS)) and nuclear export (nuclear export signal (NES)). Second, the PAS domain that contains two structural repeats (PAS A and PAS B) which are involved in AhR/Arnt dimerization (PAS A) and AhR ligand and chaperone protein heat shock protein 90 (Hsp90) binding (PAS B). Third, the C-terminal glutamine-rich (Q-rich) domain is involved in co-activator recruitment and transactivation (Denison *et al.*, 2002; Wu *et al.*, 2013).

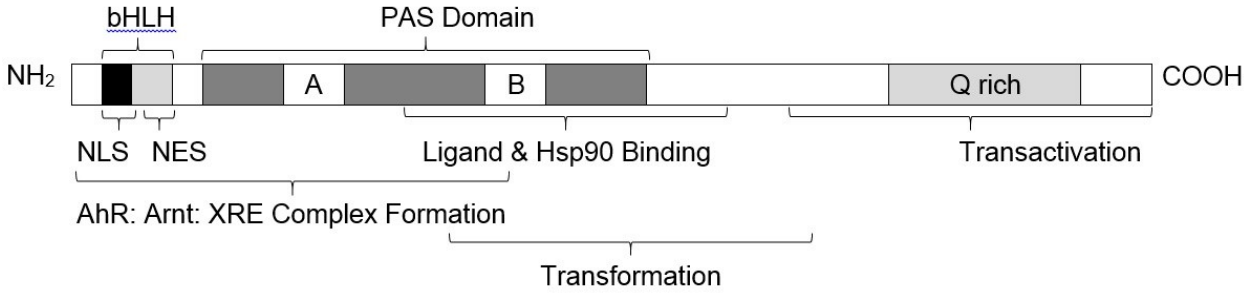


Figure 1.1 Schematic representation of domain composition of the AhR. AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon receptor nuclear translocator; bHLH, protein family of basic region helix-loop-helix; Hsp90, chaperone protein heat shock protein 90-kDa; NES, nuclear export signal; NLS, nuclear localization signal or sequence; PAS, Per-Arnt-Sim domain; Q-rich, glutamine-rich; XRE, xenobiotic response element. Adapted from (Denison *et al.*, 2002).

1.2 AhR-regulated genes

AhR is well known to regulate phase I enzymes, such as cytochrome P450 1A1 (CYP1A1), CYP1A2, CYP1B1, and CYP2S1. In addition, it participates in the regulation of phase II enzymes, such as NAD(P)H:quinone oxidoreductase 1 (NQO1; nicotinamide quinone oxidoreductase 1, DT-diaphorase, quinone reductase type 1, or menadione reductase), glutathione S-transferase alpha 1 (GSTA1; Ya), aldehyde dehydrogenase 3 (cytosolic, class 3) (ALDH3), and uridine diphosphate glucuronosyltransferase 1A6 (UGT1A6) (Nebert and Duffy, 1997; Rivera *et al.*, 2002; Brauze *et al.*, 2017). Induction of AhR-regulated genes decreases the potential toxicity of environmental pollutants by metabolizing them; however, it also mediates the toxicity of several environmental pollutants because metabolites of some xenobiotics are more toxic than their parent compounds (Ko and Shin, 2012). Phase I AhR-regulated genes CYP1A1, CYP1A2, and CYP1B1 are called "carcinogen activating enzymes", which could activate various procarcinogens and promutagens into highly carcinogenic and mutagenic substances (Moon *et al.*, 2006; Androutsopoulos *et al.*, 2009; MacDonald *et al.*, 2009). With regard to phase II AhR-regulated genes, they are considered a cytoprotective battery of genes based on their ability to detoxify carcinogens and to protect the cells against oxidative stress (Moon *et al.*, 2006; MacDonald *et al.*, 2009). However, enzymes such as NQO1 and GSTA1 are known to be associated with oxidative stress and some types of carcinomas (Siegel *et al.*, 1998; Parsons *et al.*, 2001; Takakusa *et al.*, 2008).

1.2.1 Phase I AhR-regulated genes: CYP1A1, CYP1A2, and CYP1B1

Cytochrome P450 (CYP) exists as a superfamily of heme-containing enzymes that catalyzes the oxidative metabolism of a diverse range of compounds. Indeed, CYP catalyzes pathways that serve as key clearance mechanisms, as well as terminating the actions of different endogenous and exogenous compounds including drugs from many therapeutic classes, steroid hormones, eicosanoids, cholesterol, vitamins, fatty acids and bile acids (Knights *et al.*, 2013). There are sixty known human CYP genes, which are classified into eighteen gene families and forty three subfamilies on the basis of sequence identity; of these families, enzymes of the CYP1 family are known to be responsible for the metabolism of drugs and other non-drug xenobiotics (Knights *et al.*, 2013). Members of the CYP1 family, namely CYP1A1, CYP1A2, and CYP1B1, are regulated by the AhR transcription factor (Nebert *et al.*, 2004; Go *et al.*, 2015).

1.2.1.1 CYP1A1

Constitutive CYP1A1 is mainly expressed in extrahepatic organs, especially in epithelial tissues (Bozina *et al.*, 2009). However, inducible CYP1A1 activity was found to be ubiquitous, and was detected virtually in every tissue of the body including endothelial cells of blood vessels, epithelial cells of the skin and gastrointestinal tract, and even in fetuses and embryos (Nebert *et al.*, 2004). High levels of CYP1A1 mRNA, protein, and enzyme activity are detectable following induction by polycyclic aromatic hydrocarbons (PAH), which are present in tobacco smoke for example (Nebert *et al.*, 2004). CYP1A1 catalyzes the first step in the metabolism of PAH which may lead to a formation of electrophilic carcinogenic molecules (Nebert *et al.*, 2004; Bozina *et al.*, 2009;

Go *et al.*, 2015). CYP1A1 catalyzes the oxidation of several xenobiotic chemicals such as theophylline, caffeine, 7-ethoxycoumarin, and chlorzoxazone in addition to endogenous chemicals such as 17β-estradiol and estrone (Bozina *et al.*, 2009).

1.2.1.2 CYP1A2

CYP1A2 is mainly expressed in the liver where it shows substantial constitutive expression (Nebert *et al.*, 2004; Bozina *et al.*, 2009). CYP1A2 is known to be inducible in different organs such as the liver, gastrointestinal tract, nasal epithelium, and brain (Nebert *et al.*, 2004). CYP1A2 protein levels represent 10 % to 15 % of total CYP in the human adult liver. However expression levels were found to vary about 40 to 60 times between individuals (Nebert *et al.*, 2004; Bozina *et al.*, 2009). CYP1A2 is known to be involved in the bioactivation of procarcinogenic compounds to carcinogenic derivatives, in addition to its role in drug metabolism (Go *et al.*, 2015). CYP1A2 oxidizes xenobiotic chemicals such as acetaminophen, antipyrine, caffeine, lidocaine, phenacetin, theophylline, and *R*-warfarin (Bozina *et al.*, 2009). In addition, CYP1A2 metabolizes and catalyzes the metabolic activation of several N-heterocyclic amines found in charcoal-grilled food (such as 2-amino-3-methylimidazo[4,5f]quinoline and 2-amino-1-methyl-6-phenylimidazo[4,5b] pyridine), in addition to arylamines (such as 2-acetylaminofluorene, 4-aminobiphenyl, and 2-aminoanthracene) (Nebert *et al.*, 2004; Bozina *et al.*, 2009). Interestingly, CYP1A2 catalyzes the activation of PAH diols to reactive metabolites at much slower rates than CYP1A1 and 1B1 (Bozina *et al.*, 2009).

1.2.1.3 CYP1B1

CYP1B1 often shows substantial constitutive expression (Nebert *et al.*, 2004; Go *et al.*, 2015). CYP1B1 is expressed in the endoplasmic reticulum of extrahepatic organs, predominantly in the steroidogenic tissues of the uterus, breast, ovary, testis, prostate, and adrenal gland. It is also expressed in many other extrahepatic tissues including the lung, kidney, thymus, spleen, brain, heart, vascular endothelial cells, epithelial lining of the head and neck, adrenal cortex, colon, and intestine (Nebert *et al.*, 2004; Bozina *et al.*, 2009). Similar to CYP1A1, large inter-individual differences in CYP1B1 protein levels have been reported in humans (Nebert *et al.*, 2004). CYP1B1 metabolizes numerous PAH, N-heterocyclic amines, arylamines, amino azo dyes, nitroarenes, and several other carcinogens (Nebert *et al.*, 2004); thus it plays an important role in activating diverse procarcinogens to reactive metabolites that cause DNA damage (Bozina *et al.*, 2009). In addition, CYP1B1 was found to be largely responsible for PAH-induced immunotoxicity (Nebert *et al.*, 2004). CYP1B1 converts estrogen to 4-hydroxylated metabolites that may initiate breast cancer in humans (Bozina *et al.*, 2009). Interestingly, high levels of CYP1B1 have been detected in a wide range of human cancers, such as cancers of the skin, brain, testis and breast (Nebert *et al.*, 2004; Bozina *et al.*, 2009; Go *et al.*, 2015).

1.2.2 Phase II AhR-regulated genes: NQO1, GSTA1

Phase II AhR-regulated drug metabolizing enzymes such as NQO1 and GSTA1 can increase the excretion rate of harmful xenobiotics (Warwick *et al.*, 2012). Regulation of these genes is complex and involves transcription factors other than AhR, such as nuclear factor erythroid 2-related factor

2 (Nrf2) which binds to its cognate binding site, known as antioxidant response element (ARE), and transactivates the expression of these genes (Yeager *et al.*, 2009).

1.2.2.1 NQO1

NQO1 is a flavoenzyme that catalyzes the two-electron reduction and detoxification of quinones and related compounds derived from the oxidation of phenolic metabolites of benzene (Siegel *et al.*, 1998; Moran *et al.*, 1999; Smith, 1999; Nioi and Hayes, 2004). NQO1 is highly inducible by several natural and synthetic compounds. For example, extracts of cruciferous vegetables, such as broccoli, and synthetic antioxidants, such as butylated hydroxyanisole, have been shown to be potent inducers of NQO1 (Smith, 1999). NQO1 attracted considerable attention due to its ability to detoxify several natural and synthetic compounds, in addition to activating certain anticancer agents such as mitomycin C, E09, RH1, β-lapachone, and 17AAG (Smith, 1999; Oh and Park, 2015). The effect of NQO1 in the cellular microenvironment sheds light on the important role that NQO1 might play in cancer chemoprevention (Smith, 1999; Oh and Park, 2015). Interestingly, although NQO1 is considered part of the AhR gene battery, it is also considered a prototypical target gene for Nrf2. Induction of NQO1, mechanistically, is mediated by the activation of AhR/XRE and Nrf2/ARE pathways (Nioi and Hayes, 2004; Yeager *et al.*, 2009; Lee *et al.*, 2010).

1.2.2.2 GSTA1

The glutathione S-transferases (GST) are an important class of enzymes that play a prominent role in the intracellular detoxification of reactive electrophiles and products of oxidative stress

(Parsons *et al.*, 2001). GST catalyzes nucleophilic attack by reduced glutathione (GSH) on nonpolar compounds that contain an electrophilic carbon, nitrogen, or sulfur atom (Hayes *et al.*, 2005). Conjugation of toxic and carcinogenic substances to GSH results in their inactivation and inability to form DNA adducts; thus GST could protect against neoplastic transformation (Parsons *et al.*, 2001). Examples of the GST substrates are halogenonitrobenzenes, arene oxides, quinones, and α,β-unsaturated carbonyls (Hayes *et al.*, 2005). GST was found to be induced by cruciferous vegetables due to the levels of glucosinolates, which upon hydrolysis form biologically active compounds such as indoles and isothiocyanates known for their chemoprotective effects via inducing GST (Navarro *et al.*, 2009). GST is widely distributed in nature and is composed of three major families of proteins. Two families are soluble enzymes of the cytosolic and mitochondrial GST. The third family is the microsomal GST or the membrane-associated proteins in eicosanoid and glutathione (MAPEG) metabolism (Hayes *et al.*, 2005). Cytosolic GST isoenzymes have five major classes designated as alpha, mu, pi, sigma, and theta (Parsons *et al.*, 2001). Of these GST, GSTA1 is the major hepatic GST and has a higher affinity than other GST for many carcinogens, particularly PAH and heterocyclic amines, such as 2-amino-1-methyl-6-phenylimidazaol[4,5-b]pyridine, produced in well-cooked meats and implicated in the etiology of cancers (Navarro *et al.*, 2009). Mechanistically, Nrf2 is required in addition to AhR, for the induction and activation of GSTA1 (Yeager *et al.*, 2009).

1.3 Activation of the AhR

AhR is present in the cytosolic compartment as a multiprotein complex that includes the AhR ligand binding subunit associated with two molecules of Hsp90, hepatitis B virus X-associated

protein 2 (XAP2; AhR interacting protein; AIP; AhR-activated 9; ARA9), and a 23-kDa co-chaperone protein (p23) (Denison *et al.*, 2002; Denison and Nagy, 2003; Attignon *et al.*, 2017). Subsequent to ligand binding, the AhR undergoes a conformational change that results in exposure of NLS. The complex then translocates into the nucleus, where the AhR dissociates from the protein complex, and dimerizes with a closely related nuclear bHLH–PAS protein called Arnt, together referred to as the aryl hydrocarbon receptor complex (AhRC). Thereafter, the AhR-Arnt heterodimer binds to a DNA sequence, termed the XRE, upstream in the regulatory domains of target genes such as CYP1A1 and NQO1, leading to chromatin and nucleosome disruption, increased promoter accessibility, and activation of gene transcription (Figure 1.2) (Davarinos and Pollenz, 1999; Denison *et al.*, 2002; Denison and Nagy, 2003; Kann *et al.*, 2005; Beischlag *et al.*, 2008; Mohammadi-Bardbori *et al.*, 2015).

1.3.1 Ligand-dependent and ligand-independent activation of AhR

The AhR can be activated by a structurally diverse range of chemicals, and some of these chemicals have the ability to directly bind to, and activate, the AhR through ligand-dependent mechanisms. However, some chemicals have the ability to induce CYP1A1 and/or activate the AhR and AhR-regulated gene expression indirectly through ligand-independent mechanisms (Denison and Nagy, 2003; Xiao *et al.*, 2015). Examples of chemicals that activate the AhR and induce AhR-dependent gene expression, yet do not competitively bind to the AhR, are 11-ethoxy-cyclopenta(a)phenanthrene-17-one, caffeine, canathaxanthin, carbaryl, cypermethrin, diflubenzuron, lanosperole, methylenedioxyphenyls (isosafrole, piperonyl butoxide), mevinolin, myristicin, nicotine, omeprazole, oxfendazole, primaquine, pyridines, tetrachlorvinphos,

thiabendazole, β-apo-8'-carotinal (Denison and Nagy, 2003). Three mechanisms have been proposed for the ligand-independent activation of the AhR signaling pathway. First, these chemicals could be metabolically converted into AhR ligands; second, these chemicals could have the ability to affect some cellular pathway that results in AhR activation; third, these chemicals may interact with a second binding site on the AhR distinct from the ligand binding site (Gradelet *et al.*, 1997; Ciolino *et al.*, 1998; Denison and Nagy, 2003). Noteworthy is that, although some weak AhR inducers have not been observed to competitively bind to the AhR, they may still be AhR ligands that bind with relatively low affinity. For example, carbaryl has 300,000-fold lower potency compared to TCDD, which may explain its inability to competitively displace [^{3}H]TCDD from the AhR ligand binding domain using the ligand binding assay (Denison and Nagy, 2003). However, some scientists have proposed that, carbaryl could bind to a second binding site on the AhR (Ciolino *et al.*, 1998). Similar to activators, AhR inhibitors could act directly through the AhR binding site or indirectly through other mechanisms. For example, resveratrol has an inhibitory effect on AhR/XRE binding through two possible mechanisms. First, resveratrol may interact with a second binding site on the AhR distinct from the TCDD-binding site, causing inactivation of the AhR; second, resveratrol may inhibit AhR function without altering ligand binding, indirectly by affecting one or more of the proteins involved in the regulation of the AhR signaling pathway, such as Hsp90, c-SRC, XAP2, and protein kinase C (PKC) (Ciolino *et al.*, 1998).

1.3.2 Endogenous and exogenous AhR ligands

AhR ligands can be classified into three categories. First, endogenous physiological ligands which are formed in humans or animals as a result of natural processes. Second, exogenous synthetic AhR ligands which are formed as a result of anthropogenic or non-biological activity. Third, exogenous natural (dietary) AhR ligands, which are consumed by humans and animals with dietary intake (Denison *et al.*, 2002; Denison and Nagy, 2003).

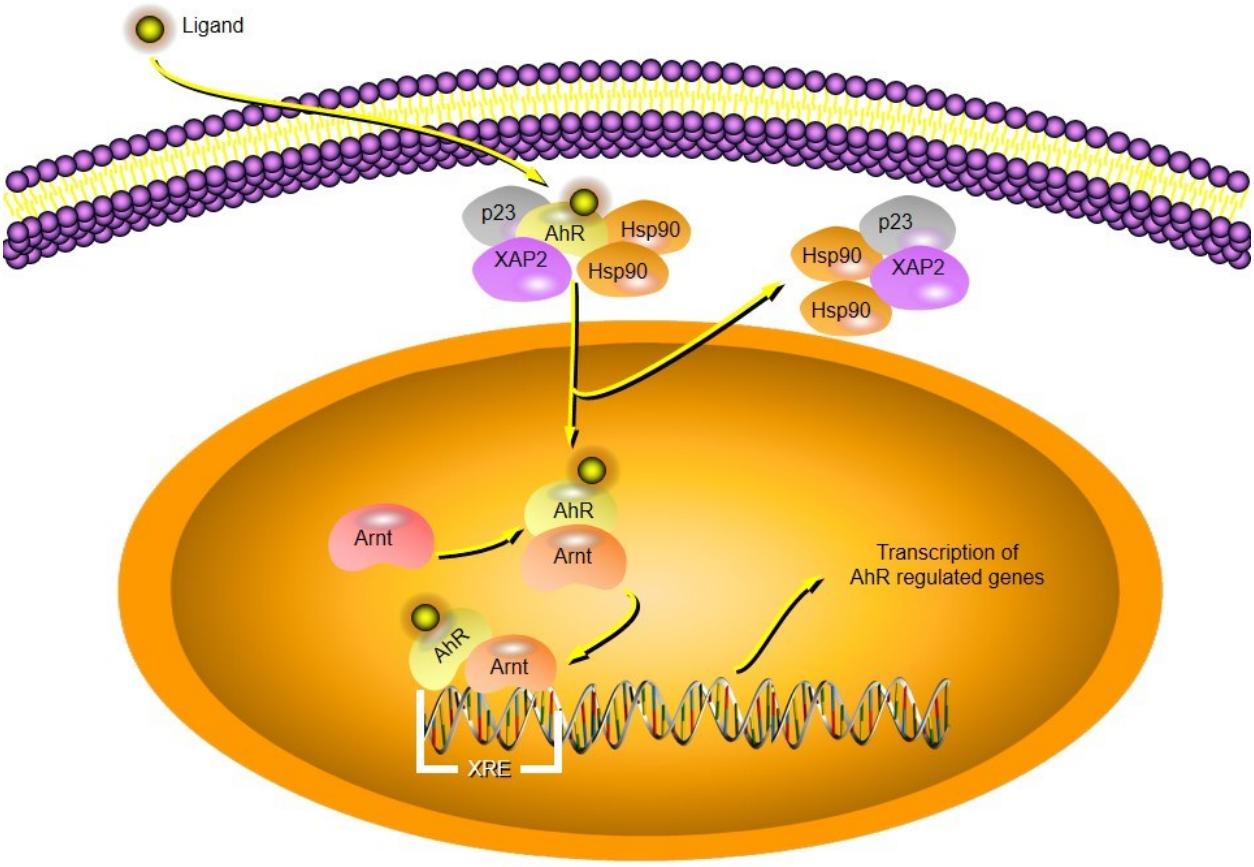


Figure 1.2 Schematic representation of the AhR signaling pathway. AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon receptor nuclear translocator; Hsp90, chaperone protein heat shock protein 90-kDa; p23, 23-kDa co-chaperone protein; XAP2, hepatitis B virus X-associated protein 2; XRE, xenobiotic response element. Adapted from (Dietrich and Kaina, 2010; Murray *et al.*, 2014).

1.3.2.1 Endogenous ligands for the AhR

Several structurally distinct classes of endogenous chemicals have shown ability to bind to AhR and activate AhR-dependent gene expression; however, the majority of these chemicals are relatively weak compared to TCDD. Examples of these chemicals are indoles (formed from tryptophan metabolism such as 6-formylindolo[3,2-b]carbazole (FICZ)), tetrapyroles (formed from heme degradation, such as bilirubin and biliverdin), and arachidonic acid metabolites (such as lipoxinA4 and prostaglandin G2) (Denison *et al.*, 2002; Denison and Nagy, 2003; Mohammadi-Bardbori *et al.*, 2015)

1.3.2.2 Exogenous synthetic ligands for the AhR

The majority of the high affinity AhR ligands that have been identified, characterized, and extensively studied include planar, hydrophobic halogenated aromatic hydrocarbons (HAH) (such as the polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans, and biphenyls, and related chemicals) and non-halogenated PAH (such as 3-methylcholanthrene (3MC), benzo[a]pyrene (BaP), benzanthracenes, and benzoflavones, rutaecarpine alkaloids, aromatic amines and related chemicals) (Denison *et al.*, 2002; Denison and Nagy, 2003; Kann *et al.*, 2005). Both HAH and PAH have some distinct features inside the body; HAH are metabolically more stable and represent the most potent class of AhR ligands, whereas PAH are metabolically more labile and bind with relatively lower affinity (Denison *et al.*, 2002; Denison and Nagy, 2003).

1.3.2.3 Exogenous natural (dietary) ligands for the AhR

Diet is the greatest source of exposure of humans and animals to AhR ligands. Previous studies have shown that a wide range of naturally occurring dietary chemicals can directly affect the AhR signaling pathway, with a resultant activation or inhibition of its downstream target genes (Denison and Nagy, 2003). However, the majority of the naturally-occurring dietary chemicals are relatively weak AhR ligands (Denison *et al.*, 2002). Flavonoids (such as flavones, flavanols, flavanones, and isoflavones) are the largest group of naturally occurring dietary AhR ligands. Although the majority of these compounds are AhR antagonists, several agonists have been identified (such as quercetin, diosmin, tangeritin, and tamarixetin) (Denison and Nagy, 2003). Other chemicals that competitively bind to the AhR and/or induce AhR-dependent gene expression are indole 3-carbinol, 7,8-dihydrorutacarpine, dibenzoylmethanes, curcumin, and carotinoids (e.g., canthaxanthin, astaxanthin, and the apo-carotinoid, β-apo-8'carotenal) (Denison and Nagy, 2003).

1.4 Cross talk between AhR and other signaling pathways

1.4.1 Cross talk between AhR and Nrf2

Previous studies suggested the presence of cross-talk between AhR and Nrf2 signaling pathways (Nioi and Hayes, 2004; Shin *et al.*, 2007; Nakahara *et al.*, 2015). Historically, the AhR has been associated with toxicity and carcinogenesis, whereas Nrf2 is associated with cytoprotection. Nrf2 is a cap'n'collar (CNC) basic leucine zipper (bZIP) transcription factor (Ma *et al.*, 2004; Shin *et al.*, 2007). Nrf2 is a central transcription factor that plays a critical role in mediating the cellular

adaptive response to oxidative stress, and thus regulates the cellular antioxidant response for protecting the cells from various insults (Pi *et al.*, 2003; Wang *et al.*, 2007; Fu *et al.*, 2010; Zhao *et al.*, 2011; Yang *et al.*, 2012; Zhao *et al.*, 2012). Nrf2 activation is known to be effective in detoxifying environmental insults and in preventing a broad spectrum of diseases induced by environmental exposure to harmful substances (Zheng *et al.*, 2012). Activation of the Nrf2/Keap1 (Kelch-like ECH-associated protein 1) signaling pathway is mediated by dissociating Nrf2 from Keap1 resulting in its translocation to the nucleus to dimerize with Maf protein. Nrf2/Maf associates with a *cis*-acting enhancer sequence located upstream in regulatory regions of antioxidant and detoxifying genes such as NQO1 gene; this sequence is known as the antioxidant response element (ARE) (He *et al.*, 2006) (Figure 1.3). Binding of Nrf2/Maf to ARE was found to control both the basal and inducible expression of the redox-sensitive genes (He *et al.*, 2006). Interestingly, Nrf2 plays a crucial role in controlling the induction of classical phase II AhR battery genes such as NQO1 and GSTA1 (Yeager *et al.*, 2009). In addition, it controls the expression of other redox sensitive phase II enzymes such as HO-1 (Mann, 2014).

Several studies proved the direct relationship between AhR and Nrf2, and this relationship was found to play an important role in coupling phase I and phase II enzymes into an integrated system to facilitate effective xenobiotic and carcinogen detoxification (Miao *et al.*, 2005). For example, TCDD, the prototypical AhR ligand, can induce NQO1 gene expression through Nrf2/ARE activation (Radjendirane and Jaiswal, 1999; Ma *et al.*, 2004). The activation of Nrf2/ARE signaling pathway by TCDD and other bi-functional inducers can only occur in the presence of AhR (Miao *et al.*, 2005). Moreover, scientists reported that, Nrf2 is a downstream target of the AhR and can be directly modulated by AhR-XRE activation (Miao *et al.*, 2005). On the other hand,

other studies showed that signaling in the opposite direction also occurs, whereby Nrf2 directly regulates the expression of AhR and thus modulates several downstream events of the AhR signaling cascade (Shin *et al.*, 2007).

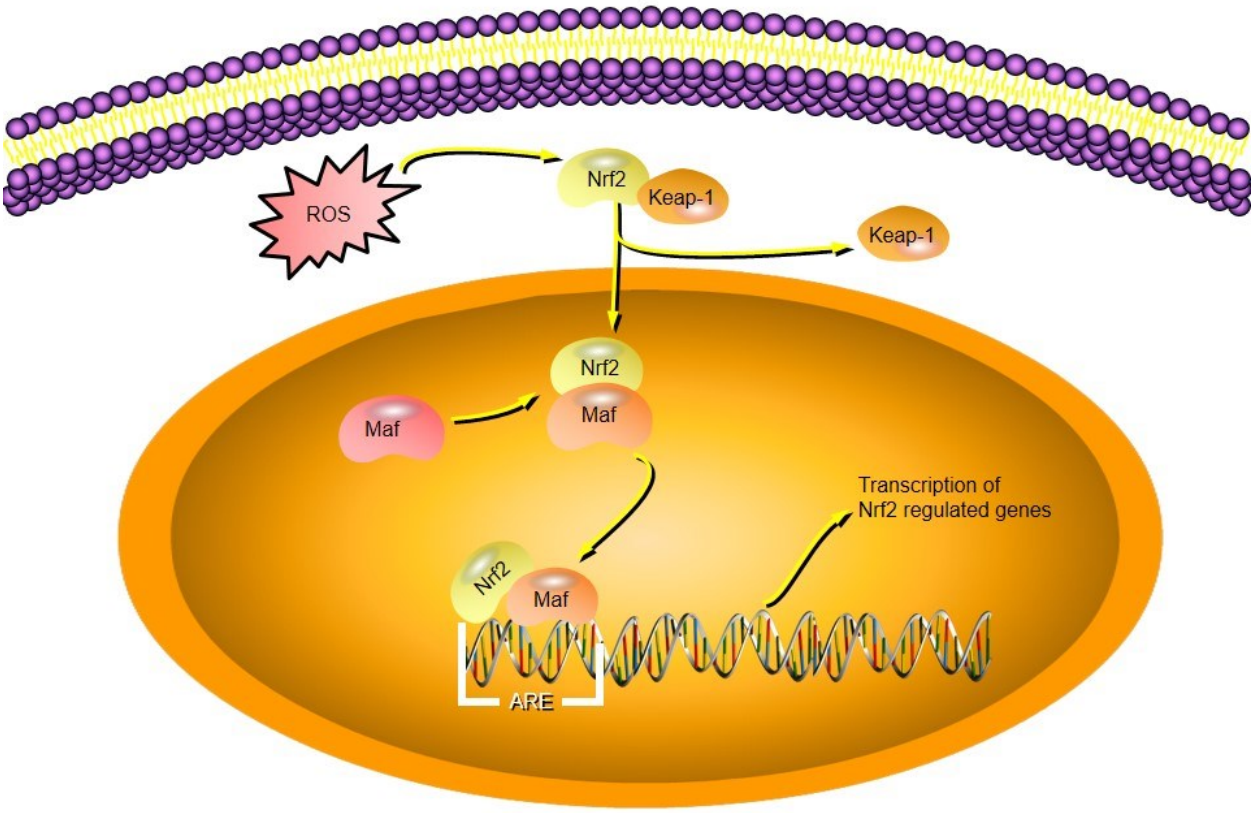


Figure 1.3 Schematic representation of the Nrf2 signaling pathway. ARE, antioxidant response element; Keap-1, kelch-like ECH-associated protein 1; Maf, small Maf proteins; Nrf2, Nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species. Adapted from (Petri *et al.*, 2012).

1.4.2 Cross talk between AhR and mitogen-activated protein kinases (MAPK)

Mitogen-activated protein kinases (MAPK) are a family of serine/threonine kinases that function as critical mediators for a variety of extracellular signals. Members of the MAPK superfamily include extracellular signal regulated kinases (ERKs), c-Jun NH_2 terminal kinases (JNKs), and p38 mitogen-activated protein kinases (p38) (Banerjee *et al.*, 2016). MAPK are activated by external stimuli, such as growth factors, cytokines, ultraviolet (UV) radiation, osmotic shock, and genotoxic or oxidative stress. MAPK phosphorylate important transcriptional factors directly or indirectly via down-stream MAPK-activated protein kinases (Henklova *et al.*, 2008). Mounting evidence has proved the mutual interactions between AhR and MAPK, where typical AhR activators, such as TCDD and BaP have been shown to activate the MAPK (Henklova *et al.*, 2008). For example, TCDD was reported to induce a p38-MAPK-dependent pathway through AhR dependent mechanisms (Weiss *et al.*, 2005). On the other hand, MAPK pathways were found to be crucial signaling mechanisms that are involved in the TCDD-mediated activation of AhR. Studies showed that TCDD induced immediate activation of ERKs and JNKs, but not p38 MAPK, and this activation did not require the AhR, since it occurred in AhR-positive and AhR-negative cells. TCDD-stimulated MAPK were found to be critical for the induction of AhR-dependent gene transcription and CYP1A1 expression, indicating that AhR ligands elicit AhR-independent non-genomic events that are essential for AhR activation and function (Tan *et al.*, 2002).

1.4.3 Cross talk between AhR and nuclear factor-kappaB (NF-κB)

Nuclear factor-kappaB (NF-κB) is a transcription factor that is known to be involved in diverse signaling pathways in responses to different xenobiotics (Tian *et al.*, 1999). The NF-κB family is

composed of five proteins: NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (p65), RelB, and c-Rel. The NF-κB proteins form dimeric complexes that bind to enhancer sequences to control gene expression. Inactive NF-κB is localized in the cytoplasm associated with inhibitory proteins referred to as IκBs (IκBα, IκBβ, IkBγ, IκBε, Bcl-3, as well as the precursors of NF-κB1 (p105) and NF-κB2 (p100)) (Tian *et al.*, 2002). AhR and RelA was reported to interact by physical and functional association resulting in mutual functional modulation of gene expression controlled by both AhR and NF-κB (Tian *et al.*, 1999; Tian *et al.*, 2002). This physical association occurs once activating signals of AhR (such as TCDD) and NF-κB (such as tumor necrosis factor (TNF)) subunits cause the dissociation of Hsp90 and IκBs from AhR and RelA, respectively (Tian *et al.*, 1999). The consequences of AhR-NF-κB physical association is contradictory; for example NF-κB RelA was shown to be a critical component regulating the expression of AhR and the induction of AhR-dependent genes in immune cells (Vogel *et al.*, 2014). In addition, TCDD was reported to activate a CYP1A1- and AhR-dependent oxidative stress signal that results in the prolonged activation of NF-κB (Puga *et al.*, 2000). However, other reports showed that AhR-NF-κB association may provide a physical basis for functional antagonism. In other words, AhR may induce suppression of NF-κB activity, while NF-κB transrepresses AhR activity (Tian *et al.*, 1999). Although the mechanism for this mutual functional repression is not completely clear, it could be attributed to the formation of an inactive complex between AhR and NF-κB RelA. In addition, it could be mediated by a transcription coactivator, such as the p300/CBP, through competition between ligand-AhR/ARNT complexes and RelA for p300/CBP binding; which would affect the levels of transcriptional activation of both pathways (Tian *et al.*, 1999).

1.4.4 Cross talk between AhR and activator protein 1 (AP-1)

AP-1 is a transcription factor consisting of either Jun homodimers or Jun/Fos heterodimers, which binds to enhancer sequences and plays a crucial role in the regulation and activation of different genes (Suh *et al.*, 2002). Mounting evidence has revealed that proper expression and activity of c-Jun and other AP-1 subunits is critical for many cellular processes such as differentiation, proliferation, and apoptosis (Weiss *et al.*, 2005). Previous studies showed that TCDD activates an AhR-dependent oxidative stress signal that results in increased mRNA and protein levels of the proto-oncogene c-Jun; thus a critical role of c-Jun in dioxin-induced tumorigenesis has been suggested (Puga *et al.*, 2000; Weiss *et al.*, 2005). Interestingly, TCDD-dependent activation of AP-1 was found to be sustained and requires functional AhR, Arnt, and CYP1A1 proteins, suggesting that a transcriptional product of AhR, possibly CYP1A1 itself, is partly responsible for the activation of AP-1 (Puga *et al.*, 2000). In addition, treatment of mouse hepatoma cells with HAH or PAH such as TCDD and BaP caused an increase in mRNA levels of the immediate early proto-oncogenes c-fos, c-jun, junB, and junD. This increase in mRNA was concomitant with an increase in the DNA-binding activity of the transcription factor AP-1. Noteworthy, induction of c-fos and junB is independent of AhR and Arnt, since it also takes place in variant hepatoma cell lines with highly reduced levels of AhR or lacking Arnt (Puga *et al.*, 1992; Hoffer *et al.*, 1996). Interestingly, the human CYP1A2 enhancer region contains two potential binding sites for AP-1, revealing that induction of AhR-regulated genes is modulated through activation of AP-1 DNA binding (Quattrochi *et al.*, 1998).

1.5 Arsenic

According to the World Health Organization (WHO) and The Agency for Toxic Substances and Disease Registry, arsenic is ranked at the top of the most hazardous contaminants of concern for human health worldwide (ATSDR, 2013; Notch *et al.*, 2015). Moreover, The International Agency for Research on Cancer (IARC) considers arsenic as a group I human carcinogen (Ruiz de Luzuriaga *et al.*, 2011; Lee and Yu, 2016). Arsenic is a known toxic metalloid, ubiquitously distributed in the environment; it is found in rocks, soil, water, food and air (Wang *et al.*, 2008; Kumagai, 2009; Hughes *et al.*, 2011; Martinez *et al.*, 2011; Sinha *et al.*, 2013). Arsenic is released from anthropogenic activities such as copper, zinc, and lead smelters; glass, pesticide, and herbicide manufacturing; cigarette smoke, arsenic-treated wood; and agricultural fertilizers (Fishbein, 1981; WHO, 2000; Patrick, 2003; McCoy *et al.*, 2015; Stice *et al.*, 2016). Arsenic has different oxidation states in the environment and can be found both in organic and inorganic compounds (Martinez *et al.*, 2011). According to WHO guidelines, the maximum acceptable concentration (MAC) for arsenic in drinking water is 10 μg/L. However, this level is higher than levels associated with negligible risk of cancer, based on limitations of available water treatment technology. Accumulating evidence has revealed that hundreds of millions of people across the world are exposed to arsenic via drinking water at doses higher than the WHO maximum contaminant level of 10 μg/L (Health-Canada, 2006; Abhyankar *et al.*, 2012; McCoy *et al.*, 2015; Notch *et al.*, 2015). The United States Geological Survey has reported that more than 25 million people in the U.S. are exposed to well water with arsenic concentrations exceeding the current US Environmental Protection Agency (EPA) standard for public water supplies (Notch *et al.*, 2015). Moreover, seafood is considered a rich source of exposure to an array of arsenicals including

inorganic and methylated arsenicals (Calderon *et al.*, 2013; Aylward *et al.*, 2014). Arsenic exposure through drinking water and food is known to be associated with chronic toxicity, which poses serious health risks to millions of people around the world (Du *et al.*, 2008; Jiang *et al.*, 2009; Stice *et al.*, 2016).

1.5.1 Arsenic toxicity and carcinogenicity

Arsenic elicits pleiotropic adverse and adaptive responses in mammalian species (He *et al.*, 2006), and it damages biological systems through multiple mechanisms (Wang *et al.*, 2007). Symptoms of acute intoxication include fever, anorexia, hepatomegaly, melanosis, cardiac arrhythmia and, in fatal cases, eventual cardiac failure. In addition acute arsenic toxicity can produce encephalopathy, with signs and symptoms of headache, lethargy, mental confusion, hallucination, seizures, and even coma (Casarett *et al.*, 2008). Chronic exposure to arsenic was found to alter the physiology of different key immune cells, particularly macrophages (Wang *et al.*, 2011a; Morzadec *et al.*, 2012) and T lymphocytes (Martin-Chouly *et al.*, 2011). In addition, it is associated with nervous system pathogenesis and vascular diseases, as well as reproductive and developmental toxicity (Massrieh *et al.*, 2006). Skin is the major target organ in chronic arsenic exposure, whereby diffuse or spotted hyperpigmentation or hypopigmentation appear between 6 months to 3 years with chronic exposure to inorganic arsenic. Palmar-plantar hyperkeratosis usually follows the initial appearance of arsenic-induced pigmentation changes within a period of years. Long term exposure to arsenic can cause liver injury manifested as jaundice, abdominal pain, and hepatomegaly. This injury may progress to cirrhosis, ascites, and hepatocellular carcinoma. Repeated exposure to low levels of inorganic arsenic can produce peripheral neuropathy in the form of sensory changes, such

as numbness in the hands and feet that later may develop into a painful "pins and needles" sensation. In addition, peripheral vascular disease, manifested by acrocyanosis and Raynaud's phenomenon which may progress to endarteritis and gangrene of the lower extremities (Blackfoot disease) have been described in populations exposed to arsenic on a chronic basis (Casarett *et al.*, 2008).

Arsenic has the potential to induce several types of cancers, and interestingly this potential was identified more than hundred years ago. Epidemiological studies have linked the exposure to arsenic to an increased incidence of human cancers in the skin, urinary bladder, liver, kidney, lung, and prostate; thus it has been classified as a known human carcinogen (Hopenhayn-Rich *et al.*, 1998; Mandal *et al.*, 2001; Casarett *et al.*, 2008). Unlike most carcinogens for which the human carcinogenicity is extrapolated from studies conducted on animal species, information on the carcinogenicity of arsenic is available mostly from humans (Cohen *et al.*, 2013).

1.5.2 Common exposure to arsenic and AhR ligands

Complex mixtures of carcinogenic metalloids, such as arsenic, and AhR ligands, such as PAH or HAH, are common environmental contaminants. For example, carcinogenic metals and AhR ligands are common contaminants of hazardous waste sites and are co-released from sources such as fossil fuel combustion and municipal waste incineration, and as components of cigarette smoke (Maier *et al.*, 2000; Kann *et al.*, 2005; Letasiova *et al.*, 2012). In addition, multiple co-exposures involving arsenicals and dioxins were reported to occur during leather tanning and processing, and in flavor and fragrance production (Humblet *et al.*, 2008). Accumulating data from ongoing

epidemiological studies showed that large populations exposed to a wide range of arsenic levels in drinking water in regions such as Taiwan and Bangladesh are also exposed to AhR ligands in the form of polychlorinated biphenyl, dioxins, and PAH. Taiwan is an industrialized island with many municipal waste incinerators. These incinerators, along with other factories with combustive processes, release unintentional byproduct dioxins into the environment, and these dioxins will eventually concentrate in the human body via environmental transport, food chains, and bioaccumulation (Ma *et al.*, 2002; Hossain *et al.*, 2008; Hsu *et al.*, 2010; Linderholm *et al.*, 2011; Naujokas *et al.*, 2013). Likewise in Bangladesh, persistent organic pollutants and heavy metals are well known environmental pollutants (Linderholm *et al.*, 2011). In addition, ship scrapping workers, at Chittagong Coastal Zone in Bangladesh, were found to be affected by heavy metals, oil residues, polychlorinated biphenyl, dioxins, or a cocktail of toxic chemicals contained in the ships (Hossain *et al.*, 2008). These factors impose environmental, health and safety concerns for populations in these areas and bring up the importance of toxicity assessment studies for human health. Although the health effects of individual chemicals may be known, the toxicity of environmental mixtures is largely unexplored and usually has unpredictable biological consequences when compared to the effects of each toxicant alone (Kann *et al.*, 2005). Co-exposure to these mixtures can disrupt the regulation of phase I and phase II detoxification genes, leading to imbalances in gene expression that may have important implications in the toxicity of complex mixtures (Maier *et al.*, 2000; Vakharia *et al.*, 2001a). For example, arsenic is not a potent mutagen by itself, however arsenite (As(III)) augments the mutagenicity of BaP, a known DNA-damaging agent, suggesting that As(III) might uncouple expression of phase I and phase II genes responsible for detoxification (Ho and Lee, 2002; Maier *et al.*, 2002; Kann *et al.*, 2005).

1.5.3 Arsenic compounds as anticancer agents

Interestingly, although arsenic is a carcinogen, some arsenic compounds have been recently rediscovered for the treatment of certain cancers. Arsenic trioxide (As_2O_3, ATO, Trisenox) has been successfully used for the treatment of acute promyelocytic leukemia (APL) with success rates up to 80%, and it is used even in refractory and relapsed cases; in addition ATO was found to have activity in multiple myeloma (Shen *et al.*, 1997; Vernhet *et al.*, 2003; Lin *et al.*, 2006; Morales *et al.*, 2009; Liu *et al.*, 2010). Recently, the potential of ATO for use as a novel therapy for ovarian cancer treatment has been increasingly recognized (Ong *et al.*, 2012). ATO has been shown to induce apoptosis in a variety of solid tumor cell lines, including non-small cell lung carcinomas (NSCLCs). However, a main challenge is that the high doses required for the treatment of these solid tumors are associated with excessive toxicity and thus hinder the clinical use of ATO in these cases (Diaz *et al.*, 2005; Park *et al.*, 2008). These potential adverse consequences include serious side effects, human carcinogenicity and the possible development of resistance, in addition to hepatotoxicity (Lee *et al.*, 2011; Qu *et al.*, 2011). Despite the extensive research conducted on ATO, there is no clear understanding of the exact mechanisms responsible for its anti-tumor effects (Liu *et al.*, 2010).

Darinaparsin (DAR, S-dimethylarsino-glutathione) is a novel organic arsenic compound that has been developed to improve the efficacy and therapeutic index of arsenic as an antineoplastic agent (Hosein *et al.*, 2012). DAR was found to be cytotoxic to prostate cancer cell lines and fresh prostate cancer cells from patients. In addition, DAR inhibits the growth of castrate resistant DU145 prostate tumor propagated as a xenograft in mice, and inhibits the tumor initiating potential of

prostate cancer cells (Bansal *et al.*, 2015). Scientists showed that DAR causes a dose-dependent cytotoxicity and apoptosis in all T-cell lymphoma (TCL) and Hodgkin lymphoma (HL) cell lines. Similarly, experiments with Jurkat (TCL) and L540 (HL)-derived lymphoma xenografts showed significant inhibition of tumor growth and improved survival in DAR-treated SCID mice (Ravi *et al.*, 2014). DAR has been used in clinical trials for hematologic malignancies and refractory solid tumors (Tsimberidou *et al.*, 2009). A phase I clinical trial of DAR conducted in patients with solid malignancies refractory to conventional therapies showed that the recommended dose for DAR is 300 mg/m^2 intravenously given daily for 5 days every 4 weeks (Tsimberidou *et al.*, 2009). In a multicenter, phase II trial, patients with relapsed or refractory Hodgkin (HL) and non-Hodgkin lymphoma (NHL) were treated with DAR 300 mg/m^2 intravenously daily for 5 consecutive days every 4 weeks, for up to six cycles. Results from this multicenter trial showed that DAR was safe and showed preliminary activity in heavily pretreated population of relapsed/refractory lymphoma patients. In addition, encouraging responses were seen in peripheral T-cell lymphoma (PTCL) (Hosein *et al.*, 2012).

1.6 Modulation of AhR and Nrf2 signaling pathways by arsenic

Arsenic is known to interact with drug-metabolizing enzymes such as CYP, which are known to be involved in the oxidative metabolism and elimination of toxic chemicals (Vernhet *et al.*, 2003; Noreault *et al.*, 2005b). Moreover, arsenic is well known to alter transcription factors such as AhR and Nrf2, in addition to their downstream targets, such as CYP1A1, CYP1A2, CYP1B1, NQO1, GSTA1, and HO-1 (Elbekai and El-Kadi, 2004; Anwar-Mohamed *et al.*, 2009; Sinha *et al.*, 2013). Hence, arsenic-mediated alteration of these targets may contribute to arsenic-induced disorders.

1.6.1 Modulation of AhR signaling pathway and its downstream targets: CYP1A1, CYP1A2, and CYP1B1

Arsenic differentially modulates the expression of various CYP1 family enzymes both in *in vivo* and *in vitro* systems. This alteration was reported to be time-, tissue-, and enzyme-specific and observed in the presence and absence of typical AhR agonists such as TCDD (Wu *et al.*, 2009; Anwar-Mohamed *et al.*, 2012). CYP1 enzymes are considered among the most important AhR-regulated genes, and their importance emerges from the fact that CYP1 enzymes have been associated with the metabolism of procarcinogenic compounds to highly carcinogenic metabolites (Anwar-Mohamed *et al.*, 2009). Arsenic is well known to modulate CYP1 enzymes by interfering with the AhR signaling pathway (Anwar-Mohamed *et al.*, 2009).

Treatment of mouse hepatoma, Hepa-1, cells with As(III) was found to induce AhR nuclear translocation and binding to the Cyp1a1 gene promoter leading to up-regulation of Cyp1a1 transcription (Kann *et al.*, 2005). In agreement, time-dependent study in Hepa-1c1c7 cells showed that As(III) significantly induced the basal Cyp1a1 mRNA, and potentiated the inducible Cyp1a1 mRNA level (Elbekai and El-Kadi, 2007). In addition, As(III) increased BaP-DNA adduct levels in Hepa-1 cells by as much as 18-fold, and this induction was even higher in cells depleted of reduced GSH (Maier *et al.*, 2002). Similarly, in mouse embryonic fibroblasts, an As(III) and BaP mixture was reported to have an additive effect on the mRNA levels of Cyp1b1 (Kann *et al.*, 2005). In C57BL/6 mice, As(III) potentiated the TCDD-mediated induction of Cyp1a protein expression, EROD, and MROD activities in the liver and lung, while inhibiting the TCDD-mediated induction of Cyp1a, EROD, and MROD activities in the kidney (Anwar-Mohamed *et al.*, 2012; Anwar-

Mohamed *et al.*, 2013a). In addition, As(III) potentiated the TCDD-mediated induction of Cyp1b1 protein expression in the liver of C57BL/6 mice (Anwar-Mohamed *et al.*, 2013a). In agreement, ICR mice showed elevation of Cyp1a1 expression and activity in the lung tissues of arsenic-exposed mice (Wu *et al.*, 2009). In rats, treatment of the rat liver cell line, TRL 1215, with As(III) increased Cyp1a1 transcription and induced apoptosis (Qu *et al.*, 2011). Animal studies have demonstrated that co-exposure to BaP and arsenic increased BaP-7,8-diol-9,10-epoxide (BPDE) adduct formation and lung tumorigenesis (Wu *et al.*, 2009).

In human hepatoma HepG2 cells, As(III) was found to decrease levels of PAH-induced CYP1A1 activities (Vakharia *et al.*, 2001a; Vakharia *et al.*, 2001b). Similarly, treatments of human hepatoma Huh7 cells and Huh7-DRE-Luc (cells transfected with a DRE-driven firefly luciferase reporter plasmid) with As(III) attenuated the TCDD-induced CYP1A1 and luciferase expression, in a concentration-dependent manner (Chao *et al.*, 2006). In primary cultures of human hepatocytes, As(III) decreased the induction of CYP1A1/2 by BaP, benzo[b]fluoranthene (BbF), dibenzo[a,h]anthracene (DBahA), benzo[a]anthracene (BaA), and benzo[k]fluoranthene (BkF) (Vakharia *et al.*, 2001a; Noreault *et al.*, 2005b). ATO inhibits CYP1A activity in primary human hepatocytes and hepatoma, Hep3B and HepG2 cells coexposed to 3MC, BaP, or dioxin and the metalloid for 24 h. Interestingly, ATO effects were abrogated by N-acetylcysteine through blocking the cellular arsenic uptake and increasing the arsenic efflux through multi-drug resistance-associated proteins (Vernhet *et al.*, 2003). Of interest, treatment of HepG2 cells with methylated arsenic derivatives in the absence and presence of TCDD significantly induced CYP1A1 at the mRNA, protein, and catalytic activity levels by activating the AhR signaling pathway (Anwar-Mohamed *et al.*, 2014b). In H1355 cells, a human lung adenocarcinoma cell line,

As(III) enhanced AhR activation leading to elevation of CYP1A1 expression and activity (Wu *et al.*, 2009). Buthionine-(S,R)-sulfoximine, a prooxidant, could further increase the arsenic-induced AhR activation and CYP1A1 enhanced expression, whereas antioxidants, such as N-acetylcysteine and catalase, in addition to AhR antagonists, such as 3',4'-dimethoxyflavone, were found to effectively block the elevation in CYP1A1 (Wu *et al.*, 2009). Interestingly, CYP1A1 polymorphisms seem to play a crucial role in individuals with higher risk of lung cancer due to arsenic exposure. For example, the CYP1A1*2A genotype (MspI) is associated with higher lung cancer risk in males (Adonis *et al.*, 2005). In addition, differences in arsenic biotransformation could play a significant role in tissue- and species-specific effects of arsenic as well as in mortality associated with arsenic-induced cancers (Adonis *et al.*, 2005).

1.6.2 Modulation of Nrf2 signaling pathway and its downstream targets

Arsenic is known to induce the Nrf2-dependent signaling pathway and to activate Nrf2-mediated cellular defense pathways, providing protection against arsenic-mediated toxicities in many cell types and tissues (Du *et al.*, 2008; Wang *et al.*, 2008; Li *et al.*, 2011; Sumi *et al.*, 2011; Yang *et al.*, 2012). Two mechanisms have been proposed for the arsenic-mediated induction of the Nrf2 signaling pathway. First, arsenic binds to cysteine residues of Keap1, a regulator of Nrf2 activity, to regulate divergent functions in Nrf2 signal transduction (He and Ma, 2009; He and Ma, 2010). Second, arsenic induces Nrf2-dependent responses by inhibiting Nrf2 ubiquitination and degradation resulting in enhancement of its protein levels (Wang *et al.*, 2008; He and Ma, 2009). It was reported that arsenic enhances the interaction between Keap1 and Cul3, a subunit of the E3 ubiquitin ligase for Nrf2, resulting in impaired dynamic assembly/disassembly of the E3 ubiquitin

ligase and thus decreases its ligase activity (Wang *et al.*, 2008; He and Ma, 2009). Interestingly, stabilization of Nrf2 protein by arsenic could result in extending its half-life from 21 to 200 min (He *et al.*, 2006). The activation of Nrf2 by arsenic results in induction of phase II and antioxidative genes which is required for neutralizing reactive oxygen species (ROS) generated from arsenic exposure (Jiang *et al.*, 2009; Kumagai, 2009; He and Ma, 2010). Of interest, although Nrf2 serves as a master regulator in cellular defense against oxidative stress and chemical detoxification, persistent activation of Nrf2 achieved by mutations in Nrf2 and/or downregulation of/or mutations in its suppressor, Keap1, was found to be associated with tumorigenicity and chemoresistance of NSCLCs (Zhan *et al.*, 2012). Similarly, other reports addressed the constitutive activation of Nrf2 as a contributor in malignant phenotypes, suggesting that constitutive Nrf2 activation may be involved in arsenic carcinogenesis (Pi *et al.*, 2008; Yang *et al.*, 2012).

Mouse embryonic stem (ES) cells treated with As(III) and arsenate (As(V)) showed significant increases in ROS generation and nuclear accumulation of the transcription factor Nrf2 (Huang *et al.*, 2009). This nuclear accumulation of Nrf2 could be a part of the cellular defense mechanism against arsenic-induced toxicities. This hypothesis was supported by previous reports that showed that pretreatment of primary mouse hepatocytes with sulforaphane (SFN), an activator of Nrf2, for 24 h prior to As(III) exposure reduced not only the arsenic accumulation in cells but also cellular toxicity of this metalloid (Shinkai *et al.*, 2006). Similarly, the wild-type mouse embryo fibroblast (WT-MEF) cells were protected from As(III)-induced toxicity following Nrf2 activation by tert-butylhydroquinone (tBHQ) or SFN, whereas neither tBHQ nor SFN conferred protection in the Nrf2(-/-) MEF cells (Wang *et al.*, 2007). In rat liver epithelial TRL 1215 cells, Nrf2 was clearly activated by As(III) exposure; however it was poorly activated in rat H9c2(2-1) cardiac myocytes,

suggesting reduced ability to metabolize and excrete arsenic in H9c2(2-1) (Sumi *et al.*, 2011). Human hepatocytes showed similar results whereby As(III) could increase the mRNA expression of Nrf2 downstream targets (Li *et al.*, 2011).

In Chang human hepatocytes, As(III) induced ROS generation in a dose- and time-dependent manner; in addition cellular Nrf2 protein level was increased rapidly after 2 h of exposure, elevated significantly at 6 h, and reached the maximum at 12 h (Li *et al.*, 2013). In a human bladder urothelial cell line, UROtsa, the activation of the Nrf2 pathway by tBHQ and SFN rendered the cells more resistant to As(III) (Wang *et al.*, 2007). On the other hand, compromised Nrf2 expression in UROtsa cells, by stably infecting the cells with Nrf2-siRNA (Small interfering RNA), resulted in sensitizing the cells to As(III)-induced toxicity (Wang *et al.*, 2007). In HaCaT cells, an immortalized human keratinocyte cell line, As(III) enhanced cellular expression of Nrf2 at the transcriptional and protein levels, in addition to increasing its nuclear accumulation resulting in activated expression of Nrf2-related genes (Pi *et al.*, 2003). Pretreatment of HaCaT cells with Nrf2 activators, such as tBHQ, protects the cells against acute As(III) toxicity, whereas selective knockdown of Nrf2 reduced the expression of many antioxidant enzymes significantly and sensitized the cells to acute cytotoxicity of As(III) (Zhao *et al.*, 2012). Consistent with the negative regulatory role of Keap1 in Nrf2 activation, Keap1-knockdown cells exhibited enhanced transcriptional activity of Nrf2 under non stressed conditions, in addition Keap1 silencing led to dramatic resistance to As(III)-induced apoptosis (Zhao *et al.*, 2012).

In vivo, Nrf2 protects against liver and bladder injury in response to six weeks of arsenic exposure in a mouse model, whereas Nrf2(-/-) mice displayed more severe pathological changes in the liver

and bladder, compared to Nrf2(+/+) mice. In addition, Nrf2(-/-) mice were more sensitive to arsenic-induced DNA hypomethylation, oxidative DNA damage, and apoptotic cell death (Jiang *et al.*, 2009). Similarly, in a mouse model, two-week exposure to arsenic-containing dust resulted in pathological alterations, oxidative DNA damage, and mild apoptotic cell death in the lung; all of which were blocked by SFN, suggesting that Nrf2 activation is a feasible approach to reduce adverse health effects associated with arsenic exposure (Zheng *et al.*, 2012). Of interest, Sprague-Dawley rats exposed to 50 mg inorganic arsenic per liter in drinking water for one year showed down-regulation of Nrf2 mRNA expression in nucleus accumbens (Rodriguez *et al.*, 2010).

Several studies have been conducted to investigate the effect of the anticancer medication, ATO, on modulation of the Nrf2 signaling pathway. In a multiple myeloma cell line, Nrf2 is expressed constitutively at the mRNA level, however the protein is not detected in untreated cells. When the cells are treated with ATO, Keap1 is inactivated and Nrf2 protein is stabilized and present in the nucleus within 6 h (Morales *et al.*, 2009). Similarly, ATO was reported to markedly alter nuclear levels of Nrf2 in human macrophages (Bourdonnay *et al.*, 2009). In arsenic-resistant ovarian cancer cells, OVCAR-3 subline (OVCAR-3/AsR), numerous gene targets of the Nrf2 transcription factor showed elevated expression resulting in increased tumor aggressiveness and chemoresistance to ATO (Ong *et al.*, 2012). In agreement, it was reported that tumor cells that are deficient for Nrf2 exhibit increased sensitivity to ATO (Liu *et al.*, 2010). Thus inhibiting the Nrf2-mediated adaptive antioxidant response is widely considered a promising strategy to prevent tumor growth and reverse chemoresistance in NSCLCs (Zhan *et al.*, 2012). It is of note that stable knockdown of Keap1 by lentiviral short hairpin RNA (shRNA), unexpectedly sensitized three independent NSCLC cell lines (A549, HTB-178, and HTB-182) to ATO despite moderately

increased Nrf2 levels (Zhan *et al.*, 2012). Similarly, contradicting results were obtained in a study examining the expression of Nrf2 and Nrf2-regulated genes, in oral squamous cell carcinoma (OSCC) *in vitro* and in xenografts. The results of this study showed that ATO had anti-cancer effects on both models with two opposite mechanisms. *In vitro,* ATO activated a silent Nrf2 pathway in cultured OSCC cells as shown by induction of Nrf2 and Nrf2-regulated genes, in a dose-dependent manner. On the contrary, ATO treatment down-regulated expression of Nrf2 and Nrf2-regulated genes in OSCC xenograft tumors which have an active Nrf2 pathway (Yang *et al.*, 2012). Mechanisms behind these discrepancies are to be determined and further studies should be conducted to define the exact molecular mechanisms involved.

1.6.2.1 Modulation of NQO1

Arsenic-mediated induction of NQO1 suggests a role in protecting the cells from injuries due to arsenic exposure (Shi *et al.*, 2010). In activated T cells, non-cytotoxic concentrations of As(III) were found to potently increase the expression of NQO1 without altering the levels of ROS (Morzadec *et al.*, 2012). Similarly, in human bronchial epithelial (BEAS-2B) cells, the NQO1 pathway was found to be modulated by high concentrations of arsenic (Chilakapati *et al.*, 2010). In human umbilical vein endothelial cells (HUVECs), mRNAs of NQO1 increased strikingly when cells were treated with a low concentration of As(III) (Shi *et al.*, 2010). In CL3, H1299, and MC-T2 cancer cell lines, As(III) pretreatment significantly enhanced the expression of NQO1 and susceptibility to antineoplastic activity of mitomycin C (MMC). As(III)-mediated MMC susceptibility was abrogated by the NQO1 inhibitor dicoumarol, suggesting that NQO1 is one of the key regulators of As(III)-mediated MMC susceptibility (Lin *et al.*, 2006). On the other hand,

inhibiting NQO1 in HeLa cells, by dicoumarol, was found to sensitize the cells to ATO-induced apoptosis through raising the ROS level (Jing *et al.*, 2004). In C57BL/6 mice, arsenic-induced NQO1 expression in the kidney, lung and heart (Wu *et al.*, 2009; Anwar-Mohamed *et al.*, 2012). However, chronic exposure to arsenic could have different effects on NQO1, whereby administration of ATO for 6 consecutive weeks via drinking water decreased the reduced GSH levels and the expressions of NQO1 mRNA in the cerebral cortex of mice, and these changes were significantly correlated with the accumulation of arsenic (Yen *et al.*, 2011).

1.6.2.2 Modulation of GSTA1

In vitro, As(III) alone or in the presence of TCDD, enhanced Gsta1 mRNA levels in murine hepatoma Hepa-1c1c7 cells, an effect which was potentiated in the presence of the prooxidant buthionine-(S,R)-sulfoximine, and abrogated with the antioxidant N-acetylcysteine (Elbekai and El-Kadi, 2004; Elbekai and El-Kadi, 2005). In agreement, As(III) showed significant increases in Gsta1 activity, in the absence and presence of TCDD, in a dose-dependent manner (Elbekai and El-Kadi, 2004). *In vivo*, a previous study which investigated the effect of exposure to As(III) in the absence and presence of TCDD, using C57BL/6 mice, reported that As(III) increased Gsta1 mRNA and protein expression and potentiated the TCDD-mediated induction of Gsta1 mRNA and protein expression in the lung and kidney (Anwar-Mohamed *et al.*, 2012). In agreement, another study utilized C57BL/6 mice and showed that Gsta1 mRNA expression and catalytic activity is induced in the kidney during and/or shortly after conditions of oxidative stress by arsenic (Seubert *et al.*, 2002). In another study, mdr1a/1b(-/-) and WT mice were exposed to As(III) in the drinking water for 10 weeks, which resulted in hepatic Gst activity that was significantly higher than in the

respective controls in both mdr1a/1b(–/–) and WT mice (Xie *et al.*, 2004). In Sprague-Dawley rats, a single dose of As(III) selectively modulates activities of GST in a tissue-selective manner, whereby As(III) increased total GST activity in the rat kidneys but not in any of the other tissues (Falkner *et al.*, 1993). Similarly, a single dose of As(III) in Hartley guinea pigs caused an increase in GST activity in the kidney (Falkner *et al.*, 1993). In Wistar rats daily exposure to arsenic via drinking water for 28 days caused an increase in hepatic GST activity (Naraharisetti *et al.*, 2008). Similarly, broiler chickens exposed daily to arsenic in drinking water for 28 days, showed increases in the activities of hepatic microsomal and cytosolic GST (Naraharisetti *et al.*, 2009).

1.6.2.3 Modulation of Heme Oxygenase 1 (HO-1)

HO-1 is an oxidative stress response gene, up-regulated by various physiological and exogenous stimuli as a cellular defense providing cytoprotective activities against stressful stimuli (Cooper *et al.*, 2007; Wang *et al.*, 2011a; Yen *et al.*, 2011). HO-1 is the first and rate-controlling enzymatic step in heme degradation into ferrous iron, carbon monoxide, and biliverdin, which is subsequently converted into bilirubin; these products are supposed to provide protection from oxidative stress (Idriss *et al.*, 2008) (Figure 1.4). HO-1 is known to be a downstream target to Nrf2 and was found to be modulated by heavy metals such as arsenic (Li *et al.*, 2011). Arsenic is reported to be a strong stimulator of HO-1 expression at the level of mRNA and protein in different cell types and tissues in time- and concentration-dependent manners (Cooper *et al.*, 2007; Meng *et al.*, 2010; Wu *et al.*, 2010). Persistent up-regulation of HO-1 was found to play a role in cellular adaptation to chronic arsenic exposure as well as in acute arsenic exposure (Cooper *et al.*, 2007). However, the exact role of HO-1 in As(III)-induced cytotoxicity is poorly understood (Abiko *et al.*, 2010).

Exposure of HepG2 cells to As(III) resulted in persistent induction of HO-1 and prolonged Nrf2 activation; whereas, siRNA-mediated knockdown of HO-1 decreased the prolonged Nrf2 activation, suggesting that As(III)-induced HO-1 appears, at least in part, to act as a positive feedback regulator of Nrf2 activation (Abiko *et al.*, 2010). Moreover, pretreatment with either HO-1 siRNA or the HO inhibitor tin protoporphyrin IX significantly enhanced As(III)-induced cytotoxicity, indicating the importance of HO-1 in diminishing arsenic-induced cytotoxicity (Abiko *et al.*, 2010). In activated T cells, As(III) rapidly and potently increased the expression of HO-1, and similar results were obtained in peripheral blood mononuclear cells (PBMC) treated with As(III) (Martin-Chouly *et al.*, 2011; Morzadec *et al.*, 2012). In Chang human hepatocytes, As(III), dramatically induced HO-1 mRNA and protein, and these effects were reported to last for as long as 24 h (Li *et al.*, 2011). In human choriocarcinoma (JAr) cells, inorganic arsenic was found to cause up-regulation of HO-1 expression as well (Massrieh *et al.*, 2006). Similarly, treatment of cultured MC3T3-E1 osteoblasts with As(III) or As(V) induced Nrf2 and resulted in transcriptional activation of HO-1 (Aono *et al.*, 2003). In vascular smooth muscle cells (VSMCs), arsenic was found to enhance the expression of HO-1, which provides a protective effect against arsenic-induced injury in VSMCs (Lee *et al.*, 2005).

In human promonocytic THP-1 cells, a non-lethal concentrations of ATO significantly induced the expression of HO-1, whereas co-treatment of cells with the HO-1 competitive inhibitor, zinc protoporphyrin (Znpp), potentiated arsenic-induced cytotoxicity (Wang *et al.*, 2011a). In glioma cells, ATO generated ROS, resulting in cell damage and induction of HO-1 expression; in addition, knockdown of Nrf2 strongly inhibited the induction of HO-1 and significantly enhanced ATO-induced oxidative damage (Liu *et al.*, 2011). Moreover, HO-1 inducers clearly protected from

ATO-induced cell death and ROS generation, whereas HO-1 inhibitors led to a significant increases in ATO-induced cell death and ROS generation, suggesting that administration of ATO in combination with either a HO-1 inhibitor or Nrf2 knockdown may act as a new approach for the treatment of glioma (Liu *et al.*, 2011).

In vivo, gestational arsenic exposure increased expression of HO-1 in the liver (Nohara *et al.*, 2012), and similarly, subcutaneous injection of mice with either As(III) or As(V) increased the expression of HO-1 by 10-fold (Liu *et al.*, 2001). Interestingly, different HO-1 alleles can play a crucial role in protection against arsenic-induced diseases. For example short (GT)n allele, in the promoter region of HO-1 gene, is associated with lower risk of developing carotid atherosclerosis in individuals exposed to high levels of arsenic in drinking water (Wu *et al.*, 2010).

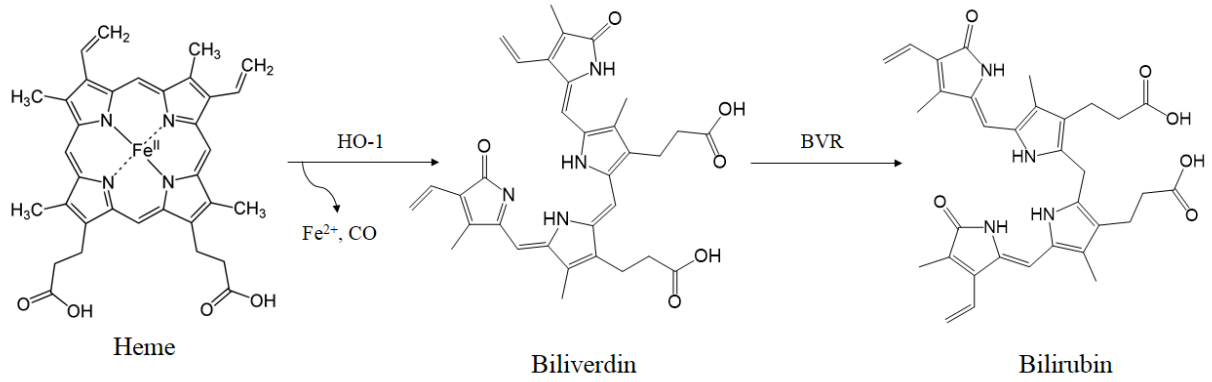


Figure 1.4 Oxidative metabolism of heme by heme oxygenase 1 (HO-1) and biliverdin reductase (BVR). CO, carbon monoxide; Fe^{2+}, ferrous iron. HO-1 metabolic products of heme are considered to be an adaptive and beneficial response to oxidative stress in a wide variety of cells. Adapted from (Erdmann *et al.*, 2008).

1.7 Organic arsenic metabolites

Acute toxicity of arsenic was found to be dependent on the chemical form and concentration of arsenic (Liu *et al.*, 2001). Arsenic is known to be widely distributed in the environment in different inorganic and organic forms in the trivalent or the pentavalent states (Hughes *et al.*, 2011; Cullen *et al.*, 2016; Sattar *et al.*, 2016). Arsenic is released into the environment from smelters, pesticide manufacturing, herbicide manufacturing, cigarette smoke, arsenic-treated wood and agricultural fertilizers (Fishbein, 1981; WHO, 2000). Inorganic arsenic is progressively biomethylated in the liver into organic derivatives; in addition, the methylation process occurs in the soil and water, resulting in the direct exposure of humans to these metabolites (Fishbein, 1981; WHO, 2000) (Figure 1.5). Inorganic arsenic is usually more harmful than organic forms (Yen *et al.*, 2011); however reports suggest that methylation of inorganic arsenic could activate the toxic and carcinogenic potential of arsenic rather than being a detoxification mechanism (Hughes, 2009; Martinez *et al.*, 2011).

1.7.1 Metabolism and disposition of inorganic arsenic to organic arsenic

Scientists agree that arsenic metabolism in the human body is an important determinant of its toxicity. Once inside the body, inorganic arsenic is easily absorbed into the blood and taken up by tissues and cells, mainly in the liver, where it undergoes several steps of reductions and oxidative methylations, by arsenic methyltransferase (AS3MT, previously CYT19), to form the pentavalent organic metabolites monomethylarsonic acid (MMA(V)), dimethylarsinic acid (DMA(V) (also known as cacodylic acid) and trimethylarsine oxide (TMAO), in addition to the trivalent

intermediates, monomethylarsonous acid (MMA(III)) and dimethylarsinous acid (DMA(III)). More specifically, As(V) is reduced to As(III), and this reduction is followed by oxidative methylation of As(III) to, MMA(V). The sequence repeats with the reduction of MMA(V) to MMA(III), followed by oxidative methylation of MMA(III) to form DMA(V). The sequence continues to form DMA(III), then TMAO (Figure 1.5) (Dombrowski *et al.*, 2005; Casarett *et al.*, 2008; Hughes, 2009; Hughes *et al.*, 2011; Cullen *et al.*, 2016). Noteworthy, the metabolism of arsenic is a complicated process, where the metabolic profile depends on the arsenic species, the route of administration, and the type of cells involved in arsenic elimination (Stice *et al.*, 2016). Arsenic was found to be metabolized in yeast, fungi, algae, plants and animals, in addition to soil, and fresh and salt water (Fishbein, 1981; WHO, 2000; Roy and Saha, 2002; Aylward *et al.*, 2014). Previous studies reported the ability of the human body to metabolize different forms of arsenicals into methylated trivalent and pentavalent arsenicals, including the metabolic end product TMAO (Marafante *et al.*, 1987; Vahter, 1999; Francesconi *et al.*, 2002; Shen *et al.*, 2003; ATSDR, 2007). Available data suggests that the methylated arsenicals are not demethylated to inorganic arsenicals in humans or in animals (Yamauchi and Yamamura, 1984; ATSDR, 2007). For years, the methylation of arsenic was considered to be a detoxification mechanism; however, recent reports revealed that the methylation process could activate the toxic and carcinogenic potential of arsenic in humans (Cohen *et al.*, 2006; Hughes, 2009; Martinez *et al.*, 2011). Studies showed that cells with higher rates of arsenic metabolism have increased susceptibility to DNA oxidative damage and induction of tumorigenesis (Pace *et al.*, 2016). Accumulating evidence has suggested that arsenic-induced carcinogenicity could be mediated by AhR-regulated genes. One of the mechanisms responsible for carcinogenicity could be the induction of the Cyp1 family, Cyp1a1, Cyp1a2, and Cyp1b1. These enzymes are known to have carcinogen activating capacity, and they

are regulated by the AhR transcription factor (McFadyen *et al.*, 2004b; Mahadevan *et al.*, 2007; Androutsopoulos *et al.*, 2009; Chang *et al.*, 2010).

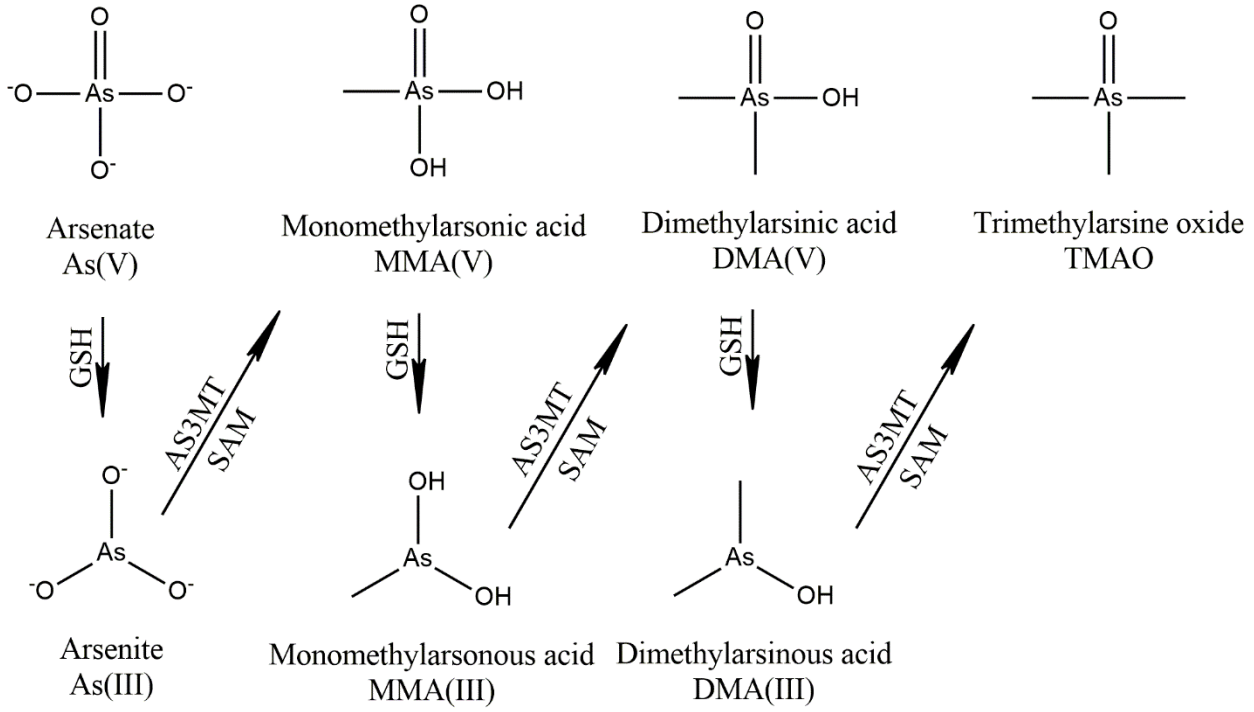


Figure 1.5 Schematic representation of arsenic metabolism via methylation. Oxidative methylation is catalyzed by arsenic methyltransferase (AS3MT; Cyt19), with S-adenosylmethionine (SAM) serving as the methyl donor. Reduction of pentavalent to trivalent forms is required for methylation, where reducing equivalents are supplied by glutathione (GSH). As(V): arsenate; As(III), arsenite; MMA(V), monomethylarsonic acid; MMA(III), monomethylarsonous acid; DMA(V), dimethylarsinic acid; DMA(III), dimethylarsinous acid; TMAO, trimethylarsine oxide. Adapted from (Cohen *et al.*, 2006; Casarett *et al.*, 2008; Ren *et al.*, 2011).

1.7.2 Toxicity of organic metabolites

It is thought that the toxicological effects of arsenicals depend on their chemical speciation, valences and chemical structures such as differences in the number of methyl substituents on the arsenic atom (Kaise *et al.*, 1989; de Francisco *et al.*, 2016). In the following subsections we will discuss the toxicity of three organic metabolites of arsenic that may play a crucial role in arsenic-mediated toxicity and carcinogenicity: first, TMAO which is known to be the metabolic end product of arsenic biotransformation; second, DMA(V) which is considered the main metabolite of arsenic metabolism; and finally, MMA(III) which is the first trivalent organic metabolite formed upon transformation of trivalent inorganic As(III).

1.7.2.1 TMAO

The metabolic end product, TMAO, is a pentavalent arsenic compound, which has three methyl groups, in addition to one oxygen atom, bonded to the arsenic atom (TRC, 2015). While there are few reports on the study of the behavior and toxicological properties of TMAO *in vivo*, it is generally accepted that TMAO is not demethylated, but remains stable inside the body (Kaise *et al.*, 1989; Yamauchi *et al.*, 1990). A previous study showed extensive absorption from the gastrointestinal tract in hamsters administered a single oral dose of TMAO (10 mg As/kg), where arsenic levels in whole blood and plasma peaked within 1 h. The assessment of TMAO clearance showed that approximately 89% of the dose was eliminated in urine within 120 h (Yamauchi *et al.*, 1989). Yamauchi et al. calculated the biological half-lives after oral administration of organoarsenicals to hamsters from many studies conducted in their laboratory, and it was reported

that the half-life of TMAO is 5.3 h (Yamauchi *et al.*, 1990). Metabolic studies in which humans specifically consumed TMAO alone rather than in seafood were not found. Hence, tissue distribution data in humans are derived from limited studies in which human volunteers have ingested labelled organoarsenicals in the form of arsenobetaine. The results showed that arsenobetaine is rapidly and widely distributed in soft tissues (Brown *et al.*, 1990). Thus further studies on the toxicity and metabolism of TMAO and its occurrence in the natural environment and in the body seem necessary.

1.7.2.2 DMA(V)

The pentavalent arsenic metabolite, DMA(V), is the major methylated metabolite of ingested arsenicals in most mammals (Yamamoto *et al.*, 1995; Nishikawa *et al.*, 2002). It is formed in humans and rodents after exposure to trivalent (As(III)) or pentavalent (As(V)) inorganic arsenic via ingestion or inhalation (Kenyon and Hughes, 2001). Scientists determined the extent to which inorganic arsenic is metabolized through examining the relative amounts of its metabolites in urine. In this regard, scientists reported that human urine typically contains 60–80% DMA(V), indicating that the intracellular metabolism of inorganic arsenic involves extensive metabolism into DMA(V) (Cohen *et al.*, 2006). Based on this data, DMA(V) has been utilized as a biological marker for inorganic arsenic exposure (Shimoda *et al.*, 2015). In an *in vitro* assay system involving rat liver cytosol, more than 90% of As(III) was converted to DMA(V) after a 90-minute incubation (Styblo *et al.*, 1995). On commercial levels, DMA(V) has been manufactured and sold as herbicide for weed control on cotton, nonbearing orchards, turf, and in noncrop areas (Cohen *et al.*, 2006).

Previously, DMA(V) was considered a detoxification product of arsenic; however evidences have accumulated in recent years indicating that, DMA(V) itself has unique toxic properties (Brown *et al.*, 1997). For example, DMA(V) induces lung-specific DNA damage in mice and rats; in addition to cultured human pulmonary cells, which was attributed to free radicals produced during the metabolism of DMA(V) (Yamanaka *et al.*, 1989; Brown *et al.*, 1997). In a multi-organ promotional study in rats, DMA(V) promoted carcinogenesis of urinary bladder, kidney, liver, and thyroid gland tumors initiated by chemical carcinogenesis initiators (Yamamoto *et al.*, 1995). Similarly, DMA(V) was reported to promote chemically-induced lung cancer in mice (Yamanaka *et al.*, 1996). Moreover, DMA(V) was found to be a carcinogenic to urinary bladder in rats. The identified mechanism for the DMA(V)-induced bladder tumors was cytotoxicity with necrosis of the bladder urothelium, which is followed by a regenerative process, leading to sustained increased cell proliferation and hyperplasia (Cohen *et al.*, 2006). This mounting evidence suggests that DMA(V) may be a complete carcinogen in some organs, which could play a role in the carcinogenesis of inorganic arsenic. Thus, the US EPA classifies DMA(V) as a probable human carcinogen (Ahmad *et al.*, 1999). In addition, IARC consider DMA(V) to be responsible for carcinogenesis in urinary bladder and lung (Shimoda *et al.*, 2015).

1.7.2.3 MMA(III)

Trivalent arsenic metabolite, MMA(III), is a key intermediate in the metabolic pathway of arsenic biomethylation (Aposhian *et al.*, 2000a; Wang *et al.*, 2015). MMA(III) is the first trivalent organic intermediate in the methylation of inorganic arsenic. The oxidation state of MMA(III) is +3, similar to its parent compound, As(III). Evidence has shown that MMA(III) is present in

appreciable concentrations in mammals, including humans, following exposure to inorganic arsenic. For example, urinary excretion of MMA(III) has been detected in human populations exposed to inorganic arsenic in their drinking water, without and with concomitant treatment with chelators (Aposhian *et al.*, 2000b). The fact that humans excrete much more MMA(III) than any other species led scientists to hypothesize that MMA(III) might be a cause of arsenic carcinogenesis; this is plausible in light of the unusually high sensitivity of humans to arsenic-induced carcinogenesis (Kitchin, 2001).

MMA(III) showed biological activities in different experimental models including enzyme inhibition, cell toxicity, and genotoxicity (Kitchin, 2001). For instance, MMA(III) toxicity in Chang human hepatocytes was even greater than inorganic arsenic (Petrick *et al.*, 2000); similarly, it was found to be more cytotoxic than As(III) in normal human hepatocytes, epidermal keratinocytes, bronchial epithelial cells and urinary bladder cells (Styblo *et al.*, 2000). Thus MMA(III) might be responsible for some of the adverse effects associated with exposure to arsenic (Styblo *et al.*, 2000). Moreover, MMA(III) nicks and breaks DNA *in vitro* without the need for exogenously added enzymatic or chemical activation, and it was found to be an effective DNA-damaging agent in human lymphocytes. Interestingly, MMA(III) is ~ 77-fold more potent as a DNA-damaging agent than As(III) (Mass *et al.*, 2001). In fact the biological activities of MMA(III) are impressive for a methylated metabolite formerly thought to be a "detoxified" form of arsenic (Kitchin, 2001).

Some efforts have been made to demonstrate the toxicity of MMA(III) *in vivo* and to explore the mechanism of toxicity in intact animals (Petrick *et al.*, 2001). For example, MMA(III) has been

identified and quantitated in the livers of male Golden Syrian hamsters following intraperitoneal (i.p.) injection of radioactive As(V) (Sampayo-Reyes *et al.*, 2000), and it has been identified in bile of rats injected with As(III) or As(V) (Gregus *et al.*, 2000). In an effort to compare the *in vivo* toxicities of MMA(III) and As(III), both compounds were injected i.p. into male Golden Syrian hamsters and the resulting median lethal dose (LD50) values were 29.3 and 112.0 μmol/kg of body weight, respectively (Petrick *et al.*, 2001). Since inorganic As(III) is less toxic *in vivo* and *in vitro* than its biotransformation product, MMA(III), it is plausible to consider formation of MMA(III) as a toxification of inorganic arsenic (Gregus *et al.*, 2000; Petrick *et al.*, 2001). Despite efforts that have been conducted to study MMA(III) toxicity and its biological effects, no attempts has been made to examine the effect of MMA(III) on drug metabolizing enzymes and its signaling pathways.

1.8 Mechanisms of arsenic-mediated toxicity and carcinogenicity

Several mechanisms have been proposed for arsenic toxicity and carcinogenicity (Figure 1.6). For example arsenic and its metabolites have been shown to cause oxidative stress, oxidative DNA damage, chromosomal aberration, aneuploidy, micronuclei formation, alteration in DNA methylation status, genomic instability, impaired DNA damage repair, and enhanced cell proliferation (Hirano *et al.*, 2003; Casarett *et al.*, 2008; Ruiz-Ramos *et al.*, 2009; Ezeh *et al.*, 2016). In addition, trivalent compounds of arsenic are thiol-reactive, and thus they can inhibit enzymes and alter proteins by reacting with thiol groups. On the other hand, pentavalent arsenate is considered an uncoupler of mitochondrial oxidative phosphorylation, through competitive substitution of As(V) for inorganic phosphate in the formation of adenosine triphosphate (ATP)

(Casarett *et al.*, 2008). In the following subsections, we will discuss three main mechanisms proposed to be involved in arsenic-mediated toxicity and carcinogenicity: first, ROS formation, second, epigenetic changes, and finally, arsenic biotransformation.

1.8.1 ROS formation

Arsenic-induced ROS generation results in the accumulation of free radicals and oxidative stress in the cells. Generation of ROS is mediated by cycling between oxidation states of metals such as arsenic and iron, in addition to interaction with antioxidants. Major arsenic-induced ROS include superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and peroxyl radicals. Several sources and mechanisms have been implicated in the generation of ROS by arsenicals (Flora, 2011). First, mitochondria are suggested to be one of the important sites of ROS production through the mitochondrial respiratory chain. Second, generation of intermediary arsenic species may produce significant amounts of free radicals. Third, methylated arsenic species can release redox-active iron from ferritin, whereby free iron plays a central role in generating harmful oxygen species through promotion of the conversion of $O_2^{\bullet -}$ and H_2O_2 into the highly reactive $^{\bullet}OH$ radical by the Haber-Weiss reaction. Fourth, ROS can be generated during oxidation of As(III) to As(V) (Flora, 2011). Arsenic-induced oxidative damage was found to be associated with skin tumors, modification of gene transcription profiles of human hyperkeratosis, affecting several cancer-relevant pathways, such as the Wnt/β-catenin and calcium signaling pathways. In addition, it was found to induce both single- and double-DNA strand breaks in most cancer types (Martinez *et al.*, 2011).

1.8.2 Epigenetic changes

Normal epigenetic transcriptional regulation is affected, directly and indirectly, by inorganic arsenic, organic metabolites, and biotransformation processes. Modulation of epigenetic regulation was reported at the level of DNA methylation, histone maintenance, and miRNA expression (Martinez *et al.*, 2011). First, at the DNA methylation level, an association was found between modulation of DNA methylation and several cancer types induced by arsenic. For example epithelial cells were reported to undergo malignant transformation after exposure to chronic, low-level arsenic, and this transformation was associated with DNA hypomethylation (Zhao *et al.*, 1997). Second, at the histone maintenance level, arsenic and its metabolites have been shown to modulate normal histone patterns. For example, chronic exposure to arsenic compounds induces malignant transformation of human non-tumorigenic urothelial cells, UROtsa. This malignant transformation was associated with changes to histone H3 acetylation (Jensen *et al.*, 2008). Third, on the level of miRNA expression, arsenic exposure can significantly alter miRNA expression levels. For example, six days treatment of arsenic alters the expression of five miRNAs (hsa-miR-210, -22, -34a, -221, and -222) in human lymphoblastoid cells (Marsit *et al.*, 2006). Similarly, prolonged exposure to low levels of As(III) in immortalized p53-knocked down human bronchial epithelial cells (p53(low)HBECs) resulted in malignant transformation that was associated with reduction in the levels of miR-200 family members. Stable expression of miR-200b completely reversed this transformation. In addition, stable expression of miR-200b in non-transformed p53(low)HBECs completely prevented malignant transformation induced by As(III) (Wang *et al.*, 2011b).

1.8.3 Biotransformation

As discussed earlier (section 1.7. Organic arsenic metabolites), biomethylation could participate in the activation of the toxic and carcinogenic potential of arsenic. Several mechanisms could be involved in the toxicity-mediated by arsenic biomethylation. First, organic metabolites of arsenic are able to affect the transcription of several genes (Martinez *et al.*, 2011). Second, trivalent organic metabolites are more cytotoxic, more genotoxic, and more potent inhibitors of the activities of some enzymes than inorganic arsenicals that contain arsenic in the trivalent oxidation state (Thomas *et al.*, 2001). Third, the biotransformation pathway uses S-adenosylmethionine (SAM), as a methyl group donor, and thus it can interfere with other cellular processes that require SAM for methylation (Martinez *et al.*, 2011).

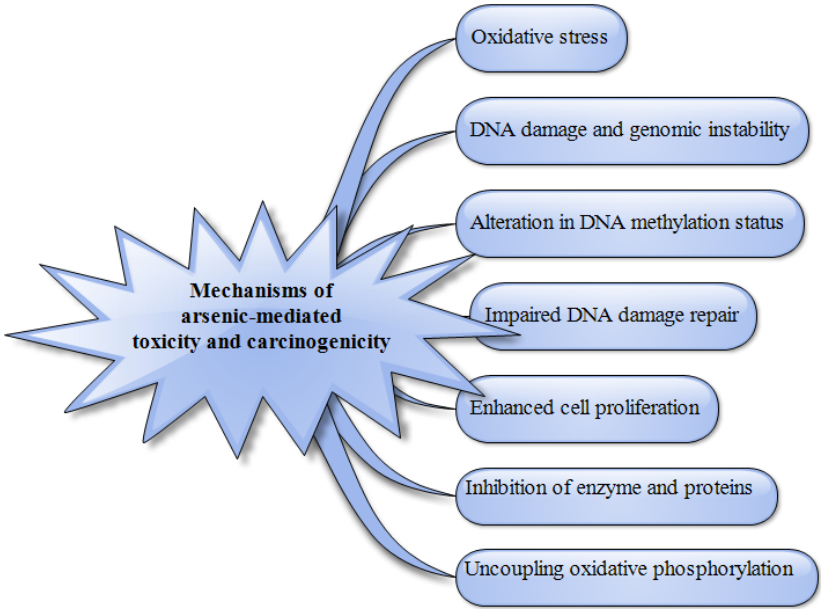


Figure 1.6 Schematic representation of the mechanisms involved in arsenic-mediated toxicity and carcinogenicity. Adapted from (Casarett *et al.*, 2008; Martinez *et al.*, 2011).

1.9 Rationale

Arsenic is a well-known modulator of drug-metabolizing enzymes such as CYP enzymes, which are known to be involved in the oxidative metabolism and elimination of a variety of chemicals (Noreault *et al.*, 2005a; Noreault *et al.*, 2005b; Naraharisetti *et al.*, 2008). This modulation of CYP enzymes is considered as a contributing factor to arsenic-induced toxicity (Noreault *et al.*, 2005b). In addition, arsenic is known to modulate signaling pathways of transcription factors responsible for regulation of CYP enzymes, such as AhR (Vakharia *et al.*, 2001a; Bessette *et al.*, 2005; Bonzo *et al.*, 2005; Chao *et al.*, 2006; Bessette *et al.*, 2009). The modulation of AhR with As(III) results in differential alteration of expression of various phase I and phase II AhR-regulated genes in *in vivo* and *in vitro* systems (Elbekai and El-Kadi, 2004; Anwar-Mohamed *et al.*, 2012; Anwar-Mohamed *et al.*, 2013a). *In vitro,* several models have been employed such as Hepa-1c1c7 cells, human HepG2 cells, and primary hepatocytes (Vakharia *et al.*, 2001b; Elbekai and El-Kadi, 2007; Anwar-Mohamed *et al.*, 2013a). *In vivo,* studies have examined the effect of As(III) in guinea pigs, rats, and mice models (Falkner *et al.*, 1993; Bashir *et al.*, 2006; Anwar-Mohamed *et al.*, 2013a). Despite all these efforts, no previous attempt, to the best of our knowledge, has been conducted to examine the effect of organic metabolites of arsenic on the regulation of AhR-regulated genes *in vivo* in the hepatic and extrahepatic tissues. In this dissertation, we will focus on understanding the potential outcome upon exposure to organic arsenicals and environmental pollutants, With regard to the expression of AhR downstream targets. Understanding of cellular signaling events associated with these downstream targets in response to arsenicals exposure could facilitate rational designing of strategies to reduce arsenic damage by modulating signaling events, which could be of great importance in the fight against arsenic-induced diseases.

1.9.1 Hypotheses

Hypothesis 1: exposure to TMAO, the metabolic end product of As(III), and the AhR ligand, TCDD, disrupt the coordinated balance of phase I and phase II AhR-regulated genes both *in vitro* and *in vivo*.

Hypothesis 2: exposure to TMAO and TCDD *in vivo* alters the expression of phase I and phase II AhR-regulated genes in extrahepatic tissues in a tissue- and enzyme-specific manner.

Hypothesis 3: exposure to DMA(V), a main pentavalent metabolite of As(III), and TCDD differentially modulate the prototypical phase I and II AhR-regulated genes, Cyp1a1 and Nqo1, respectively.

Hypothesis 4: exposure to MMA(III), the first trivalent metabolite of As(III), and TCDD modulate the prototypical AhR-regulated gene, CYP1A1, via modulation of its signaling pathway.

1.9.2 Specific objectives

1- To investigate the effect of TMAO, in the absence and presence of TCDD, on AhR-regulated phase I and phase II enzymes *in vivo* using C57BL/6 mice livers, and to investigate molecular mechanisms *in vitro* using isolated primary hepatocytes from C57BL/6 mice.

2- To investigate the tissue-, and enzyme-specific effects, upon the exposure to a single dose of TMAO, in the absence and presence of TCDD, in extrahepatic tissues, namely: lung, kidney, and heart of C57BL/6 mice.

3- To examine the effect of DMA(V), in the absence and presence of TCDD, on the modulation of the AhR signaling pathway with a resultant alteration of its prototypical phase I and phase II regulated genes, Cyp1a1 and Nqo1, in the liver, lung, and kidney of C57BL/6 mice.

4- To examine the effects of MMA(III) as compared to its parent compound, As(III), in the absence and presence of TCDD, on the constitutive and inducible levels of CYP1A1 gene expression, protein production and catalytic activity in human HepG2 cells; and to explore the underlying mechanisms involved in this modulation at the transcriptional, post-transcriptional, translational, and post-translational levels.

All experiments were conducted in the presence and absence of the TCDD, as the prototypical and the most potent AhR ligand/agonist.

1.9.3 Significance and impact on patient outcomes

This research is expected to have a great impact on the understanding of the cellular and molecular mechanisms responsible for the modulation of AhR-regulated genes by arsenicals. Better recognition of coordinating phase I and II metabolism may greatly attenuate health risks posed by CYP1A1-generated toxic intermediates and ROS. In addition, it will also reveal novel points of intervention to be exploited in the development of new therapies for the treatment and prevention of arsenic toxicity and carcinogenicity.

2 CHAPTER TWO - MATERIALS AND METHODS

Versions of this chapter have been published in:

Elshenawy, O.H., El-Kadi, A.O., 2015a. Modulation of aryl hydrocarbon receptor-regulated enzymes by trimethylarsine oxide in C57BL/6 mice: In vivo and in vitro studies. Toxicol. Lett. ***238****, 17-31.*

Elshenawy, O.H., El-Kadi, A.O., 2015b. Modulation of aryl hydrocarbon receptor regulated genes by acute administration of trimethylarsine oxide in the lung, kidney and heart of C57BL/6 mice. Xenobiotica ***45****, 930-943.*

Elshenawy, O.H., Abdelhamid, G., Althurwi, H.N., El-Kadi, A.O., 2017a. Dimethylarsinic acid modulates the aryl hydrocarbon receptor-regulated genes in C57BL/6 mice: in vivo study. Xenobiotica, 1-11. DOI 10.1080/00498254.00492017.01289423.

Elshenawy, O.H., Abdelhamid, G., Soshilov, A.A., Denison, M.S., El-Kadi, A.O., 2017b. Down-regulation of cytochrome P450 1A1 by monomethylarsonous acid in human HepG2 cells. Toxicol. Lett. ***270****, 34-50.*

2.1 Chemicals

10% neutral-buffered formalin, 1-chloro-2,4-dinitrobenzene (CDNB), 2,6-dichlorophenolindophenol (DCPIP), 20% neutral-buffered formalin, 1 diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 7-ethoxyresorufin, 7-methoxyresorufin, actinomycin D (Act-D), anti-goat IgG peroxidase secondary antibody, collagen from rat tail, collagenase, cycloheximide (CHX), dicoumarol, fluorescamine, menadione, protease inhibitor cocktail, As(III) (NaAsO2), and β-glucuronidase from Helix pomatia with sulfatase activity were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Alexa Fluor 488, DAPI (4',6-diamidino-2-phenylindole, dihydrochloride), Dulbecco's modified Eagle medium (DMEM), Lipofectamine 2000 reagent, ProLong® Gold Antifade mounting medium, and TRIzol reagent, were purchased from Invitrogen (Carlsbad, CA, USA). Dihydroethidium (DHE) was purchased from Abcam (Toronto, ON, Canada). TCDD, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). TMAO was purchased from Tri Chemical Laboratories Inc. (Yamanashi, Japan). DMA(V) and MMA(III), in the form of its precursor CH_3AsI_2 (Pace *et al.*, 2016), were purchased from Toronto Research Chemicals (Toronto, ON, Canada). 96-well optical reaction plates with optical adhesive films, SYBR Green SuperMix, and The High-Capacity Complementary DNA (cDNA) Reverse Transcription Kit, were purchased from Applied Biosystems (Foster City, CA, USA). Real-time PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Chemiluminescence Western blotting detection reagents were purchased from GE Healthcare Life Sciences (Piscataway, NJ, USA). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Primary monoclonal antibodies for Cyp1a1/2, Nqo1, Gsta1/2, Ho-1, and glyceraldehyde-3-phosphate dehydrogenase

(Gapdh), and primary polyclonal antibodies for Cyp1b1, AhR, actin, and calnexin, in addition to anti-rabbit and anti-mouse IgG peroxidase secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse IgG peroxidase secondary antibody was purchased from R&D Systems (Minneapolis, MN, USA). Glass coverslips were purchased from Electron Microscopy Sciences (Hatfield, PA, USA). Renilla luciferase plasmid pRL-CMV and dual luciferase assay reagents were obtained from Promega (Madison, WI, USA). The XRE-driven luciferase reporter plasmids, pGudLuc1.1 and pGudLuc6.1, were generously provided by Dr. M.S. Denison (University of California at Davis). All other chemicals were purchased from Fisher Scientific (Toronto, ON, Canada).

2.2 Cell model

To test the hypotheses raised in this dissertation, both human hepatoma HepG2 cells, and isolated mice primary hepatocytes were utilized. The HepG2 cells is one of the most widely used human cell lines; it is considered a potential useful model for toxicological studies (Dehn *et al.*, 2004). It was generated in the 1970s as an immortalized cell line that expresses many liver-specific genes (Soldatow *et al.*, 2013), in addition it expresses both phase I and phase II metabolizing enzymes (Grant *et al.*, 1988; Westerink and Schoonen, 2007). Thus, it is capable of performing the metabolic and biotransformation reactions required for detoxification processes for several xenobiotics (Dehn *et al.*, 2004). Interestingly, response of HepG2 cells to dioxin is similar to that observed in primary human hepatocytes (Silkworth *et al.*, 2005). Hence, HepG2 cell line is a useful model for investigating 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). On the other hand, isolated

primary hepatocytes are considered the gold standard for *in vitro* testing based on several facts. First, primary hepatocytes are a predictive model for what would occur in the whole organ as they usually responds in the same manner as an organ would respond. Second, primary hepatocytes can maintain functional activities for several days. Third, they can be used for enzyme induction and inhibition studies. Fourth, they allow for medium-throughput screening of compounds. Fifth, they are ideal for examining interspecies and inter-individual differences in metabolism (Sivaraman *et al.*, 2005; Soldatow *et al.*, 2013).

2.3 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% fetal bovine serum, 100 IU/mL penicillin, 100 µg/mL streptomycin. 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/6 mice and suspended in DMEM supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin, to obtain a final density of 5 x 10^5 cells/mL. Hepatocytes were plated onto cell culture plates (Falcon, Becton Dickinson Labware, New Jersey, USA) which were coated overnight with Type I rat-tail collagen. Cell culture plates were incubated at 37 °C in a humidified cell culture incubator with 5% CO_2. Viability was assessed by the trypan blue (0.2%) exclusion method.

2.4 Isolation of primary mouse hepatocytes

Three solutions were utilized for the isolation of mouse hepatocytes as previously described with some modifications (Seglen, 1976; El-Kadi *et al.*, 1997). Solution A contained 115 mM sodium chloride, 5 mM potassium chloride, 1 mM potassium dihydrogen phosphate, 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) sodium salt, 0.5 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), 5.5 mM glucose and 10 U/mL heparin in deionized water (pH=7.4). Solution B was composed of 115 mM sodium chloride, 5 mM potassium chloride, 1 mM potassium dihydrogen phosphate, 25 mM HEPES sodium salt, 5.5 mM glucose, 10 U/mL heparin, 1 mM calcium chloride, 0.25 μM trypsin inhibitor, and 0.05% collagenase in deionized water (pH=7.4). Solution C contained 25 mL of solution B and 75 mL of DMEM supplemented with 1.2 mM magnesium sulfate. Mice were anesthetized, and the liver was perfused via the portal vein with 25 mL of solution A at flow rate of 5 mL/min, and then with 25 mL of solution B at the same flow rate. After in situ perfusion, the liver was removed and placed in a petri dish containing 30 mL solution C. The capsule was stripped away and the cells were detached and filtered through a (70 μm) cell strainer. The cells were centrifuged at 100 g for 2 min, the supernatant was discarded and the sediment was re-suspended in DMEM supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin, to obtain a final density of 5 x 10^5 cells/mL. The isolated hepatocytes were incubated at 37 °C in a cell culture incubator with 5% CO_2.

2.5 Chemical treatment for cells and stock solutions

Cells (Human hepatoma HepG2 cells or isolated primary mouse hepatocytes) were treated in serum-free medium (SFM) with various concentrations of test compounds (TMAO, As(III) or MMA(III)) in the absence and presence of 1 nM TCDD as described in the figure legends in chapter three. TCDD and MMA(III) were dissolved in dimethyl sulfoxide (DMSO) and maintained in DMSO at −20 °C until use. As(III) was prepared in double-deionized water and maintained at −20 °C until use. TMAO was prepared fresh in double deionized water. Diluted stock solutions of test compounds were prepared fresh in SFM before each experiment. In all treatments, the DMSO concentration did not exceed 0.05% (v/v).

2.6 Biohazard precaution

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

2.7 Animal treatment protocols

2.7.1 Animal model and ethics

Male C57BL/6 mice, ages 10–12 weeks (Charles River Laboratories, Montreal, QC, Canada) were used. 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.

2.7.2 Animal treatment for TMAO experiment

Male C57BL/6 (20–25 g) mice were injected i.p. with TMAO (dissolved in saline) at 13 mg/kg with or without TCDD (dissolved in corn oil) at 15 μg/kg, also i.p. The mice were divided into four groups. The first group (n=12), control mice, received saline (0.4 mL) plus corn oil (0.4 mL). The second group (n=12), TMAO-treated mice, received TMAO dissolved in saline (0.4 mL) plus corn oil (0.4 mL). The third group (n=12), TCDD-treated mice, received TCDD dissolved in corn oil (0.4 mL) plus saline (0.4 mL). The fourth group (n=12), TMAO plus TCDD-treated mice, received TMAO dissolved in saline (0.4 mL) plus TCDD dissolved in corn oil (0.4 mL). The animals were euthanized at 6 (n=6) or 24 h (n=6) after a single injection via cervical dislocation. Liver, Lung, kidney, 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.

2.7.3 Animal treatment for DMA(V) experiment

Male C57BL/6 (20–25 g) mice were injected i.p. with DMA(V) (dissolved in saline) at 13.3 mg/kg with or without i.p. injection of 15 μg/kg TCDD (dissolved in corn oil). The mice were divided into four groups. The first group (*n*=12), control mice, received saline (0.4 mL) plus corn oil (0.4 mL). The second group (*n*=12), DMA(V)-treated mice, received DMA(V) (13.3 mg/kg) dissolved in saline (0.4 mL) plus corn oil (0.4 mL). The third group (*n*=12), TCDD-treated mice, received TCDD (15 μg/kg) dissolved in corn oil (0.4 mL) plus saline (0.4 mL). The fourth group (*n*=12), DMA(V) plus TCDD-treated mice, received DMA(V) (13.3 mg/kg) dissolved in saline (0.4 mL) plus TCDD (15 μg/kg) dissolved in corn oil (0.4 mL). Thereafter, animals were euthanized at 6 (*n*=6) or 24 h (*n*=6) after treatment via cervical dislocation. Liver, lung, and kidney tissues were excised, immediately frozen in liquid nitrogen, and stored at -80 °C until further analysis. All experimental procedures involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee.

2.8 Experimental protocols

2.8.1 RNA extraction and cDNA synthesis

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

transcription kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, 1.5 µg of total RNA from each sample was added to a mix of 2.0 µL of 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 4.2 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 for 5 min, and finally cooled to 4 °C.

2.8.2 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 Master Mix, 11.05 µL of nuclease-free water, and 1.25 µL of cDNA sample. The primers used were chosen from previously published studies (Song and Freedman, 2005; Amara *et al.*, 2012; Amara *et al.*, 2013; Yu *et al.*, 2015) and are listed in Table 2-1. Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. After the plate was sealed with an optical adhesive cover, the thermocycling conditions were initiated at 95 °C for 10 min, followed by 40 PCR cycles of denaturation at 95 °C for 15 sec and annealing/extension at 60 °C for 1 min. A melting curve (dissociation stage) was performed to ascertain the specificity of the primers and the purity of the final PCR product.

Table 2-1 Primer sequences used for real-time PCRs

Genes	**Forward primers**	**Reverse primers**
Mouse *Cyp1a1*	5′-GGT TAA CCA TGA CCG GGA ACT-3′	5′-TGC CCA AAC CAA AGA GAG TGA-3′
Mouse *Cyp1a2*	5′-TGG AGC TGG CTT TGA CAC AG-3′	5′-CGT TAG GCC ATG TCA CAA GTA GC-3′
Mouse *Cyp1b1*	5′-AAT GAG GAG TTC GGG CGC ACA-3′	5′-GGC GTG TGG AAT GGT GAC AGG-3′
Mouse *Nqo1*	5′-GGA AGC TGC AGA CCT GGT GA-3′	5′-CCT TTC AGA ATG GCT GGC A-3′
Mouse *Gsta1*	5′-CCC CTT TCC CTC TGC TGA AG-3′	5′-TGC AGC TTC ACT GAA TCT TGA AAG-3′
Mouse *Ho-1*	5′-GTG ATG GAG CGT CCA CAG C-3′	5′-TGG TGG CCT CCT TCA AGG-3′
Mouse *β-actin*	5′-TAT TGG CAA CGA GCG GTT CC-3′	5′-GGC ATA GAG GTC TTT ACG GAT GTC-3′
Human *CYP1A1*	5′-CTATCTGGGCTGTGGGCAA-3′	5′-CTGGCTCAAGCACAACTTGG-3′
Human *β-actin*	5′-CTGGCACCCAGCACAATG-3′	5′-GCCGATCCACACGGAGTACT-3′

2.8.3 Real-Time PCR data analysis

The real-time PCR data were analyzed using the relative gene expression, i.e., the $\Delta\Delta C_T$ method as described in Applied Biosystems User Bulletin No. 2 and explained further by Livak and Schmittgen (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 the control (vehicle-treated animals, untreated cells or 0 h time point) from the ΔC_T of the corresponding treatment group. The values and ranges given in the figures were determined as follows: $2^{-\Delta(\Delta CT)}$ + SEM, where SEM is the standard error of the mean of the $2^{-\Delta(\Delta CT)}$ value.

2.8.4 Determination of mRNA half-life

The half-life of CYP1A1 mRNA was determined by an Act-D chase assay. Cells were pretreated with 1 nM TCDD for 12 h. The cells were then washed and incubated with 5 μg/mL Act-D, to inhibit further RNA synthesis, immediately before treatment with 5 μM As(III) or MMA(III). Total RNA was extracted at 0, 1, 3, 6, 12, and 24 h after incubation with 5 μM As(III) or MMA(III). Real-time PCR was performed using SYBR Green PCR Master Mix. The fold change in the level of CYP1A1 (target gene) between treated and untreated cells, corrected by the level of β-actin, was determined using the equation: fold change = $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = C_{t\ (target)} - C_{t\ (\beta\text{-actin})}$ and $\Delta(\Delta Ct) = \Delta C_{t\ (treated)} - \Delta C_{t\ (untreated)}$. The mRNA half-life value was determined by applying exponential regression to a semilog plot of mRNA amount, expressed as a percentage, versus time.

2.8.5 Preparation of cell homogenates and protein extraction

After incubation with the test compounds for the indicated time interval, cells were collected in lysis buffer containing 50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% (v/v) glycerol, 1% Triton X-100, and 5 µL/mL protease inhibitor cocktail. The cell homogenates were obtained by incubating the cell lysates on ice for 1 h, with intermittent vortexing every 10 min, followed by centrifugation at 12,000 × g for 10 min at 4 °C. The total cellular lysate supernatant was collected and stored at −80 °C. Thereafter, protein concentrations were determined by the Lowry method using bovine serum albumin as a standard (Lowry *et al.*, 1951).

2.8.6 Preparation of microsomal and cytosolic protein fractions from tissues

Liver, lung, and kidney microsomal and cytosolic protein fractions were prepared by differential centrifugation of homogenized tissues as previously described (Diaz and Squires, 2000). Individual liver, lung, or kidney tissues were rapidly removed and washed in ice-cold potassium chloride (1.15% w/v). Subsequently, they were cut into pieces and homogenized separately in 0.25 M sucrose solution. The homogenate was centrifuged at 10,000 g for 20 min and the resulting supernatant was centrifuged again at 100,000 g for 60 min in order to obtain the microsomal and cytosolic fractions. The final microsomal pellets were reconstituted in sucrose and stored with supernatant cytosols at −80 °C. Thereafter, protein concentrations were determined by the Lowry method using bovine serum albumin as a standard (Lowry *et al.*, 1951).

2.8.7 Western blot analysis

Western blot analysis was performed using a previously described method (Towbin *et al.*, 1979; Elbekai and El-Kadi, 2004). Microsomal proteins (10 μg), cytosolic proteins (20 μg), primary hepatocyte total cell lysates (60 μg), or HepG2 total cell lysates (20 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), and then electrophoretically transferred to nitrocellulose membranes. Protein blots were then blocked overnight at 4 °C in blocking solution containing 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris-base (Tris-buffered saline (TBS)), 5% skim milk, 2% bovine serum albumin, and 0.5% Tween 20. After blocking, the blots were incubated for 2 h at 4 °C with primary antibodies (1:1000). Thereafter, incubation with a peroxidase-conjugated IgG secondary antibodies was carried out for 1 h at room temperature. The bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare Life Sciences). The intensity of the protein bands was quantified, relative to the signals obtained for β-actin or Gapdh, using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The values and ranges given in the figures represent relative protein intensity (%), compared to control, + SEM.

2.8.8 Determination of protein half-life

The half-life of CYP1A protein was determined by the CHX chase assay. Cells were pretreated with 1 nM TCDD for 24 h. Thereafter, cells were then washed and incubated with 10 μg/mL CHX, to inhibit further protein synthesis, immediately before treatment with 5 μM As(III) or MMA(III).

Cell homogenates were extracted at 0, 1, 3, 6, and 12 h after incubation with 5 μM As(III) or MMA(III). Cellular protein was determined using the method of Lowry (Lowry *et al.*, 1951). CYP1A protein was measured by Western blotting. The intensity of CYP1A protein bands was quantified, relative to the signals obtained for GAPDH protein, using ImageJ software. The protein half-life value was determined by applying exponential regression to a semilog plot of protein amount, expressed as a percentage, versus time.

2.8.9 Immunocytochemical analysis of AhR localization in primary hepatocytes

Plated cells on collagen-coated glass coverslips were treated for 30 min with either vehicle, TMAO, or TCDD. Treated cells were fixed using 3.7% (w/v) paraformaldehyde in CSK buffer (10 mM Pipes, pH 6.8; 10 mM NaCl; 300 mM sucrose; 3 mM $MgCl_2$; 2 mM EDTA) at room temperature. Cells were then permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature. The cells were incubated with blocking buffer (1× PBS, 1% donkey serum, 0.1% Tween 20) for 10 min at room temperature. Blocked cells were incubated with anti-AhR antibody for 1 h at 4 °C, and then incubated with Alexa Fluor 488 (green) secondary antibody. Thereafter the DNA was stained with DAPI (blue). ProLong® Gold Antifade mounting medium was added to each coverslip before it was mounted on a microscope glass slides. Images were obtained using spinning disk confocal, integrated by Quorum Technologies (Guelph, ON, Canada) on an Olympus IX-81 stand with a Yokogawa CSU-X11 confocal scan unit, with a 100×/1.4 NA lens obtaining an image pixel size of 109 nm.

2.8.10 Immunocytochemical analysis of AhR localization in HepG2 cells

HepG2 cells were plated on collagen-coated glass coverslips in 6-well plates; thereafter, cells were treated for 1 h with either vehicle, 5 μM As(III), or MMA(III) in the absence and presence of 1 nM TCDD. Treated cells were fixed using 10% neutral-buffered formalin (4% formaldehyde solution) at room temperature. Cells were then permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature. The cells were incubated with blocking buffer (1× PBS, 1% donkey serum, 0.1% Tween 20) for 10 min at room temperature. Blocked cells were incubated with anti-AhR antibody for 1 h at 4 °C, and then incubated with Alexa Fluor 488 (green) secondary antibody. Thereafter the DNA was stained with DAPI (blue) diluted in PBS (1:15000). ProLong Gold Antifade mounting medium was added to each coverslip before it was mounted on a microscope glass slides. Spinning disk confocal microscopy was performed on an Olympus IX81 microscope equipped with a Lumen200Pro illuminator (Prior Scientific, Rockland, MA, USA), a Photometrics CoolSNAP HQ2 camera (Photometrics, Tucson, AZ, USA), with a 20x/0.45 Ph1 lens. Thereafter, AhR green fluorescence intensities in representative nuclear and cytosolic areas were quantified using the ImageJ software, and the quotient of nuclear over cytosolic fluorescence was calculated (Seibel *et al.*, 2007). The values and ranges given in Table 3-5 represent relative fluorescence intensity (%) ± SEM, compared to control.

2.9 General assay methods

2.9.1 Measurement of cell viability

The effect of different test compounds on cell viability was determined using the MTT assay as described previously (Vakharia *et al.*, 2001b; Elbekai and El-Kadi, 2004). Cells (hepatocytes or HepG2) 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 test compounds, as described in the figure legends in chapter three, 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. After 2 h of incubation, the crystals that had been formed were dissolved in isopropanol. The intensity of the color in each well was measured at a wavelength of 550 nm using the Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA).

2.9.2 Determination of protein concentration using the Lowry method

Protein concentrations were determined by the Lowry method using bovine serum albumin as a standard, as previously described (Lowry *et al.*, 1951). Briefly, protein samples from different tissues (microsomal, or cytosolic fractions) or from the cells (total cell lysate) were diluted with distilled water to a final volume of 125 µL (dilution factor between 25-125 X). Thereafter 125 µL of freshly prepared reagent A (1 mL of 1% copper(II) sulfate, 1 mL of 2% potassium sodium tartrate, and 20 mL of 10% sodium carbonate anhydrous in 0.5 M Sodium hydroxide) were added for every sample, and the solution was maintained at room temperature for 10 minutes. After that,

375 µL of reagent B (10 X diluted solution of Folin-Phenol reagent) were added for each sample while vortexing, and all samples were incubated in a 50 °C water bath for 10 minutes. All samples were vortexed and 200 µL from each tube were transferred to a 96-well plate. Absorbance was measured spectrophotometrically at 550 nm using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). Protein concentrations were determined by calibrating the absorbance of the samples against a bovine serum albumin standard curve prepared and assayed at the same time as the samples.

2.9.3 Determination of CYP1A1 catalytic activity in cells

The CYP1A1-dependent 7-ethoxyresorufin O-deethylase (EROD) assay was performed on intact living cells using 7-ethoxyresorufin as previously described (Kennedy and Jones, 1994; Sinal and Bend, 1997). After incubation with test compounds for 24 h, 200 µL of 2 µM 7-ethoxyresorufin in assay buffer (0.05 M Tris, 0.1 M NaCl, pH 7.8, supplemented with 100 U β-glucuronidase for isolated hepatocytes; and PBS, pH 7.4 for HepG2) was added to each well. Fluorescence measurement using excitation and emission wavelengths of 535 nm and 585 nm, respectively, was recorded using BioTek Synergy H1Hybrid Multi-Mode Microplate Readers (BioTek Instruments, Winooski, VT, USA). The amount of resorufin formed in each well was determined by comparison to a standard curve of known concentrations. Enzymatic activity was normalized for cellular protein content, which was determined using a modified fluorescence assay, using fluorescamine as a substrate (Lorenzen and Kennedy, 1993; Sanderson *et al.*, 1996). The rate of resorufin formation was expressed as pmol/min/mg protein.

2.9.4 Direct inhibition study of CYP1A1 catalytic activity in cells

The direct inhibitory effect of As(III) and MMA(III) on the CYP1A1 enzyme was determined using a method similar to that described for the EROD assay, with slight modifications, as previously described (Aldawsari *et al.*, 2015). Briefly, HepG2 cells were incubated with 1nM TCDD for 24 h. Thereafter, the cells were washed and incubated for 1 h in fresh medium containing 5 μM As(III), increasing concentrations of MMA(III) (1-10 μM), or (E)-3,4',5-Trimethoxystilbene (TMS), as a positive control. The remaining CYP1A1 activity was detected using the EROD assay and normalized to cellular protein content using a modified fluorescence method (Lorenzen and Kennedy, 1993; Sanderson *et al.*, 1996).

2.9.5 Determination of cellular protein content

The cells were rinsed with PBS, and 50 μL of double de-ionized water was added to each well, and cell plates were placed at −80°C for 30 min to lyse the cells. Thereafter, the cell lysates were allowed to thaw, and protein levels were determined as described previously (Lorenzen and Kennedy, 1993; Sanderson *et al.*, 1996). Briefly, 100 μL of 0.1 M potassium phosphate buffer was added to each well followed by 50 μL of 1.08 mM fluorescamine and plates were shaken for five minutes. The florescence was measured using Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA) using excitation and emission wavelengths of 365 and 470 nm, respectively. To obtain sample protein concentrations, the fluorescent intensity data were calibrated against a bovine serum albumin standard curve prepared and assayed at the same time as the sample plates.

2.9.6 Microsomal incubation and measuring Cyp1a1 and Cyp1a2 catalytic activities

Cyp1a1-dependent EROD and Cyp1a2-dependent 7-methoxyresorufin O-demethylase (MROD) activities were assessed using 7-ethoxyresorufin and 7-methoxyresorufin as substrates, respectively. Microsomes from liver, lung, or kidney of various treatments (1 mg protein/mL) were incubated in the incubation buffer (5 mM magnesium chloride hexahydrate dissolved in 0.1 M potassium phosphate buffer, pH 7.4) with 7-ethoxyresorufin or 7-methoxyresorufin (2 μM final concentration) at 37 °C in a shaking water bath. A pre-equilibration period of 5 min was performed. The reaction was initiated by the addition of 1 mM NADPH (nicotinamide adenine dinucleotide phosphate hydrogen). After incubation at 37 °C (5 min for EROD and 10 min for MROD assay), the reaction was stopped by adding 0.5 mL of cold methanol. The amount of resorufin formed was measured using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA) using excitation and emission wavelengths of 535 and 585 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.

2.9.7 Cytosolic incubation and determination of Nqo1 enzymatic activity

The Nqo1 activity was determined by the continuous spectrophotometric assay to quantitate the reduction of its substrate, DCPIP, as described previously (Aleksunes *et al.*, 2006). Cytosolic protein (20 μg) of liver, lung, or kidney of different treatment groups was incubated with 200 μL of the assay buffer (40 μM DCPIP, 0.2 mM NADPH, 25 mM Tris hydrochloride, 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 100 seconds at 600 nm using Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). The Nqo1 activity was calculated as the decrease in absorbance per min per mg protein of the sample that quantitates the dicoumarol-inhibitable reduction in DCPIP. The enzyme activity was expressed as percentage relative to control, + SEM.

2.9.8 Cytosolic incubation and determination of Gst activity

Gst activity was determined spectrophotometrically using CDNB as a substrate, according to the previously described protocol (Habig *et al.*, 1974). Cytosolic protein (20 μg of liver, 20 μg of lung, or 5 μg of kidney) of different treatments was incubated with 1 mM CDNB, 1 mM reduced GSH in 0.1 M potassium phosphate buffer, pH 6.5 in a total volume of 200 μL. GST activity was measured as the amount of CDNB conjugate formed by recording the absorbance at 340 nm for 3 min using Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). The enzyme activity was expressed as percentage relative to control, + SEM

2.9.9 Microsomal incubation and determination of Ho-1 activity

Ho-1 activity was determined using a previously described method (Sinal *et al.*, 1995; Ndisang *et al.*, 2014). Incubations contained 1 mg/mL microsomal protein, 1.5 mg/mL liver cytosol, as a source of biliverdin reductase, and 25 μM hemin made up to a final volume of 200 μL with buffer (0.1 M potassium phosphate, pH 7.4). These incubation volumes were incubated at 37°C for 5

min; thereafter, reactions were started by adding NADPH (400 μM final). After incubation for 1 h at 37 ºC, with shaking, the sample volumes were scanned spectrophotometrically using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA) from 450 to 550 nm. The amount of bilirubin formed was quantitated based on the readings at 450 nm minus the backgrounds at 530 nm. The enzyme activity was expressed as percentage relative to control, + SEM.

2.9.10 Transient transfection and luciferase assay

Cells were plated onto cell culture plates (24-well cell culture plates for hepatocytes, and 12-well cell culture plates for HepG2), and each well of cells was transfected with 1.6 μg of XRE-driven luciferase reporter plasmid (pGudLuc1.1 for hepatocytes, and pGudLuc6.1 for HepG2), generously provided by Dr. M.S. Denison (University of California at Davis, USA). Cells were co-transfected with 0.1 μg of the Renilla luciferase pRL-CMV vector, for normalization. Transfection was conducted using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). After incubation with test compounds for 24 h, 100 μL of passive lysis buffer was added into each well with continuous shaking for 20 min, and then the content of each well was collected separately. Enzyme activities were determined using a Dual-Luciferase reporter assay system according to the manufacturer's instructions (Promega, Madison, WI, USA). Quantification was performed using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). Luciferase activity in each well was expressed as a ratio of the relative light units of firefly luciferase/renilla luciferase and compared to vehicle-treated cells.

2.9.11 Competitive ligand-binding assay

Ligand binding was performed in two different species using a hydroxyapatite (HAP) assay as previously described (Denison *et al.*, 1986), with slight modifications. Specifically, untreated guinea pig (guinea pig AhR is known to have high homology to the human AhR (Flaveny *et al.*, 2009)) or mouse liver cytosolic protein was diluted to 2 mg/mL in 25 mM 3-(N-morpholino)propanesulfonic acid, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol. Aliquots of 100 µL were incubated in the presence of 2 nM [^{3}H]TCDD alone, 2 nM [^{3}H]TCDD and 200 nM 2,3,7,8-tetrachlorodibenzofuran (TCDF) (100-fold excess of competitor), or 2 nM [^{3}H]TCDD in the presence of increasing concentrations of As(III), or MMA(III) (5 and 50 µM). All chemical stocks were prepared in DMSO, for which the DMSO content in reactions was adjusted to 2% (v/v) where necessary. After 1.5 h incubation at room temperature, reactions were further incubated with 250 µL of HAP suspension for an additional 30 min with gentle vortexing every 10 min. Thereafter, reactions were washed three times with 1 mL of 25 mM 3-(N-morpholino)propanesulfonic acid, pH 7.5, 1 mM EDTA, 10% (v/v) glycerol, 0.5% (v/v) Tween 80. The HAP pellets were transferred to scintillation vials, and scintillation cocktail was added and the reactions were counted in a scintillation counter. Specific binding of [^{3}H]TCDD to the AhR was computed by subtracting the amount of [^{3}H]TCDD bound in the presence of TCDF from the amount of [^{3}H]TCDD bound in the absence of competitor.

2.9.12 Determination of intracellular production of total ROS

To determine the degree of total ROS formation induced by As(III), or MMA(III), a fluorometric assay utilizing the intracellular oxidation of DCFH-DA to 2′,7′-dichlorofluorescein (DCF) has been conducted (Mary *et al.*, 2017). HepG2 cells were exposed to 5 μM As(III) or MMA(III) in the absence and presence of 1 nM TCDD for 24 h; thereafter, the cells were incubated with 10 μM DCFH-DA for 1 h, and the fluorescence of the oxidized probes DCF was measured by using a BioTek Synergy H1Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). Fluorescence was measured using an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

2.9.13 Determination of intracellular production of superoxide radical anion ($O_2^{\bullet-}$)

To determine the degree of superoxide radical anion formation induced by As(III), or MMA(III), a fluorometric assay utilizing the intracellular oxidation of DHE to 2-hydroxyethidium (2-OH-E^+) has been conducted (Mary *et al.*, 2017). HepG2 cells were exposed to 5 μM As(III) or MMA(III), in the absence and presence of 20 μM menadione, as a positive control, for 24 h. Thereafter, the cells were incubated with 10 μM DHE for 1 h, and the fluorescence of the oxidized probes 2-OH-E^+ was measured by using a BioTek Synergy H1Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). Fluorescence was measured using an excitation wavelength of 535 nm and an emission wavelength of 585 nm.

2.10 Statistical Analysis

The comparative statistical analysis of results was performed using SigmaPlot® for Windows (Systat Software, CA, USA). Control and treatment measurements were compared using the student t-test. For multiple comparisons, a one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc test were used. The differences were considered significant when $p<0.05$. Results were calculated as the mean ± SEM, and presented as the mean + SEM for clarity. The percentage was calculated relative to control designated as 100%.

3 CHAPTER THREE - RESULTS

Versions of this chapter have been published in:

Elshenawy, O.H., El-Kadi, A.O., 2015a. Modulation of aryl hydrocarbon receptor-regulated enzymes by trimethylarsine oxide in C57BL/6 mice: In vivo and in vitro studies. Toxicol. Lett. ***238****, 17-31.*

Elshenawy, O.H., El-Kadi, A.O., 2015b. Modulation of aryl hydrocarbon receptor regulated genes by acute administration of trimethylarsine oxide in the lung, kidney and heart of C57BL/6 mice. Xenobiotica ***45****, 930-943.*

Elshenawy, O.H., Abdelhamid, G., Althurwi, H.N., El-Kadi, A.O., 2017a. Dimethylarsinic acid modulates the aryl hydrocarbon receptor-regulated genes in C57BL/6 mice: in vivo study. Xenobiotica, 1-11. DOI 10.1080/00498254.00492017.01289423.

Elshenawy, O.H., Abdelhamid, G., Soshilov, A.A., Denison, M.S., El-Kadi, A.O., 2017b. Down-regulation of cytochrome P450 1A1 by monomethylarsonous acid in human HepG2 cells. Toxicol. Lett. ***270****, 34-50.*

3.1 Modulation of AhR-regulated enzymes by trimethylarsine oxide (TMAO) and TCDD in C57BL/6 mice: *in vivo* and *in vitro* studies.

3.1.1 Effect of TMAO and TCDD on Cyp1 mRNA, protein and catalytic activity levels in the liver of C57BL/6 mice

To examine the effect of TMAO treatment on the hepatic expression of Cyp1 mRNA, protein and activity levels, total RNA was extracted from livers of mice treated for 6 h, whereas microsomal proteins were prepared from livers of mice treated for 24 h. The expression level of mRNA was measured using real-time PCR; protein levels were determined using Western blot analysis and activity levels were determined using Cyp1a1-dependent EROD and Cyp1a2-dependent MROD activities.

At 6 h, TMAO alone significantly increased Cyp1a1, Cyp1a2, and Cyp1b1 mRNA levels in the liver to 178%, 126%, and 176%, respectively, compared to control. TCDD alone significantly induced Cyp1a1, Cyp1a2, and Cyp1b1 mRNA levels in the liver to 521153%, 512%, and 212%, respectively, compared to control. When animals were co-exposed to TMAO and TCDD, TMAO significantly potentiated the TCDD-mediated induction of Cyp1a1 and Cyp1b1 mRNA levels in the liver to 1419076% and 326%, respectively, whereas this co-exposure had no significant effect on Cyp1a2 mRNA levels compared to TCDD (Figure 3.1A, B, and C).

At 24 h, TMAO alone significantly increased Cyp1a1 and Cyp1a2 protein expression levels in the liver to 396%, and 209%, respectively, compared to control. TCDD alone significantly induced

Cyp1a1, Cyp1a2 and Cyp1b1 protein expression levels in the liver to 1420%, 2690%, and 948%, respectively, compared to control. When animals were co-exposed to TMAO and TCDD, TMAO significantly potentiated the TCDD-mediated induction of Cyp1a1 and Cyp1b1 protein expression levels in the liver to 2803% and 2041%, respectively, whereas this co-exposure had no significant effect on Cyp1a2 protein expression levels compared to TCDD (Figure 3.2A, B, and C).

With regard to catalytic activity, TMAO alone significantly increased Cyp1a1 and Cyp1a2 catalytic activities, as assessed by EROD and MROD activities, in the liver to 156% and 129%, respectively, compared to control. TCDD alone significantly induced EROD and MROD activities in the liver to 2100% and 161%, respectively, compared to control. When animals were co-exposed to TMAO and TCDD, TMAO significantly potentiated the TCDD-mediated induction of EROD activity in the liver to 2348% (Figure 3.3A, and B).

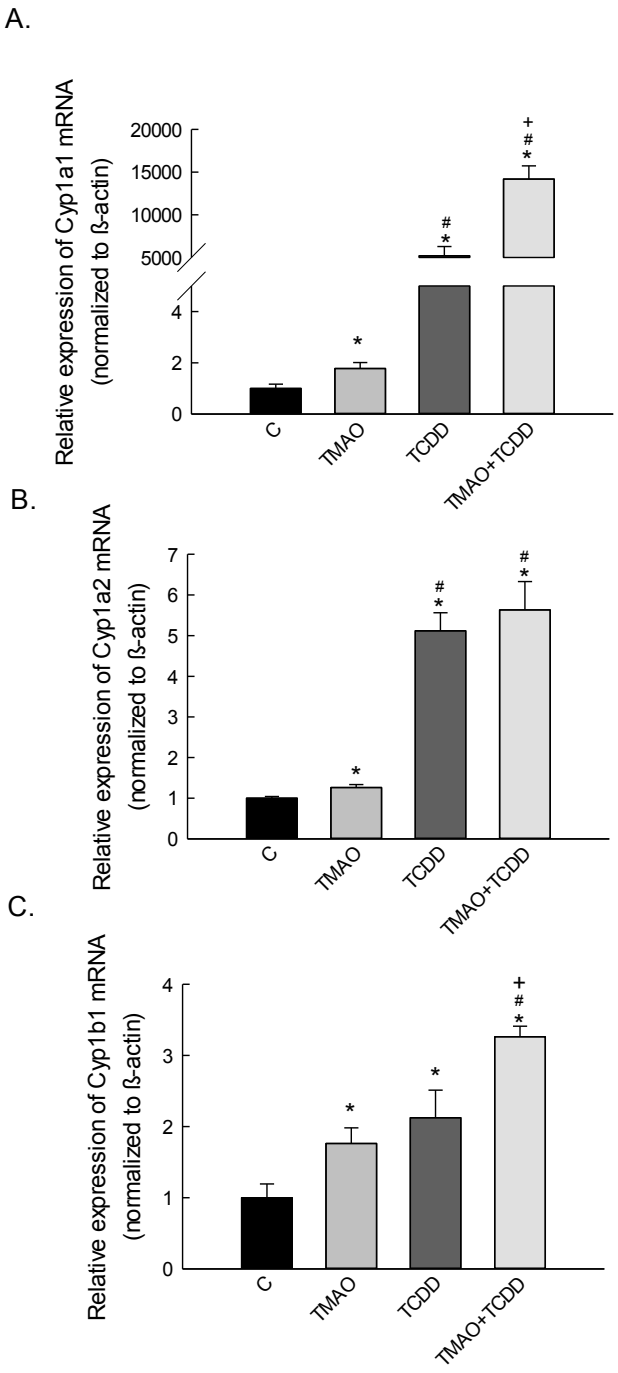


Figure 3.1 Effect of TMAO and TCDD on liver Cyp1a1, Cyp1a2, and Cyp1b1 mRNA in C57BL/6 mice. Animals were injected i.p. with 13 mg/kg TMAO with or without i.p. injection of 15 µg/kg TCDD; thereafter total liver RNA was isolated at 6 h of the treatment. The expression of Cyp1a1, Cyp1a2, and Cyp1b1 was measured using real-time PCR. Results are the mean + SEM (n = 6 mice per group). * $p<0.05$, compared to control (C); # $p<0.05$, compared to TMAO treatment; + $p<0.05$, compared to TCDD treatment.

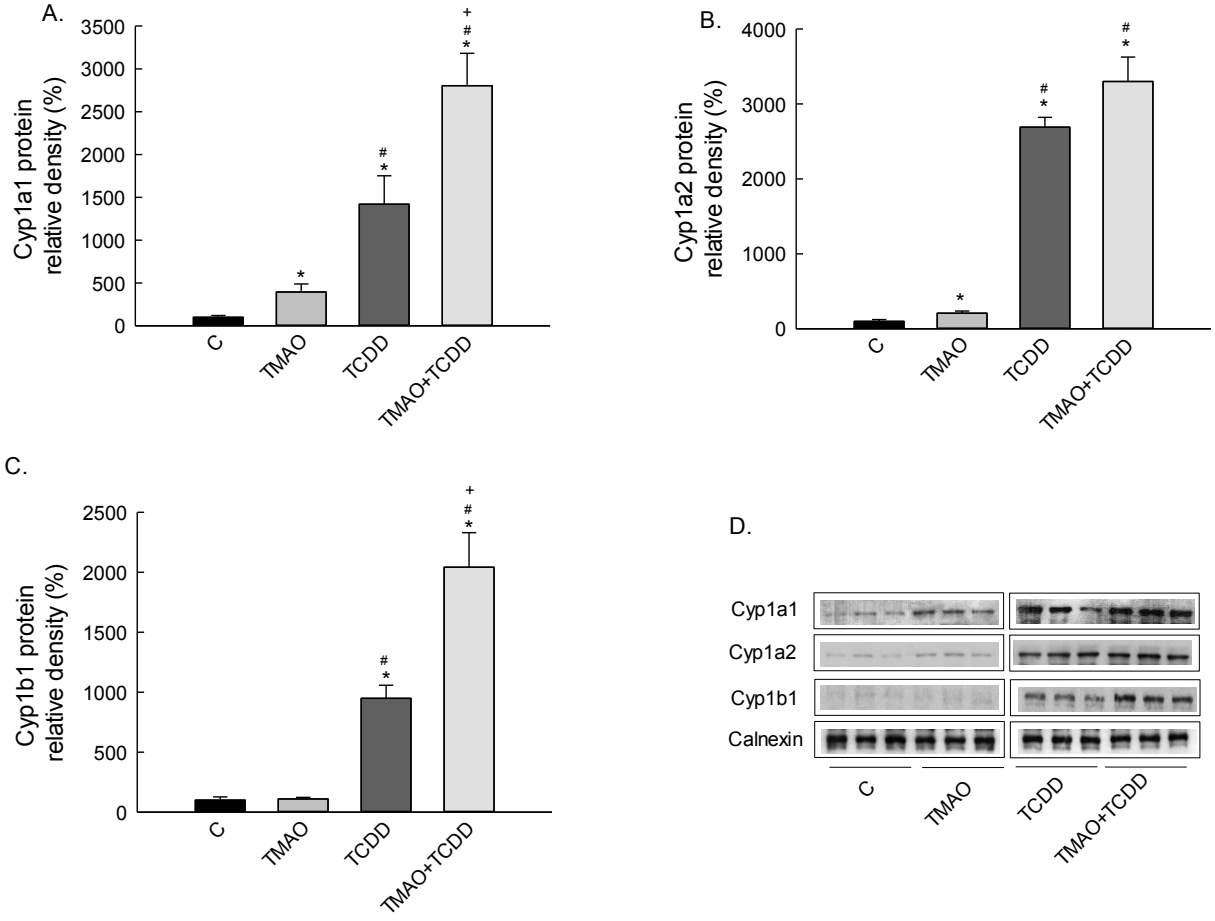
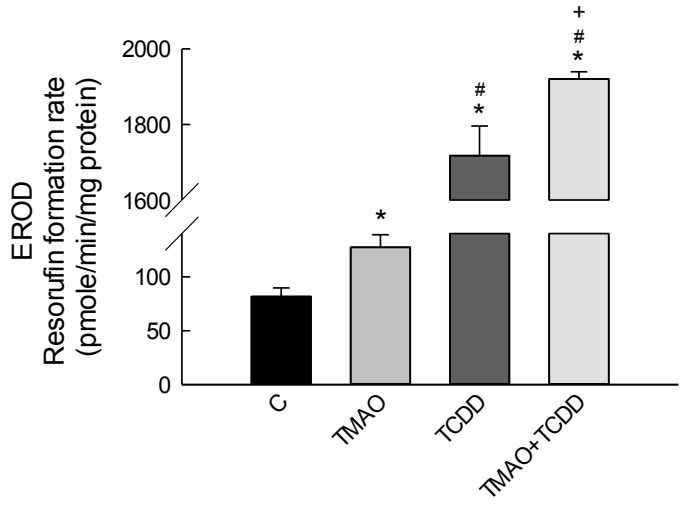


Figure 3.2 Effect of TMAO and TCDD on liver Cyp1a1, Cyp1a2, and Cyp1b1 protein expression levels in C57BL/6 mice. Animals were injected i.p. with 13 mg/kg TMAO with or without i.p. injection of 15 µg/kg TCDD; thereafter liver microsomal proteins were isolated at 24 h of the treatment. The expression of Cyp1a1, Cyp1a2, and Cyp1b1 was measured using western blot analysis. Results are the mean + SEM (images are representative of 6 mice per group). * $p<0.05$, compared to control (C); # $p<0.05$, compared to TMAO treatment; + $p<0.05$, compared to TCDD treatment.

A.



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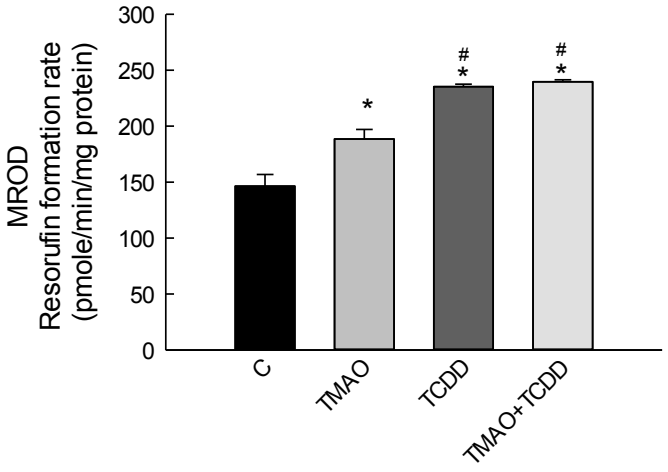


Figure 3.3 Effect of TMAO and TCDD on liver Cyp1a1 and Cyp1a2 activities in C57BL/6 mice. Animals were injected i.p. with 13 mg/kg TMAO with or without i.p. injection of 15 μg/kg TCDD; thereafter liver microsomal proteins were isolated at 24 h of the treatment. EROD and MROD activities were measured using 7-ethoxyresorufin and 7-methoxyresorufin as substrates, respectively. Results are the mean + SEM (n = 6 mice per group). * $p<0.05$, compared to control (C); # $p<0.05$, compared to TMAO treatment; + $p<0.05$, compared to TCDD treatment.

3.1.2 Effect of TMAO and TCDD on Nqo1 and Gst mRNA, protein and catalytic activity levels in the liver of C57BL/6 mice

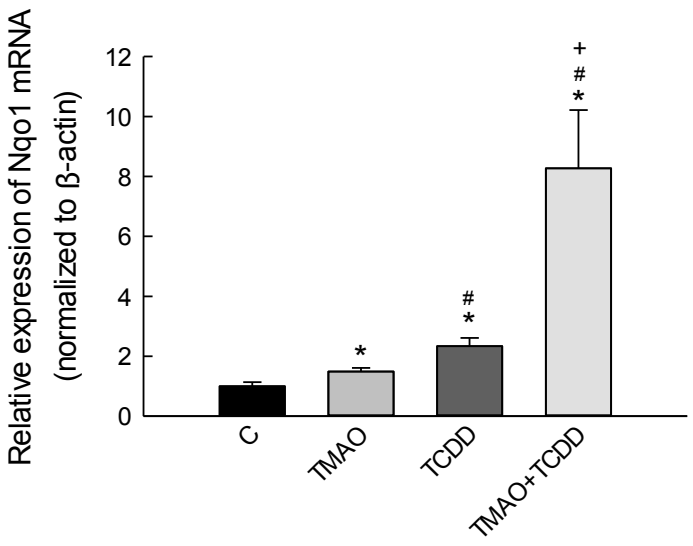
To examine the effect of TMAO treatment on the hepatic expression of Nqo1 and Gst mRNA, protein and activity levels, total RNA was extracted from mice treated for 6 h, whereas cytosolic proteins were prepared from mice treated for 24 h. The expression levels of mRNA were measured using real-time PCR, protein levels were determined using Western blot analysis, and activity levels were determined using DCPIP and CDNB as substrates, respectively.

At 6 h, TMAO alone significantly induced Nqo1 and Gsta1 mRNA levels in the liver to 149% and 212%, respectively, compared to control. TCDD alone significantly induced Nqo1, and Gsta1 mRNA levels in the liver to 234% and 269%, respectively, compared to control. When animals were co-exposed to TMAO and TCDD, TMAO significantly potentiated the TCDD-mediated induction of Nqo1 mRNA levels in the liver to 827%, whereas this co-exposure had no significant effect on Gsta1 mRNA levels compared to TCDD (Figure 3.4A, and B).

At 24 h, TMAO alone significantly increased Nqo1 and Gsta1/2 protein expression levels in the liver both to 215%, compared to control. TCDD alone significantly induced Nqo1 and Gsta1/2 protein expression levels in the liver to 243% and 392%, respectively, compared to control. When animals were co-exposed to TMAO and TCDD, TMAO had no significant effect on Gsta1/2 protein expression levels compared to TCDD, while significantly inhibiting the TCDD-mediated induction of Nqo1 protein to 178% (Figure 3.5A, B, and C).

With regard to catalytic activity, TMAO alone significantly induced Nqo1 and Gst catalytic activities to 163% and 119%, respectively, compared to control. Similarly, TCDD alone significantly induced Nqo1 and Gst catalytic activities to 212% and 137% respectively, compared to control. When animals were co-exposed to TMAO and TCDD, TMAO had no significant effect on Nqo1 and Gst catalytic activities compared to TCDD (Figure 3.6A, and B).

A.



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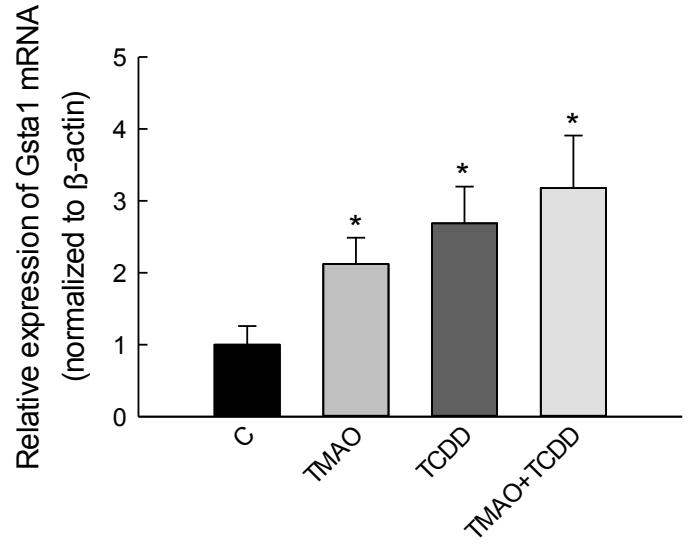


Figure 3.4 Effect of TMAO and TCDD on liver Nqo1 and Gsta1 mRNA in C57BL/6 mice. Animals were injected i.p. with 13 mg/kg TMAO with or without i.p. injection of 15 μg/kg TCDD; thereafter total liver RNA was isolated at 6 h of the treatment. The expression of Nqo1 and Gsta1 was measured using real-time PCR. Results are the mean + SEM (n = 6 mice per group). * $p<0.05$, compared to control (C); # $p<0.05$, compared to TMAO treatment; + $p<0.05$, compared to TCDD treatment.

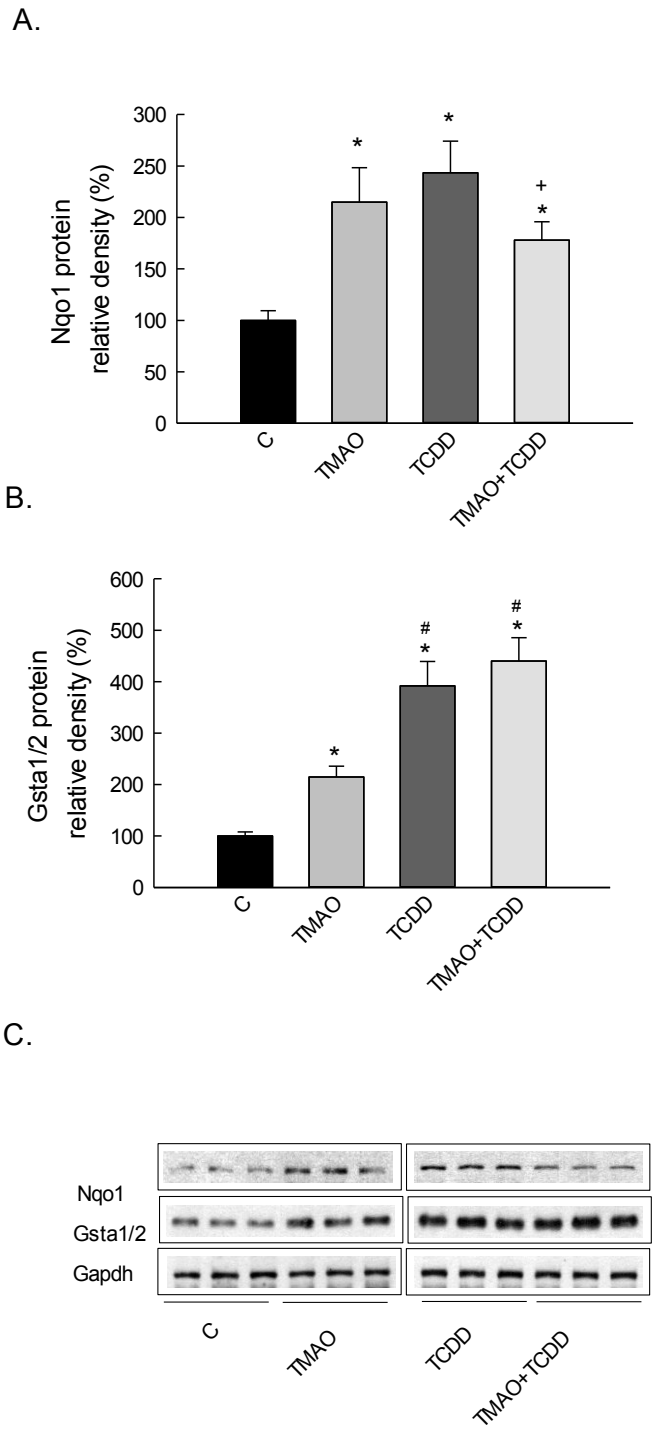
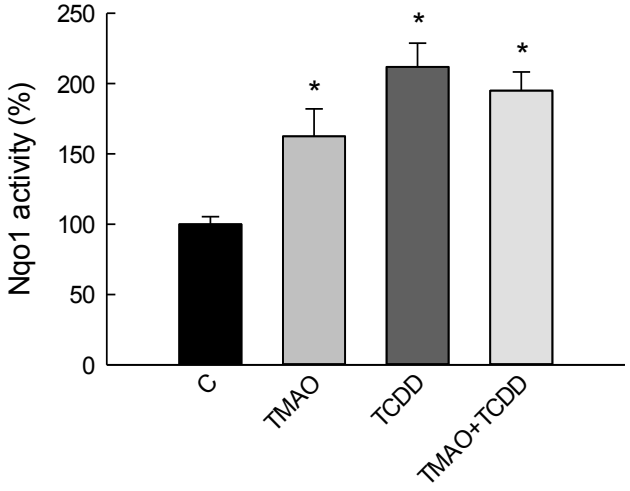


Figure 3.5 Effect of TMAO and TCDD on liver Nqo1 and Gsta1/2 protein expression levels in C57BL/6 mice. Animals were injected i.p. with 13 mg/kg TMAO with or without i.p. injection of 15 μg/kg TCDD; thereafter liver cytosolic proteins were isolated at 24 h after treatment. The expression of Nqo1 and Gsta1/2 was measured using western blot analysis. Results are the mean + SEM (images are representative of 6 mice per group). * $p<0.05$, compared to control (C); # $p<0.05$, compared to TMAO treatment; + $p<0.05$, compared to TCDD treatment.

A.



B.

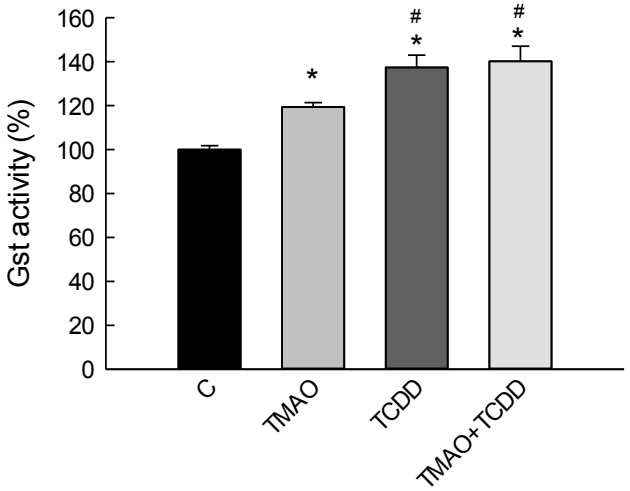


Figure 3.6 Effect of TMAO and TCDD on liver Nqo1 and Gst catalytic activities in C57BL/6 mice. Animals were injected i.p. with 13 mg/kg TMAO with or without i.p. injection of 15 μg/kg TCDD for 24 h. Liver cytosolic proteins were isolated and Nqo1 catalytic activity (A) was determined spectrophotometrically using DCPIP as substrate, and dicoumarol as a specific Nqo1 inhibitor. Gst catalytic activity (B) was determined spectrophotometrically in cytosolic fraction using CDNB as a substrate. Results are the mean + SEM (n = 6 mice per group). * $p<0.05$, compared to control (C); # $p<0.05$, compared to TMAO treatment; + $p<0.05$, compared to TCDD treatment.

3.1.3 Effect of TMAO and TCDD on Ho-1 mRNA, protein and catalytic activity levels in the liver of C57BL/6 mice

To examine the effect of TMAO treatment on the hepatic expression of Ho-1 mRNA, protein and activity levels, total RNA was extracted from mice treated for 6 h, whereas microsomal proteins were prepared from mice treated for 24 h. The expression level of mRNA was measured using real-time PCR, protein levels were determined using Western blot analysis, and activity levels were determined using hemin in the presence of biliverdin reductase.

At 6 h, TMAO alone significantly induced Ho-1 mRNA levels in the liver to 219% compared to control. TCDD alone significantly induced Ho-1 mRNA levels in the liver to 220% compared to control. When animals were co-exposed to TMAO and TCDD, TMAO had no significant effect on TCDD-mediated induction of Ho-1 mRNA levels in the liver compared to TCDD (Figure 3.7A).

At 24 h, TMAO alone significantly induced Ho-1 protein expression levels in the liver to 283% compared to control. Similarly, TCDD alone significantly induced liver Ho-1 protein expression levels in the liver to 254% compared to control. When animals were co-exposed to TMAO and TCDD, TMAO had no significant effect on Ho-1 protein expression levels compared to TCDD (Figure 3.7B). With regard to catalytic activity, TMAO alone significantly induced Ho-1 catalytic activities to 117% compared to control. Similarly, TCDD alone significantly induced liver Ho-1 activity to 117% compared to control. When animals were co-exposed to TMAO and TCDD, TMAO had no significant effect on Ho-1 activity compared to TCDD (Figure 3.7C).

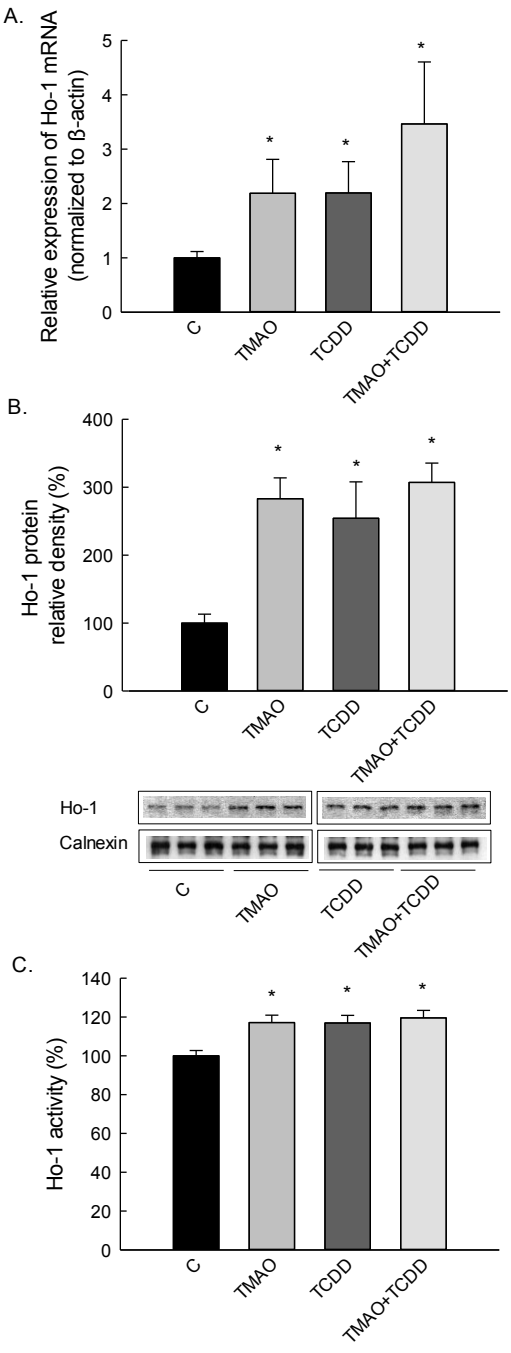


Figure 3.7 Effect of TMAO and TCDD on liver Ho-1 mRNA, Protein and catalytic activity in C57BL/6 mice. Animals were injected i.p. with 13 mg/kg TMAO with or without i.p. injection of 15 µg/kg TCDD for 6 and 24 h. The expression of Ho-1 mRNA (A) at 6 h was measured using real-time PCR. The expression of Ho-1 protein (B) at 24 h was measured using western blot analysis. Ho-1 catalytic activity (C) at 24 h was determined spectrophotometrically using hemin as substrate, in presence of biliverdin reductase. Results are the mean + SEM (n = 6 mice per group). * $p<0.05$, compared to control (C).

3.1.4 Effect of TMAO and TCDD on cell viability *in vitro* in isolated hepatocytes

To determine the nontoxic concentrations of TMAO to be utilized in this study, isolated hepatocytes from C57BL/6 mice were exposed, for 24 h, to increasing concentrations of TMAO (0 - 50 μM) in the absence and presence of 1 nM TCDD, and thereafter cytotoxicity was assessed using the MTT assay. TMAO at concentrations up to 50 μM in the presence and absence of 1 nM TCDD did not affect the cell viability (Figure 3.8). All subsequent studies were conducted using the concentration of 5 μM TMAO, in the absence and presence of 1 nM TCDD, to match concentration of the parent compound, As(III), previously used in different models (Elbekai and El-Kadi, 2007; Anwar-Mohamed and El-Kadi, 2010).

3.1.5 Effect of TMAO and TCDD on Cyp1a1 mRNA, protein and catalytic activity levels *in vitro* in isolated hepatocytes

To examine the effect of TMAO on the prototypical AhR target gene, Cyp1a1, isolated hepatocytes were treated with TMAO in the absence and presence of TCDD for 6 h. Thereafter, Cyp1a1 mRNA was measured using real-time PCR. Our results showed that, TMAO alone significantly increased Cyp1a1 mRNA levels to 228% as compared to untreated cells. TCDD alone significantly increased Cyp1a1 mRNA levels to 318% as compared to untreated cells. In cells co-exposed to TMAO and TCDD, TMAO significantly potentiated the TCDD-mediated induction of Cyp1a1 mRNA to 425% (Figure 3.9A).

To better understand the kinetics of Cyp1a1 mRNA in response to the exposure to TMAO, the time-dependent effect was determined at various time points up to 24 h after treatment of isolated hepatocytes with TMAO. TMAO was found to induce Cyp1a1 mRNA in a time-dependent manner. TMAO caused a maximal induction of the Cyp1a1 mRNA to 347% at 12 h, compared to untreated cells. Whereas TMAO caused induction of Cyp1a1 mRNA to 176% at 24 h, compared to untreated cells (Figure 3.9B).

To investigate whether the induction of Cyp1a1 mRNA by TMAO treatment was further translated to catalytically active protein, isolated hepatocytes were treated for 24 h with TMAO in the absence and presence of TCDD. Thereafter, Cyp1a1 protein expression levels were determined using Western blot analysis. With regard to activity, cells were incubated with 7-ethoxyresorufin as substrate. The metabolite, resorufin, was measured to indicate Cyp1a1-dependent EROD activity.

TMAO alone significantly induced Cyp1a1 protein to 156% compared to untreated cells. TCDD alone significantly induced Cyp1a1 protein to 516% compared to untreated cells. In cells co-exposed to TMAO and TCDD, TMAO significantly potentiated the TCDD-mediated induction of Cyp1a1 protein to 768% (Figure 3.10A). With regard to catalytic activity, TMAO alone significantly induced Cyp1a1 activity to 136% compared to untreated cells. TCDD alone significantly induced Cyp1a1 activity to 951% compared to untreated cells. In cells co-exposed to TMAO and TCDD, TMAO significantly potentiated the TCDD-mediated induction of Cyp1a1 catalytic activity to 1331% (Figure 3.10B).

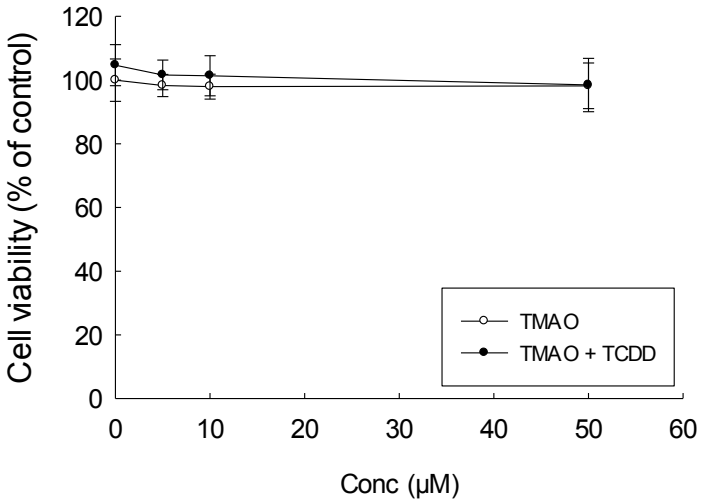


Figure 3.8 Effect of TMAO on cell viability in primary hepatocytes. Isolated hepatocytes were treated for 24 h with TMAO (0, 5, 10, and 50 μM) in the absence and presence of 1 nM TCDD. Cytotoxicity was determined using the MTT assay. Results are the mean ± SEM (n = 6).

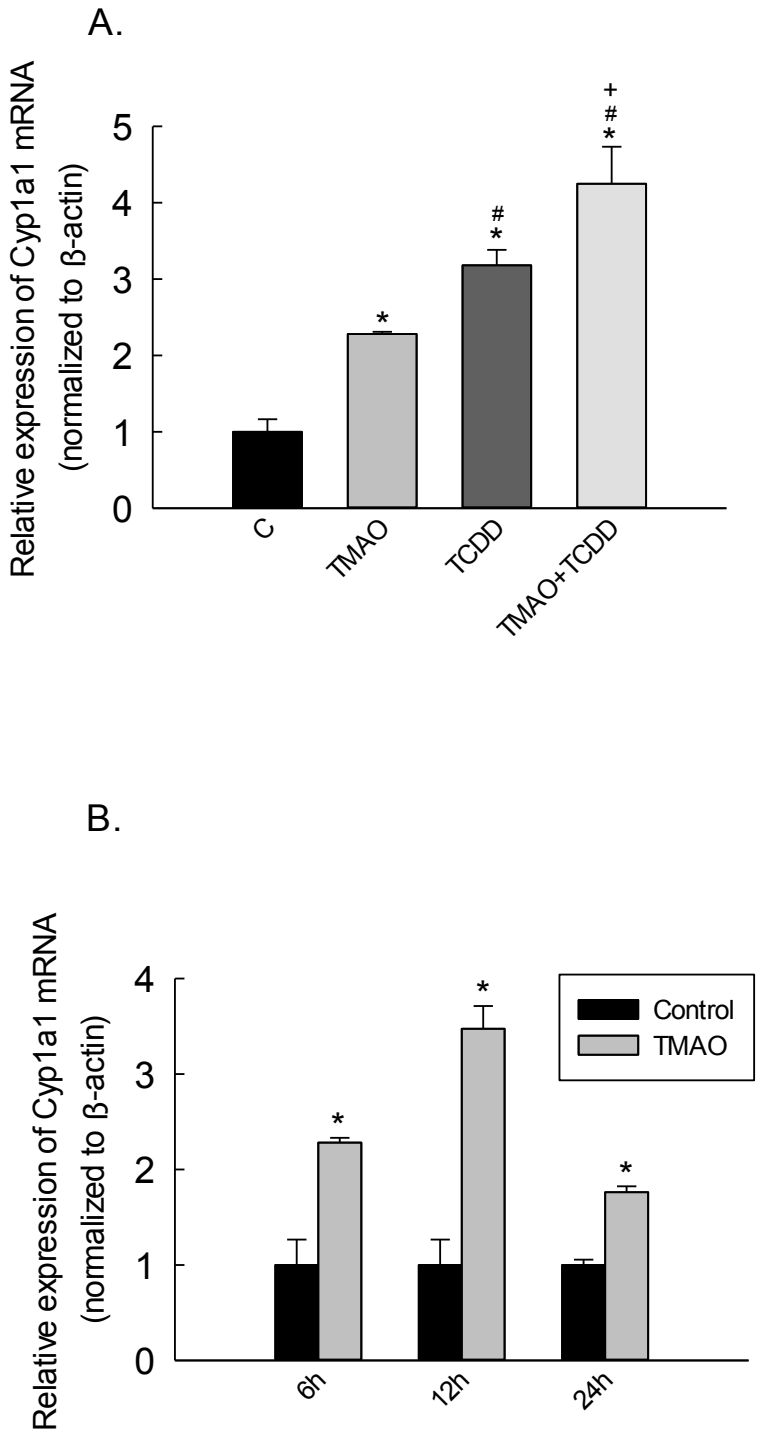


Figure 3.9 Effect of TMAO on Cyp1a1 mRNA in primary hepatocytes. Hepatocytes were treated with 5 μM TMAO in the absence and presence of 1 nM TCDD for 6 h (A) or 5 μM TMAO for various times for time-dependent effect (B). The expression of Cyp1a1 was measured using real-time PCR. Results are the mean + SEM (n = 6). * $p<0.05$, compared to control (C); # $p<0.05$, compared to TMAO treatment; + $p<0.05$, compared to TCDD treatment.

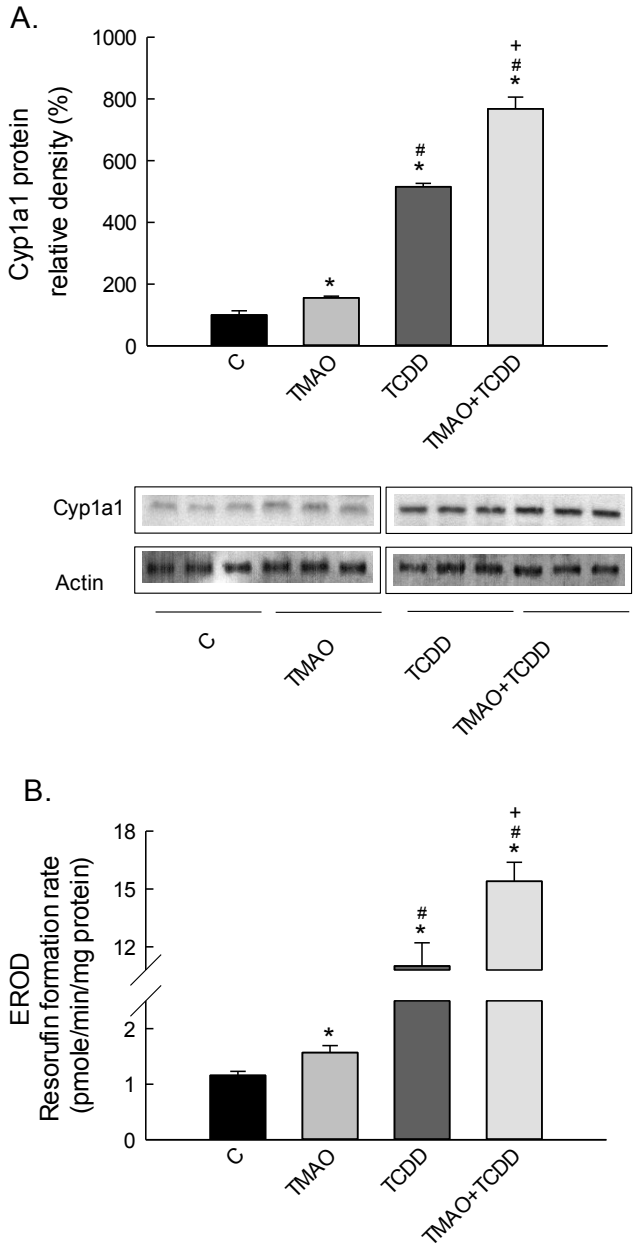


Figure 3.10 Effects of TMAO on Cyp1a1 protein and catalytic activity in isolated hepatocytes. Hepatocytes were treated with 5 μM TMAO in the absence and presence of 1 nM TCDD for 24 h to assess protein and catalytic activity. Expression of Cyp1a1 protein (A) was measured using western blot analysis. Cyp1a1 activity (B) was measured using 7-ethoxyresorufin as a substrate. Results are the mean + SEM (n = 6) * $p<0.05$, compared to control (C); # $p<0.05$, compared to TMAO treatment; + $p<0.05$, compared to TCDD treatment.

3.1.6 Effect of TMAO and TCDD on XRE–luciferase activity *in vitro* in isolated hepatocytes

To investigate the effect of TMAO on AhR-dependent transcriptional activation, isolated hepatocytes were transiently transfected with the XRE-driven luciferase reporter gene, and hepatocytes were treated for 24 h with TMAO in the absence and presence of TCDD. Thereafter, luciferase activity was measured via a luminescence-based approach.

Luciferase activity results showed that TMAO alone significantly induced the expression of the XRE-driven luciferase activity to 121% compared to control. TCDD alone caused a significant increase in XRE-driven luciferase activity to 665% compared to control. In cells co-exposed to TMAO and TCDD, TMAO significantly potentiated the TCDD-mediated induction of XRE-driven luciferase activity to 947% (Figure 3.11).

3.1.7 Effect of TMAO and TCDD on AhR protein localization *in vitro* in isolated hepatocytes

To investigate the effect of TMAO on AhR activation with a subsequent translocation to the nucleus, we examined the subcellular location of AhR upon treatment with TMAO using immunocytochemical analysis. For this purpose isolated hepatocytes were treated for 30 min with TMAO or TCDD followed by fixation, permeabilization and staining. Our results showed that, AhR is a cytosolic protein, as vehicle-treated cells did not have significant localization of AhR to the nucleus. TCDD-treated cells showed increased nuclear localization of AhR, and interestingly, TMAO was found to induce nuclear localization of AhR as well (Figure 3.12).

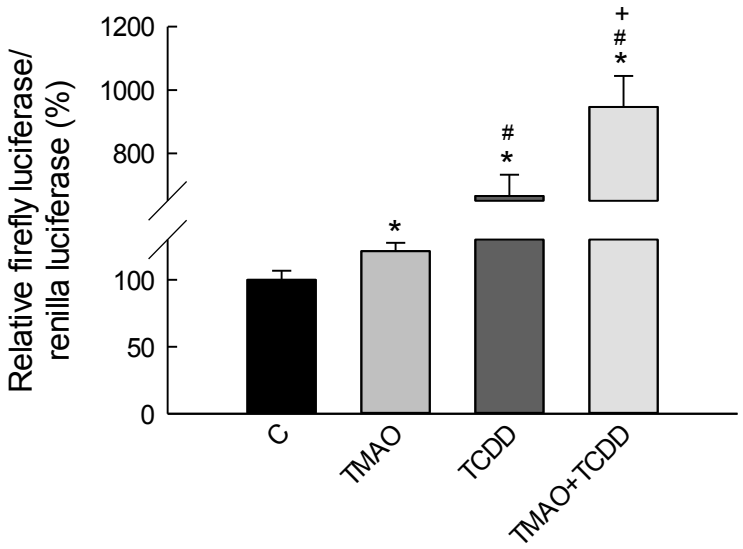


Figure 3.11 Effects of TMAO on XRE-driven luciferase activity in isolated hepatocytes. Hepatocytes were transiently transfected with the XRE–luciferase reporter plasmid pGudLuc1.1 and Renilla luciferase plasmid pRL-CMV. Cells were treated with 5 μM TMAO in the absence and presence of 1 nM TCDD for 24 h and luciferase activity was measured. Results are the mean + SEM (n = 6). * $p<0.05$, compared to control (C); # $p<0.05$, compared to TMAO treatment; + $p<0.05$, compared to TCDD treatment.

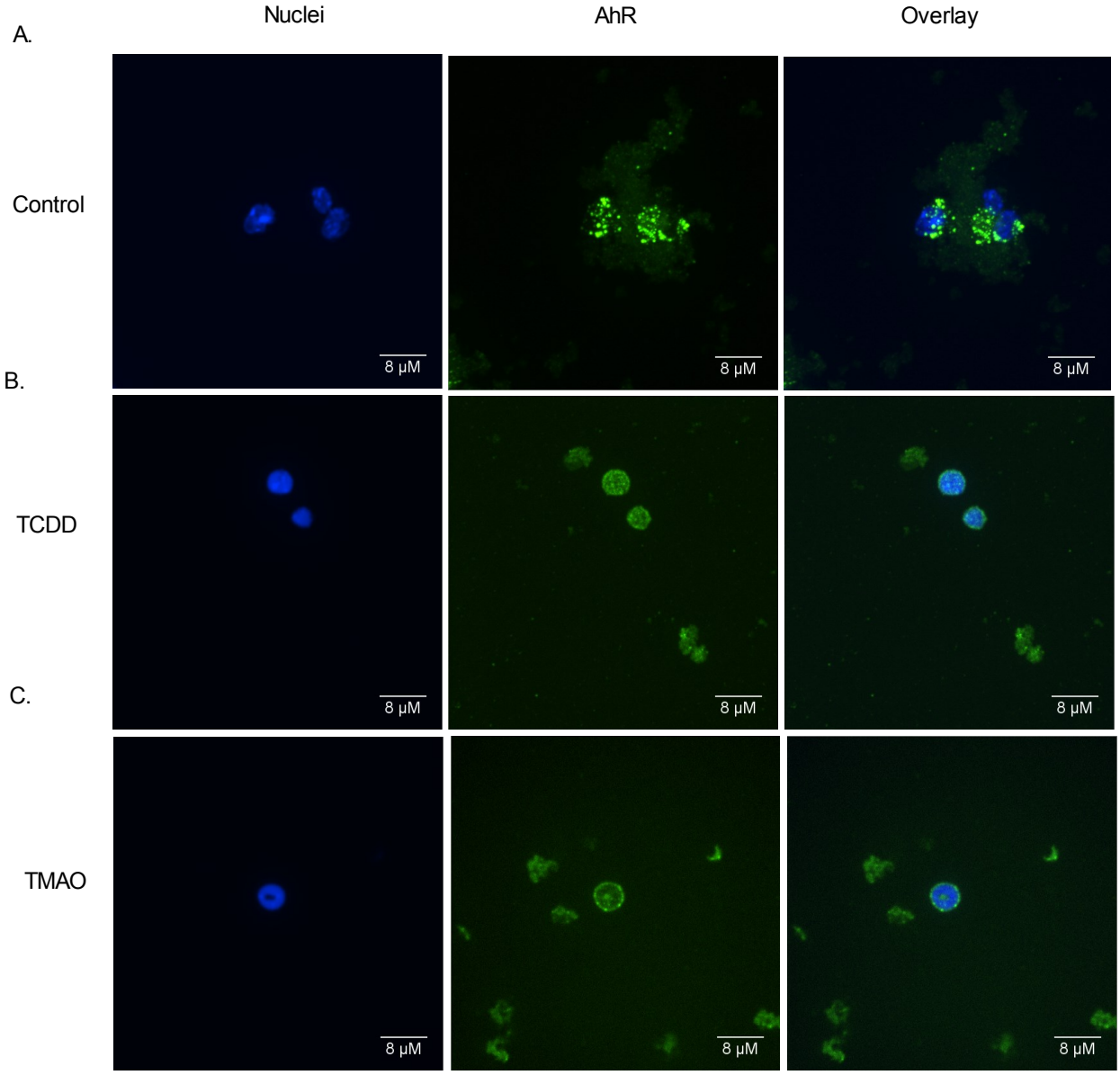


Figure 3.12 Effect of TMAO on AhR subcellular localization in isolated hepatocytes via immunocytochemical analysis. Cells were plated on collagen-coated glass coverslips, treated for 30 min with vehicle, 1 nM TCDD, or 5 µM TMAO; thereafter cells were fixed, permeabilized and stained with anti-AhR antibody, Alexa Fluor 488 (green), and DAPI (blue). Images were obtained using spinning disk confocal microscopy with a 100×/1.4 NA lens.

3.1.8 Summary of the effects of TMAO and TCDD in the liver of C57BL/6 mice

The effects of TMAO and TCDD on the expression of different genes in the liver of C57BL/6 mice have been summarized in Table 3-1.

Table 3-1 Summary of the effects of TMAO and TCDD on the expression of different genes in the liver of C57BL/6 mice.

Treatment	Gene	Liver		
		mRNA expression	**Protein levels**	**Catalytic activity**
TMAO	Cyp1a1	↑mRNA	↑protein	↑activity
	Cyp1a2	↑mRNA	↑protein	↑activity
	Cyp1b1	↑mRNA	↔protein	ND
	Nqo1	↑mRNA	↑protein	↑activity
	Gsta1	↑mRNA	↑protein	↑activity
	Ho-1	↑mRNA	↑protein	↑activity
TCDD	Cyp1a1	↑mRNA	↑protein	↑activity
	Cyp1a2	↑mRNA	↑protein	↑activity
	Cyp1b1	↑mRNA	↑protein	ND
	Nqo1	↑mRNA	↑protein	↑activity
	Gsta1	↑mRNA	↑protein	↑activity
	Ho-1	↑mRNA	↑protein	↑activity
TMAO + TCDD	Cyp1a1	↑↑mRNA	↑↑protein	↑↑activity
	Cyp1a2	↑mRNA	↑protein	↑activity
	Cyp1b1	↑↑mRNA	↑↑protein	ND
	Nqo1	↑↑mRNA	↑/↓↓protein	↑activity
	Gsta1	↑mRNA	↑protein	↑activity
	Ho-1	↑mRNA	↑protein	↑activity

(↑) increase; (↓) decrease; (↔) no change; (↑↑) potentiation; (↓↓) attenuation; (ND) not determined.

3.2 Modulation of AhR-regulated genes by acute administration of trimethylarsine oxide (TMAO) in the lung, kidney, and heart of C57BL/6 mice

3.2.1 Effect of co-exposure to TMAO and TCDD on Cyp1a1, Cyp1a2 and Cyp1b1 mRNA in the lung, kidney, and heart of C57BL/6 mice

To examine the effect of TMAO treatment on the extrahepatic expression of Cyp1a1, Cyp1a2, and Cyp1b1, total RNA was extracted from the lung, kidney, and heart of control-, TMAO-, TCDD-, TMAO/TCDD-treated mice at 6 h after administration of treatment. Thereafter, the expression level of mRNA of different genes was measured using reverse transcription followed by real-time PCR.

In the lung, TMAO alone at 6 h significantly induced Cyp1a1 and Cyp1b1 mRNA levels to 173% and 146%, respectively, while it failed to significantly affect Cyp1a2 mRNA levels compared to control. TCDD alone at 6 h significantly induced Cyp1a1, Cyp1a2, and Cyp1b1 mRNA levels to 20461%, 421%, and 369%, respectively, compared to control. When animals were co-exposed to TMAO and TCDD, TMAO at 6 h significantly potentiated the TCDD-mediated induction of Cyp1a1 and Cyp1a2 mRNA to 29719% and 1039%, respectively, while it had no effect on Cyp1b1 mRNA compared to TCDD alone (Figure 3.13A, B, and C).

In the kidney, TMAO alone at 6 h significantly induced Cyp1b1 mRNA levels to 186%, while it failed to significantly affect Cyp1a1 and Cyp1a2 mRNA levels compared to control. TCDD alone at 6 h significantly induced Cyp1a1, Cyp1a2, and Cyp1b1 mRNA levels to 4585%, 2580%, and

173%, respectively, compared to control. When animals were co-exposed to TMAO and TCDD, TMAO at 6 h significantly potentiated the TCDD-mediated induction of Cyp1a1 and Cyp1b1 mRNA to 20124% and 312%, respectively, while it had no effect on Cyp1a2 mRNA compared to TCDD alone (Figure 3.13A, B, and C).

In heart, TMAO alone at 6 h failed to significantly affect Cyp1a1, Cyp1a2, and Cyp1b1 mRNA levels. TCDD alone at 6 h significantly induced Cyp1a1, Cyp1a2, and Cyp1b1 mRNA levels to 18600%, 226%, and 163%, respectively, compared to control. When animals were co-exposed to TMAO and TCDD, TMAO at 6 h significantly potentiated the TCDD-mediated induction of Cyp1a1 and Cyp1b1 mRNA to 30579% and 283%, respectively, while it had no effect on Cyp1a2 mRNA compared to TCDD alone (Figure 3.13A, B, and C).

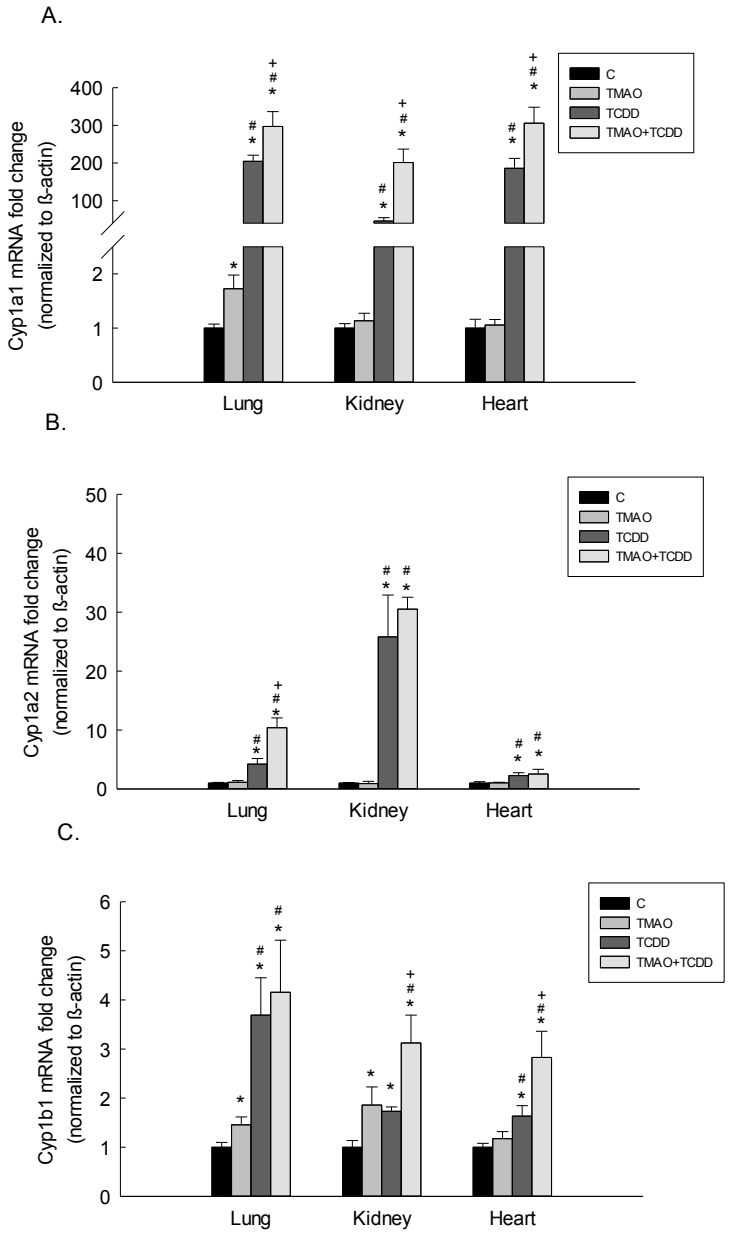


Figure 3.13 Effect of co-exposure to TMAO and TCDD on Cyp1a1, Cyp1a2, and Cyp1b1 mRNA in the lung, kidney, and heart of C57BL/6 mice. Animals were injected i.p. with 13 mg/kg TMAO with or without i.p. injection of 15 µg/kg TCDD for 6 h. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from lung, kidney, and heart and the expression of Cyp1a1, Cyp1a2, and Cyp1b1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using an ABI 7500 real-time PCR system as described under materials and methods. The values are presented as the mean + SEM. * $p<0.05$, compared to control (C); # $p<0.05$, compared to TMAO treatment; + $p<0.05$, compared to TCDD treatment.

3.2.2 Effect of co-exposure to TMAO and TCDD on Cyp1 protein expression, and catalytic activity levels in the lung and kidney of C57BL/6 mice

To investigate whether the effect of TMAO treatment on Cyp1 mRNA expression was further translated to proteins which are catalytically active, microsomal protein was prepared from lung and kidney of control-, TMAO-, TCDD-, TMAO/TCDD-treated mice at 24 h after administration of treatment. Thereafter, Cyp1a1, Cyp1a2 and Cyp1b1 protein expression levels were determined using Western blot analysis. With regard to Cyp1a1 and Cyp1a2 catalytic activities, lung and kidney microsomes were incubated with 7-ethoxyresorufin and 7-methoxyresorufin, respectively, as substrates. The metabolite, resorufin, was measured using fluorometry to indicate Cyp1a1-dependent EROD activity and Cyp1a2-dependent MROD activity.

In the lung, our results showed that TMAO alone at 24 h significantly induced Cyp1a1 and Cyp1b1 protein expression levels to 352% and 121%, respectively, while it had no effect on Cyp1a2 protein expression levels compared to control. TCDD alone significantly induced the Cyp1a1, Cyp1a2, and Cyp1b1 protein expression levels to 2191%, 677%, and 220%, respectively, compared to control. When animals were co-exposed to TMAO and TCDD, TMAO at 24 h significantly potentiated the TCDD-mediated induction of Cyp1a1 and Cyp1a2 protein expression levels to 6259% and 954%, respectively, while it had no effect on Cyp1b1 protein expression levels compared to TCDD alone (Figure 3.14A, B, and C). In the kidney, our results showed that TMAO alone at 24 h significantly induced Cyp1b1 protein expression levels to 221%, while it had no effect on Cyp1a1 and Cyp1a2 protein expression levels compared to control. TCDD alone significantly induced the Cyp1a1, Cyp1a2, and Cyp1b1 protein expression levels to 260%, 2271%,

and 244%, respectively, compared to control. When animals were co-exposed to TMAO and TCDD, TMAO at 24 h significantly potentiated the TCDD-mediated induction of Cyp1a1 and Cyp1b1 protein expression levels to 561% and 328%, respectively, while it had no effect on Cyp1a2 protein expression levels compared to TCDD alone (Figure 3.14A, B, and C).

At the catalytic activity levels, TMAO alone significantly potentiated the lung EROD activity to 117%, while it had no effect on MROD activity compared to control. TCDD alone significantly induced EROD and MROD activities to 1267% and 829%, respectively, compared to control. When animals were co-exposed to TMAO and TCDD, TMAO significantly potentiated the TCDD-mediated induction of EROD and MROD catalytic activities to 1948% and 1477%, respectively, compared to TCDD alone (Figure 3.15A, and B). In the kidney, TMAO alone had no effect on EROD and MROD activities compared to control. TCDD alone significantly induced EROD and MROD activities to 646% and 281%, respectively, compared to control. When animals were co-exposed to TMAO and TCDD, TMAO significantly potentiated the TCDD-mediated induction of EROD catalytic activity to 1392%, while it had no effect on MROD activity compared to TCDD alone (Figure 3.15A, and B).

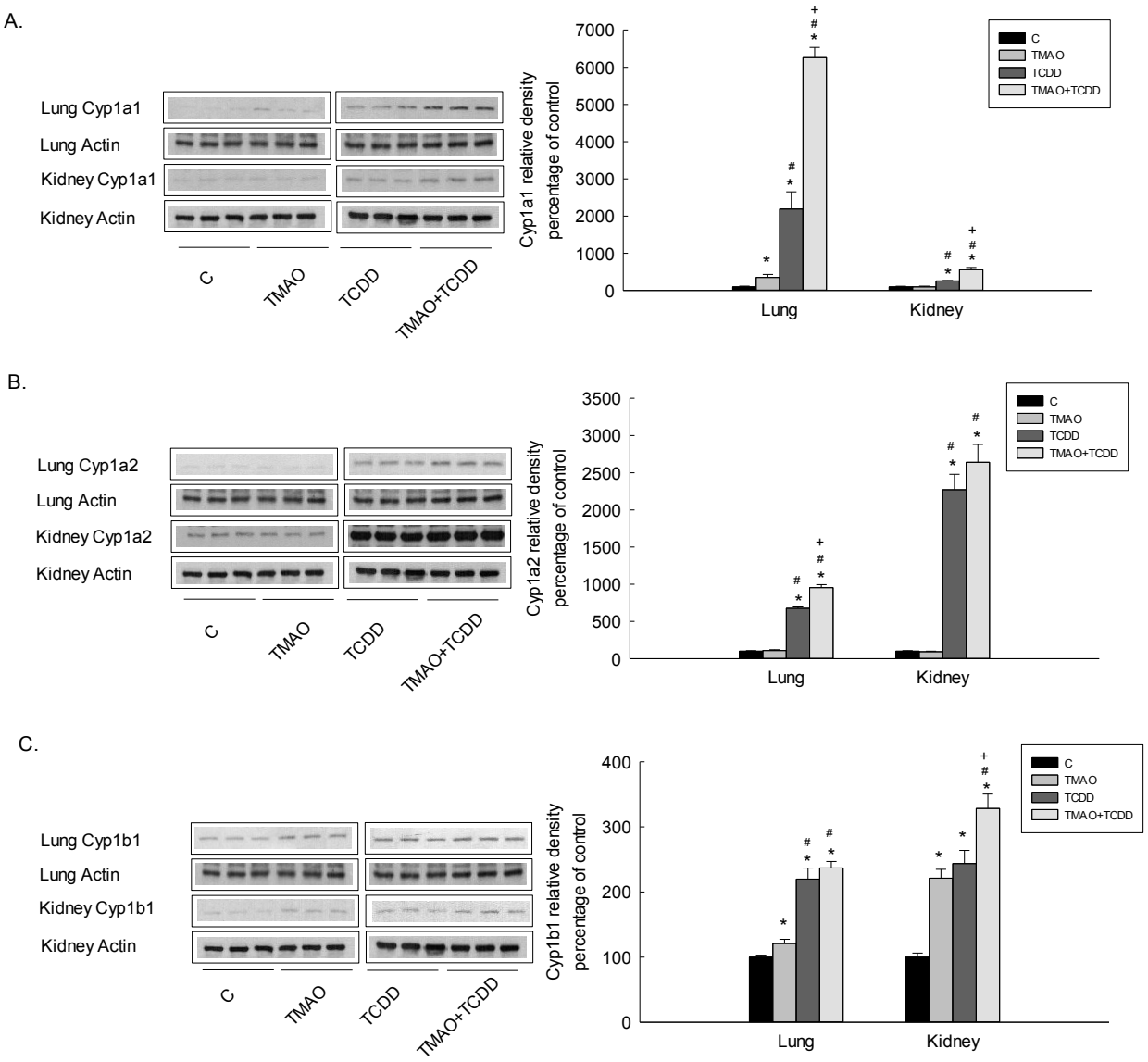


Figure 3.14 Effect of co-exposure to TMAO and TCDD on Cyp1a1, Cyp1a2, and Cyp1b1 protein expression levels in the lung, and kidney of C57BL/6 mice. Animals were injected i.p. with 13 mg/kg TMAO with or without i.p. injection of 15 μg/kg TCDD for 24 h. Lung, and kidney microsomal proteins were isolated after 24 h of treatment. Ten micrograms of microsomal proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4 °C and then incubated with a primary antibody for 2 h at 4 °C, followed by 1 h incubation with secondary antibody at room temperature. Proteins were detected using the enhanced chemiluminescence method. The intensity of the bands was normalized to β-actin signals, which were used as a loading control. The values are presented as the mean + SEM relative to the control value (set at 100%). * $p<0.05$, compared to control (C); # $p<0.05$, compared to TMAO treatment; + $p<0.05$, compared to TCDD treatment.

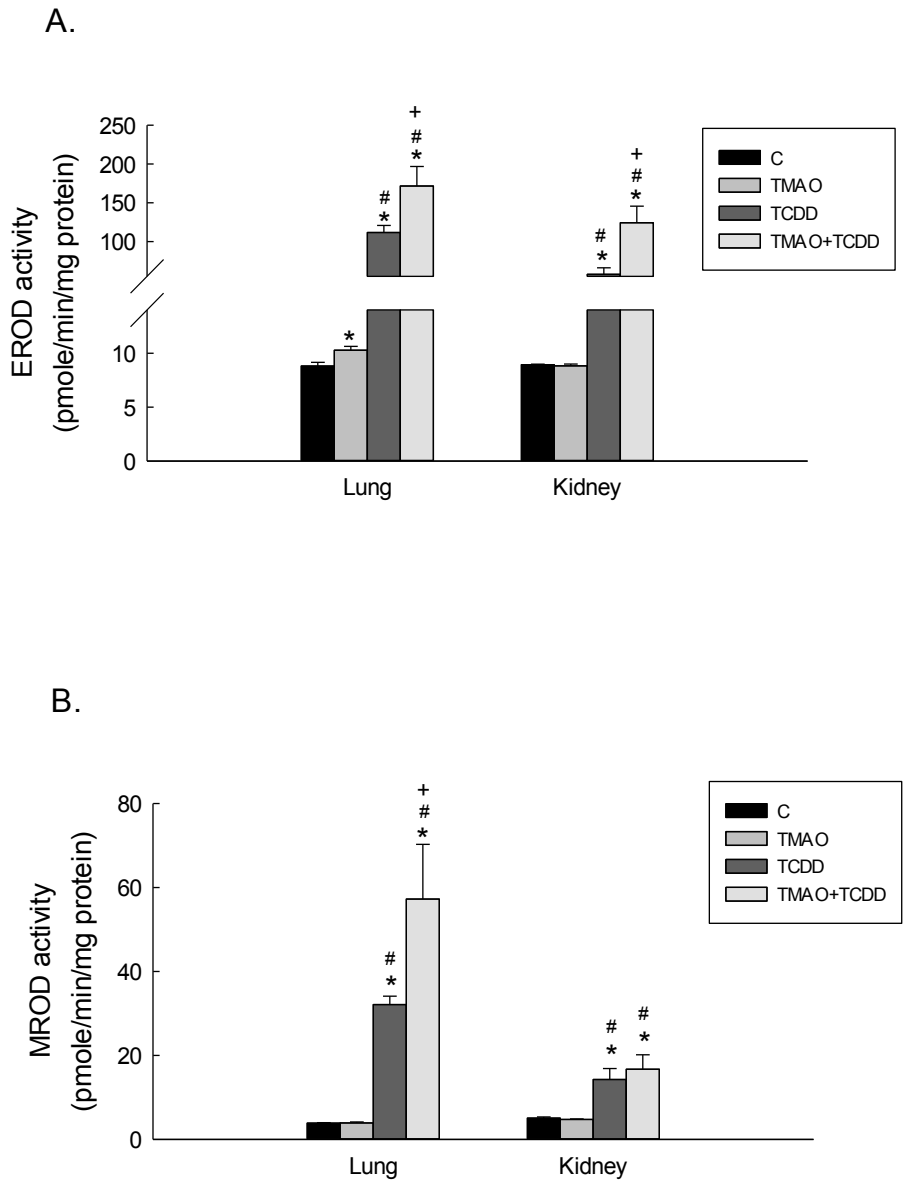


Figure 3.15 Effect of co-exposure to TMAO and TCDD on Cyp1a1 and Cyp1a2 activities in the lung and kidney of C57BL/6 mice. Animals were injected i.p. with 13 mg/kg TMAO with or without i.p. injection of 15 µg/kg TCDD for 24 h. Lung, and kidney microsomal proteins were isolated, thereafter, EROD and MROD 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 methanol. The values are presented as the mean + SEM. * $p<0.05$, compared to control (C); # $p<0.05$, compared to TMAO treatment; + $p<0.05$, compared to TCDD treatment.

3.2.3 Effect of co-exposure to TMAO and TCDD on Nqo1 and Gsta1 mRNA in the lung, kidney, and heart of C57BL/6 mice

To examine the effect of TMAO treatment on the extrahepatic expression of Nqo1 and Gsta1, total RNA was extracted from the lung, kidney, and heart of control-, TMAO-, TCDD-, TMAO/TCDD-treated mice at 6 h after administration of treatment. Thereafter, the expression level of mRNA of different genes was measured using reverse transcription followed by real-time PCR.

In the lung, TMAO alone at 6 h significantly induced Nqo1 mRNA levels to 341%, while it failed to significantly affect Gsta1 mRNA levels compared to control. TCDD alone at 6 h significantly induced Nqo1 mRNA levels to 586%, while it had no effect on Gsta1 mRNA levels compared to control. When animals were co-exposed to TMAO and TCDD, TMAO at 6 h had no effect on Nqo1 and Gsta1 mRNA levels compared to TCDD alone (Figure 3.16A, and B).

In the kidney, TMAO alone at 6 h significantly induced Nqo1 mRNA levels to 125%, while it failed to significantly affect Gsta1 mRNA levels compared to control. TCDD alone at 6 h significantly induced Nqo1 and Gsta1 mRNA levels to 152% and 177%, respectively, compared to control. When animals were co-exposed to TMAO and TCDD, TMAO at 6 h significantly potentiated the TCDD-mediated induction of Nqo1 mRNA to 232%, while it had no effect on Gsta1 compared to TCDD alone (Figure 3.16A, and B).

In the heart, TMAO alone at 6 h significantly induced Nqo1 and Gsta1 mRNA levels to 152% and 242%, respectively compared to control. TCDD alone at 6 h significantly induced Gsta1 mRNA

levels to 209%, while it had no effect on Nqo1 mRNA levels compared to control. When animals were co-exposed to TMAO and TCDD, TMAO at 6 h significantly induced Nqo1 mRNA levels to 193%, while it had no effect on Gsta1 mRNA compared to TCDD alone (Figure 3.16A, and B).

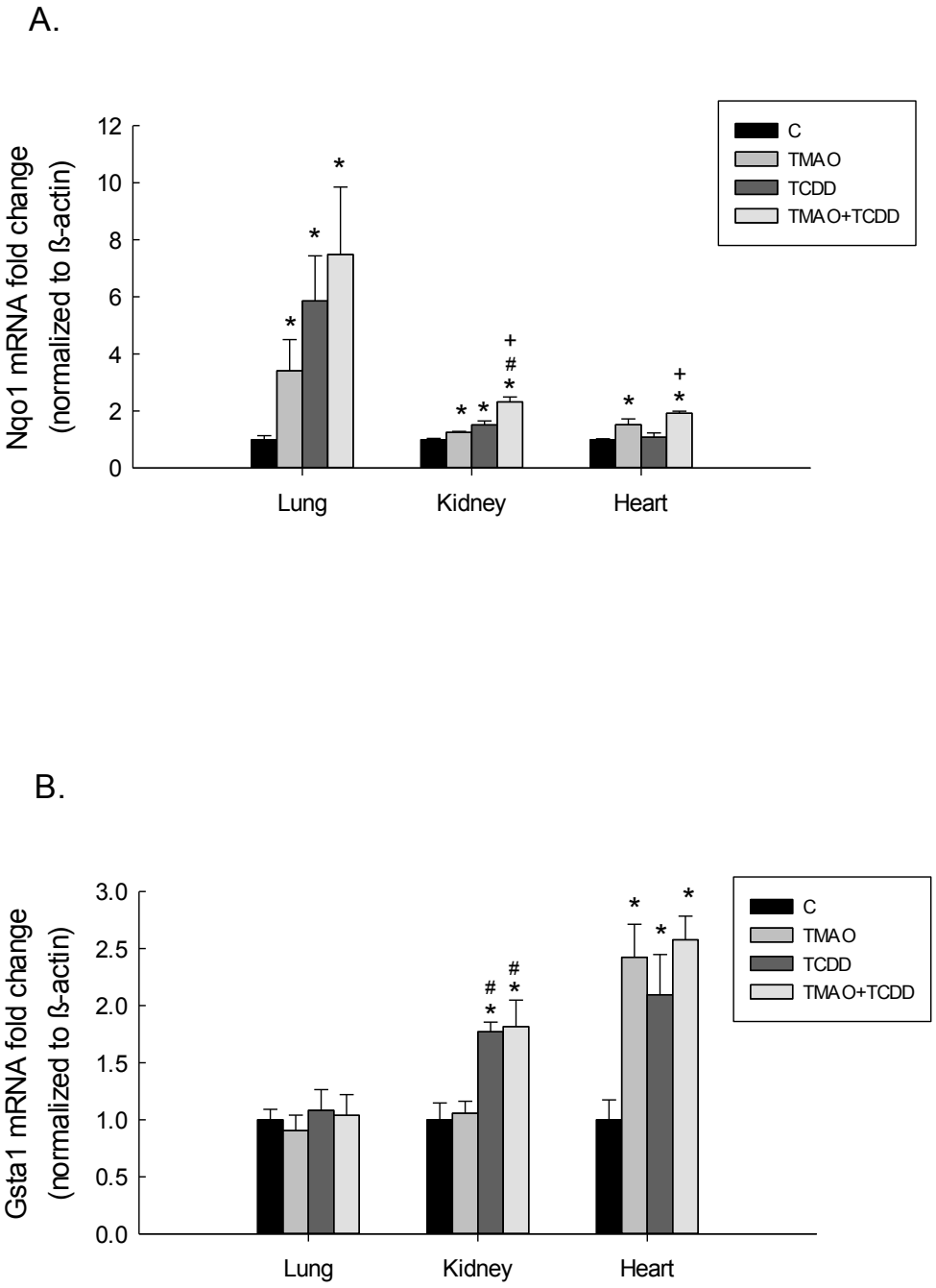


Figure 3.16 Effect of co-exposure to TMAO and TCDD on Nqo1 and Gsta1 mRNA in the lung, kidney, and heart of C57BL/6 mice. Animals were injected i.p. with 13 mg/kg TMAO with or without i.p. injection of 15 µg/kg TCDD for 6 h. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from lung, kidney, and heart and the expression of Nqo1 and Gsta1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using an ABI 7500 real-time PCR system as described under materials and methods. The values are presented as the mean + SEM. * $p<0.05$, compared to control (C); # $p<0.05$, compared to TMAO treatment; + $p<0.05$, compared to TCDD treatment.

3.2.4 Effect of co-exposure to TMAO and TCDD on Nqo1 and Gst protein expression and catalytic activity levels in the kidney and lung of C57BL/6 mice

To investigate whether the effect of TMAO treatment on Nqo1 and Gsta1 mRNA expression was further translated to proteins which are catalytically active, cytosolic protein was prepared from lung and kidney of control-, TMAO-, TCDD-, TMAO/TCDD-treated mice at 24 h after administration of treatment. Thereafter, Nqo1 and Gsta1/2 protein expression levels were determined using Western blot analysis. With regard to Nqo1 and Gst catalytic activities, lung and kidney cytosols were incubated with DCPIP and CDNB, as substrates, respectively. The reactions were monitored spectrophotometrically.

TMAO alone significantly induced the lung Nqo1 and Gsta1/2 protein expression levels to 134% and 128%, respectively, compared to control. TCDD alone significantly induced lung Nqo1 and Gsta1/2 protein expression levels to 144% and 216%, respectively, compared to control. When animals were co-exposed to TMAO and TCDD, TMAO had no significant effect on lung Nqo1 and Gsta1/2 protein expression levels compared to TCDD (Figure 3.17A, and B). In the kidney, TMAO significantly induced kidney Gsta1/2 protein expression levels to 283%, while it had no effect on kidney Nqo1 protein expression compared to control. TCDD alone significantly induced kidney Gsta1/2 protein expression levels to 153%, while it had no effect on kidney Nqo1 protein expression compared to control. When animals were co-exposed to TMAO and TCDD, TMAO significantly potentiated the TCDD-mediated induction of kidney Gsta1/2 protein expression levels to 280%, while it had no effect on kidney Nqo1 protein levels compared to TCDD alone (Figure 3.17A, and B).

In agreement with protein expression data, catalytic activity results showed that TMAO alone significantly induced lung Nqo1 and Gst activities to 143% and 119%, respectively, compared to control. TCDD alone significantly induced lung Nqo1 and Gst activities to 159% and 116%, respectively, compared to control. When animals were co-exposed to TMAO and TCDD, TMAO did not affect lung Nqo1 and Gst activities compared to TCDD alone (Figure 3.18A, and B). In the kidney, our results demonstrated that TMAO alone significantly induced the kidney Gst activity to 125%, while it had no effect on kidney Nqo1 activity compared to control. TCDD alone significantly induced kidney Gst activity to 113%, while it had no effect on kidney Nqo1 activity compared to control. When animals were co-exposed to TMAO and TCDD, TMAO significantly potentiated the TCDD-mediated induction of kidney Gst activity to 127%, while it did not affect kidney Nqo1 activity compared to TCDD alone (Figure 3.18A, and B).

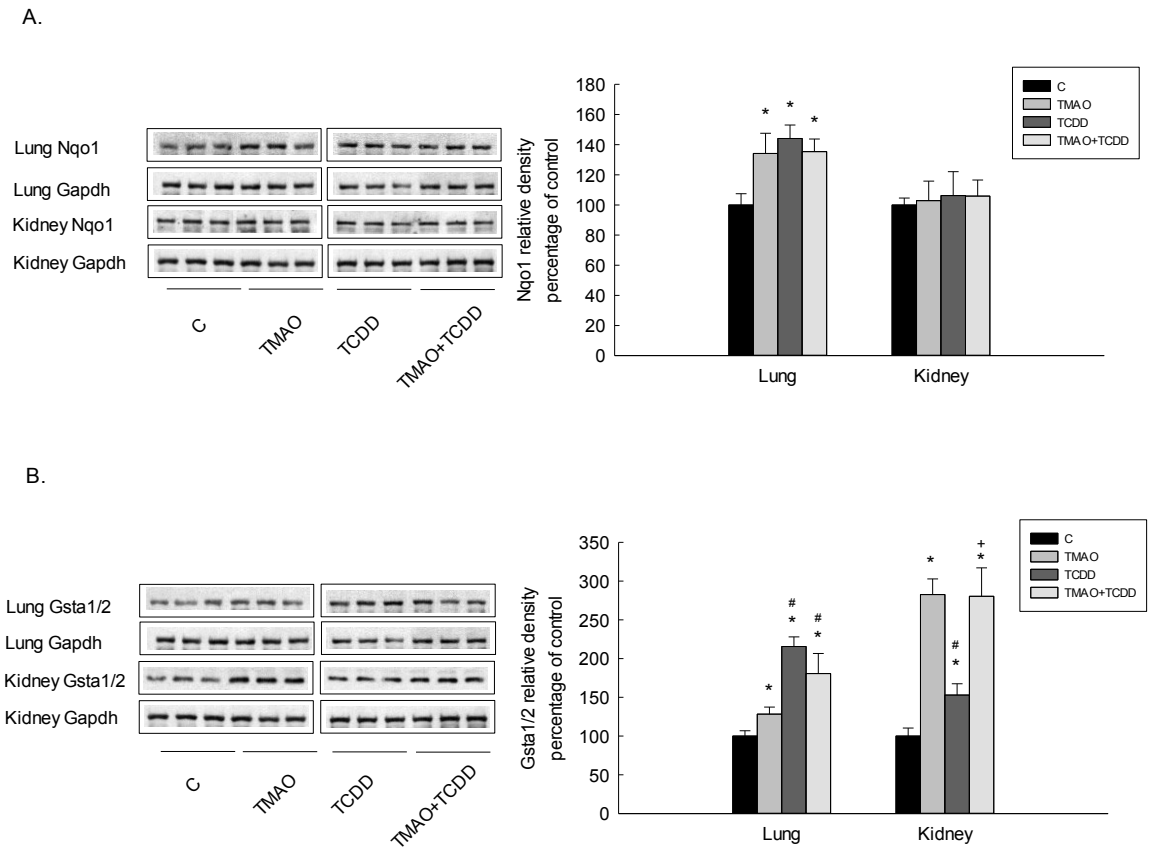


Figure 3.17 Effect of co-exposure to TMAO and TCDD on Nqo1, and Gsta1/2 protein expression levels in the lung and kidney of C57BL/6 mice. Animals were injected i.p. with 13 mg/kg TMAO with or without i.p. injection of 15 μg/kg TCDD for 24 h. Lung and kidney cytosolic proteins were isolated after 24 h of treatment. Ten micrograms of cytosolic proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4 °C and then incubated with a primary antibody for 2 h at 4 °C, followed by 1 h incubation with secondary antibody at room temperature. Proteins were detected using the enhanced chemiluminescence method. The intensity of the bands was normalized to β-actin signals, which were used as a loading control. The values are presented as the mean + SEM relative to the control values (set at 100%). * $p<0.05$, compared to control (C); # $p<0.05$, compared to TMAO treatment; + $p<0.05$, compared to TCDD treatment.

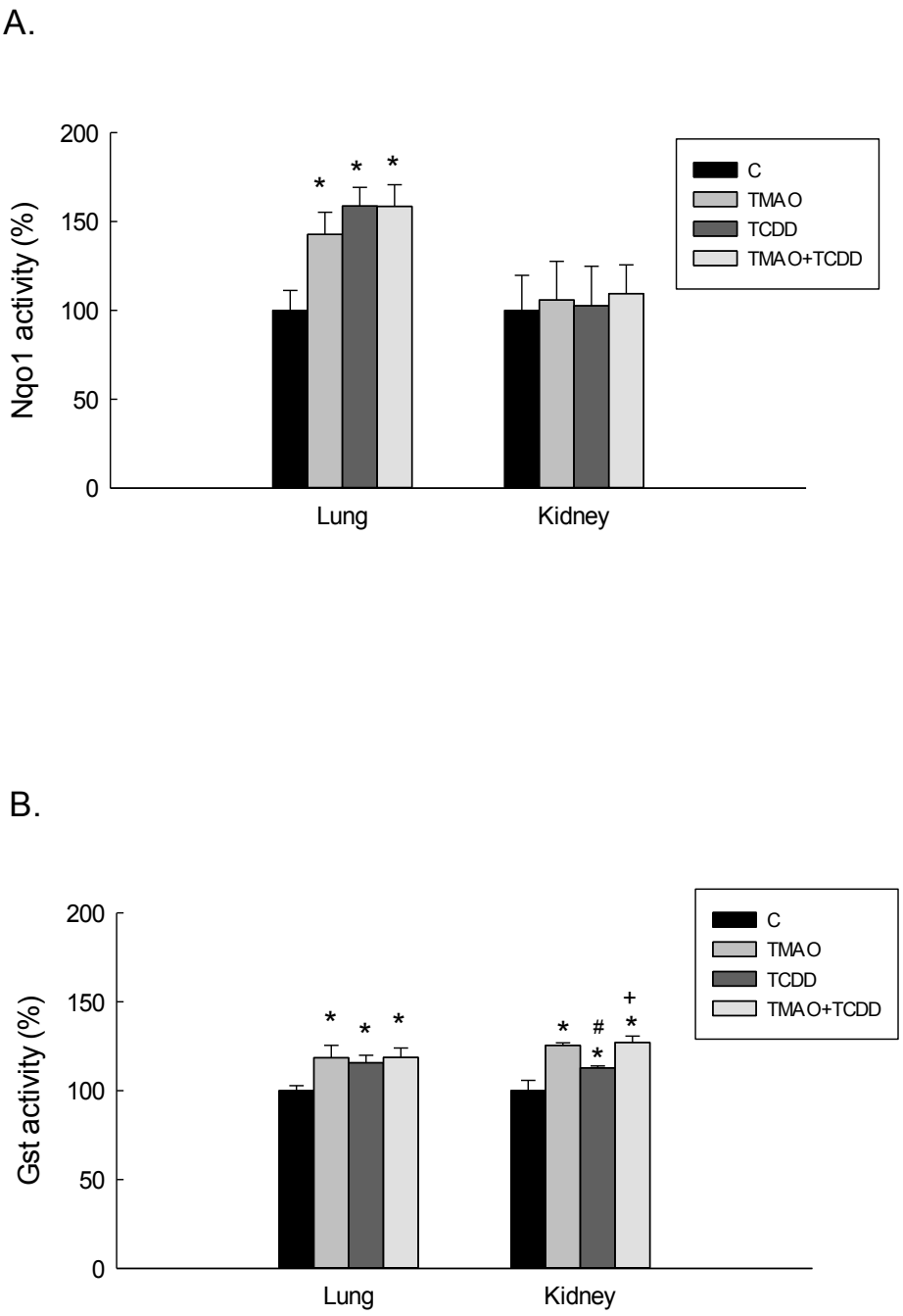


Figure 3.18 Effect of co-exposure to TMAO and TCDD on Nqo1 and Gst catalytic activities in the lung and kidney of C57BL/6 mice. Animals were injected i.p. with 13 mg/kg TMAO with or without i.p. injection of 15 μg/kg TCDD for 24 h. Lung and kidney cytosolic proteins were isolated and Nqo1 catalytic activity (A) was determined spectrophotometrically using DCPIP as substrate, and dicoumarol as a specific Nqo1 inhibitor. Gst catalytic activity (B) was determined spectrophotometrically in cytosolic fraction using CDNB as a substrate. The values are presented as mean + SEM. * $p<0.05$, compared to control (C); # $p<0.05$, compared to TMAO treatment; + $p<0.05$, compared to TCDD treatment.

3.2.5 Effect of co-exposure to TMAO and TCDD on Ho-1 mRNA levels in the lung, kidney, and heart

To examine the effect of TMAO treatment on the extrahepatic expression of Ho-1, total RNA was extracted from the lung, kidney, and heart of control-, TMAO-, TCDD-, TMAO/TCDD-treated mice at 6 h after administration of treatment. Thereafter, the expression level of Ho-1 mRNA was measured using reverse transcription followed by real-time PCR.

Our results demonstrated that TMAO alone was able to induce Ho-1 mRNA levels in the kidney and heart after 6 h of treatment to 143% and 245%, respectively, while it did not affect lung Ho-1 mRNA levels compared to control. TCDD alone, after 6 h of treatment, significantly induced Ho-1 mRNA levels in the lung, kidney, and heart to 155%, 152%, and 203%, respectively, compared to control. When the animals were co-exposed to TMAO and TCDD for 6 h, TMAO at 6 h significantly potentiated the TCDD-mediated induction of Ho-1 mRNA in the kidney and heart to 258%, 445%, while it had no effect on lung Ho-1 mRNA compared to TCDD alone (Figure 3.19).

3.2.6 Summary of the effects of TMAO and TCDD in the lung, kidney, and heart of C57BL/6 mice

The effects of TMAO and TCDD on the expression of different genes in the lung, kidney, and heart of C57BL/6 mice have been summarized in Table 3-2.

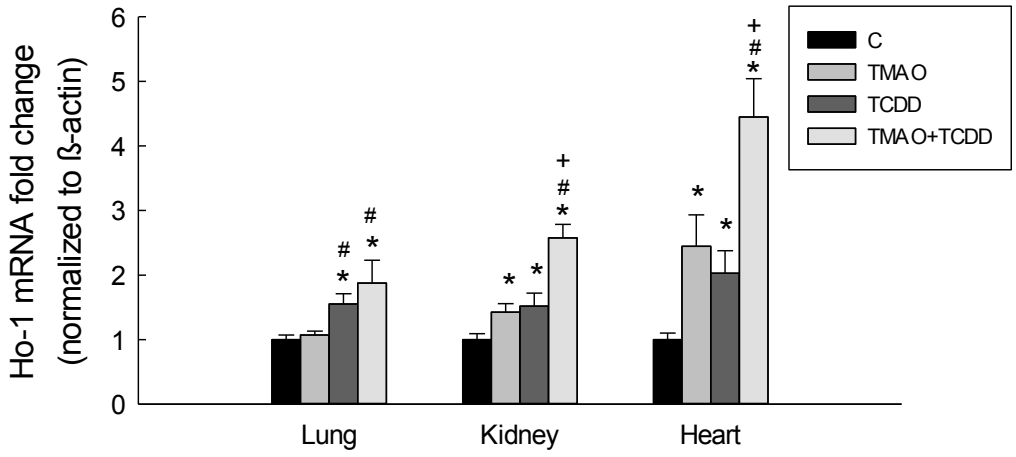


Figure 3.19 Effect of co-exposure to TMAO and TCDD on lung, kidney, and heart Ho-1 mRNA in C57BL/6 mice. Animals were injected i.p. with 13 mg/kg TMAO with or without i.p. injection of 15 µg/kg TCDD for 6 h. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from lung, kidney, and heart; and the expression of Ho-1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using an ABI 7500 real-time PCR system as described under materials and methods. The values are presented as the mean + SEM. * $p<0.05$, compared to control (C); # $p<0.05$, compared to TMAO treatment; + $p<0.05$, compared to TCDD treatment.

Table 3-2 Summary of the effects of TMAO and TCDD on the expression of different genes in the lung, kidney, and heart.

Treatment	Gene	Tissue		
		Lung	**Kidney**	**Heart**
TMAO	Cyp1a1	↑mRNA, ↑protein, ↑activity	↔mRNA, ↔protein, ↔activity	↔mRNA
	Cyp1a2	↔mRNA, ↔protein, ↔activity	↔mRNA, ↔protein, ↔activity	↔mRNA
	Cyp1b1	↑mRNA, ↑protein	↑mRNA, ↑protein	↔mRNA
	Nqo1	↑mRNA, ↑protein, ↑activity	↑mRNA, ↔protein, ↔activity	↑mRNA
	Gsta1	↔mRNA, ↑protein, ↑activity	↔mRNA, ↑protein, ↑activity	↑mRNA
	Ho-1	↔mRNA	↑mRNA	↑mRNA
TCDD	Cyp1a1	↑mRNA, ↑protein, ↑activity	↑mRNA, ↑protein, ↑activity	↑mRNA
	Cyp1a2	↑mRNA, ↑protein, ↑activity	↑mRNA, ↑protein, ↑activity	↑mRNA
	Cyp1b1	↑mRNA, ↑protein	↑mRNA, ↑protein	↑mRNA
	Nqo1	↑mRNA, ↑protein, ↑activity	↑mRNA, ↔protein, ↔activity	↔mRNA
	Gsta1	↔mRNA, ↑protein, ↑activity	↑mRNA, ↑protein, ↑activity	↑mRNA
	Ho-1	↑mRNA	↑mRNA	↑mRNA
TMAO + TCDD	Cyp1a1	↑↑mRNA, ↑↑protein, ↑↑activity	↑↑mRNA, ↑↑protein, ↑↑activity	↑↑mRNA
	Cyp1a2	↑↑mRNA, ↑↑protein, ↑↑activity	↑mRNA, ↑protein, ↑activity	↑mRNA
	Cyp1b1	↑mRNA, ↑protein	↑↑mRNA, ↑↑protein	↑↑mRNA
	Nqo1	↑mRNA, ↑protein, ↑activity	↑↑mRNA, ↔protein, ↔activity	↑↑mRNA
	Gsta1	↔mRNA, ↑protein, ↑activity	↑mRNA, ↑↑protein, ↑↑activity	↑mRNA
	Ho-1	↑mRNA	↑↑mRNA	↑↑mRNA

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

3.3 Dimethylarsinic acid (DMA(V)) modulates the AhR-regulated genes in C57BL/6 mice: *in vivo* study

3.3.1 Effect of DMA(V) and TCDD on Cyp1a1 and Cyp1a2 mRNA expression in the liver, lung, and kidney of C57BL/6 mice

To examine the effect of DMA(V) treatment on the hepatic and extrahepatic expression of Cyp1a1 and Cyp1a2 mRNA, total RNA was extracted from the liver, lung, and kidney of different treatment groups at 6 h. Thereafter, the expression level of Cyp1a1 and Cyp1a2 mRNA was measured using reverse transcription followed by real-time PCR.

Our results showed that DMA(V) alone had no significant effect on Cyp1a1 mRNA levels compared to control in the liver, lung, or kidney. TCDD alone significantly induced Cyp1a1 mRNA levels in the liver, lung, and kidney to 1107703%, 14279%, and 84135%, respectively, compared to control. When animals were co-treated with DMA(V) and TCDD, DMA(V) had no significant effect on the TCDD-mediated induction of Cyp1a1 mRNA in the liver compared to TCDD alone. On the other hand, DMA(V) significantly potentiated the TCDD-mediated induction of Cyp1a1 mRNA in the lung to 19507%. In contrast, DMA(V) significantly inhibited the TCDD-mediated induction of Cyp1a1 mRNA in the kidney to 30236% (Figure 3.20A, B, and C).

Figure 3.21 shows that DMA(V) alone had no significant effect on the liver, lung, or kidney Cyp1a2 mRNA levels compared to control. TCDD alone significantly induced Cyp1a2 mRNA levels in the liver, lung, and kidney to 1007%, 412%, and 665%, compared to control. When

animals were co-treated with DMA(V) and TCDD, DMA(V) had no significant effect on the TCDD-mediated induction of the liver Cyp1a2 mRNA compared to TCDD alone. However, DMA(V) significantly potentiated the TCDD-mediated induction of Cyp1a2 mRNA in the lung to 1068%. While DMA(V) significantly inhibited the TCDD-mediated induction of Cyp1a2 mRNA in the kidney to 133% (Figure 3.21A, B, and C).

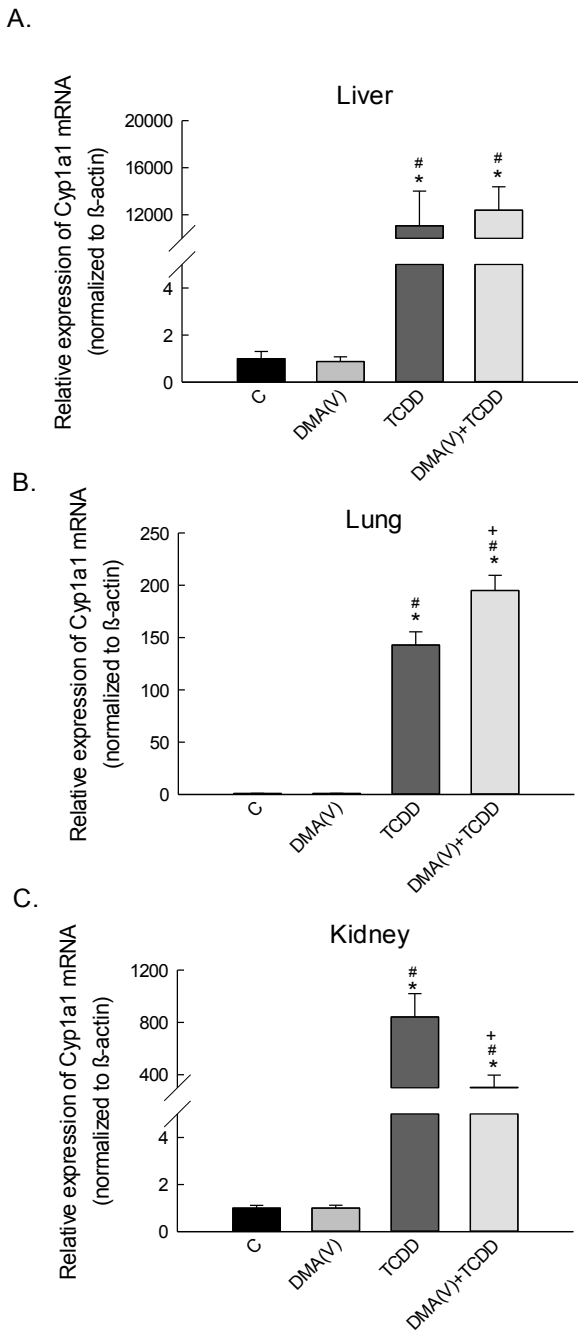


Figure 3.20 Effect of co-exposure to DMA(V) and TCDD on Cyp1a1 mRNA in the liver, lung, and kidney of C57BL/6 mice. Animals were injected i.p. with 13.3 mg/kg DMA(V) with or without i.p. injection of 15 µg/kg TCDD for 6 h. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from the liver, lung, and kidney and the expression of Cyp1a1 mRNA was measured using real-time PCR. cDNA fragments were amplified and quantitated using an ABI 7500 real-time PCR system as described under materials and methods. The values are presented as the mean + SEM. * $p<0.05$, compared to control (C); # $p<0.05$, compared to DMA(V) treatment; + $p<0.05$, compared to TCDD treatment.

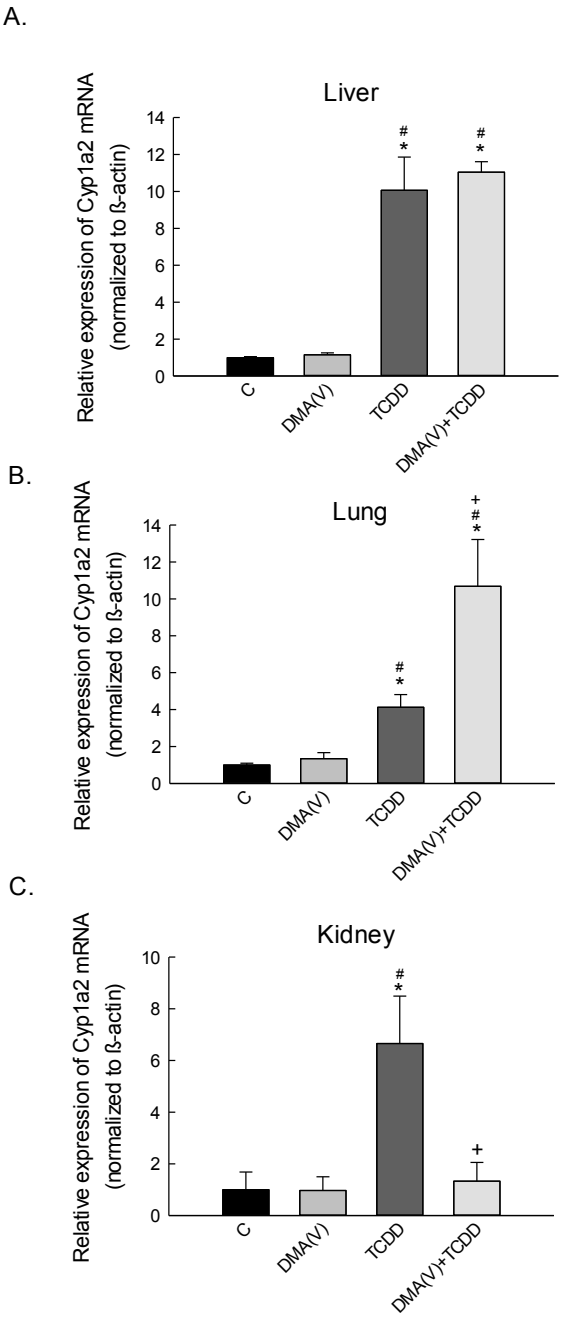


Figure 3.21 Effect of co-exposure to DMA(V) and TCDD on Cyp1a2 mRNA in the liver, lung, and kidney of C57BL/6 mice. Animals were injected i.p. with 13.3 mg/kg DMA(V) with or without i.p. injection of 15 µg/kg TCDD for 6 h. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from the liver, lung, and kidney and the expression of Cyp1a2 mRNA was measured using real-time PCR. cDNA fragments were amplified and quantitated using an ABI 7500 real-time PCR system as described under materials and methods. The values are presented as the mean + SEM. * $p<0.05$, compared to control (C); # $p<0.05$, compared to DMA(V) treatment; + $p<0.05$, compared to TCDD treatment.

3.3.2 Effect of DMA(V) and TCDD on Cyp1a protein levels in the liver, lung, and kidney of C57BL/6 mice

To examine whether the effect of DMA(V) treatment on Cyp1a mRNA expression was further translated to proteins, microsomal proteins were prepared from the liver, lung and kidney of different treatment groups at 24 h. Thereafter, Cyp1a protein expression levels were determined using Western blot analysis.

In accordance with mRNA expression, Western blot results showed that DMA(V) alone had no significant effect on Cyp1a protein expression levels in the liver, lung, or kidney, compared to control. TCDD alone significantly induced the Cyp1a protein expression levels in the liver, lung, and kidney to 1089%, 495%, and 3347%, respectively, compared to control. When animals were co-treated with DMA(V) and TCDD, DMA(V) had no significant effect on the TCDD-mediated induction of Cyp1a protein expression levels in the liver compared to TCDD alone. However, DMA(V) significantly potentiated the TCDD-mediated induction of Cyp1a protein expression levels in the lung to 1161%. In contrast, DMA(V) significantly inhibited the TCDD-mediated induction of Cyp1a protein expression levels in the kidney to 1176% (Figure 3.22A, B, and C).

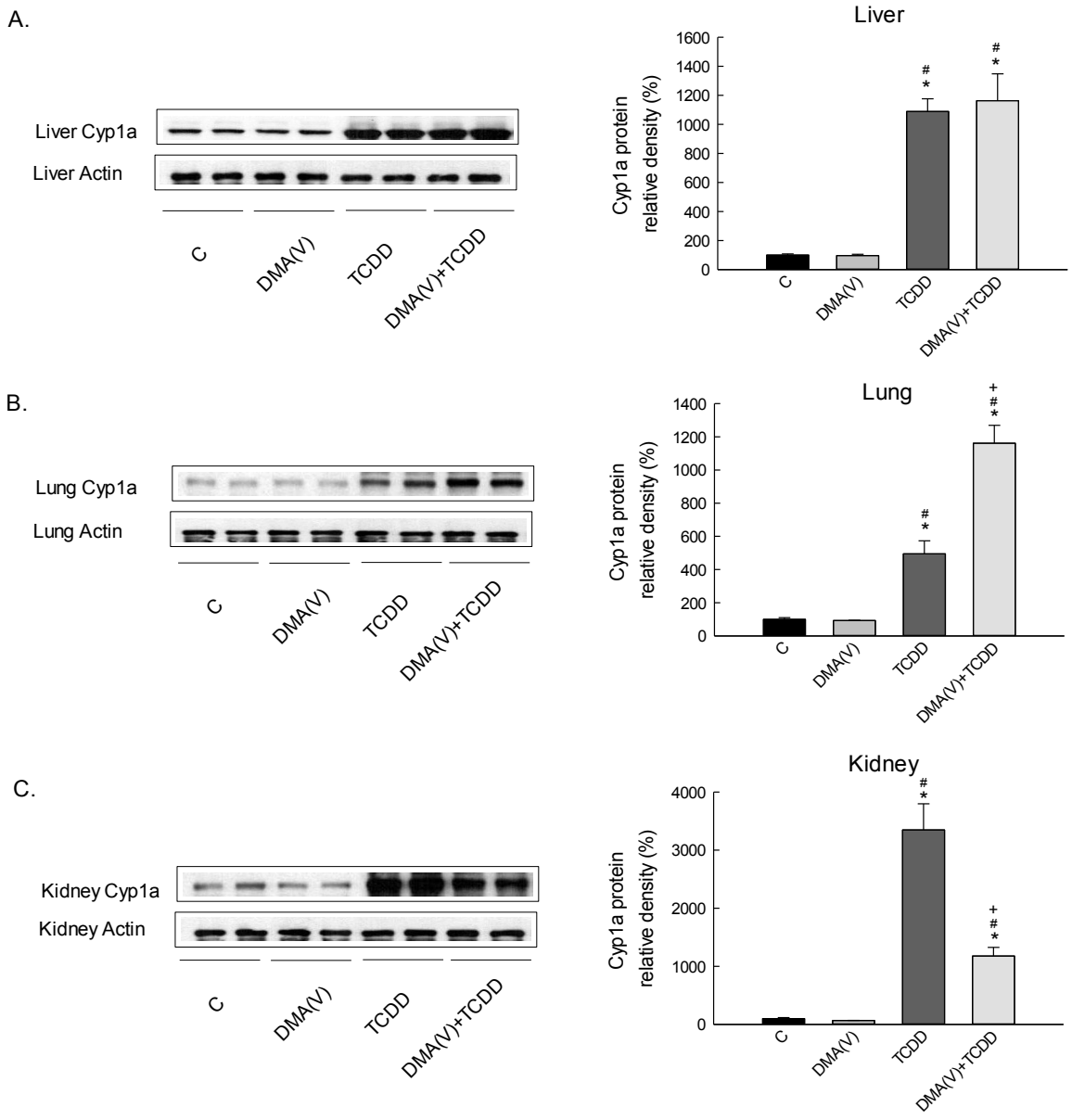


Figure 3.22 Effect of co-exposure to DMA(V) and TCDD on Cyp1a protein expression levels the liver, lung, and kidney of C57BL/6 mice. Animals were injected i.p. with 13.3 mg/kg DMA(V) with or without i.p. injection of 15 µg/kg TCDD for 24 h. Liver, lung, and kidney microsomal proteins were isolated after 24 h of treatment. Ten micrograms of microsomal proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4 °C and then incubated with a primary antibody for 2 h at 4 °C, followed by 1 h incubation with the secondary antibody at room temperature. Proteins were detected using the enhanced chemiluminescence method. The intensity of the bands was normalized to loading control signals. The values are presented as the mean + SEM relative to the control values (set at 100%). * $p<0.05$, compared to control (C); # $p<0.05$, compared to DMA(V) treatment; + $p<0.05$, compared to TCDD treatment.

3.3.3 Effect of DMA(V) and TCDD on Cyp1a catalytic activity levels in the liver, lung, and kidney of C57BL/6 mice

To examine whether the effect of DMA(V) treatment on Cyp1a mRNA and protein expression levels was further reflected in the catalytic activity, microsomal proteins were prepared from the liver, lung and kidney of different treatment groups at 24 h. Thereafter, Cyp1a1-dependent EROD and Cyp1a2-dependent MROD catalytic activities were measured by incubating the microsomes with 7-ethoxyresorufin and 7-methoxyresorufin as substrates, respectively.

Our results showed that DMA(V) alone had no significant effect on EROD activity in the liver, lung, or kidney compared to control. TCDD alone significantly induced EROD activity in the liver, lung, and kidney to 1211%, 528%, and 2327%, respectively, compared to control. When animals were co-treated with DMA(V) and TCDD, DMA(V) had no significant effect on TCDD-mediated induction of EROD catalytic activity in the liver compared to TCDD alone. However, DMA(V) significantly potentiated the TCDD-mediated induction of EROD catalytic activity in the lung to 837%. On the other hand, DMA(V) significantly inhibited the TCDD-mediated induction of EROD catalytic activity in the kidney to 923% (Figure 3.23A, B, and C).

Figure 3.24 shows that DMA(V) alone had no significant effect on MROD activity in the liver, lung, and kidney compared to control. TCDD alone significantly induced MROD activity in the liver, lung, and kidney to 190%, 410%, and 1129%, respectively compared to control. When animals were co-treated with DMA(V) and TCDD, DMA(V) had no significant effect on TCDD-mediated induction of MROD catalytic activity in the liver, compared to TCDD alone. However

DMA(V) significantly potentiated the TCDD-mediated induction of MROD catalytic activity in the lung to 640%. On the other hand, DMA(V) significantly inhibited the TCDD-mediated induction of MROD catalytic activity in the kidney to 574% (Figure 3.24A, B, and C).

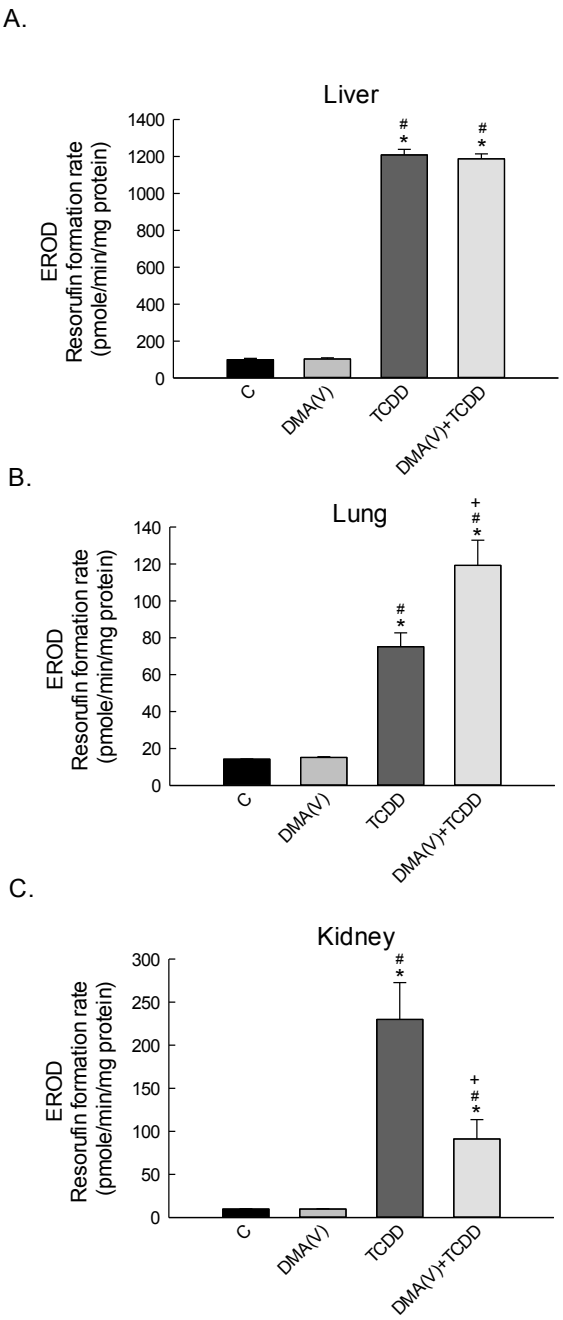


Figure 3.23 Effect of co-exposure to DMA(V) and TCDD on Cyp1a1 activity in the liver, lung, and kidney of C57BL/6 mice. Animals were injected i.p. with 13.3 mg/kg DMA(V) with or without i.p. injection of 15 µg/kg TCDD for 24 h. Liver, lung, and kidney microsomal proteins were isolated; thereafter, EROD activities were measured using 7-ethoxyresorufin as substrate. The reaction was started by the addition of 1 mM NADPH and lasted for 5 min. The reaction was terminated by the addition of ice-cold methanol. The values are presented as the mean + SEM. * $p<0.05$, compared to control (C); # $p<0.05$, compared to DMA(V) treatment; + $p<0.05$, compared to TCDD treatment.

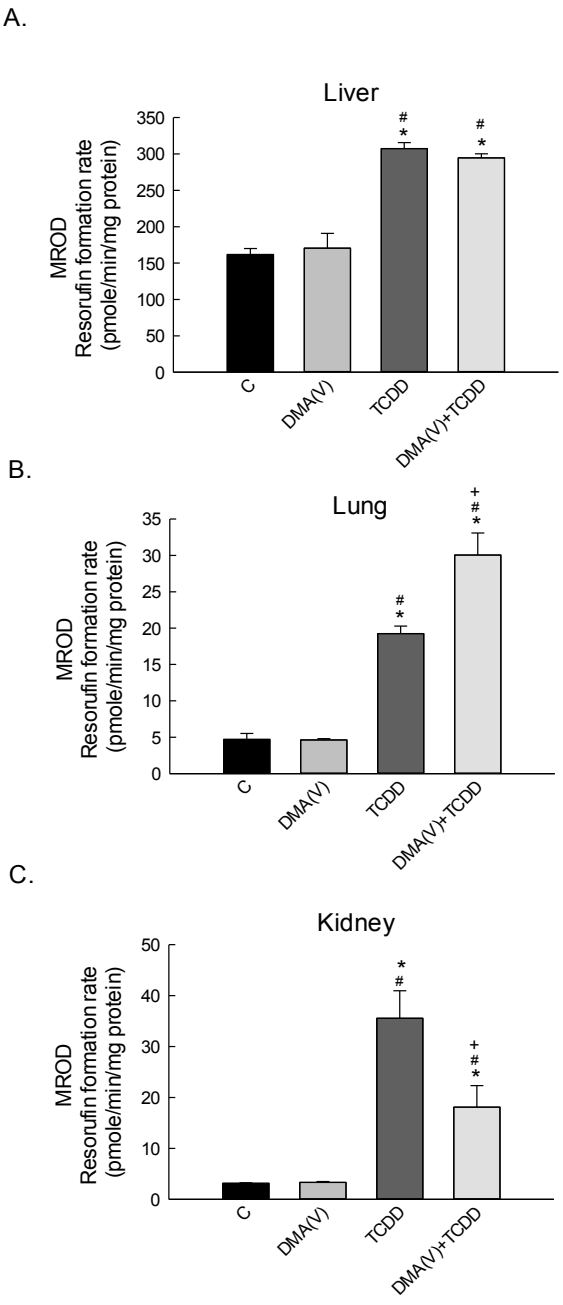


Figure 3.24 Effect of co-exposure to DMA(V) and TCDD on Cyp1a2 activity in the liver, lung, and kidney of C57BL/6 mice. Animals were injected i.p. with 13.3 mg/kg DMA(V) with or without i.p. injection of 15 µg/kg TCDD for 24 h. Liver, lung, and kidney microsomal proteins were isolated; thereafter, MROD activities were measured using 7-methoxyresorufin as substrate. The reaction was started by the addition of 1 mM NADPH and lasted for 10 min. The reaction was terminated by the addition of ice-cold methanol. The values are presented as the mean + SEM. * $p<0.05$, compared to control (C); # $p<0.05$, compared to DMA(V) treatment; + $p<0.05$, compared to TCDD treatment.

3.3.4 Effect of DMA(V) and TCDD on Nqo1 mRNA expression in the liver, lung, and kidney of C57BL/6 mice

To examine the effect of DMA(V) treatment on the hepatic and extrahepatic expression of Nqo1 mRNA, total RNA was extracted from the liver, lung, and kidney of different treatment groups at 6 h. Thereafter, the expression level of Nqo1 mRNA was measured using reverse transcription followed by real time-PCR.

DMA(V) alone had no significant effect on Nqo1 mRNA levels in the liver, lung, or kidney compared to control. TCDD alone significantly induced Nqo1 mRNA levels in the liver, lung, and kidney to 367%, 1670%, and 136%, respectively, compared to control. When animals were co-treated with DMA(V) and TCDD, DMA(V) had no significant effect on the TCDD-mediated induction of Nqo1 mRNA in the liver, lung, or kidney compared to TCDD alone (Figure 3.25A, B, and C).

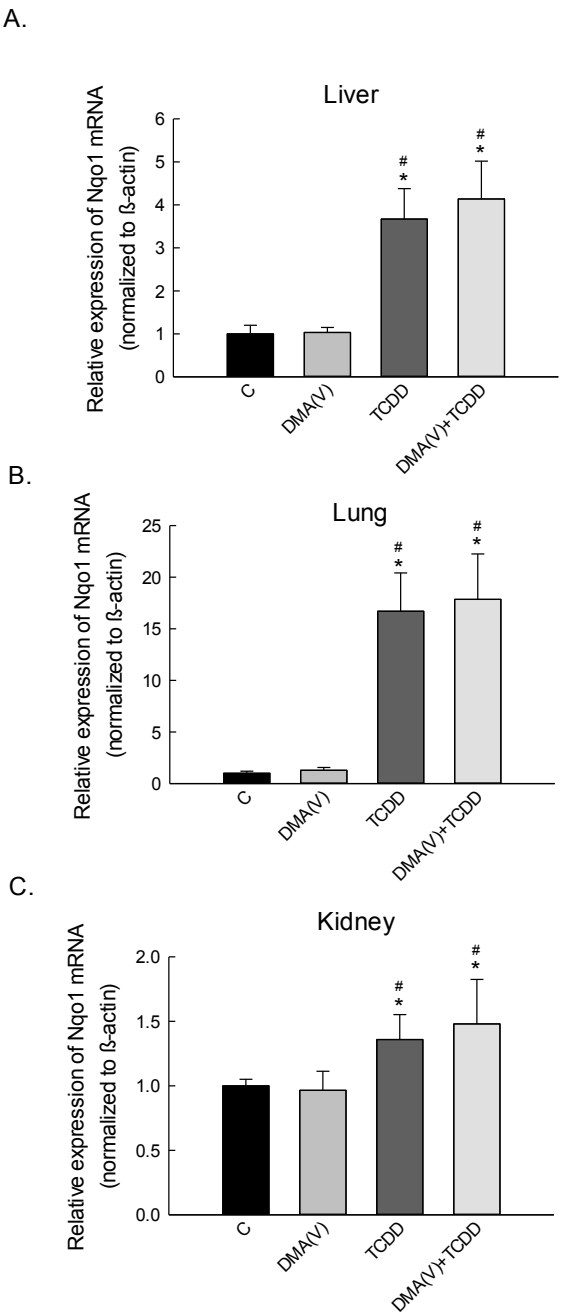


Figure 3.25 Effect of co-exposure to DMA(V) and TCDD on Nqo1 mRNA in the liver, lung, and kidney of C57BL/6 mice. Animals were injected i.p. with 13.3 mg/kg DMA(V) with or without i.p. injection of 15 µg/kg TCDD for 6 h. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from the liver, lung, and kidney and the expression of Nqo1 mRNA was measured using real-time PCR. cDNA fragments were amplified and quantitated using an ABI 7500 real-time PCR system as described under materials and methods. The values are presented as the mean + SEM. * $p<0.05$, compared to control (C); # $p<0.05$, compared to DMA(V) treatment; + $p<0.05$, compared to TCDD treatment.

3.3.5 Effect of DMA(V) and TCDD on Nqo1 protein levels in the liver, lung, and kidney of C57BL/6 mice

To examine whether DMA(V) treatment has an effect on Nqo1 protein expression via non-transcriptional mechanisms (such as post-transcriptional or post-translational mechanisms), cytosolic proteins were prepared from the liver, lung, and kidney of different treatment groups at 24 h. Thereafter, Nqo1 protein expression levels were determined using Western blot analysis.

Our results showed that DMA(V) alone had no significant effect on Nqo1 protein expression levels in the liver, lung, or kidney compared to control. TCDD alone significantly induced the Nqo1 protein expression levels in the liver, and lung to 159% and 178%, respectively, compared to control. However TCDD alone had no significant effect on Nqo1 protein expression levels in the kidney compared to control. When animals were co-treated with DMA(V) and TCDD, DMA(V) had no significant effect on Nqo1 protein expression levels in the liver, lung, or kidney compared to TCDD alone (Figure 3.26A, B, and C).

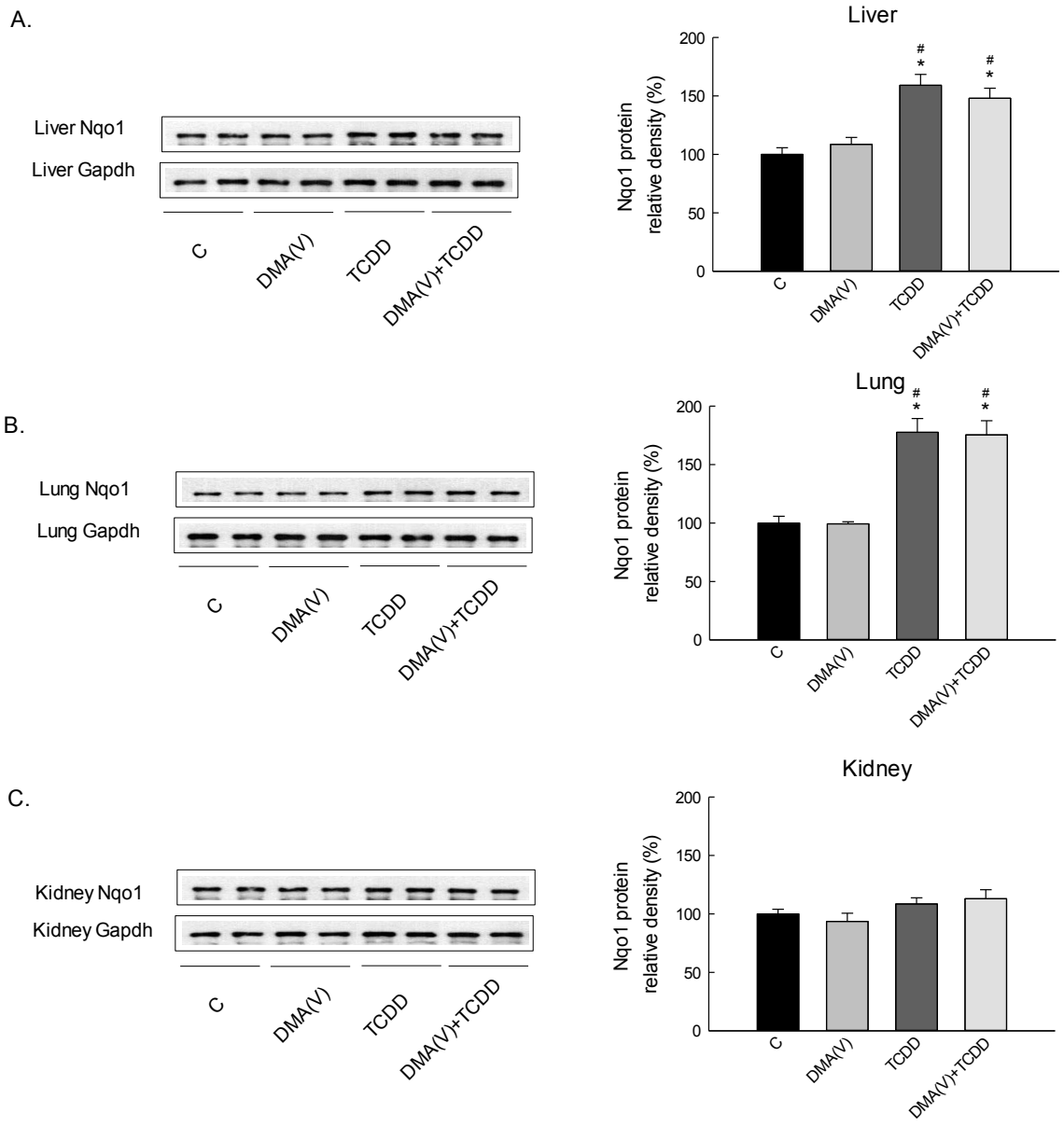


Figure 3.26 Effect of co-exposure to DMA(V) and TCDD on Nqo1 protein expression in the liver, lung, and kidney of C57BL/6 mice. Animals were injected i.p. with 13.3 mg/kg DMA(V) with or without i.p. injection of 15 µg/kg TCDD for 24 h. Liver, lung, and kidney cytosolic proteins were isolated after 24 h of treatment. Ten micrograms of cytosolic proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose membrane. Protein blots were blocked overnight at 4 °C and then incubated with a primary antibody for 2 h at 4 °C, followed by 1 h incubation with secondary antibody at room temperature. Proteins were detected using the enhanced chemiluminescence method. The intensity of the bands was normalized to loading control signals. The values are presented as the mean + SEM relative to the control values (set at 100%). * $p<0.05$, compared to control (C); # $p<0.05$, compared to DMA(V) treatment; + $p<0.05$, compared to TCDD treatment.

3.3.6 Effect of DMA(V) and TCDD on Nqo1 catalytic activity levels in the liver, lung, and kidney of C57BL/6 mice

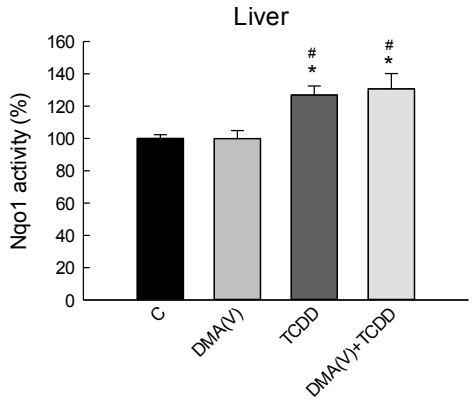
To examine whether DMA(V) treatment has an effect on Nqo1 catalytic activity via non-transcriptional or non-translational mechanisms (possibly by post-translational mechanisms), cytosolic proteins were prepared from the liver, lung and kidney of different treatment groups at 24 h. Thereafter, Nqo1 activity levels were determined using DCPIP as substrate.

In line with Western blot analysis, activity results showed that DMA(V) alone had no significant effect on Nqo1 catalytic activity levels in the liver, lung, or kidney, compared to control. TCDD alone significantly induced the Nqo1 catalytic activity levels in the liver and lung to 127% and 126%, respectively, compared to control. However TCDD alone had no significant effect on the kidney Nqo1 catalytic activity compared to control. When animals were co-treated with DMA(V) and TCDD, DMA(V) had no significant effect on Nqo1 catalytic activity levels in the liver, lung, or kidney, compared to TCDD alone (Figure 3.27A, B, C).

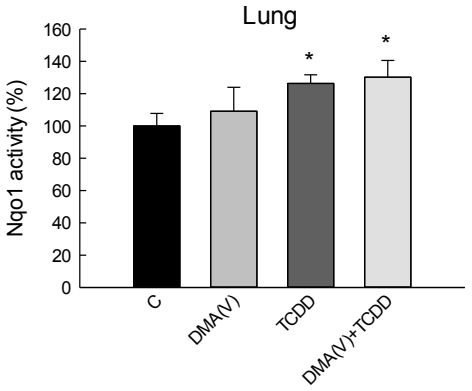
3.3.7 Summary of the effects of DMA(V) and TCDD in the liver, lung, and kidney of C57BL/6 mice

The effects of DMA(V) and TCDD on the expression of different genes in the liver, lung, and kidney of C57BL/6 mice have been summarized in Table 3-3.

A.



B.



C.

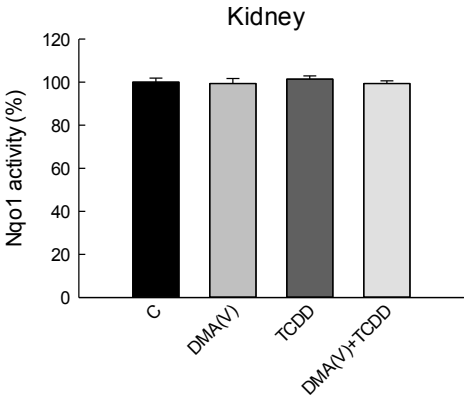


Figure 3.27 Effect of co-exposure to DMA(V) and TCDD on Nqo1 catalytic activity levels in the liver, lung, and kidney of C57BL/6 mice. Animals were injected i.p. with 13.3 mg/kg DMA(V) with or without i.p. injection of 15 μg/kg TCDD for 24 h. Liver, lung, and kidney cytosolic proteins were isolated after 24 h of treatment. Nqo1 catalytic activity was determined spectrophotometrically using DCPIP as substrate, and dicoumarol as a specific Nqo1 inhibitor. The values are presented as the mean + SEM relative to the control values (set at 100%). * $p<0.05$, *compared to control (C);* # $p<0.05$, *compared to DMA(V) treatment;* + $p<0.05$, *compared to TCDD treatment.*

Table 3-3 Summary of the effects of DMA(V) and TCDD on the expression of different genes in the liver, lung, and kidney.

Treatment	Gene	Tissue		
		Liver	**Lung**	**Kidney**
DMA(V)	Cyp1a1	↔mRNA, ↔protein, ↔activity	↔mRNA, ↔protein, ↔activity	↔mRNA, ↔protein, ↔activity
	Cyp1a2	↔mRNA, ↔protein, ↔activity	↔mRNA, ↔protein, ↔activity	↔mRNA, ↔protein, ↔activity
	Nqo1	↔mRNA, ↔protein, ↔activity	↔mRNA, ↔protein, ↔activity	↔mRNA, ↔protein, ↔activity
TCDD	Cyp1a1	↑mRNA, ↑protein, ↑activity	↑mRNA, ↑protein, ↑activity	↑mRNA, ↑protein, ↑activity
	Cyp1a2	↑mRNA, ↑protein, ↑activity	↑mRNA, ↑protein, ↑activity	↑mRNA, ↑protein, ↑activity
	Nqo1	↑mRNA, ↑protein, ↑activity	↑mRNA, ↑protein, ↑activity	↑mRNA, ↔protein, ↔activity
DMA(V) + TCDD	Cyp1a1	↑mRNA, ↑protein, ↑activity	↑↑mRNA, ↑↑protein, ↑↑activity	↓↓mRNA, ↓↓protein, ↓↓activity
	Cyp1a2	↑mRNA, ↑protein, ↑activity	↑↑mRNA, ↑↑protein, ↑↑activity	↓↓mRNA, ↓↓protein, ↓↓activity
	Nqo1	↑mRNA, ↑protein, ↑activity	↑mRNA, ↑protein, ↑activity	↑mRNA, ↔protein, ↔activity

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

3.4 Modulation of Cytochrome P450 1A1 by monomethylarsonous acid (MMA(III)) in human HepG2 cells

3.4.1 Effect of exposure to As(III) and MMA(III) in the absence and presence of TCDD on cell viability

To determine the nontoxic concentrations of As(III) and MMA(III) to be utilized in this study, HepG2 cells were exposed for 24 h with increasing concentrations of As(III) or MMA(III) (1–10 μM) in the absence and presence of 1 nM TCDD; thereafter cytotoxicity was assessed using the MTT assay. Figure 3.28A, and B show that As(III) and MMA(III) at concentrations of 1–10 μM in the absence and presence of 1 nM TCDD were not cytotoxic (cell viability above 80%, pursuant to ISO 10993-5 (ISO, 2009; Lopez-Garcia *et al.*, 2014)). Therefore, all subsequent studies were conducted using concentrations of 1–10 μM.

3.4.2 Effect of As(III) and MMA(III) on CYP1A1 mRNA

To examine the effect of As(III) and MMA(III) on CYP1A1 mRNA, HepG2 cells were treated with 5 μM As(III) or increasing concentrations of MMA(III) (1-10 μM), in the absence and presence of 1 nM TCDD. Thereafter, CYP1A1 mRNA was assessed using real-time PCR. As(III) alone significantly reduced CYP1A1 mRNA to 60%, compared to control. Similarly, increasing concentrations (1, 2.5, 5, or 10 μM) of MMA(III) alone significantly reduced CYP1A1 mRNA to 63%, 42%, 31%, and 21%, respectively, compared to control (Figure 3.29A). In contrast, TCDD alone significantly induced CYP1A1 mRNA levels to 5239%, compared to control, which was

decreased by As(III) to 2075%, compared to control. Increasing concentrations (1, 2.5, 5, or 10 μM) of MMA(IIII), reduced TCDD-mediated induction of CYP1A1 mRNA levels to 3378%, 2934%, 2627%, and 2505% respectively, compared to control (Figure 3.29B).

3.4.3 Time-dependent effect of As(III) and MMA(III) on CYP1A1 mRNA

To better understand the kinetics of CYP1A1 mRNA in response to As(III) and MMA(III), the time-dependent effect was determined at various time points up to 24 h after treatment of HepG2 cells with 1nM TCDD in the absence and presence of 5 μM As(III) or MMA(III). Our results clearly demonstrated that TCDD alone caused a time-dependent increase in CYP1A1 mRNA levels over a 24 h period to reach 12575%. In contrast, when HepG2 cells were co-exposed to As(III) or MMA(III) and TCDD, there was a significant decrease in the CYP1A1 mRNA levels at all tested time points. Both As(III) and MMA(III) resulted in a significant reduction of TCDD-mediated induction of CYP1A1 mRNA as early as 1 h, suggesting an effect on gene transcription. After 24 h of treatment, MMA(III) reduced TCDD-mediated induction of CYP1A1 mRNA by 84%, while As(III) showed only 50% reduction in CYP1A1 mRNA, compared to TCDD (Figure 3.30A, and B). The result demonstrated that MMA(III) has a more sustained and prominent effect compared to As(III) at later time points.

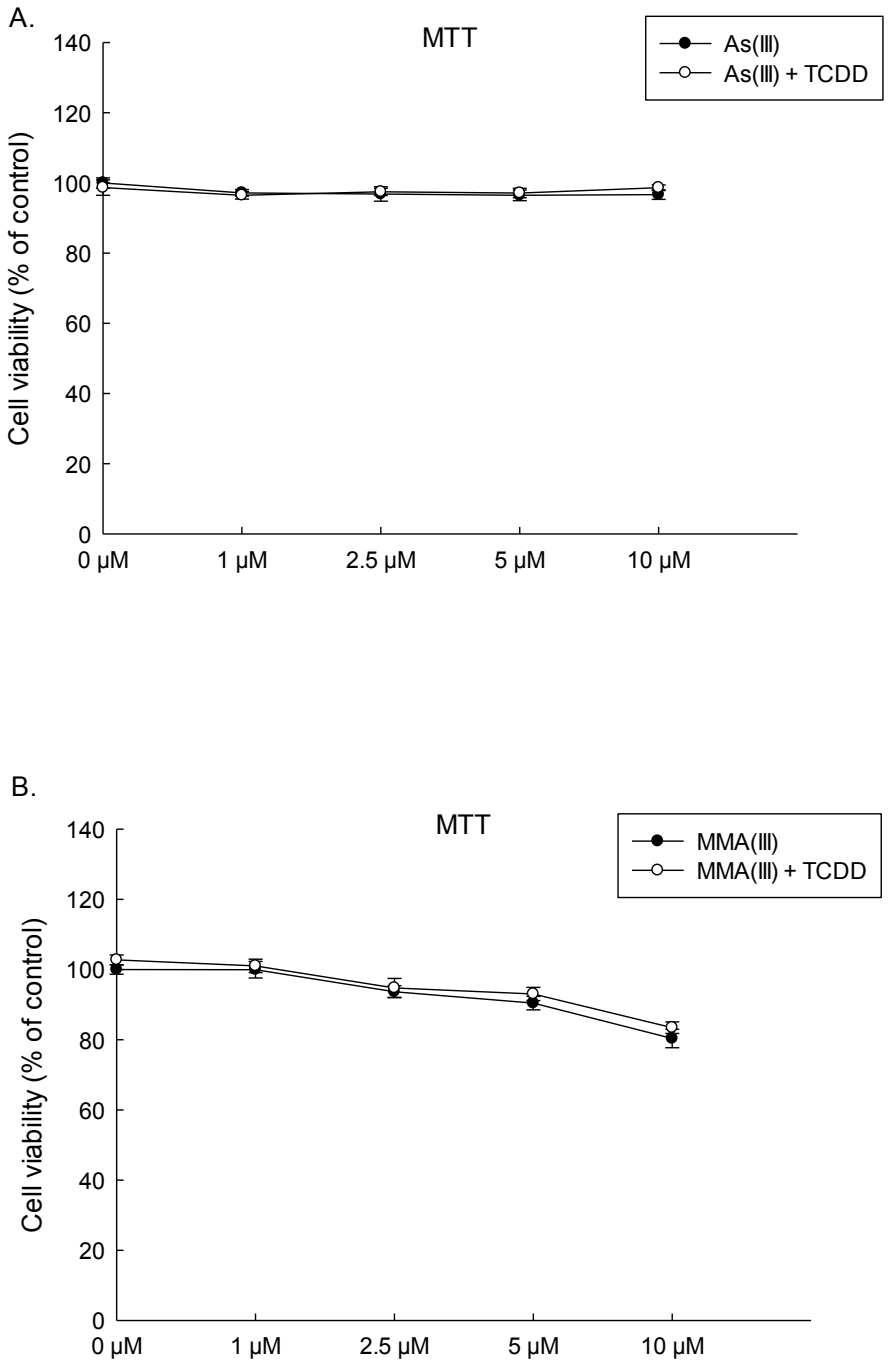


Figure 3.28 Effect of exposure to MMA(III) and its parent compound As(III), in the absence or presence of TCDD, on cell viability in HepG2 cells. HepG2 cells were treated for 24 h with As(III) (1-10 µM) (A) or MMA(III) (1-10 µM) (B) in the absence and presence of 1 nM TCDD. Cell cytotoxicity was determined using the MTT assay. Data are expressed as the percentage of untreated control (set at 100%) ± SEM (n = 8).

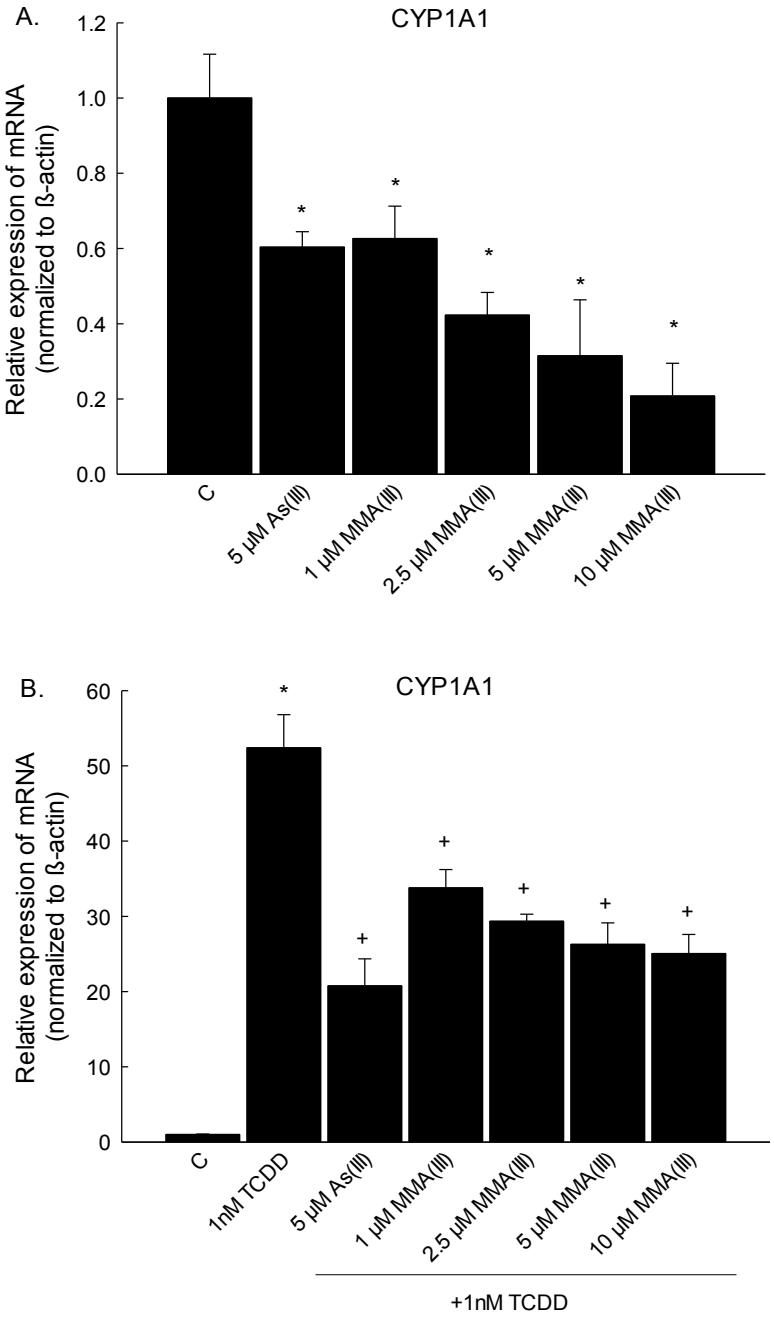


Figure 3.29 Effect of exposure to MMA(III) and its parent compound As(III), in the absence or presence of TCDD, on CYP1A1 mRNA in HepG2 cells. HepG2 cells were treated with 5 µM As(III) or increasing concentrations of MMA(III) (1-10 µM) in the absence (A) and presence (B) of 1 nM TCDD for 6 h. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using the ABI 7500 real-time PCR system as described under materials and methods. Values are presented as the mean + SEM (n=6). * $p<0.05$, compared to control (C); + $p<0.05$, compared to TCDD treatment.

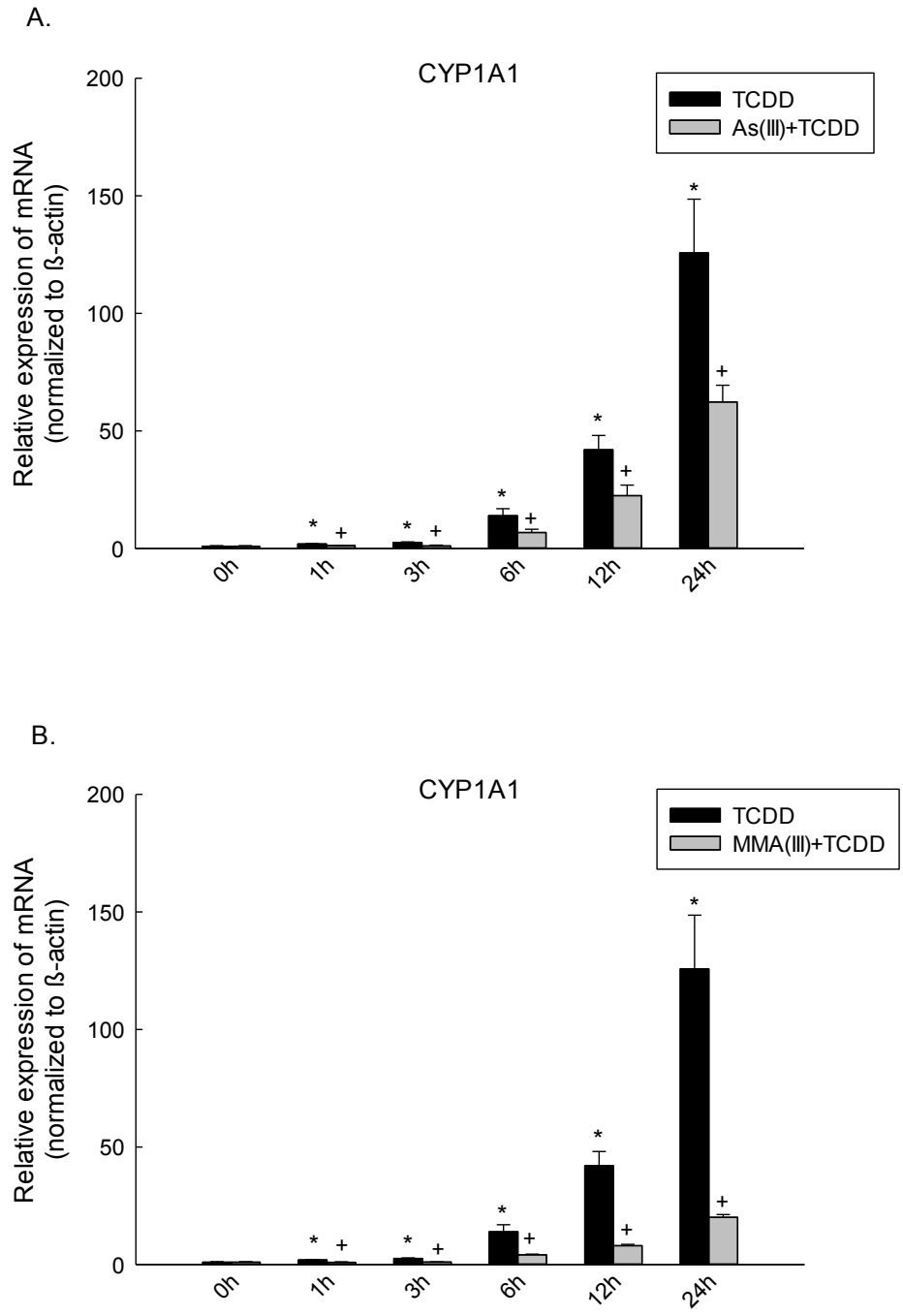


Figure 3.30 Time-dependent effect of exposure to As(III) and MMA(III) in the presence of TCDD, on CYP1A1 mRNA in HepG2 cells. HepG2 cells were treated with 1 nM TCDD in the absence and presence of 5 μM As(III) (A) or 5 μM MMA(III) (B) for various time points (0 to 24 h). First-strand cDNA was synthesized from total RNA (1.5 μg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using the ABI 7500 real-time PCR system as described under materials and methods. Values are presented as the mean + SEM (n=6). * $p<0.05$, compared to 0h treatment; + $p<0.05$, compared to TCDD treatment.

3.4.4 Effect of As(III) and MMA(III) on CYP1A1 mRNA stability

To examine the effect of As(III) and MMA(III) on CYP1A1 mRNA stability, we performed an Act-D chase experiment using intact viable HepG2 cells. The level of mRNA expression is not only a function of the transcription rate, but is also dependent on the elimination rate, through processing or degradation. If As(III) or MMA(III) decrease CYP1A1 mRNA by decreasing its stability, a decrease in half-life would be observed. All half-lives we reported were determined from goodness-of-fit criterion using log-linear graphs with regression coefficients greater than 0.8. Our results showed that CYP1A1 mRNA decayed with a half-life of 5.80 h. Furthermore, co-exposure to As(III) or MMA(III) and TCDD did not significantly alter the CYP1A1 mRNA half-life, which was 5.76 and 6.19 h, respectively (Figure 3.31). These results demonstrated that the decrease in CYP1A1 mRNA transcripts in response to As(III) or MMA(III) was not due to any increase in its degradation.

3.4.5 Effects of As(III) and MMA(III) on CYP1A protein

To investigate whether the observed reduction of CYP1A1 mRNA levels in response to As(III) and MMA(III) is reflected at the protein level, HepG2 cells were treated for 24 h with 5 μM As(III), or increasing concentrations of MMA(III) (1-10 μM), in the absence and presence of 1 nM TCDD. Our results showed that As(III) alone was able to significantly reduce CYP1A protein levels to 26% compared to control. Similarly, increasing concentrations (1, 2.5, 5, or 10 μM) of MMA(III) alone significantly reduced CYP1A protein levels to 62%, 49%, 15%, and 13%, respectively, compared to control (Figure 3.32A). On the other hand, TCDD alone significantly induced CYP1A

protein levels to 627% compared to control. When HepG2 cells were coexposed to As(III) and TCDD, there was a reduction of TCDD-mediated induction of CYP1A protein levels to 32% of that of the control. Similarly, increasing concentrations (1, 2.5, 5, or 10 μM) of MMA(IIII), reduced TCDD-mediated induction of CYP1A protein levels to 246%, 135%, 14%, and 12%, respectively, compared to control (Figure 3.32B).

3.4.6 Effect of As(III) and MMA(III) on CYP1A protein stability

To examine the effect of As(III) and MMA(III) on the CYP1A protein stability, we performed a CHX chase experiment using intact, viable HepG2 cells. The level of protein expression is not only a function of the concentration of mRNA transcripts, but is also dependent on the turnover rate of the final gene product, the CYP1A protein. If As(III) or MMA(III) decrease CYP1A protein by decreasing its stability, a decrease in its half-life would be observed. All half-lives we reported were determined from goodness-of-fit criterion using log-linear graphs with regression coefficients greater than 0.8. Our results showed that CYP1A protein decayed with a half-life of 8.91 h. Furthermore, co-exposure to As(III) and TCDD did not significantly alter protein half-life of CYP1A, which was 8.42 h. Interestingly, co-exposure to MMA(III) and TCDD significantly increased the degradation of CYP1A protein, which degraded with a half-life of 5.81 h (Figure 3.33). These results indicate that the reduction in CYP1A protein in response to MMA(III) was, in part, due to an increase in its degradation.

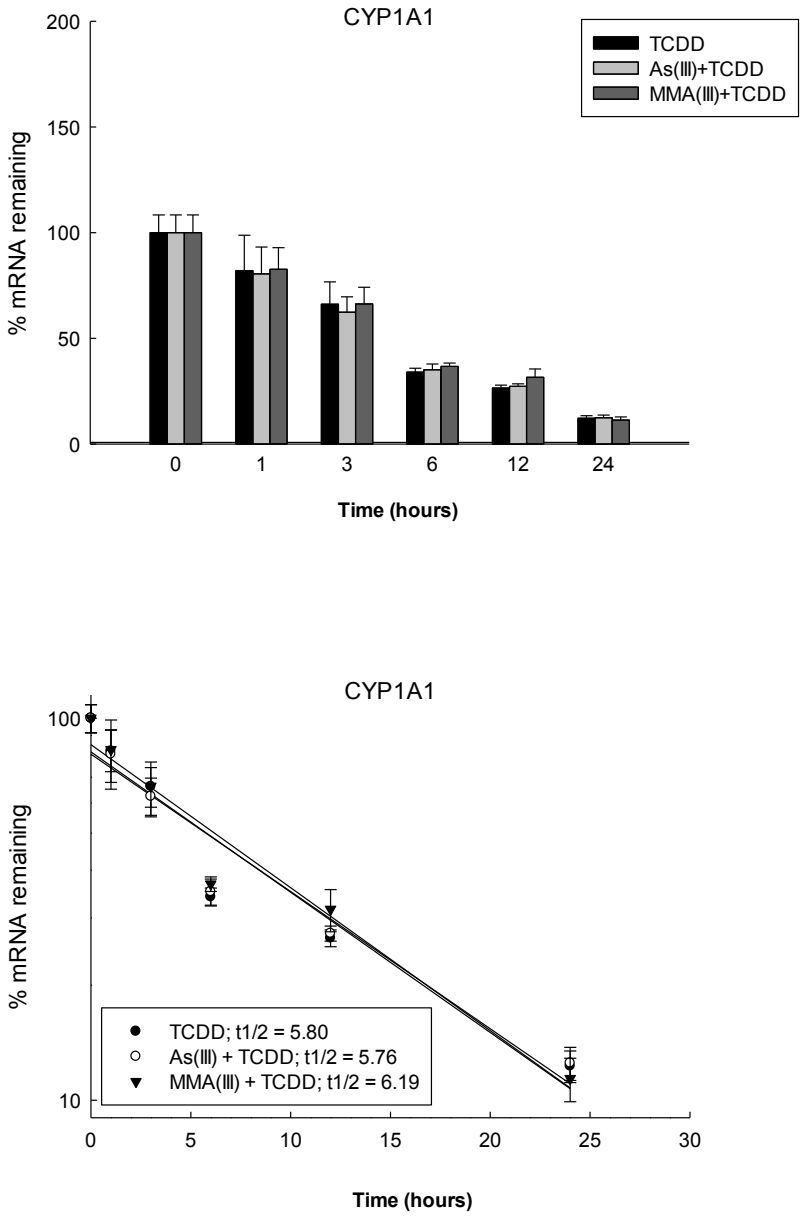


Figure 3.31 Effect of exposure to MMA(III) and its parent compound As(III) on CYP1A1 mRNA stability in HepG2 cells. HepG2 cells were treated with 1nM TCDD for 12 h. The cells were then washed and incubated in fresh medium containing 5 µg/mL Act-D, a RNA synthesis inhibitor, alone or in addition to 5 µM As(III) or 5 µM MMA(III) for various time points (0 to 24 h). First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using the ABI 7500 real-time PCR system as described under materials and methods. The half-life was estimated from the slope of a straight line fitted by exponential regression to a semilog plot of mRNA amount, expressed as a percentage of treatment at time 0 h level (maximum, 100%), versus time. Values are presented as the mean + SEM (n=6). TCDD: $y = -0.0367x + 1.9117$, $R^2 = 0.9321$; As(III): $y = -0.0359x + 1.9056$, $R^2 = 0.9379$; MMA(III): $y = -0.0374x + 1.9305$, $R^2 = 0.957$.

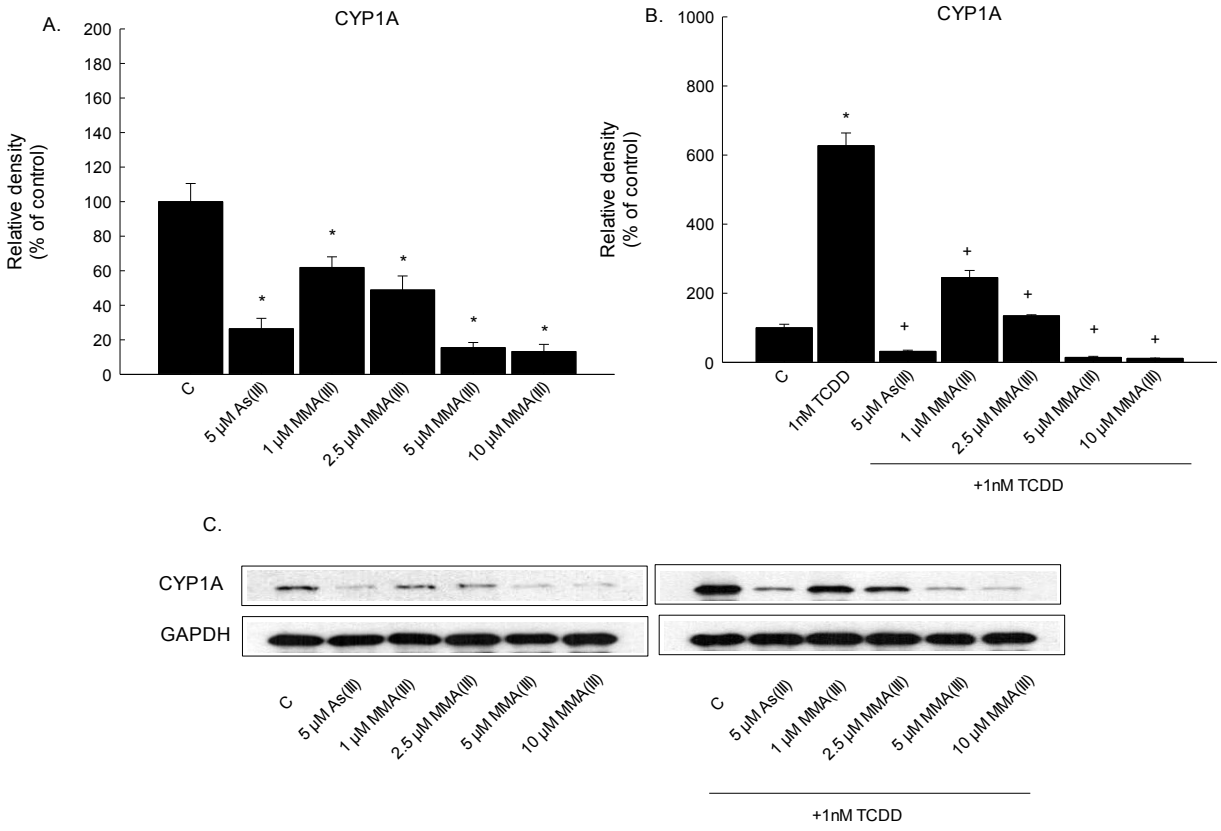


Figure 3.32 Effect of exposure to MMA(III) and its parent compound As(III), in the absence or presence of TCDD, on CYP1A protein in HepG2 cells. HepG2 cells were treated with 5 μM As(III) or increasing concentrations of MMA(III) (1-10 μM) in the absence (A) and presence (B) of 1 nM TCDD for 24 h. Twenty micrograms of total cell lysate were separated by 10% SDS–PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4 °C and then incubated with a primary CYP1A antibody for 2 h at 4 °C, followed by 1 h incubation with the secondary antibody at room temperature. CYP1A protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to GAPDH signals, which were used as a loading control. Values are presented as the mean + SEM (n=3). * $p<0.05$, compared to control (C); + $p<0.05$, compared to TCDD treatment.

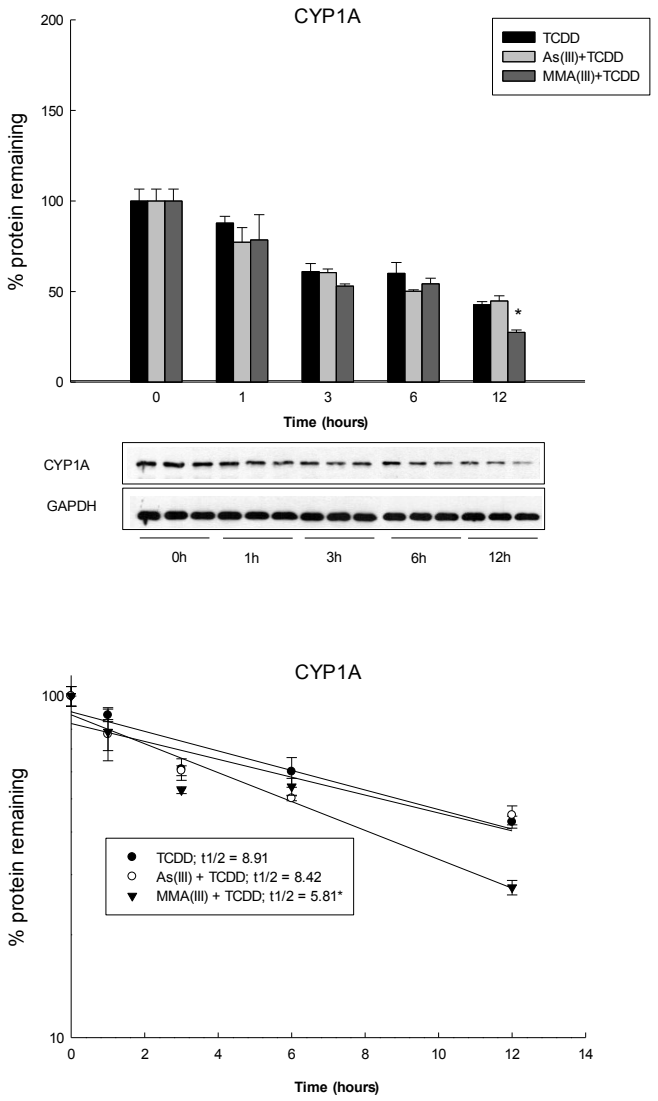


Figure 3.33 Effect of exposure to MMA(III) and its parent compound As(III) on CYP1A1 protein stability in HepG2 cells. HepG2 cells were treated with 1nM TCDD for 24 h. The cells were then washed and incubated in fresh medium containing 10 μg/mL CHX, a protein translation inhibitor, alone or in addition to 5 μM As(III) or 5 μM MMA(III) for various time points (0 to 12 h). Twenty micrograms of total cell lysate were separated by 10% SDS–PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4 °C and then incubated with a primary CYP1A antibody for 2 h at 4 °C, followed by 1 h incubation with the secondary antibody at room temperature. CYP1A protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to GAPDH signals, which were used as a loading control. The half-life was estimated from the slope of a straight line fitted by exponential regression to a semilog plot of protein amount, expressed as a percentage of treatment at time = 0 h level (maximum, 100%), versus time. Values are presented as the mean + SEM (n=3). * $p<0.05$, compared to TCDD treatment. TCDD: $y = -0.0286x + 1.9538$, $R^2 = 0.8877$; As(III): $y = -0.0262x + 1.9196$, $R^2 = 0.8$; MMA(III): $y = -0.0423x + 1.9449$, $R^2 = 0.9237$.

3.4.7 Effects of As(III) and MMA(III) on CYP1A1 catalytic activity

To investigate whether the observed reduction of CYP1A1 mRNA and protein levels in response to As(III) and MMA(III) is further reflected by a reduction in CYP1A1 enzymatic activity, HepG2 cells were treated for 24 h with 5 μM As(III), or increasing concentrations of MMA(III) (1-10 μM), in the absence and presence of 1 nM TCDD, and EROD activity was determined. Our results showed that As(III) alone significantly decreased CYP1A1 catalytic activity to 61%, compared to control. Similarly, increasing concentrations (1, 2.5, 5, or 10 μM) of MMA(III) alone resulted in a significant concentration-dependent decrease in CYP1A1 catalytic activity to 85%, 78%, 64%, and 54%, respectively, compared to control (Figure 3.34A). While TCDD alone significantly induced CYP1A1 catalytic activity to 1315% compared to control, co-exposure of HepG2 cells to TCDD and As(III) resulted in a reduction in the TCDD-induced EROD activity to only 282% of control. Increasing concentrations (1, 2.5, 5, or 10 μM) of MMA(IIII), reduced TCDD-mediated induction of CYP1A1 catalytic activity to 1074%, 875%, 380%, and 97%, respectively, compared to control (Figure 3.34B).

3.4.8 Direct effect of As(III) and MMA(III) on CYP1A1 catalytic activity

To investigate whether the observed reduction in CYP1A1 activity was due to a direct inhibitory interaction between As(III) or MMA(III) and CYP1A1 enzyme, the effect of As(III) and MMA(III) on EROD activity were determined. HepG2 cells were treated for 24 h with 1 nM TCDD, after which the cells were washed and incubated for 1 h in fresh medium containing 5 μM As(III), increasing concentrations of MMA(III) (1-10 μM), or 5 μM (E)-3,4',5-trimethoxystilbene

(TMS), included as a positive control, and EROD activity was measured. While TMS significantly reduced EROD activity to 66%, compared to control, neither As(III) nor MMA(III) in concentrations up to 5 μM reduced EROD activity. Interestingly, 10 μM MMA(III) significantly reduced EROD activity to 67%, compared to control (Figure 3.35).

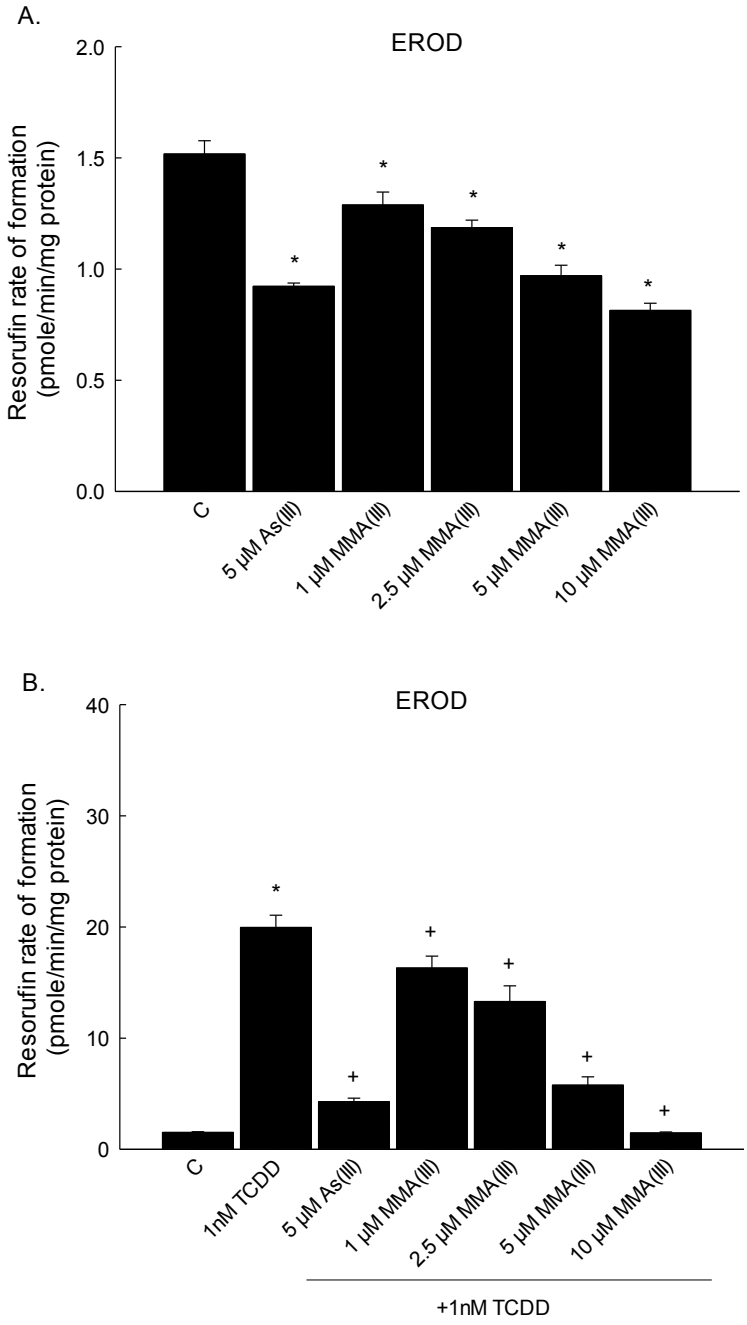


Figure 3.34 Effect of exposure to MMA(III) and its parent compound As(III), in the absence or presence of TCDD, on CYP1A1 catalytic activity levels in HepG2 cells. HepG2 cells were treated with 5 µM As(III) or increasing concentrations of MMA(III) (1-10 µM) in the absence (A) and presence (B) of 1 nM TCDD for 24 h. CYP1A1 activity was measured in intact living cells using 7-ethoxyresorufin as a substrate. Values are presented as the mean + SEM (n=8). * $p<0.05$, compared to control (C); + $p<0.05$, compared to TCDD treatment.

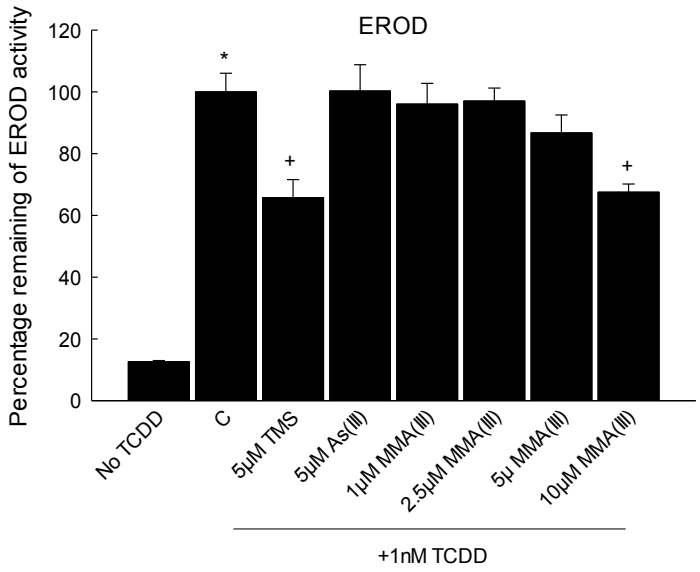


Figure 3.35 Direct inhibitory effect of MMA(III) and its parent compound As(III), on EROD activity in HepG2 cells. HepG2 cells were treated with 1nM TCDD for 24 h. The cells were then washed and incubated in fresh medium containing 5 μM As(III) or increasing concentrations of MMA(III) (1-10 μM) for 1h. (E)-3,4',5-Trimethoxystilbene (TMS) was used as a positive control. EROD activity was measured in intact living cells using 7-ethoxyresorufin as a substrate. Values are presented as the mean + SEM (n=8). * $p<0.05$, compared to control (C); + $p<0.05$, compared to TCDD treatment.

3.4.9 Effects of As(III) and MMA(III) on XRE-driven luciferase reporter activity

To determine if the observed effects of As(III) and MMA(III) occur through an AhR-dependent mechanism, HepG2 cells were transiently cotransfected with the XRE-driven luciferase reporter plasmid and its normalizing control construct, Renilla luciferase. Thereafter the cells were treated with 5 μM As(III), or MMA(III) in the absence and presence of 1 nM TCDD for 24 h. As(III) and MMA(III) alone significantly reduced background luciferase activity to 62%, and 61%, respectively, compared to control, and significantly reduced TCDD-mediated induction of XRE-driven luciferase reporter activity from 6587% to 847%, and 804%, respectively, compared to control (Table 3-4).

3.4.10 Effects of exposure to As(III) and MMA(III) on AhR protein localization

To further examine the ability of As(III) and MMA(III) to reduce CYP1A1 induction through AhR-dependent mechanisms, we examined the effect of As(III) and MMA(III) on subcellular location of AhR using immunocytochemical analysis. HepG2 cells were treated with 5 μM As(III) or MMA(III) in the absence and presence of 1 nM TCDD for 1 h followed by fixation and staining. The results show that AhR is mainly localized in the cytosol in vehicle-, As(III)-, and MMA(III)-treated cells. In contrast, TCDD significantly increased nuclear localization of the AhR to 766% compared to control. On the other hand, As(III) and MMA(III) significantly reduced TCDD stimulated AhR nuclear localization to 92% and 89%, respectively, compared to control (Figure 3.36, and Table 3-5).

Table 3-4 Effect of exposure to MMA(III) and its parent compound As(III), in the absence or presence of TCDD, on XRE-driven luciferase reporter activity in HepG2 cells.

Treatment	**Firefly Luciferase/Renilla Luciferase (%) ± SEM**
C	100.00 ± 10.11
5 μM As(III)	62.37 ± 7.80*
5 μM MMA(III)	60.66 ± 4.72*
1nM TCDD	6586.98 ± 364.08*
5 μM As(III) + 1nM TCDD	847.17 ± 235.75+
5 μM MMA(III) + 1nM TCDD	803.67 ± 222.11+

HepG2 cells were transiently transfected with the XRE-luciferase reporter plasmid pGudLuc6.1 and Renilla luciferase plasmid pRL-CMV for 6h, and thereafter the cells were treated with 5 μM As(III), or MMA(III) in the absence and presence of 1 nM TCDD for 24 h. Cells were lysed and luciferase activity was measured using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions. Luciferase activity is reported as relative light units. Values are presented as the mean ± SEM (n=6). *$p<0.05$, compared to control; +$p<0.05$, compared to TCDD treatment.

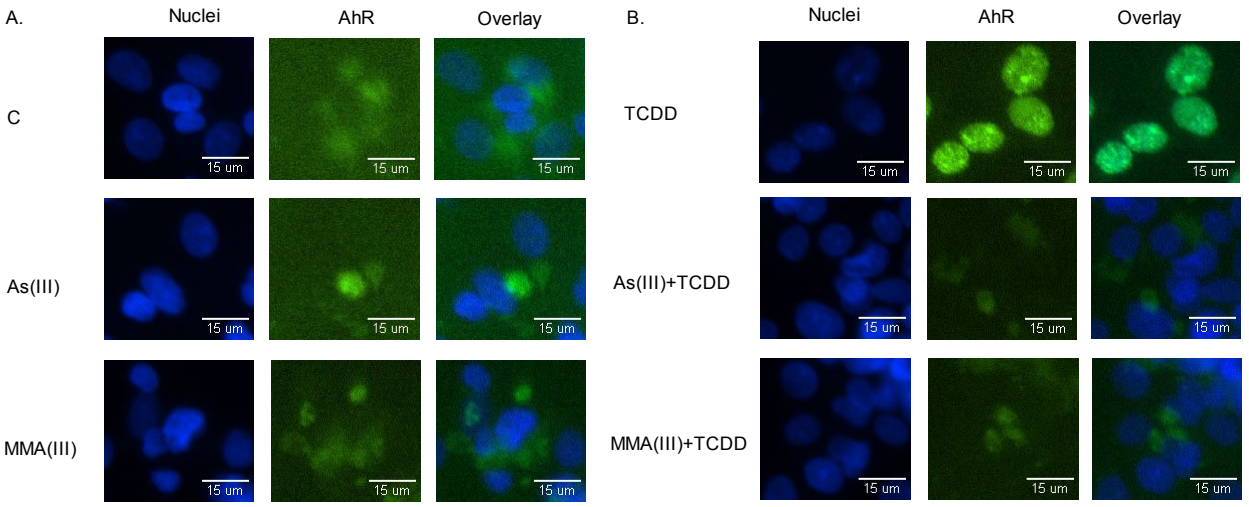


Figure 3.36 Effects of exposure to MMA(III) and its parent compound As(III) on subcellular localization of AhR, in the absence or presence of TCDD, in HepG2 cells. HepG2 cells were plated on collagen-coated glass coverslips and treated with 5 μM As(III) or MMA(III) in the absence (A) and presence (B) of 1 nM TCDD for 1 h. Thereafter, the cells were fixed and stained, as described under materials and methods, with anti-AhR antibody, Alexa Fluor 488 (green), and DAPI (blue). Images were obtained using confocal microscopy with a 20x/0.45 Ph1 lens.

Table 3-5 Effects of exposure to MMA(III) and its parent compound As(III) on nuclear localization of AhR, in the absence or presence of TCDD, in HepG2 cells.

Treatment	**Nuclear fluorescence/Cytosolic fluorescence (%) ± SEM**
C	100 ± 8.50
5 μM As(III)	86.76 ± 15.60
5 μM MMA(III)	91.14 ± 17.18
1nM TCDD	766.07 ± 16.11*
5 μM As(III) + 1nM TCDD	92.03 ± 8.63^{+}
5 μM MMA(III) + 1nM TCDD	89.21 ± 8.43^{+}

HepG2 cells were treated with 5 μM As(III) or MMA(III) in the absence and presence of 1 nM TCDD for 1 h. Thereafter, the cells were fixed and stained. Images were obtained using a confocal microscopy. Thereafter, the ratio of nuclear over cytosolic fluorescence of AhR was calculated using the ImageJ software. Values are presented as the mean ± SEM. *p<0.05, compared to control; +p<0.05, compared to TCDD treatment.

3.4.11 Reduction of CYP1A1 by As(III) and MMA(III) is through a ligand-independent mechanism

The ability of As(III) and MMA(III) to decrease both AhR- and TCDD-dependent induction of CYP1A1 prompted us to examine these compounds as potential AhR antagonists. To establish whether As(III) or MMA(III) are AhR ligands, a [^{3}H]TCDD competition binding assay was performed in two different species, using guinea pig liver cytosol and mouse liver cytosol. Total specific binding of [^{3}H]TCDD to the AhR was defined as the amount of [^{3}H]TCDD competitively displaced by a 100-fold molar excess of TCDF, another high affinity AhR ligand/agonist. The addition of 5 or 50 μM As(III) or MMA(III) to the [^{3}H]TCDD binding reactions did not reduce [^{3}H]TCDD binding to the AhR in guinea pig liver cytosol (Figure 3.37A) or mouse liver cytosol (Figure 3.37B). Thus, the reducing effects of As(III) and MMA(III) on the AhR and AhR nuclear localization likely occur through a ligand-independent mechanism.

3.4.12 Effects of As(III) and MMA(III) on HO-1 mRNA

To confirm the role of HO-1 in the down-regulation of CYP1A1, we examined the effect of exposure to As(III) and MMA(III) in the absence and presence of TCDD on HO-1 mRNA. HepG2 cells were treated with 5 μM As(III), or increasing concentrations of MMA(III) (1-10 μM), in the absence and presence of 1 nM TCDD (Figure 3.38A, and B). Thereafter, HO-1 mRNA was assessed using real-time PCR. As(III) alone significantly induced HO-1 mRNA to 1614% compared to control, while increasing concentrations of MMA(III) had no effect on HO-1 mRNA expression (Figure 3.38A). TCDD alone had no effect on HO-1 mRNA expression, while As(III)

in the presence of TCDD significantly induced HO-1 mRNA to 1741% compared to control. Increasing concentrations of MMA(III) in the presence of TCDD had no effect on HO-1 mRNA expression (Figure 3.38B).

3.4.13 Effects of As(III) and MMA(III) on total ROS formation

To verify the role of oxidative stress in the modulation of CYP1A1, we investigated the capacity of As(III) and MMA(III) to induce intracellular ROS in the HepG2 cells based on quantification of the oxidation-activated fluorescent dye, DCFH-DA. HepG2 cells were treated with 5 μM As(III) or MMA(III) in the absence and presence of 1 nM TCDD for 24 h and the oxidation of DCFH-DA to its fluorescent product, DCF, was measured. Our results showed that As(III), MMA (III) and TCDD increased DCF fluorescence to 113%, 126%, 124%, respectively, compared to control fluorescence. Co-exposure to As(III) and TCDD did not significantly alter DCF fluorescence compared to As(III) or TCDD alone. Interestingly, co-exposure to MMA(III) and TCDD resulted in a DCF fluorescence greater than MMA(III) or TCDD alone (136% compared to control) (Figure 3.39A).

3.4.14 Effects of As(III) and MMA(III) on intracellular superoxide radical anion formation

The capacity of As(III) and MMA(III) to induce superoxide radical anion formation in HepG2 cells was investigated using a DHE probe. HepG2 cells were treated with 5 μM As(III) or MMA(III) in the absence and presence of 20 μM menadione, a superoxide radical generator, for 24 h and the fluorescence intensity was measured. Our results showed that while As(III) and MMA

(III) did not significantly alter the superoxide radical levels, menadione significantly increased superoxide radicals to 125% compared to control (Figure 3.39B). Co-exposure to As(III) and menadione did not significantly alter superoxide radical generation greater than menadione alone. Interestingly, co-exposure to MMA(III) and menadione significantly increased superoxide radical generation to a level greater than MMA(III) or menadione alone (149% compared to control) (Figure 3.39B).

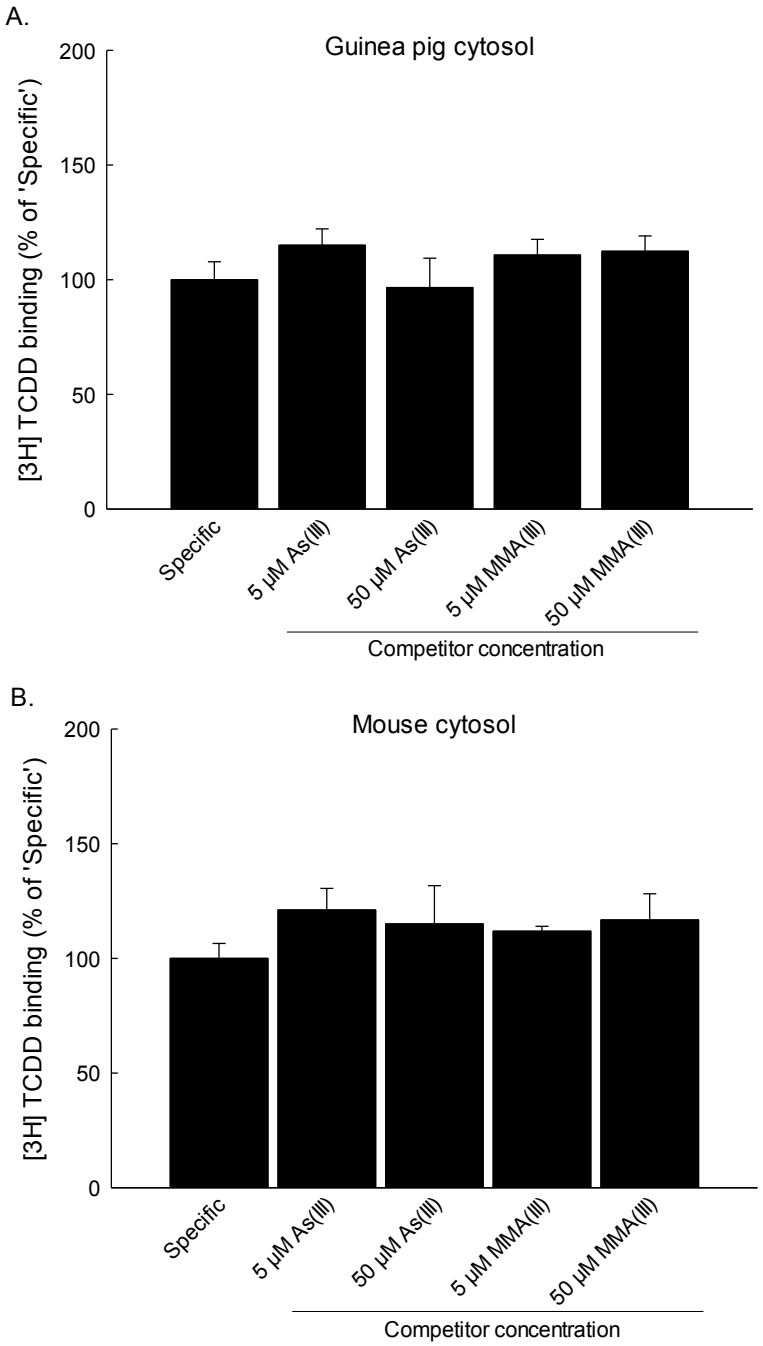


Figure 3.37 Effect of MMA(III) and its parent compound As(III), on AhR-ligand binding assay in guinea pig liver cytosol and mouse liver cytosol. Untreated guinea pig (A) or mouse (B) hepatic cytosol (2 mg/mL) was incubated with 2 nM [^{3}H]TCDD alone (total binding), 2 nM [^{3}H]TCDD and 200 nM TCDF (100-fold excess of competitor), or 2 nM [^{3}H]TCDD in the presence of 5 or 50 µM As(III) or MMA(III) and the samples were analyzed by the HAP assay as described under materials and methods. Values were expressed as % specific binding. Values are presented as the mean + SEM (n=3).

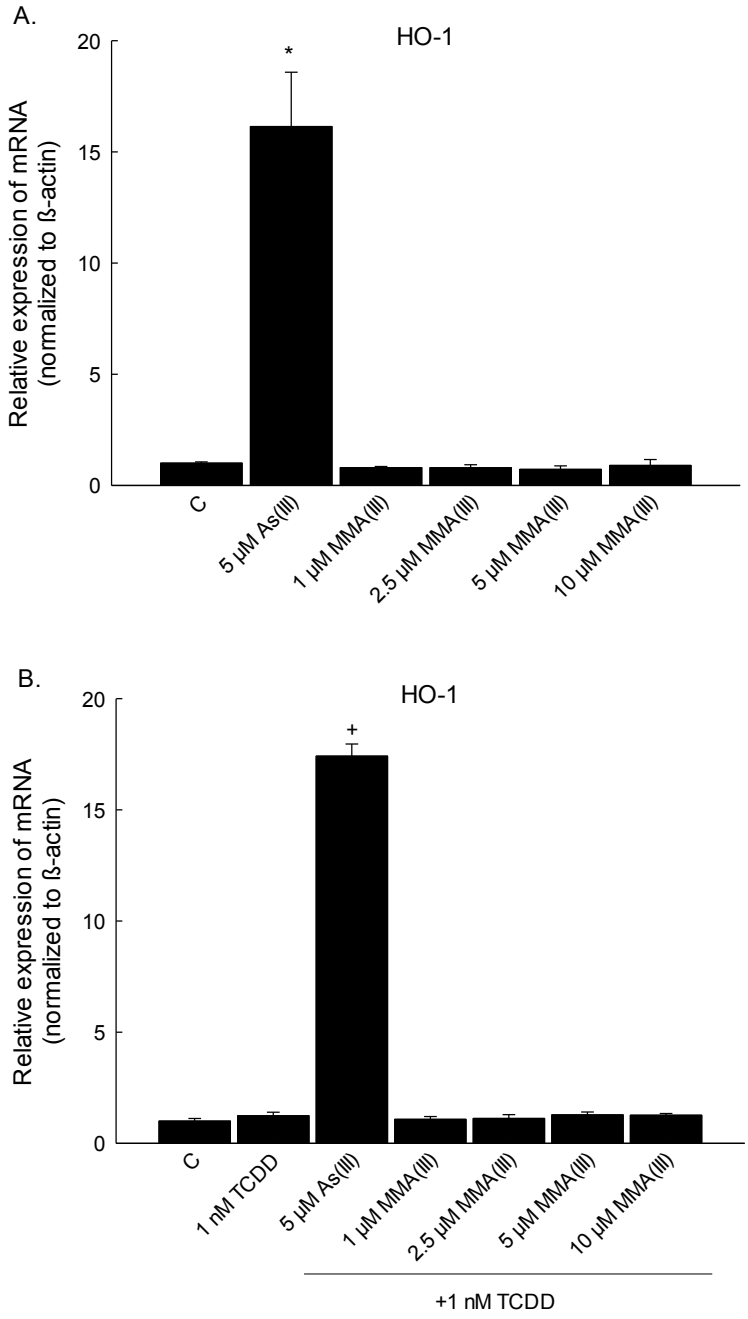


Figure 3.38 Effect of exposure to MMA(III) and its parent compound As(III), in the absence or presence of TCDD, on HO-1 mRNA in HepG2 cells. HepG2 cells were treated with 5 µM As(III) or increasing concentrations of MMA(III) (1-10 µM) in the absence (A) and presence (B) of 1 nM TCDD for 6 h. First-strand cDNA was synthesized from total RNA (1.5 µg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using the ABI 7500 real-time PCR system as described under materials and methods. Values are presented as the mean + SEM (n=6). * $p<0.05$, compared to control (C); + $p<0.05$, compared to TCDD treatment.

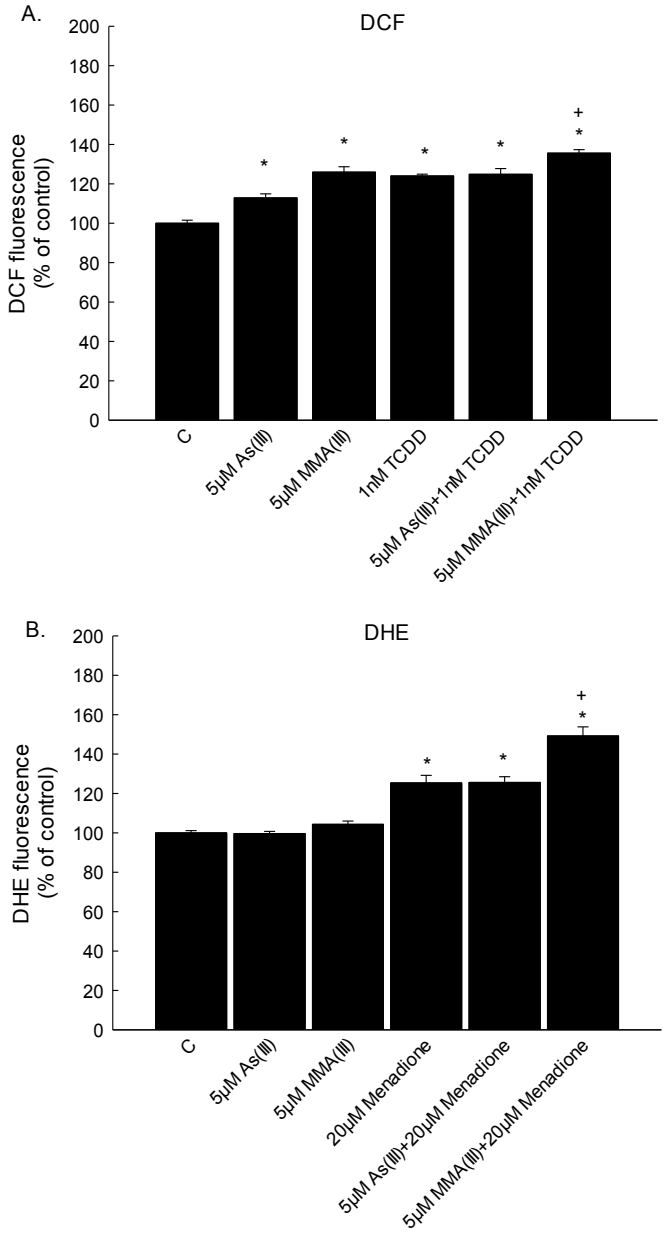


Figure 3.39 Effect of exposure to MMA(III) and its parent compound As(III), on production of total ROS and superoxide radical anion ($O_2^{\bullet-}$) in HepG2 cells. (A) HepG2 cells were treated with 5 μM As(III) or MMA(III) in the absence and presence of 1 nM TCDD for 24 h and total ROS generation was measured using DCFH-DA. Fluorescence was then measured using excitation and emission wavelengths of 485 and 535 nm, respectively. (B) HepG2 cells were treated with 5 μM As(III) or MMA(III) in the absence and presence of 20 μM menadione for 24 h and superoxide radical anion generation was measured using DHE. Fluorescence was then measured using excitation and emission wavelengths of 535 and 585 nm, respectively. Values are presented as the mean + SEM (n=8). * $p<0.05$, compared to control (C); + $p<0.05$, compared to TCDD or menadione treatment.

4 CHAPTER FOUR - DISCUSSION

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4.1 Modulation of AhR-regulated enzymes by trimethylarsine oxide (TMAO) in C57BL/6 mice: *in vivo* and *in vitro* studies.

Despite extensive research on the toxicity of arsenic, many questions remain unanswered, making risk assessment of different arsenicals of great importance (Shen *et al.*, 2003). Although pentavalent arsenicals were found to be less cytotoxic than their trivalent analogs, investigations found that pentavalent metabolites of inorganic arsenic are multiorgan tumor promoters (Hughes, 2009). Although limited work has been conducted on TMAO, some studies reported the toxicity of TMAO in both animal and human models. For example, TMAO was reported to induce a dose-dependent increase in hepatocellular adenoma and multiplicity in rats, with a significant increase in 8-hydroxydeoxyguanosine (8-OHdG), a known promutagenic DNA lesion (Shen *et al.*, 2003; Liu and Waalkes, 2008; IARC, 2012). The potential modes of action for TMAO-induced tumorigenesis involves initiation and promotion stages. In the initiation stage, TMAO exerts multiple effects by direct attack of ROS, leading to oxidative DNA damage and subsequent mutations. Then, in the promotion stage, TMAO acts via increased cell proliferation in the liver (Shen *et al.*, 2003). As for the ROS formation, it was previously reported that methylated arsenic species can release redox-active iron from ferritin. Free iron is known to play a central role in generating harmful oxygen species by promoting the conversion of superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) into the highly reactive hydroxyl ($^{\bullet}OH$) radical through the Haber–Weiss reaction (Flora, 2011). Another study also examined the liver tumor promoting effects of TMAO in rats after carcinogenic initiation with combined diethylnitrosamine and partial hepatectomy. The number and area of hepatic glutathione S-transferase-pi (GST-pi) positive foci, indicative of early premalignant lesions, were significantly increased following TMAO treatment

(Nishikawa *et al.*, 2002; Liu and Waalkes, 2008). Moreover, a previous study reported that TMAO could induce chromosomal aberrations and disruption of centromeres in human fibloblasts (Oya-Ohta *et al.*, 1996). Although, Marafante et al. had reported that only 3.5% - 6.4% of the DMA dose is excreted in urine as TMAO (Marafante *et al.*, 1987), this small amount of TMAO could possibly participate, at least in part, in the toxicity of arsenic. Further studies on the acute and chronic exposure to TMAO and risk assessment are required. Recently, clinical studies measuring environmental chemical concentrations in urine samples from a civilian, non-institutionalized US population found that TMAO concentrations are related to an increased risk of high blood pressure (Shiue, 2014). Another recent clinical study showed that people with abnormal occiput-to-wall distance, a surrogate marker of kyphosis, were found to have higher urinary TMAO concentrations (Shiue, 2015).

Arsenic is a known modulator of drug-metabolizing enzymes such as Cyp, which are known to be involved in the oxidative metabolism and elimination of toxic chemicals. Arsenic-induced modulation of Cyp is believed to be a key player in arsenic-mediated toxicities (Vernhet *et al.*, 2003; Noreault *et al.*, 2005b; Anwar-Mohamed *et al.*, 2013b; Anwar-Mohamed *et al.*, 2014a). Recently it has been reported that TMAO increases CYP1A1 in human hepatoma, HepG2, cells (Anwar-Mohamed *et al.*, 2014b). However, further studies are required to determine whether TMAO has the same effect *in vivo* and *in vitro*, in a primary cell model. In this study we hypothesized that TMAO activates an AhR signaling pathway with resultant activation of its target genes *in vivo* and in primary hepatocytes. Thus the objectives of this study were: (i) to examine the effects of TMAO on the constitutive and inducible expression levels of phase I AhR-regulated genes (Cyp1a1, Cyp1a2, and Cyp1b1) in C57BL/6 mice; (ii) to examine the effects of TMAO on

the constitutive and inducible expression levels of phase II AhR-regulated genes (Nqo1 and Gsta1) in C57BL/6 mice; (iii) to examine the effects of TMAO on the constitutive and inducible expression levels of the prototypical AhR target gene, Cyp1a1, in isolated primary hepatocytes from C57BL/6 mice; and (iv) to explore the underlying mechanisms involved in this modulation. The doses and concentrations of TMAO and TCDD were chosen based on previous studies (Yamauchi *et al.*, 1989; Kimura *et al.*, 2006; Elbekai and El-Kadi, 2007; Anwar-Mohamed *et al.*, 2013a; Anwar-Mohamed *et al.*, 2014b). In addition, our cell viability results showed that both TMAO and TCDD do not pose any cytotoxicity to isolated hepatocytes at the concentrations tested.

We have demonstrated that TMAO alone could modulate phase I and phase II AhR-regulated genes through AhR-dependent mechanisms. Regarding AhR-regulated phase I enzymes, TMAO induced liver Cyp1a1, Cyp1a2, and Cyp1b1 at the mRNA, and induced Cyp1a1, and Cyp1a2 at the protein and catalytic activity levels. Furthermore, TMAO potentiated the TCDD-mediated induction of liver Cyp1a1 and Cyp1b1 mRNA and protein levels, with a concomitant potentiation in the EROD catalytic activity levels (Figure 3.1 - Figure 3.3). Several factors could explain the observed disparity between mRNA and protein levels in the *in vivo* experiments. First, the measurement time of mRNA and protein could cause a discrepancy, as mRNA was measured 6 h after treatment, whereas, protein levels and activity were determined 24 h after treatment. Second, RNA and protein stability play a crucial role in determining the levels of mRNA and protein. Third, post-transcriptional and post-translational modifications in addition to possible microRNA regulatory effects on gene expression (Althurwi *et al.*, 2014). Our results showed that TCDD can induce Cyp1a1 mRNA to a level that is greatly higher than levels associated with TCDD-mediated

induction of Cyp1a2 and Cyp1b1 mRNA, and these results are in agreement with previous reports (Abdelhamid *et al.*, 2013; Amara *et al.*, 2013; Anwar-Mohamed *et al.*, 2013a). It is well known that the magnitude of induction of individual Cyp1 isoenzymes is different from each other due to complex transcriptional regulation of these enzymes, however the exact mechanisms involved need to be further investigated (Zhu, 2010).

Although this is the first report that examined the effect of TMAO on the constitutive and TCDD-inducible liver Cyp1 mRNA, protein expression, and catalytic activities, previous reports that utilized As(III) showed results supporting our findings. For example, a recent study showed that As(III) inhibits the TCDD-mediated induction of liver Cyp1a1 mRNA levels at 6 h while significantly potentiating the TCDD-mediated induction of Cyp1a1 mRNA levels at 24 h. One of the main factors that may contribute to the opposing effects could be the biomethylation of arsenic (Anwar-Mohamed *et al.*, 2013a). This means that the effects observed at 6 h are due to the direct effect of non-metabolized As(III), whereas the effects observed after 24 h are indirect effects that could be attributed to the metabolism of As(III) in the liver to its methylated derivatives. The fact that Cyp1a1 protein was induced to levels higher than that observed with EROD activity could be attributed to the induction of Ho-1, which could play an important role in the down regulation of Cyp1a1 activity through heme destruction and enzyme inactivation (Elbekai and El-Kadi, 2007; Anwar-Mohamed and El-Kadi, 2010).

AhR-regulated Cyp enzymes play a crucial role in metabolic activation of chemical carcinogens to highly reactive products, resulting in deleterious effects on human health. Such reactive metabolites cause carcinogenicity in experimental animals and humans, whereas their

corresponding parent compounds are chemically inactive (Androutsopoulos *et al.*, 2009). Reports have shown that substrate specificities of Cyp1a1 and Cyp1b1 towards various procarcinogens and promutagens are very similar (Androutsopoulos *et al.*, 2009). Interestingly, Cyp1a1 and Cyp1a2 have very different basal regulation, but they share induction via AhR, similar to Cyp1b1 (Rodriguez-Antona and Ingelman-Sundberg, 2006), whereas Cyp1b1 expression is subject to hormonal control, with multiple hormones playing a role in addition to AhR-mediated regulation (Leung *et al.*, 2009; Anwar-Mohamed *et al.*, 2013b).

Cyp1a1 metabolizes carcinogens to epoxide intermediates, which are further activated to diol epoxides by the enzyme epoxide hydrolase. This is the process involved in the activation of the carcinogen BaP. The carcinogenic potential of Cyp1a1 in the activation of PAH has been well documented both *in vitro* and *in vivo* (Androutsopoulos *et al.*, 2009). In addition, clinical trials in cigarette smokers, have established a correlation between the high Cyp1 inducibility phenotype and cancer of the lung, larynx, and oral cavity (Nebert *et al.*, 2004). Moreover, Cyp1a1 and Cyp1a2 metabolize estradiol to the 4-hydroxy estradiol in human liver, which is believed to be an important mediator for mammary carcinogenesis (Gao *et al.*, 2004). With regard to Cyp1b1, it has been identified to be highly expressed in a wide range of malignant tumors of different histogenetic types, including cancers of the breast, colon, lung, esophagus, skin, lymph node, brain, testis, prostate, kidney, and ovaries. It also was found to be highly expressed in metastatic disease (Murray *et al.*, 1997; McFadyen *et al.*, 2004b; Rodriguez-Antona and Ingelman-Sundberg, 2006). Cyp1b1 metabolizes steroid hormones and may play a role in susceptibility to hormone-dependent cancers such as those in the breast and prostate (Rodriguez-Antona and Ingelman-Sundberg, 2006). In addition, an accumulating body of data showed the differential expression of Cyp

enzymes within the tumor microenvironment compared with the surrounding normal tissue, which is considered a part of a pleiotropic response to tumor development (McFadyen *et al.*, 2004b).

With regard to AhR-regulated phase II enzymes, TMAO alone significantly induced Nqo1 and Gsta1 mRNA, protein expression, and catalytic activities in the liver (Figure 3.4 - Figure 3.6). Our results are in line with previous studies that showed that arsenic compounds could induce Nqo1 and Gsta1 in the murine Hepa-1c1c7 cell line and in human hepatocytes, in addition to C57BL/6 mice (Elbekai and El-Kadi, 2004; Elbekai and El-Kadi, 2008; Anwar-Mohamed *et al.*, 2012; Liu *et al.*, 2013). Interestingly, animals co-exposed to TMAO and TCDD had lower Nqo1 protein levels compared to TCDD alone. Several processes could be responsible for this discrepancy, such as RNA and protein stability, post-transcriptional and post-translational modifications, and microRNA (Althurwi *et al.*, 2014). Moreover, co-exposure to TMAO and TCDD could pose oxidative stress that is beyond the limits that could be scavenged through the induction of Nqo1, which could activate other redox sensitive transcription factors. These transcription factors in return might play counterbalancing effects to remove oxidative stress caused by TMAO and TCDD.

The regulation of Nqo1 and Gsta1 involves other signaling pathways, in addition to the AhR signaling pathway, such as the Nrf2 pathway (Chanas *et al.*, 2002; Yeager *et al.*, 2009). Furthermore, potential regulatory elements have been identified in the human GSTA1 promoter, including AP-1 and AP-2 consensus sequences and a glucocorticoid response element (Whalen and Boyer, 1998; Romero *et al.*, 2006). Both Nqo1 and Gsta1 are known to increase in response to oxidative stress products, and this supports the concept that cells are subjected to localized

increase in oxidative stress (Parsons *et al.*, 2001; Takakusa *et al.*, 2008). Moreover, previous reports have shown elevated Nqo1 activities in carcinomas such as liver, colon, breast, brain, and lung carcinomas (Siegel *et al.*, 1998). In the current study we also demonstrated that TMAO was able to induce Ho-1 mRNA, protein expression, and catalytic activity in the liver (Figure 3.7). Indeed, it has been reported that the induction of Ho-1 may increase the transcription of Cyp1a1 through the increasing synthesis of bilirubin, a known inducer of Cyp1a1 transcriptional activity (Elbekai and El-Kadi, 2007). Accumulating evidence showed that induction of Ho-1 is a hallmark to oxidative insult under pathologic conditions (Wondrak *et al.*, 2010). Our results are in agreement with previous studies that showed induction of Ho-1 with different forms of arsenic, indicating that oxidative stress is mediated by arsenicals (Liu *et al.*, 2001; Anwar-Mohamed *et al.*, 2013a).

To confirm the role of AhR in the induction of phase I and phase II AhR-regulated genes we have conducted *in vitro* experiments in isolated primary hepatocytes from C57BL/6 mice. We tested the effect of TMAO on the prototypical target of the AhR signaling pathway, Cyp1a1. The transcriptional regulation of Cyp1a1 mRNA expression by TMAO was also investigated through a luciferase reporter assay and immunocytochemical analysis. Our results showed that TMAO increased Cyp1a1 mRNA, protein, and catalytic activity levels in isolated hepatocytes treated with TMAO in the absence and presence of TCDD (Figure 3.9 - Figure 3.10). Moreover, TMAO alone or in the presence of TCDD was able to significantly increase the AhR-dependent XRE-driven luciferase reporter activity (Figure 3.11). In addition, TMAO was able to increase AhR nuclear accumulation as evidenced by immunocytochemical analysis of AhR localization (Figure 3.12). Other mechanisms, such as mRNA and protein stability, could still play an important role in the modulation of AhR-regulated genes. Our results are in agreement with previous reports that

showed that TMAO could activate the AhR/XRE signaling pathway in the human hepatoma HepG2 cell line (Anwar-Mohamed *et al.*, 2014b). Thus, targeting the AhR activated signaling pathway could help in fighting against arsenic-induced toxicity and carcinogenicity.

In conclusion, this study demonstrated that TMAO induces phase I and phase II AhR-regulated genes at the mRNA, protein, and activity levels in C57BL/6 mice. This activation is mediated by AhR accumulation in the nucleus and subsequent transcriptional activation. The activation of the AhR signaling pathway could potentially participate, at least in part, in arsenic-induced toxicity and carcinogenicity.

4.2 Modulation of AhR-regulated genes by acute administration of trimethylarsine oxide (TMAO) in the lung, kidney, and heart of C57BL/6 mice

There have been no previous attempts to investigate the effects of TMAO on the expression of AhR-regulated genes in the extrahepatic tissues. Thus, in this study we hypothesized that, TMAO modulates the AhR-regulated phase I and phase II metabolizing enzymes in extrahepatic tissues. Accordingly, the objectives of this study were to examine the effects of TMAO on the constitutive and inducible expression levels of Cyp1a1, Cyp1a2, Cyp1b1, Nqo1, and Gsta1 in addition to Ho-1, in extrahepatic tissues, namely, lung, kidney, and heart. The doses of TMAO and TCDD were chosen to match those of As(III)/TCDD that were used previously in C57BL/6 and BALB/c mice (Kimura *et al.*, 2006; Uno *et al.*, 2008; Kimura *et al.*, 2010; Wong *et al.*, 2010; Anwar-Mohamed *et al.*, 2012).

Our results showed that, the AhR-regulated enzymes Cyp1a1, Cyp1a2, Cyp1b1, Nqo1, and Gsta1 in addition to Ho-1 are constitutively expressed in all the examined tissues. We have demonstrated that TMAO modulates the constitutive and TCDD-inducible AhR-regulated enzymes in a tissue-, and AhR-regulated enzyme-specific manner. TMAO significantly increased the Cyp1a1 mRNA in the lung but not in the kidney or heart of C57BL/6 mice. TMAO failed to alter Cyp1a2 mRNA in all tested tissues. Furthermore TMAO significantly increased the Cyp1b1 mRNA in the lung and kidney but not in the heart. At the protein and catalytic activity levels, TMAO induced lung Cyp1a1 protein expression levels with a subsequent increase in EROD catalytic activity, while it had no effect on kidney Cyp1a1 protein expression or catalytic activity. TMAO had no effect on lung and kidney Cyp1a2 protein expression and MROD catalytic activity. Furthermore, TMAO induced lung and kidney Cyp1b1 protein expression levels (Figure 3.13 - Figure 3.15). The limitation of minimal protein quantities that could be extracted from the heart samples hindered us from measuring protein expression and catalytic activities of AhR-regulated genes in this organ. Our results are in line with previous reports which showed that, the parent compound As(III) was able to induce Cyp1a1 mRNA levels in human lung-derived cell lines, and lung tissues of arsenic-exposed mice (Wu *et al.*, 2009). Similarly and in agreement with our results, As(III) also failed to cause similar induction of Cyp1a1 mRNA levels in the kidney of C57BL/6 mice (Seubert *et al.*, 2002).

TMAO significantly potentiated the TCDD-mediated induction of Cyp1a1 mRNA in the lung, kidney, and heart. TMAO significantly potentiated the TCDD-mediated induction of Cyp1a2 mRNA in the lung but not in the kidney or heart. Furthermore TMAO potentiated the TCDD-mediated induction of Cyp1b1 mRNA in the kidney and heart but not in the lung. At the protein

and catalytic activity levels, TMAO significantly potentiated the TCDD-mediated induction of Cyp1a1 protein in the lung and kidney with a subsequent increase in EROD catalytic activity. TMAO significantly potentiated the TCDD-mediated induction of lung Cyp1a2 protein expression levels with a subsequent increase in MROD catalytic activity, while it had no effect on TCDD-mediated induction of kidney Cyp1a2 protein expression or catalytic activity. TMAO induced TCDD-mediated induction of Cyp1b1 protein expression in the kidney but not in the lung. Previous reports have demonstrated similar results with As(III), which potentiates the TCDD-mediated induction of Cyp1a1 mRNA and protein expression levels in the mouse liver-derived cell line, Hepa-1c1c7 (Elbekai and El-Kadi, 2007). Furthermore, As(III) was reported to potentiate the TCDD-mediated induction of Cyp1a1 and Cyp1a2 in the lung of mice co-exposed to arsenic and TCDD on the level of mRNA and protein expression, in addition to catalytic activity levels (Anwar-Mohamed *et al.*, 2012).

The Cyp1 family was one of the first Cyp families to be identified and subsequently purified; Cyp1 is probably the most inducible family in the liver and extrahepatic tissues of animals and humans (Ioannides and Lewis, 2004). Although Cyp1a1 and Cyp1a2 have very different basal regulation, they share induction via the AhR, similar to Cyp1b1 (Rodriguez-Antona and Ingelman-Sundberg, 2006). Of interest is that Cyp1b1 expression is subjected to hormonal control with multiple hormones playing a role, in addition to AhR-mediated regulation (Leung *et al.*, 2009; Anwar-Mohamed *et al.*, 2013b). Cyp1 family members were shown to be present in the human lung and lung-derived cell lines and they contribute to *in situ* activation of pulmonary procarcinogens (Raunio *et al.*, 1999). Clear positive relationships have been established between lung cancer and Cyp1a1 in the lung of smokers, where those who develop tumors display high Cyp1a1 activity

(McLemore *et al.*, 1990; Ioannides and Lewis, 2004). Interindividual differences in the expression of Cyp1 may contribute to the risk of developing lung cancer and other pulmonary diseases initiated by agents that require metabolic activation (Raunio *et al.*, 1999). Furthermore, the presence of active Cyp1b1 was reported in 70% of renal cell carcinomas (McFadyen *et al.*, 2004a). Both Cyp1a2 and Cyp1b1 were shown to be important catalysts of the 4-hydroxylation of estradiol to form the 4-hydroxy estradiol which was proven to induce tumors in the hamster kidney. The 4-hydroxyestrogen can directly interact covalently with DNA or be a precursor for genotoxic metabolites such as quinones and semiquinones (Ioannides and Lewis, 2004).

Although the carcinogenicity of arsenic has been extensively studied for decades, there is no definitive understanding of the mechanism underlying this effect (Crebelli and Leopardi, 2012). One of the main factors contributing to the gap in knowledge is the lack of complete understanding of the mechanisms of arsenic biotransformation, where methylation was considered a detoxification mechanism (Hughes, 2009). Hence, methylated arsenicals have not received adequate attention compared to inorganic arsenicals, and it was never reported before whether TMAO would affect constitutive and TCDD-mediated induction of AhR-regulated phase I enzymes. The current study demonstrated that TMAO alters Cyp1a1, Cyp1a2, and Cyp1b1 mRNA, protein, and catalytic activity levels in the extrahepatic tissues of C57BL/6 mice in a tissue-, and isoform-specific pattern. Thus, the effect of TMAO on one isoform cannot be generalized to include the other. Moreover, the activation of AhR-regulated carcinogen activating enzymes might be a main contributor to arsenic-induced toxicity and carcinogenicity.

With regard to AhR-regulated phase II enzymes, TMAO alone significantly induced Nqo1 mRNA in the lung, kidney, and heart. TMAO also significantly induced Gsta1 mRNA in the heart but not in the lung or kidney. Co-exposure to TMAO and TCDD significantly induced Nqo1 mRNA in the kidney and heart, but not lung compared to TCDD. Whereas, the co-exposure had no effect on Gsta1 mRNA in the lung, kidney, or heart compared to TCDD. At the protein and catalytic activity levels, TMAO induced lung Nqo1 protein expression with a subsequent increase in Nqo1 catalytic activity, while it had no effect on kidney Nqo1 protein expression and catalytic activity levels. TMAO induced lung and kidney Gsta1/2 protein expression levels with a subsequent increase in Gst catalytic activities. The co-exposure to TMAO and TCDD had no effect on lung and kidney Nqo1 protein expression and catalytic activity compared to TCDD. Co-exposure to TMAO and TCDD significantly induced Gsta1/2 protein expression and Gst catalytic activity in the kidney, but not in the lung compared to TCDD (Figure 3.16 - Figure 3.18). Our results are in line with previous reports that showed the parent compound As(III) was able to differentially induce AhR-regulated phase II enzymes in extrahepatic tissues of C57/BL6 mice (Anwar-Mohamed *et al.*, 2012).

The regulation of Nqo1 and Gsta1 involves, in addition to the AhR–XRE pathway, the Nrf2–ARE pathway. It is still unknown whether mouse Gsta1 regulation involves an additional pathway (Anwar-Mohamed *et al.*, 2013a). However, potential regulatory elements have been identified in the human GSTA1 promoter, including AP-1 and AP-2 consensus sequences and a glucocorticoid response element (Whalen and Boyer, 1998; Romero *et al.*, 2006). Both Nqo1 and Gsta1 are known to increase in response to oxidative stress products, and this supports the concept that cells are subjected to a localized increases in oxidative stress (Parsons *et al.*, 2001; Takakusa *et al.*,

2008). Moreover, previous reports have shown elevated Nqo1 activities in carcinomas such as liver, colon, breast, brain, and lung carcinomas (Siegel *et al.*, 1998).

In the current study, we have demonstrated that TMAO alone or in the presence of TCDD, was able to induce Ho-1 mRNA levels as early as 6 h in the kidney and heart but not in the lung (Figure 3.19). Induction of Ho-1 is considered a hallmark to oxidative insult under pathologic conditions (Wondrak *et al.*, 2010). In agreement with our results, previous studies showed induction of Ho-1 with different forms of arsenic, indicating that oxidative stress is mediated by arsenicals (Liu *et al.*, 2001; Anwar-Mohamed *et al.*, 2013a). In addition, and in line with our results, the parent compound As(III) was reported to induce Ho-1 mRNA levels in the kidney, heart, and lung of C57BL/6 mice (Anwar-Mohamed *et al.*, 2012).

In conclusion, the present study demonstrates, for the first time, that TMAO modulates constitutive and TCDD-induced AhR-regulated genes in a tissue-, and AhR-regulated enzyme-specific manner. The effect on one of these enzymes could not be generalized to other enzymes even in the same organ, as there are multiple factors that could interplay to cause differential effects. Furthermore, induction of AhR-regulated carcinogen activating enzymes by TMAO could play an important and essential role in arsenic-induced toxicity and carcinogenicity, and hence it could be a great target in developing new therapeutic strategies for arsenic-induced carcinogenesis.

4.3 Dimethylarsinic acid (DMA(V)) modulates the AhR-regulated genes in C57BL/6 mice: *in vivo* study

DMA(V) is the main metabolite of arsenic metabolism which is excreted in human urine (Marafante *et al.*, 1987; Yoshida *et al.*, 1997). Interestingly, recent scientific views revealed that the metabolic methylation pathway for inorganic arsenic to DMA(V) might be a toxicity-enhancing process rather than a detoxification process (Shimoda *et al.*, 2015). To the best of our knowledge, there have been no previous attempts to examine the effect of DMA(V) on the modulation of phase I and phase II AhR-regulated genes in hepatic and extrahepatic tissues. Thus, the objectives of this study were to examine the effects of DMA(V) on the constitutive and TCDD-inducible expression of phase I AhR-regulated genes, typified by Cyp1a, in addition to phase II AhR-regulated genes, typified by Nqo1, in the liver, lung and kidney of C57BL/6 mice. The dose of DMA(V) utilized in this study was selected to match doses of the parent compound, As(III), utilized in previous studies in C57BL/6 and BALB/c mice (Kimura *et al.*, 2006; Anwar-Mohamed *et al.*, 2012; Anwar-Mohamed *et al.*, 2013a). The TCDD dose was also selected based on previous *in vivo* studies (Uno *et al.*, 2008; Wong *et al.*, 2010).

We have demonstrated that DMA(V) modulates the AhR-regulated enzymes in a tissue-, and enzyme-specific manner. In the liver, DMA(V) had no significant effect on the constitutive and inducible Cyp1a mRNA and protein expression or EROD and MROD catalytic activities (Figure 3.20 - Figure 3.24). Previous reports showed that the parent compound, As(III), similar to DMA(V), did not alter the constitutive expression of Cyp1a mRNA and protein expression, or catalytic activity (Anwar-Mohamed *et al.*, 2013a). However, unlike DMA(V), As(III) did inhibit

TCDD-mediated induction of Cyp1a mRNA expression at 6 h, while it potentiated the TCDD-mediated induction of Cyp1a protein expression, in addition to EROD and MROD catalytic activities at 24 h (Anwar-Mohamed *et al.*, 2013a). The difference in effect between DMA(V) and its parent compound, As(III), can be attributed to the uptake of the arsenic species by the liver cells, where As(III) is known to be highly uptaken by the liver cells and extensively metabolized to its methylated metabolites (Cohen *et al.*, 2006). This proposed explanation is supported by a previous study showing that As(III) (85 mmol/kg, single subcutaneous injection) altered EROD activity in the liver of C57BL/6 mice at 24 h after treatment, while it did not alter the EROD activity after 48 or 96 h of treatment, which could be a result of As(III) metabolism and excretion (Seubert *et al.*, 2002). It has been previously shown that DMA(V) modulated CYP1A1 expression *in vitro,* in the human hepatoma HepG2 cell line (Anwar-Mohamed *et al.*, 2014b). However, the discrepancy between the *in vitro* and *in vivo* results could be attributed to different factors, such as the differences between species in the two studies, the differences between *in vivo* and *in vitro* models, and the elimination of DMA(V) from the whole animal as compared to limited elimination in cell line models.

In the lung, DMA(V) had no significant effect on Cyp1a mRNA and protein expression or catalytic activity levels. However, DMA(V) potentiated the TCDD-mediated induction of Cyp1a mRNA and protein expression, with subsequent increases in EROD and MROD catalytic activities (Figure 3.20 - Figure 3.24). Previous studies have shown that As(III), in an equivalent dose to DMA(V), produced similar effects on the mRNA, protein, and activity levels, in the lung tissues *in vivo* (Anwar-Mohamed *et al.*, 2012). Moreover, arsenic is known to induce Cyp1a expression and activity *in vitro* in lung cells (Wu *et al.*, 2009). Our findings show that potentiation of Cyp1a

in the lung tissues with As(III) treatment could be mediated by its main metabolite, DMA(V). In the kidney, DMA(V) did not significantly alter the Cyp1a mRNA and protein expression or activity. However, DMA(V) significantly inhibited the TCDD-mediated induction of Cyp1a mRNA and protein expression, with subsequent decreases of EROD and MROD catalytic activities (Figure 3.20 - Figure 3.24). These results are in agreement with previous reports showing that As(III) significantly inhibited the TCDD-mediated induction of Cyp1a mRNA expression at 6 h, in addition to Cyp1a protein and EROD and MROD activities at 24 h in the kidney (Anwar-Mohamed *et al.*, 2012). The similarity between As(III) and DMA(V) effects in the kidney confirms that part of the As(III) effect could be attributed to the effect of its main metabolite, DMA(V). The tissue-specific effects observed with DMA(V) are in agreement with previous studies demonstrating the ability of DMA(V) to induce lung-specific DNA damage in both rats and mice, in addition to lung cancer in humans (Brown *et al.*, 1997; Kenyon and Hughes, 2001). Previous studies have shown similar tissue-specific effects for DMA(V) on ornithine decarboxylase (ODC) activity in rats, where DMA(V) administration decreased ODC activity in the lung, while it had no effect on ODC in the liver tissue (Brown *et al.*, 1997). This organ-specific effect was attributed to the ability of the lung to generate different arsenic metabolites and free radicals (Brown *et al.*, 1997; Kenyon and Hughes, 2001).

The transcriptional regulation of phase I AhR-regulated genes of the Cyp1 family has received special attention from researchers because of its association with the etiology of several cancers which could be attributed to the formation of adducts between DNA and the oxidized products of Cyp1-catalyzed reactions, and thus these enzymes are called “carcinogen activating enzymes” (Danielson, 2002; Androutsopoulos *et al.*, 2009). Interestingly, it is thought that the Cyp1 family

is responsible for the activation of more than 90% of known carcinogenic chemicals, because most ubiquitous environmental and dietary carcinogens to which humans are frequently exposed (such as PAH, heterocyclic amines and mycotoxins) are molecularly planar in nature. Therefore they are favored substrates of the Cyp1 family, which can activate these compounds to carcinogens (Ioannides and Lewis, 2004; Androutsopoulos *et al.*, 2009).

In the current work we have investigated the effect of DMA(V) on phase II AhR-regulated genes, typified by Nqo1. These genes are known to have complex regulatory mechanisms that involve transcription factors other than AhR, such as nuclear factor erythroid 2-related factor 2 (Nrf2) (Yeager *et al.*, 2009; Zhou *et al.*, 2016). DMA(V) did not significantly alter the constitutive or inducible Nqo1 mRNA and protein expression, or catalytic activity in the liver, lung or kidney (Figure 3.25 - Figure 3.27). Induction of Nqo1 is an important part of the antioxidant defense, where it provides the cell with protection against environmental insults through different mechanisms. First, Nqo1 detoxifies highly reactive metabolites that can produce reactive oxygen species through redox cycling. Second, Nqo1 stabilizes the tumor suppressor, p53, in response to DNA-damaging stimuli. Third, induction of Nqo1 *in vivo* and *in vitro* is correlated with the induction of other protective phase II enzymes (Sorensen *et al.*, 2003; Nioi and Hayes, 2004; Lee *et al.*, 2010). Low Nqo1 activity has been correlated with increased susceptibility for the development of human cancers (Siegel *et al.*, 1998; Oh and Park, 2015). Thus the inability of DMA(V) to induce phase II AhR-regulated antioxidant genes, such as Nqo1, while modulating phase I AhR-regulated genes could play a major role in DMA(V)-induced toxicity and carcinogenicity.

In conclusion, our work demonstrates, for the first time, that DMA(V) can modulate AhR-regulated genes in a tissue- and enzyme-specific manner. The modulation of each specific enzyme in each tissue is unique, and many factors could contribute to this specific differential modulation. Alteration of AhR-regulated genes could be a part of the pleotropic effect of arsenic-induced toxicities and carcinogenicities, thus it could be a target for novel treatment strategies.

4.4 Modulation of Cytochrome P450 1A1 by monomethylarsonous acid (MMA(III)) in human HepG2 cells

In the current study we have demonstrated that As(III) and MMA(III) inhibits CYP1A1 through AhR-dependent and AhR-independent mechanisms. As(III) and MMA(III) reduced both basal and TCDD-induced CYP1A1 mRNA and protein expression and CYP1A1 catalytic (EROD) activity (Figure 3.29, Figure 3.32, and Figure 3.34). The effect of MMA(III) on these endpoints was shown to be concentration-dependent. The concentrations of As(III) and MMA(III) used in this work were chosen to match the tested concentrations previously used in different models (Eblin *et al.*, 2006; Elbekai and El-Kadi, 2007; Anwar-Mohamed and El-Kadi, 2010; Soh *et al.*, 2011) and are in accordance with at-risk human populations chronically exposed to arsenic in drinking water (Ahsan *et al.*, 2000; Chiou *et al.*, 2001; National Research Council, 2001; Chen *et al.*, 2013). Previous studies showed that rates and patterns of arsenic methylation by HepG2 cells resemble those described in cultured primary human hepatocytes (Drobna *et al.*, 2006). Interestingly, despite the fact that HepG2 cells are relatively efficient methylators of arsenic, it requires 72 h of incubation to methylate 63% of the total amount of inorganic arsenic (Drobna *et al.*, 2006). Moreover, speciation analyses of arsenic metabolites in HepG2 cells, incubated with 5 μM

arsenite, indicated that more than 90% of cellular arsenic species remain as unmethylated inorganic arsenic after 24 h of incubation (Watanabe *et al.*, 2011). Since all experiments in the current thesis were conducted in the range of 1h to 24h, it would be plausible to conclude that the observed effects are the direct result of the individual arsenic species rather than their metabolic products.

Although this is the first report that examined the effect of MMA(III) on both basal and TCDD-induced CYP1A1 mRNA, protein, and catalytic activity, previous reports that utilized As(III) showed results consistent with our findings. For example, it was shown that As(III) inhibited the TCDD-mediated induction of CYP1A1 at the mRNA, protein and catalytic activity levels in a concentration dependent manner in HepG2 and Huh7 human cell lines (Bonzo *et al.*, 2005; Chao *et al.*, 2006; Anwar-Mohamed and El-Kadi, 2010). Similarly, As(III) decreased BkF-induced CYP1A1 in HepG2 cells (Bessette *et al.*, 2005; Bessette *et al.*, 2009). Low concentrations of As(III) have also been shown to produce a concentration-dependent reduction in TCDD-induced mouse Cyp1a1 as well as human CYP1A1 in primary hepatocytes cultured from transgenic CYP1A1N(+/-) mice (Bonzo *et al.*, 2005). Several mechanisms have been proposed for the inhibition of the TCDD-mediated induction of CYP1A1. For example, As(III) might interrupt cell cycle control by initiating G(2)/M phase arrest with a resultant inhibition of AhR-mediated induction of CYP1A1 (Santini *et al.*, 2001). In addition, this inhibition may involve other mechanisms such as blocking the recruitment of RNA polymerase II to the CYP1A1 proximal promoter, thus reducing its transcription (Bonzo *et al.*, 2005; Beedanagari *et al.*, 2009).

We observed here that As(III) and MMA(III) alone or in the presence of TCDD were able to significantly decrease AhR-dependent, XRE-driven luciferase reporter gene expression

(Table 3-4). In agreement, immunocytochemical analysis revealed that both As(III) and MMA(III) were able to inhibit TCDD-dependent AhR nuclear accumulation (Figure 3.36 and Table 3-5), although the exact mechanism remains to be elucidated. Our results are in agreement with previous findings conducted using As(III). For example, As(III) was reported to significantly inhibit BkF- and TCDD-mediated induction of XRE-driven luciferase reporter gene expression in HepG2 cells (Bessette *et al.*, 2005; Anwar-Mohamed and El-Kadi, 2010) and was found to attenuate the TCDD-induced XRE-driven luciferase expression in Huh7 cells (Chao *et al.*, 2006). While the inhibition of TCDD-mediated induction of CYP1A1 expression through an AhR-dependent mechanisms may suggest that both As(III) and MMA(III) may be AhR antagonists, we found that they were unable to compete with [^{3}H]TCDD for binding to the AhR (Figure 3.37). These results are consistent with a previous study which showed that As(III) is not a competitive AhR ligand (Anwar-Mohamed *et al.*, 2014b).

Overall, these results suggest that the underlying mechanism of the As(III) and MMA(III) inhibitory effects is due to a different mechanism that does not involve ligand binding. There are numerous possibilities to explain this inhibitory response. It is possible that As(III) and MMA(III) bind to a second binding site on the AhR distinct from the TCDD-binding site, yet modify its functional activity. Alternatively, As(III) and MMA(III) may indirectly affect the AhR and/or AhR mechanism of action (Ciolino *et al.*, 1998). This could include effects of As(III) and MMA(III) on other proteins important in AhR action, including (but not limited to): Hsp90, XAP2, p23, c-SRC, and PKC, as well as effects on proteins in the CYP1A1 preinitiation complex (Enan and Matsumura, 1996; Long *et al.*, 1998; Denison and Nagy, 2003; Beedanagari *et al.*, 2009). As(III) and MMA(III) may inhibit AhR function by affecting one or more of these target proteins without

affecting the ligand binding site of the AhR. Alternatively, As(III) and MMA(III) may affect nuclear translocation of the AhR via an action on the AhR and/or nuclear pore complex, reducing its nuclear localization and gene expression response.

We also investigated the possibility of the involvement of post-transcriptional and post-translational mechanisms in the down regulation of CYP1A1 protein expression and catalytic activity. For this purpose, we examined the effect of As(III) and MMA(III) on CYP1A1 mRNA and protein stability, HO-1 expression, and the ability to directly inhibit CYP1A1 catalytic activity. While our results showed that As(III) and MMA(III) had no significant effect on CYP1A1 mRNA stability, and As(III) had no effect on CYP1A protein stability, MMA(III) was able to significantly decrease the half-life of the CYP1A protein (Figure 3.31 and Figure 3.33). These results are consistent with previous data showing that As(III) did not significantly alter Cyp1a protein half-life in mouse hepatoma Hepa1c1c7 cells (Elbekai and El-Kadi, 2007). These results suggest that, MMA(III) acts through a post-translational mechanism to inhibit CYP1A1 expression by decreasing CYP1A protein stability. Previous studies demonstrated a role of HO-1 in the As(III)-mediated decrease in CYP1A1 catalytic activity via decreasing the cellular heme pool (Elbekai and El-Kadi, 2007; Bessette *et al.*, 2009; Anwar-Mohamed and El-Kadi, 2010). Interestingly, the ability of As(III) but not MMA(III) to significantly induce HO-1 mRNA levels suggests that the MMA(III)-dependent decrease in CYP1A1 activity is not due to an effect on cellular heme content (Figure 3.38). We also tested the ability of As(III) and MMA(III) to directly inhibit CYP1A1 catalytic activity and found that As(III) or MMA(III) at concentrations up to 5 μM had no direct inhibitory effects on EROD activity. Interestingly, MMA(III) at a concentration of 10 μM, could directly inhibit EROD activity (Figure 3.35).

Previous studies have reported that oxidative stress can modify AhR-dependent gene expression; thus exposure to pro-oxidant environmental pollutants could disrupt the coordinated regulation of these genes (Kann *et al.*, 2005). Increased ROS levels are known to decrease CYP1A1 activity by two possible mechanisms: first, ROS may oxidize thiol groups in cysteine residues of the CYP1A1 protein, causing loss of protein function; second, ROS may also interact with the heme Fe^{2+}, leading to heme destruction and enzyme inactivation (Elbekai and El-Kadi, 2007). Accordingly, to examine the role that ROS may play in the reduction response of As(III) and MMA(III), we measured the intracellular production of total ROS and superoxide radical anions in response to As(III) and MMA(III) using DCFH-DA and DHE, respectively. Our results revealed that both As(III) and MMA(III) increased DCF fluorescence intensity, a widely used indicator of total ROS content, which was in agreement with previous studies that confirmed the generation of ROS after exposure to As(III) or MMA(III) (Shi *et al.*, 2004; Valko *et al.*, 2005). Interestingly, although As(III) and MMA(III) failed to increase superoxide radical anion generation, as measured by the DHE probe, MMA(III) resulted in a significant increases in menadione-mediated generation of superoxide radical anions (Figure 3.39). In fact, previous studies showed that in comparison to As(III), much lower concentrations of MMA(III) over shorter periods of time can alter the activity of glutathione peroxidase and catalase, and induce expression of stress proteins and metallothioneins (Pace *et al.*, 2016). In addition, MMA(III) is a much more potent inhibitor of glutathione reductase and thioredoxin reductase compared to As(III) (Mass *et al.*, 2001; Petrick *et al.*, 2001).

In conclusion, the results presented in this study demonstrated that MMA(III) down-regulates CYP1A1 through both transcriptional and post-translational mechanisms. This modulation is mediated by inhibition of TCDD-dependent nuclear accumulation of the AhR, and inhibition of transcriptional activation, and via post-translational mechanisms, leading to decreased protein stability and increased ROS generation. The modulation of the AhR signaling pathway by As(III) and MMA(III) demonstrates that trivalent metabolites of arsenic are highly reactive, and can affect important signaling pathways which could contribute, at least in part, to arsenic-induced toxicity.

4.5 General conclusion

Arsenic is a ubiquitous metalloid that has long been known as a human carcinogen. Millions of individuals worldwide are chronically exposed to arsenic with consequences ranging from acute toxicities to development of malignancies. Despite well-known arsenic-related health effects, the molecular mechanisms involved are not fully understood. Great efforts were taken to understand the potential outcome upon exposure to arsenic and environmental pollutants on the expression of AhR-regulated genes, as AhR is known to be associated with toxicity and carcinogenesis. However, although arsenic biotransformation is thought to play a key role in arsenic-mediated toxicities, the effects of different organic derivatives of arsenic on modulation of phase I and phase II AhR-regulated genes are not fully investigated yet. The biomethylation of arsenic could be involved in tissue- and species-specific toxicities associated with arsenic exposure. Elucidation of the mechanisms underlying the initiation and promotion of toxicity and carcinogenesis related to arsenic and its metabolites could provide new insights to understand the mechanism of receptor-mediated toxic responses to environmental toxins, and hence could introduce new approaches for

designing better strategies for prevention of arsenic-induced diseases worldwide. Thus, in this dissertation we focused on understanding the differential effect of different organic metabolites of arsenic on the modulation of phase I and phase II AhR-regulated genes, both *in vivo* and *in vitro*.

In an attempt to understand the role of TMAO, as the metabolic end product of arsenic biotransformation in mediating arsenic toxicities and carcinogenicity, we investigated whether TMAO modulates phase I and phase II AhR-regulated genes *in vivo* in the liver. Our work revealed that TMAO alone or in the presence of TCDD induced Cyp1a1 mRNA, with concomitant induction of Cyp1a1 protein expression and EROD activity. TMAO induced Cyp1a2 mRNA expression with resultant increase in protein expression and MROD catalytic activity. TMAO alone or in the presence of TCDD induced Cyp1b1 mRNA expression and potentiated the TCDD-mediated induction of Cyp1b1 protein expression. As for phase II enzymes, TMAO induced Nqo1, Gsta1, and Ho-1 mRNA expression, protein expression and catalytic activities. To investigate the molecular mechanisms behind modulation of these enzymes, primary hepatocytes were utilized, and the modulation of the prototypical AhR downstream target, Cyp1a1, was studied. *In vitro* work showed that TMAO induced Cyp1a1 mRNA and protein expression, in addition to catalytic activity. This modulation was mediated through an AhR-dependent signaling pathway as evidenced by the ability of TMAO to induce the XRE-driven luciferase reporter activity, and to induce AhR nuclear localization. Thus, our work revealed the ability of TMAO to activate AhR signaling pathways both *in vivo* and *in vitro*, with a resultant activation of carcinogen activating enzymes of the Cyp1 family. This activation of the Cyp1 family could play a crucial role in the activation of the carcinogenic potential of arsenic.

In order to know whether the effect of TMAO on AhR-regulated genes was specific to the liver, we investigated the effect of TMAO on phase I and phase II AhR-regulated genes in extrahepatic tissues. Our work showed that TMAO could modulate phase I and phase II AhR-regulated genes in a tissue- and enzyme-specific patterns. In the lung, TMAO alone induced Cyp1a1 and Cyp1b1 mRNA expression with a concomitant increases in protein expression and EROD catalytic activity. It also potentiated the TCDD-mediated induction of Cyp1a1 and Cyp1a2 mRNA and protein expression, in addition to EROD and MROD activity. In the kidney, TMAO alone induced Cyp1b1 mRNA and protein expression. It also potentiated the TCDD-mediated induction of Cyp1a1 and Cyp1b1 mRNA with a resultant potentiation of protein expression and EROD activity. As for phase II enzymes, TMAO induced lung Nqo1 mRNA and protein expression and activity in addition to Gsta1 protein and activity. In the kidney, TMAO induced Nqo1 mRNA expression in addition to Gsta1 protein expression and activity. This study showed that TMAO modulates AhR-regulated genes in a tissue- and enzyme-specific pattern in extrahepatic tissues. This modulation of AhR enzymes could participate in arsenic-induced toxicity and carcinogenicity, especially via activation of the carcinogen activating enzymes in the Cyp1 family.

In order to further understand the role of organic metabolites in arsenic-induced toxicity and carcinogenicity, we investigated the effect of DMA(V), as the main metabolite of arsenic biotransformation, on modulation of Cyp1a1, Cyp1a2, and Nqo1, as the prototypical AhR-regulated enzymes, in the liver and extrahepatic tissues. Our results demonstrated that, DMA(V) had no effect on the liver Cyp1a1 and Cyp1a2 mRNA and protein expression, or on EROD and MROD activities. However, DMA(V) potentiated the TCDD-mediated induction of Cyp1a1 and Cyp1a2 mRNA expression in the lung, with a resultant increase in protein expression, in addition

to EROD and MROD activities. In the kidney, DMA(V) inhibited the TCDD-mediated induction of Cyp1a1 and Cyp1a2 mRNA expression with concomitant inhibition of TCDD-mediated induction of protein expression, and EROD and MROD activities. As for Nqo1, DMA(V) had no effect on mRNA and protein expression, or activity in the liver, lung, or kidney. Our results, showed that DMA(V) modulates AhR-regulated genes in a tissue- and enzyme-specific manner. The differential effect of DMA(V) could be attributed to different factors such as the degree of uptake of DMA(V) by different tissues, being the main metabolite excreted in the urine, in addition to the generation of tissue specific intermediary metabolites that could participate in organ-specific toxicity and carcinogenicity mediated by arsenic.

In order to investigate whether MMA(III), the first trivalent metabolite of arsenic biotransformation, is less reactive than its parent trivalent compound, As(III), we investigated the effect of MMA(III), compared to As(III), on the modulation of the AhR signaling pathway *in vitro*. We have shown that MMA(III) and its parent compound, As(III), inhibit CYP1A1 through AhR-dependent and AhR-independent mechanisms. Both compounds inhibited the constitutive and the TCDD-induced CYP1A1 mRNA expression, protein expression, and EROD activity. This down-regulation was mediated by inhibition of CYP1A1 transcription, as evidenced by the inhibition of AhR-dependent XRE-driven, luciferase reporter gene expression and inhibition of AhR nuclear localization. Both compounds were unable to compete with [^{3}H]TCDD for binding to the AhR, implying that they are not AhR antagonists for the binding site. Both compounds did not affect CYP1A1 mRNA stability and As(III) also had no effect on CYP1A protein stability. However, MMA(III) decreased CYP1A protein stability with a resultant decrease in its half-life. While As(III) significantly induced HO-1 mRNA levels, MMA(III) had no significant effect on HO-1

mRNA levels. Both compounds at concentrations up to 5 μM did not affect EROD activity directly, however, 10 μM MMA(III) directly inhibited EROD activity. Moreover, both compounds increased total ROS generation, but not superoxide radical anion generation. However, MMA(III) potentiated menadione-mediated generation of superoxide radical anions. Our findings suggest that different post-translational mechanisms, in addition to transcriptional mechanisms, play a significant role in the modulation of AhR-regulated genes by MMA(III) and As(III). Moreover, we have shown that trivalent metabolites of arsenic are highly reactive and can modulate important signaling pathways which might contribute to arsenic-induced toxicity. The ability of MMA(III) to down-regulate CYP1A1 *in vitro* similar to its parent compound, As(III), sheds light on the importance of investigating whether MMA(III) can, on contrary, induce carcinogen activating enzymes of CYP1 family *in vivo* similar to As(III). A main contributor to the disparity between *in vivo* and *in vitro* effects of As(III) could be the limited capacity of the cell to metabolize arsenicals *in vitro* compared to the extensive metabolism of arsenicals *in vivo* (Cohen *et al.*, 2006; Watanabe *et al.*, 2011).

This research is expected to have a great impact on the understanding of the cellular and molecular mechanisms of arsenic-mediated toxicity and carcinogenicity. Elucidation of the mechanisms underlying the initiation and promotion of carcinogenesis related to arsenic and its metabolites could provide new insights to greatly attenuate health risks posed by arsenic exposure. Our work opened new avenues for implementing new therapeutic strategies for people exposed to high levels of arsenic, and those suffering from arsenic-induced carcinogenesis. These new strategies would be based on using CYP1-activatied prodrugs, which are nontoxic to normal cells but are activated to cytotoxic agents only at the site of the tumor by individual CYP1 enzymes, endogenously

induced by arsenicals, within the tumor cells. Several CYP1-activatied prodrugs are available and they are classified into four main groups: benzothiazoles, flavonoids, stilbenes and alkylating agents (McFadyen *et al.*, 2004b; Rodriguez-Antona and Ingelman-Sundberg, 2006; Bruno and Njar, 2007; Cui and Li, 2014). Therefore, our research could provide important clues for the development of rational therapies for the prevention and treatment of arsenic-induced carcinogenicity. The development of these rational therapies will assist in the prevention of arsenic-induced diseases, and could decrease morbidity and mortality, and hence diminish health care costs.

4.6 Future directions and studies

The results obtained from our work answered several questions previously raised about interactions between arsenicals and AhR-regulated genes. It has also highlighted the important role that could be played by AhR-regulated genes in different arsenic-induced diseases and arsenic-induced carcinogenicity. However, more efforts are needed to answer several other questions that need to be addressed in order to increase our understanding of potential interactions between arsenicals and AhR downstream targets. This better understanding of the changes associated with arsenic toxicities would advance the results of this work into clinical practice. Therefore, further research needs to be conducted to address the following points:

- To investigate the effect of acute exposure to trivalent organic metabolites of arsenic on the modulation of AhR-regulated phase I and phase II enzymes *in vivo*.

- To examine the effect of chronic exposure to arsenic and its trivalent and pentavalent organic metabolites with and without AhR-ligands on the AhR-regulated phase I and phase II enzymes *in vivo*.
- To examine the role of transporters and arsenic uptake in mediating arsenic differential effects on AhR-regulated genes *in vivo*.
- To characterize the cross-talk between AhR and Nrf2 transcription factor proteins, and to identify their role in the modulation of AhR-regulated genes by arsenicals.
- To examine the role of AP-1 and NF-κB signaling in the modulation of AhR-regulated genes by inorganic and organic arsenic metabolites.
- To determine the role of co-regulatory proteins such as co-activator and co-repressor proteins in the interactions between arsenicals and different transcription factors (AhR, Nrf2, AP-1, and/or NF-κB)

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