Differential Modulation of Aryl Hydrocarbon Receptor Regulated Genes by Chromium

By

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Abstract

Several studies have examined the toxic effects of individual aryl hydrocarbon receptor (AhR) ligands, yet there are relatively few reports of the combined toxic effects of AhR ligands and other environmental co-contaminants, such as heavy metals. Chromium (Cr^{+6}) is one of the major environmental toxic metal contaminants and a potent human toxin, mutagen, and carcinogen. Heavy metals alter the carcinogenicity of AhR ligands by modulating the cytochrome P450 1 (Cyp1) enzyme; however, the mechanism(s) remain unresolved. The objective of the current study was to investigate the effect of Cr^{+6} on expression and activity, of Cyp1a1, NQO1 and HO-1 in C57BL/6 mouse liver. C57BL/6 mice were injected intraperitoneally with Cr^{+6} (20 mg/kg) in the absence and presence of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (15 μg/kg) for 24 h. The mice were segregated into 4 experimental groups. The first group was control mice, and they received saline plus corn oil. The second group was Cr^{+6}-treated mice, and they received Cr^{+6} dissolved in saline plus corn oil. The third group was TCDD-treated mice, and they received TCDD dissolved in corn oil plus saline. The fourth group was Cr^{+6} plus TCDD–treated mice, and they received Cr^{+6} dissolved in saline plus TCDD dissolved in corn oil. Moreover, real-time PCR and Western blot were used to measure mRNA and protein expression, respectively. EROD was used to measure Cyp1a1 activity. Cr^{+6} alone did not significantly alter Cyp1a1, NQO1 or HO-1 at mRNA, protein, or catalytic activity levels. Upon co-exposure to Cr^{+6} and TCDD, Cr^{+6} significantly inhibited the TCDD-mediated induction of the Cyp1a1 mRNA, protein, or catalytic activity levels, whereas it significantly potentiated the induction of NQO1 and HO-1 mediated by TCDD at the mRNA, protein and catalytic activity levels at 24 h. We demonstrated that Cr^{+6} inhibits the AhR-ligand -mediated effect on the carcinogen-activating enzymes whereas it potentiated the carcinogen detoxifying enzymes NQO1 and HO-1.

This work is dedicated to my parents

Dr. Yousif Abdu Asiri

&

Kheria Saleh AlJanadi

My Brothers and Sisters

Abdulaziz, Moath, Abdullah, Bushra and Yusra

For Their Endless Love, Support and Encouragement.

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List of Abbreviations

3MC	3 –Methylcholanthrene
7ER	7-Ethoxyresorufin
Act-D	Actinomycin D
AHH	Aryl hydrocarbon hydroxylase
AhR	Aryl hydrocarbon receptor
ALDH-3	Aldehyde dehydrogenase 3
Arnt	AhR nuclear translocator
ARE	Antioxidant-responsive element
ATPase	Adenosine tri-phosphatase
bHLH	Basic-helix-loop-helix
BaP	Benzo[a]pyrene
Cr^{3+}	Chromium (III) ion tri chromium ion
Cr^{6+}	Chromium (IV) ion hexa chromium ion
c-AMP	Cyclic adenosine monophosphate
CHX	Cycloheximide
CYP or Cyp	Cyptochrome P450
CYPlAl or Cyplal	Cytochrome P4501A1
CYPlA2 or Cypla2	Cytochrome P4501A2
CYPlBl or Cyplbl	Cytochrome P4501B1
CYP2Sl or Cyp2sl	Cytochrome P4502S1
DCPIP	2,6-Dichlorophenolindophenol

DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EMSA	Gel electrophoretic mobility shift assay
EROD	7-Ethoxyresorufin O-deethylation
Gapdh	Glyceraldehyde-3 -phosphate dehydrogenase
GSTA1	Glutathione transferase Al
HAH	Halogenated aromatic hydrocarbon
Hepa lclc7	Murine hepatoma Hepa lclc7
HO-1	Heme oxygenase 1
HSP90	90 kDa heat-shock proteins
MG-132	Carbobenzoxy-L-leucyl-L-leucyl-leucinal
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NES	Nuclear export signals
NLS	Nuclear localization signals
NQO1 or Nqol	NAD(P)H:quinone oxidoreductase 1
NRE	Negative regulatory elements
Nrf2	Nuclear factor erythroid 2-related factor-2
PAGE	Polyacrylamide gel electrophoresis
PAHs	Polycyclic aromatic hydrocarbons
PAS	Per-ARNT-Sim
PBS	Phosphate buffered saline
PKA	Protein kinase A
PKC	Protein kinase C

ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
tl/2	Half-life
tBHQ	*tert*-Butyl hydroquinone
TBS	Tris-buffered saline
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
UGT1A6	Uridine diphosphate glucuronosyltransferases 1A6
WT	Wild-type
XRE	Xenobiotic responsive element

Chapter 1- Introduction

1.1. Aryl Hydrocarbon Receptor

1.1.1. Historical Background

The first studies on the regulation of cytochrome P450 (CYP) were conducted in the 1970s, and demonstrated a stimulation of a varied function oxygenase enzyme activity when reacted with an environmental toxicant such as benzo[a]pyrene (BaP) [1, 2]. Initially this enzyme was termed BaP hydroxylase but later the enzyme name was once changed again to be aryl hydrocarbon hydroxylase [3]. The nomenclature aryl hydrocarbon hydroxylase is preferred, since the mixed function oxygenase obtained from hamster fetal cells grown in culture or from rat liver microsomes converts a variety of polycyclic hydrocarbons to phenolic derivatives and is not specific for benzo[a]pyrene. However, the substrate specificity of either the constitutive or the induced hydroxylase system from the various mammalian tissues has not been determined. For example, endogenous substrates such as steroids may be hydroxylated by this same enzyme system [1].

Benz[a]anthracene (BA), which is a well-known PAH, was shown to be able to bind to cellular material for the first two minutes when exposed to mouse fetal cells [3]. Studies in different mouse strains suggested that there are variations in the extent of AHH activity, which suggested that these cells from these strains retain a different number of receptor sites for the inducer [1].

In 1976 the presence of a small pool of high affinity stereospecific binding sites (receptors) was identified by Poland and coworkers in the cytosolic fraction from liver of C57BL/6 mice [4]. It was found that these receptors are reversibly bound to radiolabeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a halogenated aromatic hydrocarbon (HAH) and is a well-known and potent

inducer of AHH enzyme activity [4]. From then on, it was declared that this receptor be known as the aryl hydrocarbon receptor (AhR).

1.1.2. Molecular characterization of the AhR

AhR is a cytosolic ligand-activated transcriptional factor that belongs to the basic-helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) family of transcription proteins that are involved in cell differentiation and proliferation parameters [5, 6]. The AhR nuclear translocator (ARNT) protein, the *Drosophila* circadian rhythm protein period (Per) and the *Drosophila* neurogenic protein single-minded (Sim) are also members of this family [7, 8]. Roughly 250 amino acids with the presence of the Per-ARNT-Sim (PAS) domain tend to characterize these proteins. (Fig 1.1)

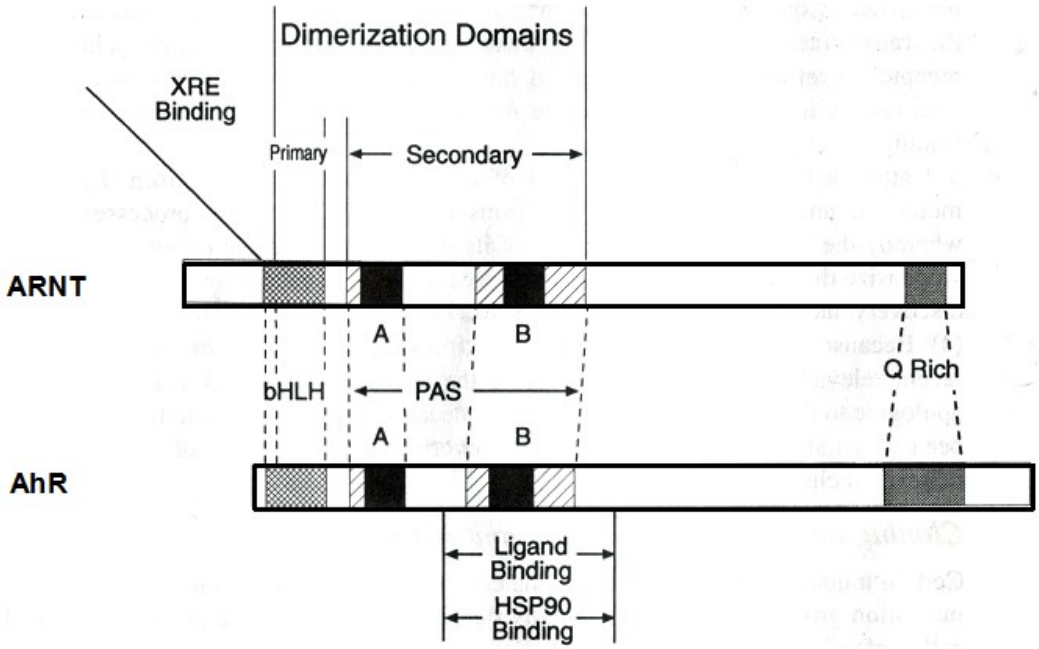

Fig. 1.1. Functional and structural domains of mouse AhR and ARNT [3].

The *AhR* gene is made up of 11 exons of approximately 30 kB of DNA, where the HLH domain sequence can be found encoded within exon 2 and the PAS domain sequence can be found encoded within exons 3-9; however the binding domain is encoded by exons 7 and 8 [7, 8]. Studies conducting sequence analysis on murine *AhR* gene showed the presence of DNA recognition sites for several transcription factors, for example the metal responsive element, xenobiotic responsive element (XRE), activator protein 1 (AP-1) and glucocorticoide responsive element binding site [9], which suggests that the *AhR* gene expression may be affected by these regulatory elements.

The nuclear localization signal (NLS) inside the AhR is made up of two basic amino acid segments, AhR (13–16:RKRR) and AhR (37–39:KRH), located one amino acid upstream from each of the two sections protein kinase C (PKC) sites of Ser-12 and Ser-36 [10]. It has been established that phosphorylated NLS nullifies the ligand-dependent nuclear import, whereas the interaction with dephosphorylated NLS stimulates its interaction with these receptors [10].

These proteins have several physiological effects which are facilitated through forming hetero- or homodimeric complexes with different transcription factors in DNA and the activity of protein binding [11, 12]. Fig. 1.1 displays an illustration of the functional domain of AhR and ARNT. The essential domain for activation of the AhR is the PAS domain, which also activates the heterodimerization with ARNT. Additionally, export signals and nuclear localization (NES and NLS) in the NH_2-terminal region have been found in AhR in the cytoplasm and nucleus and they play a role in shuttling between them [13]. Generally, the N-terminal half of the AhR is responsible for dimerization and ligand and DNA binding; it also contains the bHLH and PAS domains, while the C-terminal half is responsible of transactivation [14].

Well-designed studies in diverse species showed that AhR varies in amino acid sequence identity and may also vary in molecular size. For example, studies using cloning methods showed that human AhR molecular size is roughly 106 kDa, which is a 10 kDa difference from that of the mouse AhR which is roughly 95 kDa [15]. Nevertheless, there is a 100% amino acid sequence match in the N-terminus when comparing human AhR to murine AhR in the basic region, while there was only a 60% match in the C-terminus when comparing human to murine AhR [7]. Then again, the ARNT protein seems to be more preserved between the two species than the AhR when looking at the amino acid sequence match [7].

Dissimilarities in the induction ability of AhR-dependent genes in response to various PAHs and HAHs have been revealed between responsive C67BL/6 and non-responsive DBA/2 mouse strains [16]. There were about 78 amino acid differences discovered at their C-terminus after conducting sequence and cloning analysis studies of the AhR from these strains [17]. Molecular analysis in the coding region of the AhR cDNA from sensitive Long-Evans (L/E) and resistant Han/Wistar (H/W) rats to TCDD toxicity showed changes in the molecular size, 106 and 98 kDa, in that order, also a change in amino acid (VAL497 to ALA497) in the transactivation domain [18, 19]. Also, a mechanism was proposed after a mutation appeared in exon 10, which in turn modulates ligand-binding properties [20].

1.1.3. Tissue and cellular expression of the AhR

Different cell types and tissues tend to have different AhR protein content in the various development stages [11, 12]. The AhR protein is generally expressed in most tissues; it can be found in the liver, kidney, lung and placenta, where the mRNA and protein are highly expressed,

although in the heart lower levels are expressed [9, 11, 21-24]. The sensitivity of different organs to AhR and ARNT may decrease due to the low expression of ARNT protein levels in certain tissues, even though AhR and ARNT are expressed in a fundamentally organized manner across the tissues [21].

1.2. AhR regulated genes

Up to now, there are four phase I xenobiotic metabolizing enzymes for the AhR-regulated genes code: cytochromes P4501A1 (CYP1A1), CYP1A2, CYP1B1 and CYP2S1. There are also four phase II xenobiotic metabolizing enzymes, namely NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione transferase A1 (GSTA1), uridine diphosphate glucuronosyltransferase 1A6 (UGT1A6), and aldehyde dehydrogenase 3 (ALDH3). Even though the initiation of phase I enzymes bioactivates procarcinogens into their crucial carcinogenic and genotoxic metabolites, the initiation of phase II aids as a detoxification mechanism.

1.2.1. Phase I AhR-regulated genes

Cytochromes P450 (CYPs) play a crucial role in the oxidative metabolism of a wide variety of endogenous and xenobiotic compounds; they are made up of single polypeptide membrane-bound heme proteins [25]. Microsomal CYPs are attached to the membrane through a hydrophobic transmembrane helix at the N-terminus of the protein [26]. CYPs contain approximately 500 amino acids with a molecular size ranging from 45 to60 kDa, and a single

heme group coordinated to cysteine molecule that is essential for thiol-ligand for the heme iron [27]. Nearly, every human and animal organ expresses the CYPs.

Classification of CYPs is based on the sequencing of purified primary amino acids [28, 29]. They are required to have more than 40% amino acid sequence identity to be a member of the same gene family and 55% amino acid sequence identity or greater to be a member of the same subfamily and they reside within the same cluster on a chromosome. Hence, the family is represented by an Arabic numeral, while the subfamily is represented by a capital letter, followed by an Arabic numeral, which represents each individual member separately [28, 29]. Additionally, to describe mouse enzymes small letters are to be used, for example Cyp1a1; and to describe or refer to a gene associated with the enzyme an italic font is used, for example *CYP1A1*.

On the whole, there are many CYPs of different families that partake in oxidative metabolism of many endogenous molecules, for example eicosanoids, fatty acids, and steroids; although of all of the CYPs, only the mammalian CYP1, 2, and 3 families contribute to the metabolism of xenobiotics such as carcinogens, environmental contaminants, and drugs [30]. The xenobiotics that activate and bind to explicit intracellular receptors from a number of the CYP family genes and lead to initiation of their gene transcription are considered strong inducers [31]. There are at the very least three nuclear receptor mechanisms for the activation of transcriptional factors for most of the CYPs. They are: the AhR for CYP1A1, CYP1A2, CYP1B1, and CYP2S1, the constitutive androstane receptor (CAR) for the CYP2 family; the pregnane X receptor (PXR) for the CYP3 family; and the peroxisome proliferator-activated receptor α (PPARα) for the CYP4 family [30].

1.2.1.1. CYP1A1

CYP1A1 is considered an extremely inducible enzyme among AhR-regulated CYPs by a wide-range of xenobiotics, for example HAHs and PAHs through an AhR-XRE-mediated gene transcription pathway [32]. It is considered one of the most proficient CYPs for bio activating the environmental and toxic contaminants HAHs and PAHs into their carcinogenic metabolites. Actually, it has been stated that there is a firm connection between cancer and the induction of CYP1A1 [33]. Numerous studies have validated that the activation of the AhR is the first phase in a chain of molecular events leading to the induction of CYP1A1. As a result, CYP1A1 and its level of expression can be used as a useful biomarker of exposure to environmental HAHs and PAHs [34].

Even though in extrahepatic tissues for example in the lung and placenta, CYP1A1 is expressed at low levels [35, 36], in most mammalian species (human, mouse, rat and rabbits) it is highly inducible in extrahepatic tissues and in the liver [37]. An investigation conducted on the flanking region of CYP1A1 gene showed the existence of a number of sequences that affect the expression the of CYP1A in a positive or negative manner. This includes AP-1 responsive element, c-AMP responsive element, XRE, MRE, and negative regulatory elements (NRE) [34, 38]. As a result, these outcomes indicate that the expression of the *CYP1A1* gene can be modulated through a number of different factors.

1.2.1.2. CYP1A2

O-dealkylation of 7-ethoxyresorufin and 7-methoxyresorufin is catalyzed by CYP1A2 [39, 40]. CYP1A2 also metabolizes a number of common compounds like acetaminophen, caffeine [41], and theophylline [42]. In addition, the expression of CYP1A2 in the mouse liver but no expression in AhR knockout mice indicates a connection between AhR regulation and CYP1A2 [39, 43]. In primary human hepatocytes, TCDD induces CYP1A2 through an AhR-dependent mechanism which gives further support to the connection [44]. Conversely, it has been reported previously that there is an AhR-independent regulation of CYP1A2 [43]; studies showed the presence of two sequence homology to the binding site of the AP-1, also to the XRE, after conducting a sequence analysis studies of the human *CYP1A2* gene [43].

1.2.1.3. CYP1B1

CYP1B1 is fundamentally expressed in extrahepatic tissues and is considered to be a tumor-related form of CYP and is significantly overexpressed in a wide variety of primary tumors [45]. The presence of CYP1B1 in tumor tissues may be of importance in the modulation of these tumors by anti-cancer drug [46, 47]. On this subject, the absence of expression of CYP1B1 in normal tissues and the high expression levels in tumor tissues appears to be partially regulated through proteasomal degradation of the enzyme [48].

It has been shown that transcriptional and post-translation mechanisms both control CYP1B1 expression [47]. A wide range of toxic and carcinogenic chemicals and endogenous substances are metabolized by CYP1B1, and as a result it plays a strategic role in the metabolic bioactivation of abundant procarcinogens such as HAHs and PAHs. The expression of AhR

mRNA does not show a relationship with the inducible expression of CYP1B1 mRNA, as many studies have revealed. Furthermore, ARNT-deficient murine hepatoma cells showed a constitutive Cyp1b1 mRNA and protein expression when compared with wild-type (WT) cells [49]. These outcomes suggest that non-AhR-mediated pathways and/or post-transcriptional mechanisms as well as other mechanisms that possibly contribute to the regulation of CYP1B1.

1.2.2. Phase II AhR-Regulated Genes

The conjugation reactions necessary for xenobiotic metabolism or for further metabolism of phase I enzyme products are catalyzed by phase II metabolizing enzymes like NQO1, GSTA1, UGT1A6, and ALDH3 [50]. As a result, these enzymes play a crucial role in the detoxification of carcinogenic and xenobiotic metabolites [51, 52]. A number of studies have revealed a complex regulation of these genes, wherein their transcriptional activation is regulated by both ARE and XRE [52-55].

1.2.2.1. NQO1

Lars Ernster discovered quinone oxidoreductase in 1958 in the rat liver cytosol and labeled it as DT diaporase, presently known as NQO1 [56]. NQO1 is a cytosolic dimeric flavoprotein expressed fundamentally in a wide range of mammalian tissue and cell lines. Two-electron reduction of numerous endogenous and environmental contaminants and electrophilic compounds are catalyzed by NQO1 [57]. NQO1 is the utmost comprehensively studied enzyme of the three different forms of NQOs recognized up to the present time. A crucial role is played

by NQO1 in the defense against free radicals and mutagenicity; therefore it is considered as a cellular defense mechanism [58]. A direct relationship between the inhibition of NQO1 activities and the increased risk of carcinogenesis has been demonstrated in numerous studies [59].

The expression of NQO1 is known as a tissue type-specific, where the liver and kidneys show a maximum induction of NQO1 mRNA followed by the lungs and heart [57, 60]. A wide range of xenobiotics induce the gene expression of NQO1, like HAHs and PAHs [57] and also antioxidants like tert-butyl hydroquinone (tBHQ) [59, 61, 62], and heavy metals [63]. Up to the present time, several cis-acting regulatory elements have been after analysis of the 5'-flanking region of NQO1 gene; they mediate the transcriptional activation of NQO1 gene and include the antioxidant responsive element (ARE), AP-1, nuclear factor-κB (NF-κB), and the XRE [62,64, 65]. The transcriptional activation of NQO1 through the ARE pathway is usually a consequence of perturbation in the redox status of the cell. This perturbation in the redox status of the cell activates the nuclear factor erythroid 2-related factor-2 (Nrf2), a redoxsensitive member of the cap 'n' collar basic leucine zipper (CNC bZip) family of transcription factors [66]. Subsequently, Nrf2 dissociates from its cytoplasmic tethering polypeptide, Kelch-like ECH associating protein 1 (Keapl), and then translocates into the nucleus, dimerizes with a musculoaponeurotic fibrosarcoma (MAF) protein, and thereafter binds to and activate ARE [67, 68]. In this regard, phenolic antioxidants, such as tBHQ induce NQO1 gene expression through the ARE-mediated mechanism by the activation of Nrf2 [54,59,69].

1.3. Activation of the AhR

1.3.1. Ligand-Dependent Activation of the AhR

Among bHLH/PAS superfamily, the AhR is the only protein that requires activation by a ligand. It exists mostly in the cytoplasm as part of a multimeric protein complex of approximately 280 kDa in the absence of a ligand [70]; in addition, there are two 90 kDa heat-shock proteins (HSP90) and other AhR inhibitory proteins (AIP) of approximately 46 kDa (Fig. 1.2) [70, 71]. The binding site within the AhR overlaps the ligand-binding site, as shown in *in vitro* studies [6], and conceals the AhR-NLS (Fig. 1.2) [13, 72]. As a result, it has been hypothesized that HSP90 functions to keep the AhR in a conformation capable of high-affinity ligand binding and to prevent nuclear translocation of the AhR [13, 63].

Since the molecular structure of the AhR is not known yet, quantitative structure-activity relationships are commonly used to gain insights into the nature of the ligand-receptor interactions. In theory, there are two hypotheses about AhR interaction with its ligands has [73]. First, electrostatic interaction, wherein the active interaction of the ligand with the receptor hinges on the molecular electrostatic potential near the ligand [73]. For instance, it has been established that all dioxin compounds that were capable of activating the AhR share a distinctive molecular charge distribution pattern, which was intensely changed by chlorination patterns [73]. The second hypothesis is based on the molecular polarizability and the distance between the receptor and the ligand [73]. Incidentally, it has been proposed that the AhR pocket can bind planner ligands with maximum dimensions of 14 Å × 12 Å × 5 Å, which depends essentially on the ligand's electronic and thermodynamic features [32].

Binding of AhR to ligands causes detachment of HSP90 and AIP from the activated receptor and subsequent translocation to the nucleus. In the nucleus, the activated AhR heterodimerizes with an 87 kDa nuclear transcriptional factor protein, ARNT [14]. Although ARNT and AhR of each species are about 20% identical in amino acid sequence, ARNT does not have any ligand binding capacity and therefore appears to be at liberty from any suppressive effect by HSP90 [14, 74]. Some data recommend that ARNT endorses detachment of the AhR-HSP90 complex and targets the AhR to its nuclear site of action [75].

The AhR-ARNT complex then binds to a specific DNA recognition sequence, GCGTG, within a responsive element known as XRE [76]. The XRE is located in the promoter region of a number of genes known as the *AhR* gene battery including *CYP1A1*, *CYP1A2*, *CYP1B1*, *CYP2S1*, *NQO1*, *GSTA1*, *ALDH3*, and *UGT1A6*. The AhR-ARNT-XRE complex is then capable of commencing regulatory control of these genes in a positive or negative way [75, 77].

Alternatively, an XRE-independent regulation of phase II genes has been validated through the activation of and binding to a DNA recognition sequence located in a close proximity to XRE in the promoter regions of the phase II genes known as antioxidant responsive elements (ARE) [78, 79]. ARE was first identified by Rushmore and Pickett in the rat *GSTA1* gene that is in charge of the induction of GSTA1 by electrophilic antioxidants and hydrogen peroxide (H_2O_2) [80]. Using a gel retardation assay, Vasiliou *et al.* have confirmed that AhR can bind to both XRE and AER consensus sequences in the promoter region of phase II genes [79]. Further studies have exposed the existence of a number of binding sites for reduction/oxidation (redox)-sensitive transcription factors, such as AP-1 and Nrf2 [66].

1.3.2. Ligand-Independent Activation of the AhR

Earlier studies showed that inhibition of nuclear export of AhR by leptomycin B, the nuclear export inhibitor, or by mutation of the AhR NES resulted in nuclear accumulation of AhR in the absence of exogenous ligand [81]. Yet, although binding to ligands increases the rate of nuclear import of AhR, it does not eradicate its nuclear transfer [81]. These studies suggest that AhR travels between the nucleus and the cytosol in the absence of exogenous ligand, and hence activation of AhR could be a ligand-independent process.

Several of reports have revealed the capability of quite a few chemical compounds such as omeprazole, to induce the AhR-dependent expression of gene such as the CYP1A1 without direct binding to the AhR [82, 83]. In this regard, it has been established that transient expression of AhR and ARNT in AhR-deficient kidney CV-1 cells leads to increased AhR-ARNT-dependent luciferase gene expression [84]. Other studies showed that loss of mouse C3H10T1/2 cell–cell contact in the absence of any AhR ligands permits AhR nuclear translocation and activation and subsequent CYP1B1 induction, whereas the AhR antagonist, α-naphthoflavone, did not affect activation [85].

Even though the particular mechanisms leading to the ligand-independent activation of AhR are still not obvious, it has been proposed that metabolic activation of these compounds into AhR ligands or their abilities to stimulate endogenous AhR ligands could be part of the cause [86-88]. Furthermore, it has been stated that activation of the cyclic adenosine monophosphate (cAMP) mediator [89] or mitogen-activated protein kinases (MAPKs) signaling pathways [10] increases

AhR translocation in one way or another similar to, but functionally unlike, those TCDD-mediated mechanisms. Also, activation of B lymphocytes with CD40 has been proposed to activate the AhR with succeeding induction of the CYP1A1 in the lack of exogenous ligands [81]. An additional condition in which CYP1A1 can be induced in the absence of ligand is through oxidative stress mediated effects or induction of cell differentiation that matches an increase in the AhR transcript [90]. These results propose the presence of a cross-talk between AhR and other signaling pathways that can both positively or negatively regulate its regulation.

1.3.3. Negative Regulation of the AhR and its Regulated Genes

Previous studies have proposed the presence of NREs and associated repressor proteins in promoter regions of more than a few AhR-regulated genes, and that these negatively modulate the expression of these genes [91, 92]. Studies conducted on human and rat cells have recognized a NRE in the *CYP1A1* gene promoter that seems to negatively modulate its transcriptional activity by down-regulating a heterologous promoter/enhancer containing particular nuclear protein binding [93, 94]. This was determined from the information that mutations in the associated repressor protein would inhibit DNA-protein binding, resulting in a 2- to 3-fold increase in the CYP1A1 inducibility in response to AhR ligand [91, 95].

Additionally, super-inducibility of CYP1A1 mRNA by TCDD in Hepa 1c1c7 and human breast cancer MCF10A cells treated with the protein synthesis inhibitor, cycloheximide (CHX), support the presence and participation of negative regulatory proteins in the regulation of *CYP1A1* gene

expression [96]. Incidentally, quite a few studies have recognized nuclear transcription proteins, such as OCT-1 and NF-Y [91, 92, 97], that constitutively bind to the NREs of the *CYP1A1* gene and therefore affect the relative TCDD-induced activity in human hepatoma HepG2, but not MCF7, cells [94]. Moreover, the interaction of the AhR-ARNT complex with corepressor transcriptional proteins, for example silencing mediator for retinoic acid and thyroid hormone receptor (SMRT), decreased the XRE binding affinity, as confirmed by a gel electrophoretic mobility shift assay (EMSA) [75, 98]

The probable presence of a negative feedback and protective mechanism against TCDD toxicity is due to the huge intra- and inter-species variances in the susceptibility to TCDD toxicity. For instance, it has been shown that the HW rat strain is 1000-fold less sensitive to TCDD toxicity than the LE strains [99]. These annotations propose the presence of a negative cellular factor that is involved in the transcriptional control of AhR-regulated genes, perchance AhR repressor (AhRR) (Fig. 1.2). AhRR, which shares structural similarities with AhR and ARNT, dimerizes with ARNT and thus may strive with the AhR to bind XRE. The subsequent AhRR-ARNT complex is not capable of transactivating gene expression, but capable of binding with XRE [12, 77, 99, 100]. Remarkably, real time-polymerase chain reaction (RT-PCR) quantitative analysis of the constitutive expression of AhRR mRNA in LE [99] and AhR WT [101] mice showed a 3- and 5-fold higher expression in the heart than in the liver, respectively. In addition, it has been suggested that AhRR may facilitate AhR degradation through enhancing the release of AhR-ARNT heterodimer from the XRE sequence, resulting in repression of AhR function [102].

1.3.4. Physiological and Toxicological Consequences of AhR Activation

AhR possesses xenobiotic-independent functions, suggested from studies conducted on the AhR regulation and expression. Though AhR has been associated with several disorders of environmental etiology as well as atherosclerosis, chloracne, immunosuppression, thymic atrophy, and malignancies [103, 104], it has numerous physiological functions, as well, such as cell proliferation, apoptosis, and embryogenesis [104].

Initial pathological studies using knockout mice resulted in embryonic death when the ARNT expression was eliminated using a null allele; however elimination of AhR expression resulted in pathology of several organs, but not in death [8, 105, 106]. This suggests that the AhR-ARNT-mediated signaling pathway plays a dynamic role in quite a few organ systems. Contradictory data on the role of AhR in cell cycle advancement has been reported in a cell type-dependent method. For instance, it has been made known that AhR inhibited the human breast cancer MCF7 cells growth, while promoting HepG2 cell proliferation [107]. Furthermore, it has been confirmed that AhR is involved in the regulation of normal liver growth [8] and development of prostate, thymus, and ovaries [108], and is necessary for normal developmental closure of the ductus venous [109]. Also, AhR–null female mice showed complications in upholding normal pregnancy [110], suggesting that AhR in the reproductive system has a physiological role. Additionally, Hushka and coworkers showed that AhR is involved in the development of mammary gland, yet ligand-dependent activation of AhR overwhelms this process independently of CYP1A1 or CYP1B1 induction [111].

A large-scale mortality analysis study involving 5132 chemical workers routinely exposed to PAHs and HAHs showed a statistically significant trend for the development of cancer and various diseases [112]. AhR has been shown to induce renal disorders, such as hydronephrosis and reduced kidney size, in WT, but not in AhR-deficient, mice [113]. The role of AhR in carcinogenesis and tumor promotion is recognized. Acute TCDD toxicity has been shown to act as a potent tumor promoter in a model of liver cancer and to act as a complete carcinogen in chronic toxicity studies [114]. BaP has been shown to be a potent carcinogen to experimental animals in that intrathecal administration of BaP to mice resulted in an increase in the covalent binding to the lung DNA [115], yet AhR-null mice exposed were resistant to the carcinogenic effect.

One of the anticipated mechanisms for the carcinogenic effects of PAHs and HAHs included increased CYP1-mediated metabolic bioactivation of these compounds or other carcinogens [7]. These findings were reinforced by the observations that metabolic activation of BaP to its carcinogenic metabolites was noticeably inhibited by antibodies to CYP1A1, although not affected by antibodies against CYP2E1 [116]. Furthermore, a correlation has been reported between CYP1 catalytic activity and the activation of BaP to its carcinogenic metabolites [117]. Altogether, the wide range of toxicities following AhR activation suggests a possibility of cross-talk amongst the AhR and other transcription factors.

1.4. Mechanisms Involved in the Modulation of AhR-Regulated Genes

1.4.1. Transcriptional Mechanisms

In general, the induction of AhR-regulated genes is primarily organized at the transcriptional level through activation of numerous transcription factors that bind to particular DNA sequences to begin gene transcription. Studies using actinomycin D (Act-D) and CHX, which inhibit AhR-regulated gene RNA and protein syntheses, respectively, suggest a transcriptional regulatory mechanism is involved in the induction of CYP1A1 [30]. Furthermore, recent studies have demonstrated that the superinduction of *Cyp1a1* gene by CHX or MG-132, a 26S proteasome inhibitor, is a transcriptional mechanism and reveals a change in the synthesis, rather than equilibrium, of Cyp1a1 mRNA [96, 118, 119].

In contrast, ARE-dependent transcriptional activation of *Nqo1* and *Gsta1* genes requires the activation of a CHX-sensitive transcription factor, Nrf2 [52, 65]. On this detail, it has been stated that treatment of Hepa 1c1c7 cells with CHX inhibited the newly synthesized Nqo1 mRNA but had no effect on the existing mRNA levels [67, 120, 121].

1.4.2. Post-Transcriptional Mechanisms

The balance between the rate of mRNA synthesis and the rate of mRNA degradation is referred to as a steady state mRNA levels, hence changing the rate of mRNA degradation will absolutely affect its steady state concentration and regulate how rapidly it can be expressed [122]. The

exonucleases that catalyze mRNA mainly control mRNA decay, while sheltered by a specific terminal structure poly(A) tail at the 3' end [123]. Moreover, other studies have verified stabilizing mRNA through blocking the translation [123]. The constancy of CYP mRNA is usually mirrored by their mRNA half-lives ($t_{1/2}$). A prior study on HepG2 cells examining the decay of CYP1A1, CYP1A2, and CYP1B1 mRNAs after treatment with TCDD showed that CYP1A2 and 1B1 are prolonged CYPs with estimated half life ($t_{1/2}$ >24 h) [122].

Post-transcriptional regulatory intonations of the AhR-regulated genes are still not understood. Lee and Safe have verified that inhibition of CYP1A1 mRNA expression in T47D cells in response to resveratrol, a polyphenolic plant extract, is due to increased rate of CYP1A1 mRNA degradation [124].

1.4.3. Translational and Post-Translational Mechanisms

Post-translational modification could be defined as any variance between functional protein and linear polypeptide sequence encoded between the initiation and the termination codons of the structural gene [125]. Almost all of these amino acid modifications happen after release of polypeptide from the ribosome throughout the biosynthesis of proteins [125]. Modifications like these include noncovalent incorporation of cofactors to form an oligomeric protein and covalent modification that takes in cleavage of single peptide and/or altering amino acid residues, such as phosphorylation, glycosylation, and methylation [26].

1.4.3.1. Phsophorylation

A reversible cellular process responsible for transfer of phosphate from adenosine triphosphate (ATP) molecule to the acceptor protein via protein kinases and phosphatases, this is referred to as protein phosphorylation [125]. Some of the most common phosphorylated amino acids are Ser, Thr, and Tyr [125]. In the 1980s, Pyerin and coworkers were the first to validate the likely involvement of phosphorylation in the modulation of CYP genes. The first confirmation was reinforced by the finding that joining purified rabbit CYP2B4 with purified protein kinase A (PKA) phosphorylated theSer128 amino acid [126, 127]. Also, the topological localization of CYP and PKA in the same cellular endomembrane fraction give supplementary support to the possibility that CYP2B4 will be a substrate of PKA [128].

Additionally, it has been made known that incubation of hepatocytes isolated from phenobarbital-treated rats, to induce CYP2B1/2, with glucagon, as a stimulant of PKA and c-AMP, resulted in increased incorporation of radiolabeled phosphate in CYP2B1/2 enzymes, as verified by Western blot analysis [129]. Yet, this was complemented by a marked decrease in the catalytic activity, while neither a change in CYP2B1 protein levels nor an increase in enzyme inactive P420 forms were detected, which proposes that the loss of activity is not facilitated through phosphorylation-dependent degradation of protein [130].

Further studies on phosphorylation of CYP2E1 showed debatable results. It has been shown that c-AMP-dependent phosphorylation produced an intense decrease in both activity and protein

degradation, though PKA-dependent phosphorylation of CYP2E1 leads to a marked decrease in the activity without an increase in the rate of protein degradation [131].

In contrast to the effect of phosphorylation on CYP2B1/2 or 2E1, CYP1A1 and 1A2-formed metabolites in the rat liver hepatocytes were significantly lowered by Ser/Thr protein phosphatase inhibitor and by ortho-vanadate [89, 126], Nevertheless, no absorption of radiolabeled ATP pool into CYP1A1 or CYP1A2 was observed. These observations suggest strongly that the AhR could experience fluctuations upon PKA activation. This was reinforced by the observations that treatment of Hepa 1c17 cells with c-AMP resulted in activation of AhR and subsequent translocation to the nucleus [89]. Moreover, studies on the regulation of CYP1A1 displayed that AhR-ARNT heterodimerization entails phosphorylation of only ARNT, where binding of the AhR-ARNT to XRE involves phosphorylation of both AhR and ARNT proteins [132, 133].

1.4.3.2. Total Heme Content

Enhancement or suppression of CYP enzymatic activity levels could be caused by the cellular heme contents. Numerous studies have shown that the mechanism of CYP monooxygenase induction is attributed to enhancing of δ-aminolevulinate synthase, which is considered a rate-limiting step in the biosynthesis of heme [134]. Also, modulation of the expression of HO-1, a rate-limiting step in the heme degradation, has been shown to alter cellular heme content and hence the enzyme activity [135]. As a result, we can say that the stability between these two pathways could govern the level of CYP enzyme activity.

HOs are known to be stress-responsive enzymes that catalyze the degradation of the porphyrin ring to yield bilivurdin, free heme iron, and carbon monoxide [135]. There are three different HO isoenzymes (HO 1-3) that have been identified to date, and are ubiquitously expressed in a wide range of mammalian tissues [134, 135]. Amongst those, HO-1 is the inducible form that anchored to the endoplasmic reticulum membrane via a stretch of hydrophobic residues at the C-terminus [136]. Expression of HO-1 can be induced by oxidative stress stimuli, such as hypoxia, inflammation, heavy metals, and hydrogen peroxide.

1.4.4. Oxidative Stress

It has been shown that AhR-mediated toxicological effects can be mediated through the oxidative stress, which is described as an increase in cellular oxidation state to create an oxidative stress response. Hence, increased production of ROS and activation of several redox-sensitive transcription factors can directly regulate the expression of AhR-regulated genes.

1.4.4.1. ROS

Reactive Oxygen Species (ROS) production has been presented to be one of the mechanisms by which CYP1A1 induction leads to toxicity. For instance, it has been previously reported that CYP catalytic cycle is associated with generation of H_2O_2 that can be released by monoxygenase enzymes causing oxidative stress [137]. Consequently, this oxidative stress causes oxidation of

more than a few biological macromolecules, for instance DNA and proteins. This is supported by the annotations that TCDD and BaP cause oxidative stress in various tissues [31].

Additionally, it has been shown that AhR ligands increase ROS production in Hepa 1c1c7 cells through an AhR-dependent mechanism. This showed no effect on mRNA or protein expressions but demonstrated a decrease in the Cyp1a1 catalytic activity [137]. The AhR ligand-mediated decrease in Cyp1a1 activity was reversed by the antioxidant *N*-acetylcysteine; this tells us that there is a role for ROS in the modulation of AhR-regulated genes [137]. Subsequently, generation of ROS by Cyp1a1 activates NF-κB signaling pathway that is well known to suppress AhR activation due to multiple mechanismscausing the suppression of AhR-regulated genes transcription[31].

1.4.4.2. Cross-Talk between AhR and Redox-Sensitive Transcription Factors

With the wide array of toxic responses to PAHs and HAHs that range from cell proliferation to carcinogenesis, that some are not directly AhR-dependent, and numerous reports have suggested that AhR is likely to interact with other transcription factors to cause such varied effects [138]. Even though some of PAHs provoke irreplaceable signal transduction pathways, it has been presented that most of these ligands could trigger other common signaling pathways in the cells. Roughly, more than 20 redox-sensitive transcription factors have been identified and characterized [139]. These transcription factors are accountable for changes in the redox status of the cell in response to stimulants. Amongst these factors, Nrf2, NF-κB, and AP-1 have been

shown to influence expression of several genes that alter the activity of many metabolic processes.

1.4.4.2.1. Nrf2

As a result from an XRE-independent process, recent studies have demonstrated that gene expression of phase II metabolizing enzymes gene expressions are regulated by a labile protein transcriptional factor, Nrf2. These studies have characterized Nrf2 as the central transcription factor involved in the regulation and expression of many antioxidants and detoxifying phase II enzymes, such as NQO1 and GSTA1, against oxidative damage. In Nrf2-null mice, it has been shown that inducible, but not constitutive, *Nqo1* and *Gstp* gene expressions were eliminated, although in AhR- and Nrf2-double knockout mice, both constitutive and inducible expressions of *Nqo1* and *Gstp* genes were completely inhibited [140, 141]. These results not only support the notion that AhR- and Nrf2- mediated pathways could play an fundamental role in the regulation of *Nqo1* and *Gsta1* genes, but also propose the existence of cross-talk between these pathways. This conclusion was reinforced by the annotations of Ma and Marchand who showed that *Nrf2* gene expression is directly regulated through AhR activation and the *NQO1* gene expression is controlled by CYP1A1 activity [67, 142].

More than a few pieces of evidence support a direct relationship between AhR and Nrf2. First, *Nrf2* is a target gene for the AhR, in which three functional XRE and two ARE have been identified in the mouse, rat, and human *Nrf2* promoter [53, 143]. This is supported by the annotations that TCDD increased Nrf2 protein levels in a time-dependent manner [53], and

studies using Nrf2-deficient cell, discovered that induction of NQO1 by TCDD requires functional Nrf2 [67]. Second, Nrf2 can be activated indirectly by Cyp1a1-generated extremely reactive electrophiles that mediate the induction of phase II enzymes such as NQO1 [53, 142]. Third, a direct interaction between ARE-XRE and Nrf2-ARE signaling pathways has been characterized. Sequence analysis of the enhancer region of mouse *Nqo1* showed that the presumed ARE and XRE sequences are located near each other, which suggests a probable functional overlap between their mediated signaling pathways [67]. Several scenarios have been proposed, in that ARE and XRE function as a combined response element to which both AhR and Nrf2 bind and mediate the induction of NQO1 by TCDD. In addition, AhR and Nrf2 may interact with each other directly or over a connecter protein; such interactions are mandatory for induction of NQO1 by TCDD [67, 79, 143].

1.5. Heavy Metals

Even though there are several studies inspecting the toxic effects of individual AhR ligand forms, there are relatively few reports of the shared toxic effects of AhR ligands and other environmental co-contaminants. Among these, environmental cocontaminants of most concern are heavy metals, represented by arsenite (As^{3+}), cadmium (Cd^{2+}), and chromium (Cr^{6+}). Heavy metals are found in air, water, soil, and food. Both As^{3+} and Cd^{2+} are among the top seven most hazardous environmental contaminants listed in the Agency for Toxic Substances and Disease Registry (ATSDR) [144]

Generally, metals are considered the oldest toxic substances known to humans. Lead (Pb^{2+}) is possibly the oldest metal, used since 2000 B.C [145]. Amongst metals, heavy metals are defined as the metallic elements that are able to form polyvalent cations and retain a molecular size of more than 50 Da [146]. According to the physiological and toxicological effects of heavy metals, they have been classified into four classes [146]. Class A includes heavy metals such as iron that play a vital role on physiological functions such as enzyme activities. Class B consists of heavy metals, such as strontium, which have no physiological role, yet are slightly toxic in very low concentrations. Heavy metals that are necessary for living systems, such as zinc, nickel, and copper (Cu^{2+}), belong to class C; however, they are considered very toxic at moderately high concentrations. Finally, class D consists of heavy metals that are highly toxic at very low concentrations but have no biological functions, such as As^{3+}, Hg^{2+}, Pb^{2+}, and Cd^{2+} [146, 147].

The frequent persistent incidence and buildup of heavy metals in the environment and their possible exposure to humans, from several sources, including contaminated air, water, soil and food, make them ranked highly as the most hazardous and toxic substances in the environment by the ASTDR [148] and the Canadian Environmental Protection Act Registry (CEPA) [149]. Among all heavy metals, Hg^{2+}, Pb^{2+}, and Cu^{2+} ranked the highest in these lists.

1.5.1. Cr^{6+}

Chromium is naturally rich in the earth's crust, mainly in the form of Cr^{3+} [150], while various industrial processes using chromite ore release chromium into the atmosphere in the form of Cr^{6+}. Chromite ore is converted into sodium chromate and dichromate to be used as an anticorrosive agent in cooking instruments; also for the production of chromium alloys, chrome pigment, chrome salts for tanning leather, and wood preservation (Klaassen 2001). The main

source of Cr^{6+} in human exposure is through food, as it is estimated that humans ingest less than 100 μg of Cr^{6+} daily (Klaassen 2001). In areas of low Cr exposure, average blood Cr^{6+} levels are 20-30 μg/L, a level that is markedly increased in severely polluted regions (Klaassen 2001).

Cr^{3+} and Cr^{6+} both possess different biological and chemical properties, yet still both are of significance to the well-being of human health. Cr^{3+} is required as an essential mineral for the maintenance of normal carbohydrate metabolism [150] as it is supposed to improve insulin-stimulated insulin receptor tyrosine kinase activity (Davis and Vincent, 1997). Intrinsically, The Food and Nutrition Board of the National Research Council (NRC) has recommended a safe and sufficient intake of chromium of 50-200 μg/day (National Research Council 1989).

The human body's absorption of chromium compounds differs depending on the chemical species, ranging from 0.5-2% for dietary Cr^{3+} compounds, to 2-10% for chromates (CrO_4^{2-}). Overall the oral absorption of Cr^{3+} compounds is much lower than Cr^{6+}compounds. Chromates go into the cells through anion channels whereas Cr^{3+} compounds are absorbed by passive diffusion and phagocytosis. Absorption also takes place through the lungs and dermal routes but is commonly very poor. Once absorbed, chromium compounds are distributed to all of the body organs and are eventually excreted in the urine with a half-life of 30-40 h [151].

While minimal amounts of Cr^{3+} are absorbed orally, an oral dose of 50-70 mg/kg body weight of chromates is considered fatal in humans. Death usually happens due to severe diarrhea and

hemorrhage into the gastrointestinal tract, caused by the corrosive nature of Cr^{6+}, which if it occurs causes cardiovascular shock ([150], [152]). Liver and kidney necrosis can also be induced if a lethal oral dose of Cr^{6+} is consumed [153]. Allergic contact dermatitis occurs with exposure to Cr^{6+} and is not dependent on the dose [154]. Allergic reactions also occur in the respiratory tract, stimulated in the form of asthma, rhinitis, bronchospasm, and pneumonia. Chronic exposure to Cr^{6+} is also associated with the materialization of tumors of the respiratory tract ([150], [153]).

1.5.2. Cr^{6+} toxicity and oxidative stress

All chromium toxicities that are known are due to the hexavalent form. Cr^{3+} does not enter the cells as readily as Cr^{6+}. At physiological pH, Cr^{6+} occurs as a chromate ion, with an overall -2 charge, resembling sulfate and phosphate ions which explains why it is transported across the cell membranes via the anionic transporters [155]. Cr^{6+} is in due course is reduced to Cr^{3+} by various mechanisms that include glutathione and cysteine, ascorbic acid (AscA), Cyp450, hemoglobin, and glutathione reductase [156]. Eventually, all Cr^{6+} is converted to Cr^{3+} , but the nature and concentration of the reducing agent determines whether Cr^{6+} is converted straight to Cr^{3+} or whether it is converted to numerous intermediates, such as Cr^{5+} and Cr^{4+}, before being converted to Cr^{3+} [157]. Interestingly, Cr^{6+}-induced DNA strand breaks, DNA-DNA and DNA-protein cross-links do not take place in cell-free systems in the absence of reducing agents [150]. While Cr^{3+} interacts straight with phosphate groups and nitrogen bases in DNA [158], the formation of ROS during the reduction procedure is believed to mediate the detected genotoxicity [159]. O_2^- and H_2O_2 are formed when Cr^{6+} is reduced to Cr^{5+}. This also suggested that Cr reduction intermediates may undergo Haber-Weiss and Fenton-type reactions, resulting

in the production of O_2^-, OH, and OH^-. Hypothetical reactions that comprise the different valence forms of Cr include ([160], [159]):

$Cr^{6+} + GSH \rightarrow GS^{\cdot} + Cr^{5+}$

$GS^{\cdot} + GSH \rightarrow GSSG^{\cdot-} + H^+$

$GSSG^{\cdot-} + O_2 \rightarrow O_2^{\cdot-} + GSSG$

$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$

$Cr^{3/4/5/6+} + O_2^{\cdot-} \rightarrow Cr^{3/4/5+} + O_2$

$Cr^{3/4/5+} + H_2O_2 \rightarrow Cr^{4/5/6+} + OH^{\cdot} + OH^-$

1.6. Rationale, Hypotheses and Objectives

1.6.1. Rationale

Classical AhR ligands typified by HAHs and PAHs produce a wide range of toxic effects. AhR a cytosolic receptor to which these contaminants bind has been shown to mediate most of the toxic effects produced by these hazardous contaminants. Once bound to the AhR, these AhR ligands induce the transcription of CYPs responsible for their metabolism into toxic intermediates. These toxic intermediates then act as second messengers for the induction of phase II drug metabolizing enzymes. Thus, the toxicity of these AhR ligands cannot be assessed by measuring CYP induction alone, because it has been shown that these AhR ligands will also induce the phase II

AhR regulated genes as a counterproductive mechanism to this process. Even though numerous studies have examined the toxic effects of individual AhR ligands, there are relatively few reports of the combined toxic effects of AhR ligands and other environmental contaminants, typified by heavy metals. Hence, it was of great importance to evaluate the combined toxic effects, in particular the AhR-driven carcinogenicity and mutagenicity of these AhR ligands typified by TCDD and Cr^{6+}, a common co-contaminant of TCDD.

1.6.2. Hypotheses

Hypothesis 1: co-exposure to Cr^{6+} and TCDD disrupts the coordinated balance of AhR-regulated genes *in vivo* in C57BL/6 mice and *in vitro* using human HepG2 cells.

Hypothesis 2: co-exposure to $Cr^{6+}$and TCDD *in vivo* alters AhR-regulated genes in a time-, tissue-, and AhR-regulated gene-dependent manner.

Hypothesis 3: Cr^{6+} differentially modulates the exemplary phase I and II AhR-regulated genes *CYP1A1* and *NQO1* through affecting their upstream signaling pathways.

1.6.3. Specific Objectives

1- To determine the possible effects of Cr^{6+} on the TCDD-mediated induction of Cyp1a1 *in vivo* in C57BL/6 and *in vitro* using human HepG2 cells and to investigate the underlying molecular mechanisms involved in this alteration.

2- To determine the effects of Cr^{6+} on AhR- regulated genes in extrahepatic tissues: kidney and lung.

3- To determine the effect of Cr^{6+} on NQO1 and HO-1 in C57BL/6J, and to investigate the underlying molecular mechanisms involved in this alteration.

1.6.4. Significance

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

Chapter 2- Materials and Methods

2.1. Chemicals

Chromium trioxide (Cr^{6+}), 3-methylcholanthrene (3MC), β-nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), bovine serum albumin, cumene hydroperoxide (CHP), 1-chloro-2,4-dinitrobenzene (CDNB), Dulbecco's Modified Eagle's Medium (DMEM), 7-ethoxyresorufin (7ER), fluorescamine, glucose, reduced glutathione, glutathione reductase, 2,6-dichlorophenolindophenol (DCPIP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 7-methoxyresorufin (7MR), protease inhibitor cocktail, 1,9-pyrazoloanthrone (SP600125), phorbol 12-myristate 13-acetate (PMA), pyrrolidinedithiocarbamate (PDTC), diethyl pyrocarbonate (DEPC), polyvinylpyrrolidone, phenylmethanesulphonylfluoride (PMSF), and anti-goat IgG peroxidase secondary antibody were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). Tris hydrochloride, agarose, formamide and sodium azide were purchased from EM Science (Gibbstown, NJ). Tween-20 was purchased from BDH Inc. (Toronto, ON). Cupric sulfate, amphotericin B, resorufin, β-naphthoflavone (βNF), dithiothreitol (DTT), and 100× vitamin supplements were purchased from ICN Biomedicals Canada (Montreal, QC). Gentamicin sulfate, penicillin–strepromycin, L-glutamine, MEM non-essential amino acids solution, fetal bovine serum, TRIzol reagent, T4 polynucleotide kinase, and the random primers DNA labeling system were purchased from Invitrogen Co. (Grand Island, NY). Hybond-N-nylon membranes, poly(dI.dC), and chemiluminescence Western blotting detection reagents were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Bromophenol blue, β-mercaptoethanol, glycine, acrylamide, *N′N′*-bis-methylene-acrylamide, ammonium persulphate, nitrocellulose

membrane (0.45 μm), sodium dodecyl sulfate (SDS), and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA). CYP1A1/1A2 goat anti-mouse polyclonal primary antibody (G-18, and anti-rabbit IgG peroxidase secondary antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Cyp1b1 polyclonal primary antibody was purchased from BD Biosciences (Mississauga, ON). Rabbit NQO1 anti-human polyclonal primary antibody was generously provided by Dr. David Ross, University of Colorado Health Sciences Center (Denver, CO). HO-1 mouse monoclonal primary antibody was purchased from Abcam (Cambridge, MA, USA). Skim milk was obtained from DIFCO Laboratories (Detroit, MI). All other chemicals were purchased from Fisher Scientific Co. (Toronto, ON).

2.2. Methods

2.2.1. Chemical treatments of *in vitro* HepG2 cells

Cells were treated in serum free medium only or with an AhR ligand dissolved in DMSO, metals dissolved in double de-ionized water, or both. The metal was added 30 min prior to treatment with an AhR ligand, when applicable. The AhR ligands were maintained in DMSO at -20 °C until use, but fresh metals were prepared for each treatment. In all treatments, the concentration of double de-ionized water in the treatment medium did not exceed 0.5% (v/v) while the DMSO concentration did not exceed 0.05% (v/v). For enzyme activity assays, the duration of chemical exposure was 24 h. RNA was extracted only 6 h after chemical treatment.

2.2.2. Animal Model

Male C57BL/6 (22–30 g) mice were obtained from Charles River, Canada (Montreal, QC, Canada). Animals were group-housed under standard conditions, three to five mice per cage with food and water available at any point 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.

2.2.3. Animal Treatment

Animals were injected intraperitoneally (i.p.) with Cr^{6+} (as hexavalent chromium dissolved in saline) at 20mg/kg in the absence and presence of 15 μg/kg TCDD (dissolved in corn oil) injected i.p. The mice were divided into 4 groups. The first group (n=12) (control mice) received saline (0.4 mL) plus corn oil (0.4 mL). The second group (n=12) (Cr^{6+} treated mice) received Cr^{6+} 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) (Cr^{6+} plus TCDD treated mice) received Cr^{6+} dissolved in saline (0.4 mL) plus TCDD dissolved in corn oil (0.4 mL). Thereafter, the animals were euthanized after a single injection at 6 h (n=6) and 24 h (n=6) via cervical dislocation. Liver, heart, lung, and kidney tissues were

harvested, instantly frozen in liquid nitrogen, and stored at -80°C until further analysis. All experimental procedures involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee.

2.2.4. RNA Extraction and cDNA Synthesis

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm. RNA quality was determined by measuring the 260/280 ratio. Thereafter, first strand cDNA synthesis was performed by 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 3.2 µl of nuclease-free water. The final reaction mix was kept at 25°C for 10 min, heated to 37°C for 120 min, heated for 85°C for 5 s, and finally cooled to 4°C.

2.2.5. Quantification by Real-Time PCR

Quantitative analysis of specific mRNA expression was performed using real-time PCR by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems). The 25-µl reaction mix contained 0.1 µl of 10 µM forward primer and 0.1 µl of 10 µM reverse primer (40 nM final concentration of each primer), 12.5 µl of SYBR Green Universal Mastermix, 11.05 µl of nuclease-free water, and 1.25

µl of cDNA sample. The primers used in the current study are listed in Table 1. Assay controls were incorporated onto the same plate, namely no-template controls, to test for the contamination of any assay reagents. After sealing the plate with an optical adhesive cover, the thermocycling conditions were initiated at 95°C for 10 min, followed by 40 PCR cycles of denaturation at 95°C for 15s and annealing/extension at 60°C for 1 min. Melting curve (dissociation stage) was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product.

2.2.6. Real-Time PCR Data Analysis

The real time-PCR data were analyzed using the relative gene expression i.e. ($\Delta\Delta C_T$) method as described in Applied Biosystems User Bulletin No.2 and explained further by Livak and Schmittgen [38]. Briefly, the ΔC_T values were calculated in every sample for each gene of interest as follows: $C_{T\ \text{gene of interest}} - C_{T\ \text{reporter gene}}$, with β-actin as the reporter gene. Calculation of relative changes in the expression level of one specific gene ($\Delta\Delta C_T$) was performed by subtraction of ΔC_T of control (untreated control) from the ΔC_T of the corresponding treatment groups. The values and ranges given in different figures were determined as follows: $2^{-\Delta\Delta CT}$ with $\Delta\Delta C_T$ + S.E. and $\Delta\Delta C_T$ – S.E., where S.E. is the standard error of the mean of the $\Delta\Delta C_T$ value.

2.2.7. Determination of Cyp1a1 and Cyp1a2 Enzymes Activities

EROD and MROD activities were performed on subcellular fractions, as previously described[161]. Microsomes from livers of different treatments (1 mg protein/ml) were incubated

in the incubation buffer (5 mM magnesium chloride hexahydrate dissolved in 0.5 M potassium phosphate buffer pH = 7.4) at 37 °C in a shaking water bath (50 rpm). A pre-equilibration period of 5 min was performed. The reaction was initiated by the addition of 1 mM NADPH. The concentrations of substrate were 2 μM of 7-ethoxyresorufin or 7-methoxyresorufin for Cyp1a1 and Cyp1a2, respectively. After incubation at 37 °C (5 min for EROD, and 10 min for MROD assays), the reaction was stopped by adding 0.5 ml of cold methanol. The amount of resorufin formed in the resulting supernatant was measured using the Baxter 96-well fluorescence plate reader using excitation and emission wavelengths of 545 and 575 nm, respectively. Formation of resorufin was linear with incubation time and protein amount. Enzymatic activities were expressed as picomoles of resorufin formed per minute and per milligram of microsomal proteins.

2.2.8. Determination of Nqo1 Enzyme Activity

Nqo1 activity were determined by quantitation of the reduction rate of DCPIP using the continuous spectrophotometric assay of Ernster [56, 162] which quantitates the reduction of its substrate DCPIP. Approximately 10 μg cell homogenate protein was incubated with 1 ml of the assay buffer (40 μM DCPIP, 0.2 mM NADPH, 5 μM FAD, 25 mM Tris–HCl, pH 7.8, 0.1% (v/v), Tween-20, and 0.023% bovine serum albumin), and the rate of DCPIP reduction was monitored over 1.5 min at 600 nm with an extinction coefficient (ε) of 2.1 mM^{-1} cm^{-1}. Nqo1 activity was calculated as the decrease in absorbance per min per mg total protein of the sample.

2.2.9. Cell culture

HepG2 cell line, ATCC number HB-8065 (Manassas, VA), was maintained in Dulbecco's modified Eagle's medium (DMEM) with phenol red, supplemented with 10% heat-inactivated fetal bovine serum, 20 μM L-glutamine, 50 μg/ml amikacin, 100 IU/ml penicillin, 10 μg/ml streptomycin, 25 ng/ml amphotericin B, 0.1 mM non-essential amino acids, and vitamin supplement solution. Cells were grown in 75-cm^2 cell culture flasks at 37 °C in a 5% CO_2 humidified incubator.

2.2.10. Chemical treatments

Cells were treated in serum free medium with various concentrations of Cr^{6+} (1 - 25 μM) in the absence and presence of 1 nM TCDD. TCDD was dissolved in dimethylsulfoxide (DMSO) and maintained in DMSO at −20 °C until use. Cr^{6+} was prepared freshly in double de-ionized water. In all treatments, the DMSO concentration did not exceed 0.05% (v/v).

2.2.11. Effect of Cr^{6+} on cell viability

The effect of Cr^{6+} on cell viability was determined using the MTT assay as described previously [163]. MTT assay measures the conversion of MTT to formazan in living cells via mitochondrial enzymes of viable cells. In brief, HepG2 cells were seeded onto 96-well microtiter cell culture plates and incubated for 24 h at 37 °C in a 5% CO_2 humidified incubator. Cells were treated with various concentrations of Cr^{6+} (1-25 μM) in the absence and presence of 1 nM TCDD. After 24 h

incubation, the medium was removed and replaced with cell culture medium containing 1.2 mM MTT dissolved in phosphate buffered saline (PBS) (pH 7.4). After 2 h of incubation, the formed crystals were dissolved in isopropanol. The intensity of the color in each well was measured at a wavelength of 550 nm using the Bio-Tek EL 312e microplate reader (Bio-Tek Instruments, Winooski, VT).

2.2.12. RNA extraction and quantitative real-time PCR

After incubation with the test compounds for the specified time periods, total cellular RNA was isolated using TRIzol reagent, according to manufacturer's instructions (Invitrogen), and quantified by measuring the absorbance at 260 nm. For reverse transcription-polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized from 1.0 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit with random primers. Real-time PCR reactions were performed on an ABI 7500 real-time PCR system (Applied Biosystems), using SYBR® Green PCR Master Mix (Applied Biosystems). The amplification reactions were performed as follows: 10 min at 95°C, and 40 cycles of 94°C for 15 sec and 60°C for 1 min. Primers and probes for human CYP1A1 were: Forward primer 5`- CTA TCT GGG CTG TGG GCA A -3`, reverse primer 5`- CTG GCT CAA GCA CAA CTT GG -3`. Heme oxygenase-1 (HO-1): forward primer 5`- ATG GCC TCC CTG TAC CAC ATC -3`, reverse primer 5`- TGT TGC GCT CAA TCT CCT CCT -3` and for β-actin: forward primer 5`- CTG GCA CCC AGG ACA ATG -3`, reverse primer 5`- GCC GAT CCA CAC GGA GTA -3` were purchased from Integrated DNA technologies (IDT, Coralville, IA). The fold change in the level of *CYP1A1* or *HO-1* (target genes) between treated and untreated cells, corrected by the level of ß-actin, was determined

using the following equation: Fold change = $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{(target)} - Ct_{(\beta\text{-actin})}$ and $\Delta(\Delta Ct) = \Delta Ct_{(treated)} - \Delta Ct_{(untreated)}$.

2.2.13. Protein extraction and Western blot analysis

Twenty-four hours after incubation with the test compounds, cells were collected in lysis buffer containing 50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EDTA, 10 % (v/v) glycerol, 1 % Triton X-100, and 5 µl/ml of protease inhibitor cocktail. The cell homogenates were obtained by incubating the cell lysates on ice for 1 h, with intermittent vortexing every 10 min, followed by centrifugation at 12,000 × *g* for 10 min at 4 °C. Proteins (50 µg) were resolved by denaturing electrophoresis, as described previously [164]. Briefly, the cell homogenates were dissolved in 1X sample buffer, boiled for 5 min, separated by 10 % SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. Protein blots were blocked for 24 h at 4 °C in blocking buffer containing 5 % skim milk powder, 2 % bovine serum albumin and 0.05 % (v/v) Tween-20 in tris-buffered saline solution (TBS; 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris–base). After blocking, the blots were incubated with a primary polyclonal goat anti-rat CYP1A1 antibody for 2 h at room temperature, HO-1 primary monoclonal mouse anti-human antibody for overnight at 4 °C, or primary polyclonal rabbit anti-human GAPDH antibody for overnight at 4 °C in TBS containing 0.05% (v/v) Tween-20 and 0.02 % sodium azide. Incubation with a peroxidase-conjugated rabbit anti-goat IgG secondary antibody for CYP1A1 and GAPDH, or peroxidase-conjugated rabbit anti-mosue IgG secondary antibody for HO-1 was carried out in blocking buffer for 2 h at room temperature. The bands were visualized with the enhanced chemiluminescence method according to

manufacturer's instructions (Amersham, Arlington Heights, IL). The intensity of CYP1A1 and HO-1 protein bands was quantified, relative to the signals obtained for GAPDH protein, using ImageJ software.

2.2.14. Transient transfection and luciferase assay

HepG2 cells were plated onto 12-well cell culture plates. Each well of cells was transfected with 1.6 µg of XRE-driven luciferase reporter plasmid pGudLuc6.1, generously provided by Dr. M. S. Denison (University of California, Davies), using lipofectamine 2000 reagent according to manufacturer's instructions (Invitrogen). Luciferase assay was performed according to manufacturer's instructions (Promega) as described previously [165]. Briefly, after incubation with test compounds for 24 h, cells were washed with PBS and a 200 µl of 1X lysis buffer were added into each well with continuous shaking for at least 20 min; then the content of each well was collected separately in 1.5 ml microcentrifuge tubes. The tubes were then centrifuged to precipitate cellular waste, 100 µl cell lysate was then incubated with 100 µl of stabilized luciferase reagent and luciferase activity was quantified using TD-20/20 luminometer (Turner BioSystems).

2.3. Statistical Analysis

All results are presented as mean ± SEM. The comparative analysis of the results from various experimental groups with their corresponding controls was performed using SigmaStat for Windows, Systat Software Inc., (San Jose, CA). One-way analysis of variance (ANOVA)

followed by Student–Newman–Keul's test was carried out to assess which treatment groups showed a significant difference from the control group. The differences were considered significant when $p<0.05$.

Chapter 3 - Results

3.1. Effect of Cr^{6+} on the Expression of AhR-Regulated Genes

3.1.1. Effect of co-exposure to Cr^{6+} and TCDD on Cyp1a1 mRNA levels in the liver of C57Bl/6 mice

Our results showed that Cr^{6+} alone did not significantly affect Cyp1a1 mRNA levels in the liver (Fig.3.1.). On the other hand, TCDD alone significantly induced Cyp1a1 mRNA levels in the liver by 5,900-fold, compared to the control (Fig. 3.1.). When animals were co-exposed to Cr^{6+} and TCDD, Cr^{6+} significantly inhibited the TCDD-mediated induction of Cyp1a1 mRNA levels in the liver to 1153.5-fold, compared to control (Fig. 3.1.).

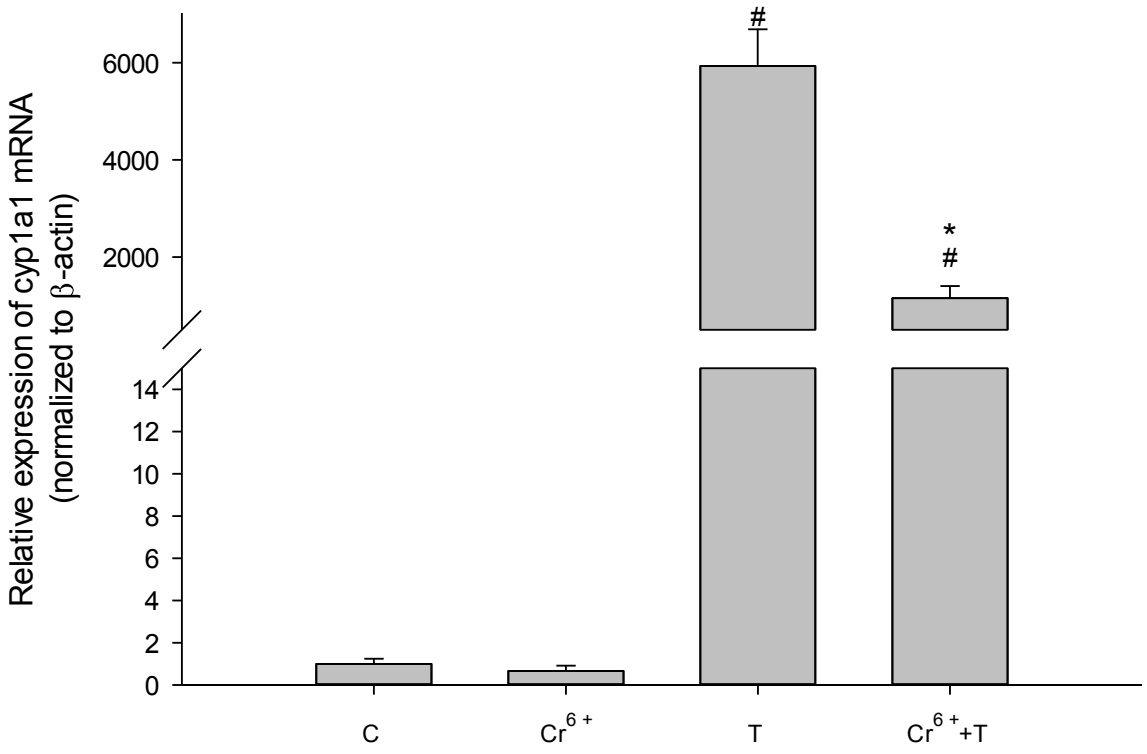

Fig. 3.1. Effect of co-exposure to Cr^{6+} and TCDD on Cyp1a1 mRNA levels in the liver of C57BL/6 mice.

Animals were injected i.p. with 20 mg/kg Cr^{6+} in the absence and presence of 15 μg/kg TCDD. First-strand cDNA was synthesized from total RNA (1.5 μg) extracted from liver and the expression of Cyp1a1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. (#)$P<0.05$, compared to control (C; vehicle treated animals); (*)$P<0.05$, compared to respective TCDD (T) treatment.

3.1.2. Effect of co-exposure to Cr^{6+} and TCDD on Cyp1a1 protein expression levels in the liver of C57BL/6 mice.

In an attempt to examine whether the observed effects at the mRNA levels will be further translated to the protein expression levels, we examined the effect of co-exposure to Cr^{6+} and TCDD at the protein expression level. Our results showed that Cr^{6+} alone did not significantly affect Cyp1a1 protein expression levels in the liver (Fig. 3.2.). TCDD alone significantly induced Cyp1a1 protein expression levels in the liver by 3.4-fold, respectively, compared to control (Fig. 3.2.). On the other hand when animals were co-exposed to Cr^{6+} and TCDD, Cr^{6+} significantly inhibited the TCDD-mediated induction of Cyp1a1 protein expression levels in the liver to 1.4-fold, respectively, compared to control (Fig. 3.2.).

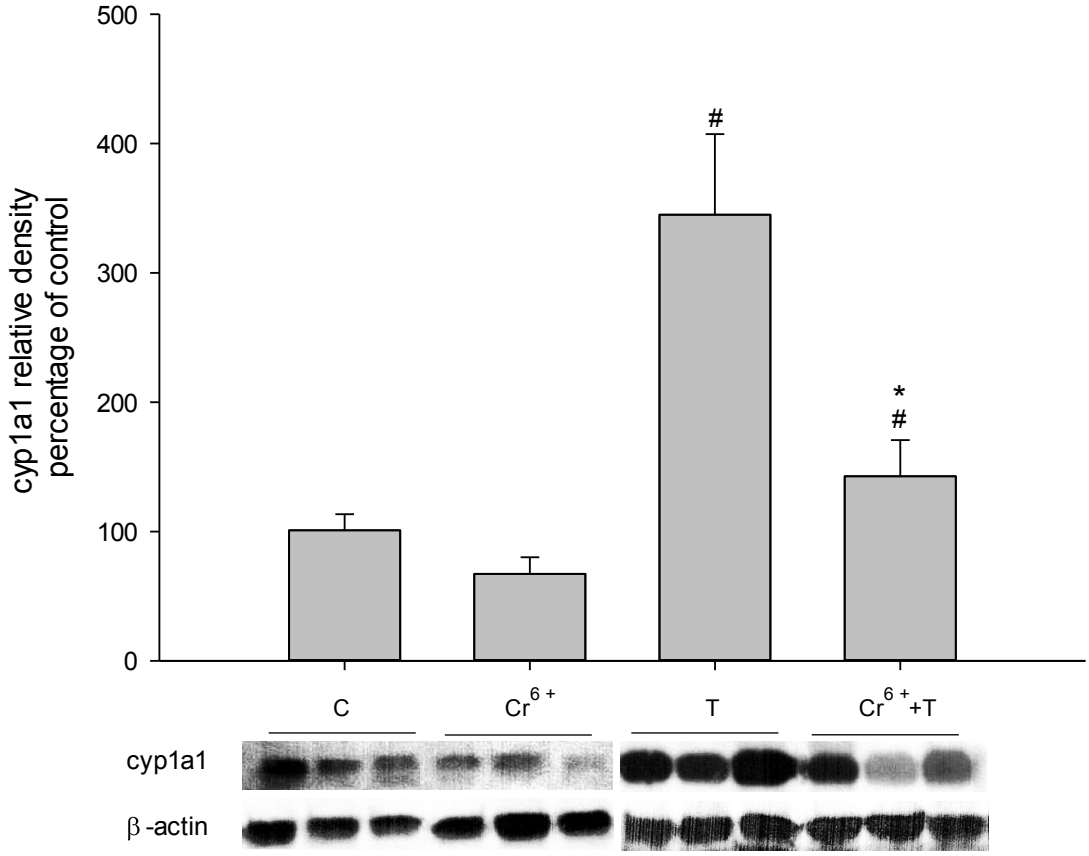

Fig. 3. 2. Effect of co-exposure to Cr^{6+} and TCDD on Cyp1a1 protein expression levels in the liver of C57BL/6 mice.

Liver microsomal proteins were isolated after 24 h of treatment. Thirty micrograms of microsomal protein were separated by 10% SDS–PAGE. Proteins were detected using the enhanced chemiluminescence method. The graph represents the relative amounts of protein normalized to actin signals (mean ± SEM, n=6), and the results are expressed as a percentage of the control values taken as 100%. Values are presented as the mean ± SEM (n=6). (#)$P<0.05$, compared to control (C; vehicle treated animals); (*)$P<0.05$, compared to respective TCDD (T) treatment.

3.1.3. Effect of co-exposure to Cr^{6+} and TCDD on EROD catalytic activity in the liver of C57BL/6 mice.

At the catalytic activity levels, Cr^{6+} alone did not significantly affect EROD activity in the liver (Fig. 3.3.). However, TCDD alone significantly induced EROD activity in the liver by 26.7-fold, compared to control (Fig. 3.3.). When animals were co-exposed to Cr^{6+} and TCDD, Cr^{6+} significantly inhibited the TCDD-mediated induction of EROD activity in the liver to 17.1-fold, compared to control (Fig. 3.3.).

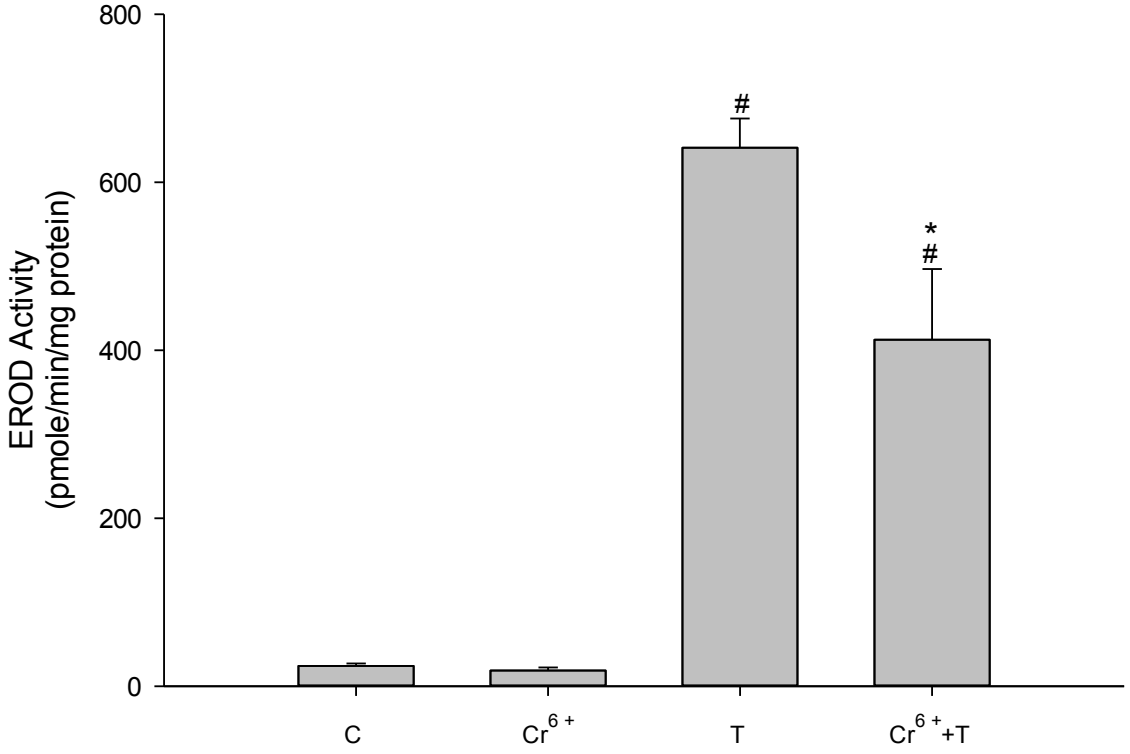

Fig. 3.3. Effect of co-exposure to Cr^{6+} and TCDD on EROD catalytic activity levels in the liver of C57BL/6 mice.

EROD activity was 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 acetonitrile. Values are presented as the mean ± SEM (*n*=6). (#)P<0.05, compared to control (C; vehicle treated animals); (*)P<0.05, compared to respective TCDD (T) treatment.

3.1.4. Effect of co-exposure to Cr^{6+} and TCDD on HepG2 cell viability

To determine the non-toxic concentrations of Cr^{6+} to be utilized in the current study, HepG2 cells were exposed for 24 h with increasing concentrations of Cr^{6+} (1 – 25 μM) in the absence and presence of 1 nM TCDD; thereafter cytotoxicity was assessed using the MTT assay. Figure 3.4. shows that Cr^{6+} at concentrations of 1 – 25 μM in the presence and absence of 1 nM TCDD did not significantly affect cell viability. Therefore, all subsequent studies were conducted using the concentrations of 1 – 25 μM.

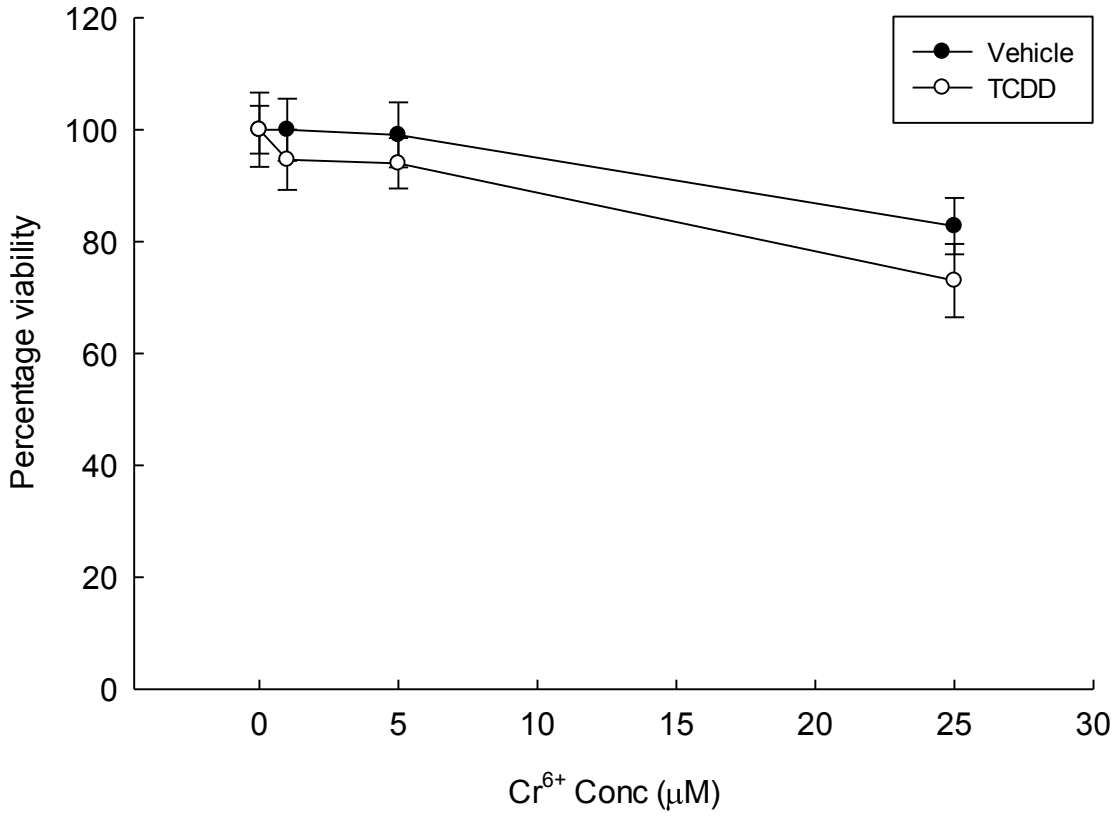

Fig. 3.4. Effect of Cr^{6+} on cell viability.

HepG2 cells were treated for 24 h with Cr^{6+} (0, 1, 5, and 25 μM) in the absence and presence of 1 nM TCDD. Cell cytotoxicity was determined using the MTT assay. Data are expressed as the percentage of untreated control (set at 100%) ± SEM (n=8).

3.1.5. Concentration-dependent effect of co-exposure to Cr^{6+} and TCDD on inducible CYP1A1 mRNA in HepG2 cells

To examine the effect of co-exposure to Cr^{6+} and TCDD on CYP1A1 mRNA, HepG2 cells were treated with various concentrations of Cr^{6+} in the absence and presence of 1 nM TCDD (Fig. 3.5.). Thereafter, CYP1A1 mRNA was assessed using real-time PCR. Cr^{6+} alone at all concentrations tested did not significantly affect CYP1A1 mRNA levels (Fig. 3.5.). TCDD alone significantly induced CYP1A1 mRNA levels by 36.5-fold, compared to control (Fig. 3.5.). Cr^{6+} at the concentrations of 1 μM and 5 μM did not significantly affect the TCDD-mediated induction of CYP1A1 mRNA. However, at the highest concentration tested, 25 μM, Cr^{6+} significantly inhibited the TCDD-mediated induction of CYP1A1 mRNA levels to 19.8-fold, compared to control (Fig. 3.5.).

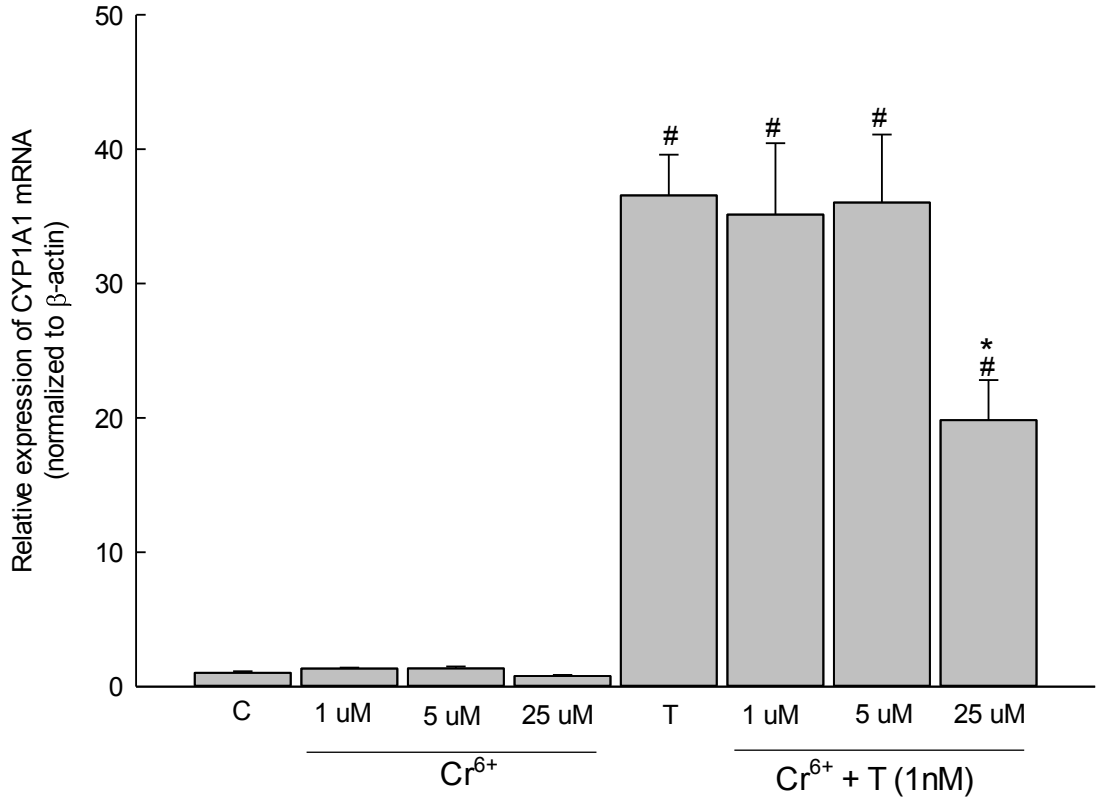

Fig. 3.5. Effect of Cr^{6+} on CYP1A1 mRNA levels in HepG2 cells.

HepG2 cells were treated with increasing concentrations of Cr^{6+} in the presence of 1 nM TCDD for 6 h for mRNA or 24 h for protein and catalytic activity. First-strand cDNA was synthesized from total RNA (1 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. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. Results were calculated as mean ± SEM (n = 6). (#) $P < 0.05$, compared to control (C; concentration 0 μM); (∗)$P < 0.05$, compared to respective TCDD treatment.

3.1.6. Concentration-dependent effect of co-exposure to Cr^{6+} and TCDD on CYP1A1 protein expression levels in HepG2 cells

To examine whether the observed inhibition of the TCDD-mediated induction of CYP1A1 mRNA by Cr^{6+} is further translated to the protein levels, HepG2 cells were treated for 24 h with increasing concentrations of Cr^{6+} in the presence of 1 nM TCDD. Similar to the observations at the mRNA levels, Cr^{6+} alone, at all tested concentrations, did not significantly affect CYP1A1 protein expression levels (Fig. 3.6.). TCDD alone significantly induced CYP1A1 protein expression levels by 3.8-fold, compared to control (Fig. 3.6.). Of interest, Cr^{6+} at 1 and 5 μM did not significantly affect the TCDD-mediated induction of CYP1A1 protein levels. Cr^{6+} at 25 μM significantly inhibited the TCDD-mediated induction of CYP1A1 protein expression levels to 1.2-fold, compared to control (Fig. 3.6.).

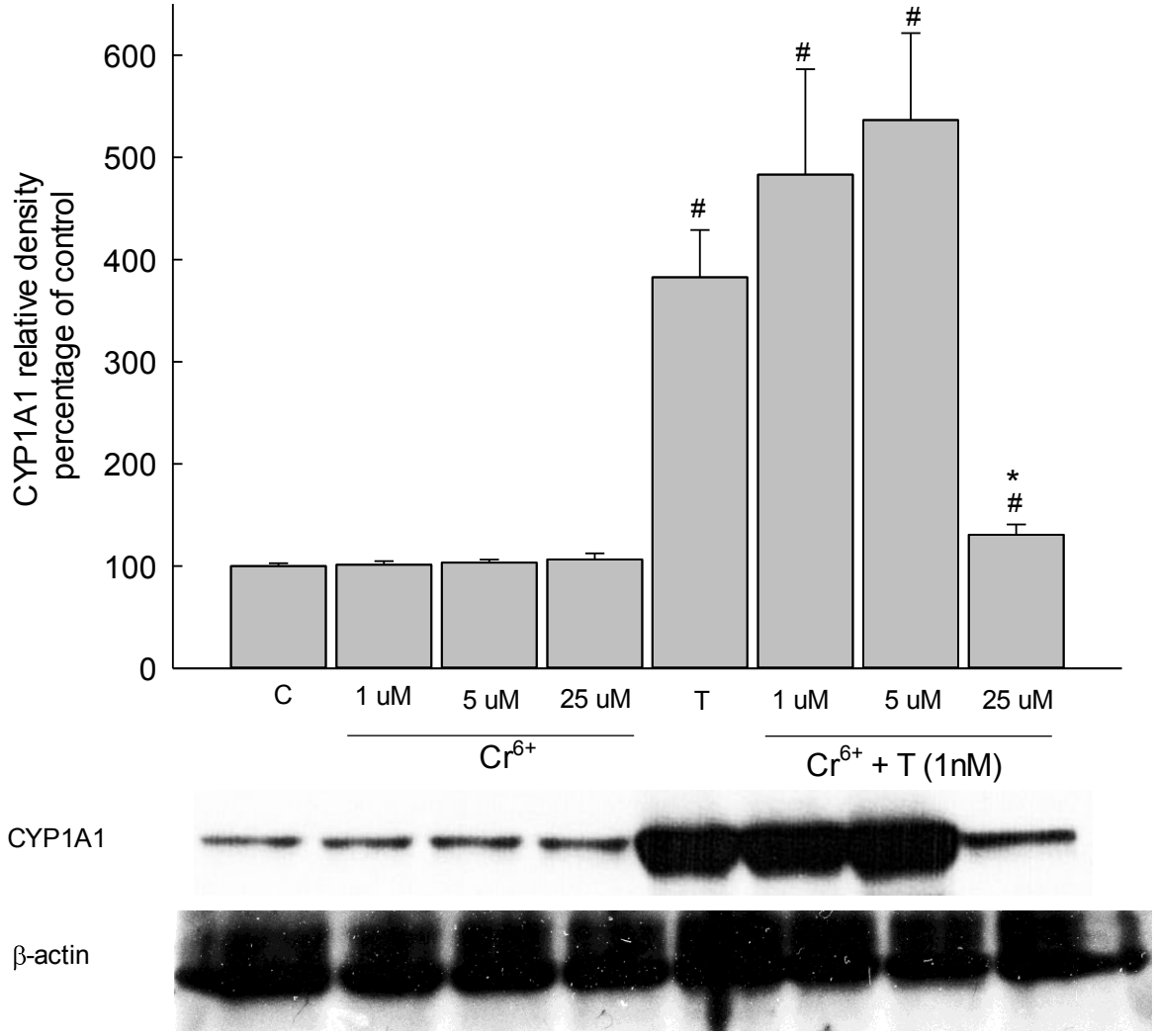

Fig. 3.6. Effect of Cr^{6+} on CYP1A1 proteinexpression levels in HepG2 cells.

Protein (50 μg) was separated by 10% SDS–PAGE and transferred to nitrocellulose membrane. Protein blots were blocked overnight at 4 °C and then incubated with a primary CYP1A1 antibody for 24 h at 4 °C, followed by 1 h incubation with secondary antibody at room temperature. CYP1A1 protein was detected using the enhanced chemiluminescence method. The intensity of the bands was normalized to β-actin signal, which was used as the loading control. One of three representative experiments is shown. Results were calculated as mean ± SEM (n = 6). (#) $P < 0.05$, compared to control (C; concentration 0 μM); (∗)$P < 0.05$, compared to respective TCDD treatment.

3.1.7. Concentration-dependent effect of co-exposure to Cr^{6+} and TCDD on CYP1A1 catalytic activity in HepG2 cells

To examine whether the observed inhibition of the TCDD-mediated induction of CYP1A1 mRNA by Cr^{6+} is further translated to activity, HepG2 cells were treated for 24 h with increasing concentrations of Cr^{6+} in the absence and presence of 1 nM TCDD. Cr^{6+} alone, at all concentrations tested, did not significantly affect CYP1A1 catalytic activity (Fig. 3.7.). TCDD alone caused a 52.7-fold increase in CYP1A1 catalytic activity. Cr^{6+} at 1 and 5 μM did not significantly affect the TCDD-mediated induction of CYP1A1 catalytic levels. On the other hand, Cr^{6+} decreased the TCDD-mediated induction of CYP1A1 catalytic activity levels only at 25 μM to 0.75-fold, compared to control (Fig. 3.7.).

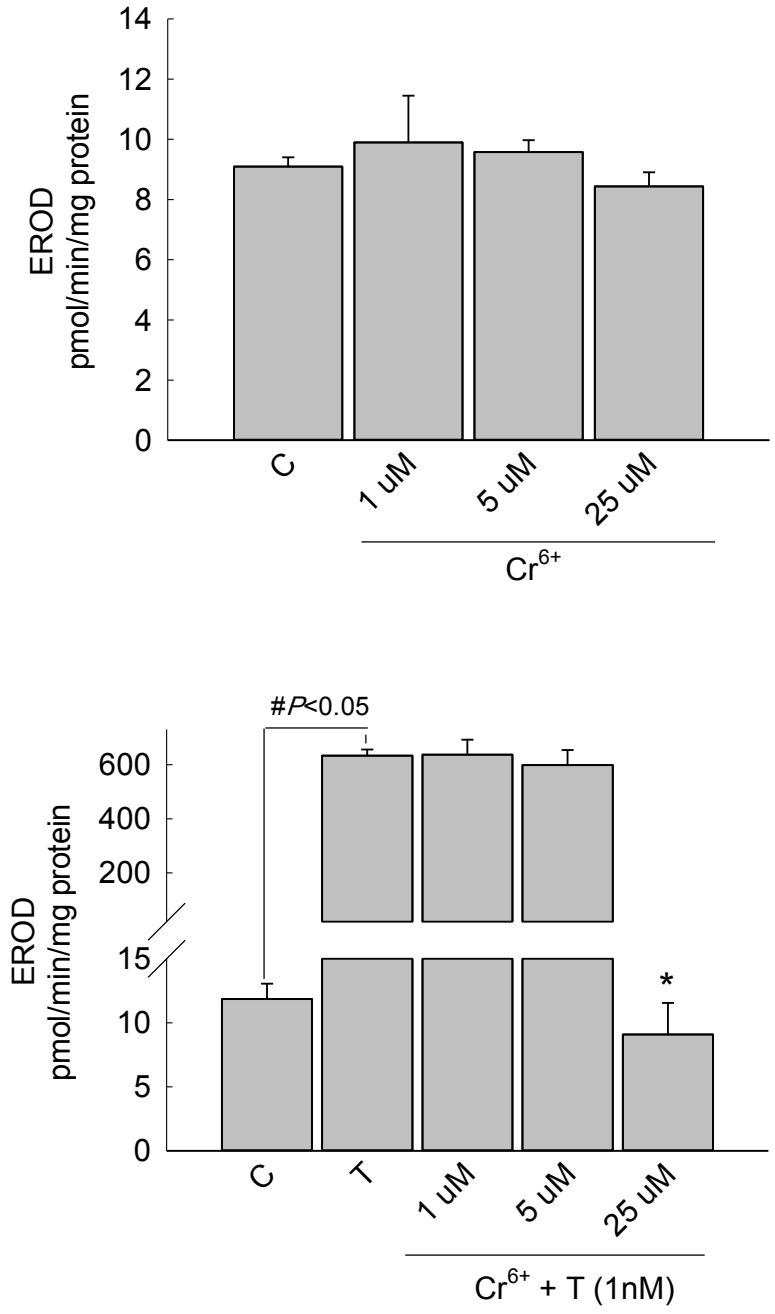

Fig. 3.7. Effect of Cr^{6+} on CYP1A1 catalytic activity levels in HepG2 cells.

CYP1A1 activity was measured in intact living cells treated with increasing concentrations of Cr^{6+}, in the absence and presence of 1 nM TCDD, for 24 h. CYP1A1 activity was measured using 7-ethoxyresorufin as a substrate. Values are presented as means ± SEM (n=6). (#)P<0.05, compared to control (C; concentration 0 μM); (*)P<0.05, compared to respective TCDD (T) treatment.

3.1.8. Transcriptional inhibition of CYP1A1 gene by Cr^{6+} in HepG2 cells,

In order to study the effect of Cr^{6+} on the AhR-dependent transcriptional activation, HepG2 cells were transiently transfected with the XRE-driven luciferase reporter gene. Luciferase activity results showed that Cr^{6+} alone, at all concentrations tested, did not significantly affect the constitutive expression of the luciferase activity (Fig. 3.8.). On the other hand, TCDD alone caused a significant increase of luciferase activity by 11.5-fold, compared to control (Fig. 3.8.). Interestingly, co-treatment with Cr^{6+} and TCDD significantly decreased the TCDD-mediated induction of luciferase activity at 1, 5, and 25 μM, to 8.7-, 7.7-, and 4.2-fold, respectively, compared to control (Fig. 3.8.).

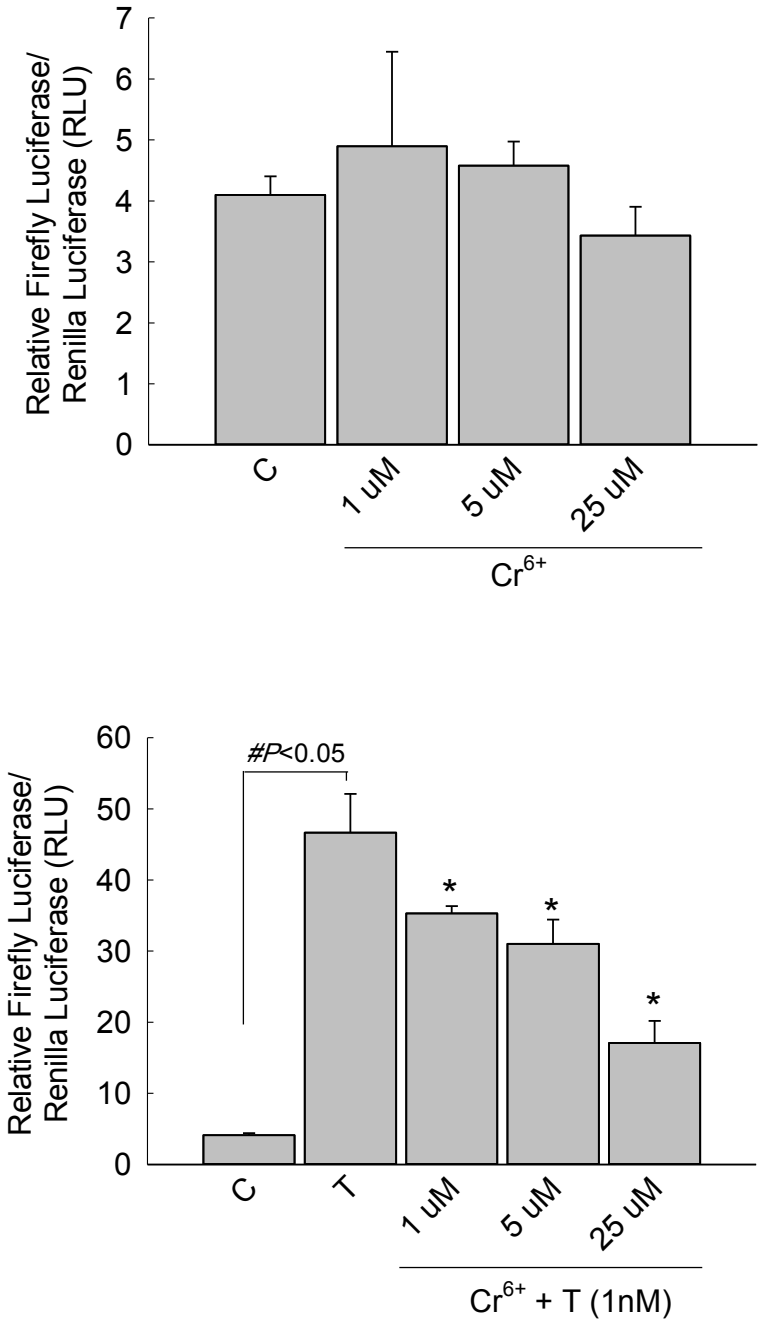

Fig. 3.8. Effect of Cr^{6+} on luciferase activity in HepG2 cells.

HepG2 cells were transiently transfected with the XRE-luciferase transporter plasmid pGudLuc 6.1. Cells were treated with vehicle, TCDD (1 nM), Cr^{6+} (5 μM), or TCDD (1 nM)+ Cr^{6+} (5 μM) for 24 h. Cells were lysed and luciferase activity was measured according to the manufacturer's instructions. Luciferase activity is reported as relative light units. Values are presented as means ± SEM (n=6). (#)P<0.05, compared to control (C); (*)P<0.05, compared to respective TCDD (T) treatment.

3.1.9. Effect of co-exposure to Cr^{6+} and TCDD on Cyp1a1 mRNA in the kidney of C57BL/6 mice.

In the kidney, our results showed that Cr^{6+} alone did not significantly affect the Cyp1a1 mRNA levels compared to the control (Fig. 3.9.). TCDD alone significantly induced Cyp1a1 mRNA levels in the kidney by 148.3-fold, (Fig. 3.9.). When animals were co-exposed to Cr^{6+} and TCDD, Cr^{6+} significantly inhibited the TCDD-mediated induction of Cyp1a1 mRNA levels in the kidney to 50.4-fold, compared to control (Fig. 3.9.).

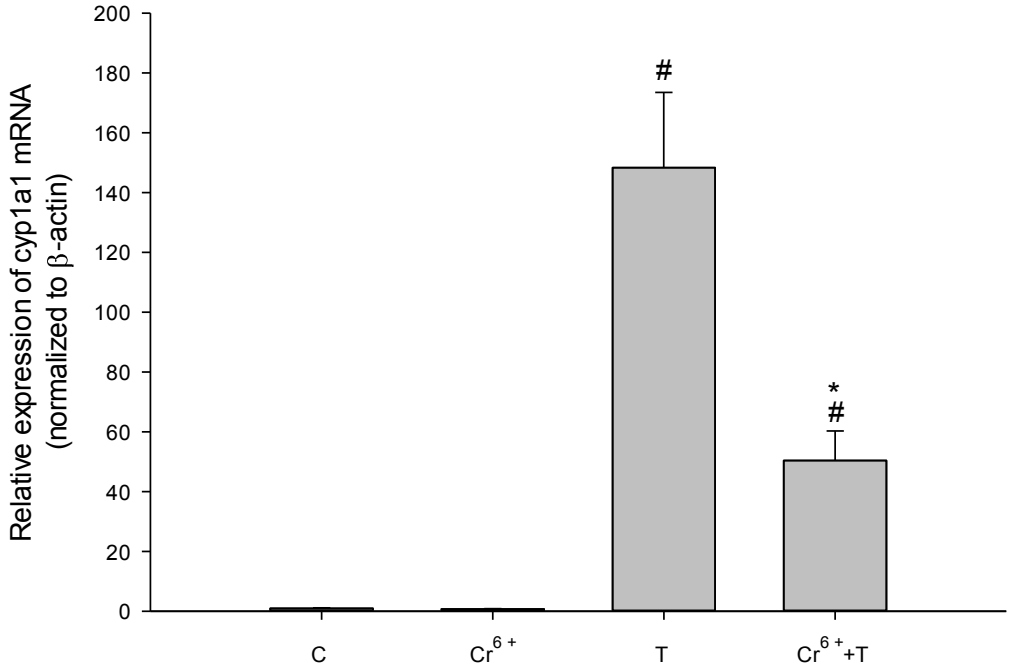

Fig. 3.9. Effect of co-exposure to Cr^{6+} and TCDD on Cyp1a1 mRNA levels in the kidney of C57Bl/6 mice.

Animals were injected i.p. with 20 mg/kg Cr^{6+} in the absence and presence of 15 μg/kg TCDD. First-strand cDNA was synthesized from total RNA (1.5 μg) extracted from kidney and lung and the expression of Cyp1a1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. Results were calculated as mean ± S.E.M. (n = 6). (#)$P<0.05$, compared to control (C; vehicle treated animals); (*)$P<0.05$, compared to respective TCDD (T) treatment.

3.1.10. Effect of co-exposure to Cr^{6+} and TCDD on Cyp1a1 protein expression levels in the kidney of C57BL/6 mice.

In an attempt to examine whether the observed effects at the mRNA levels will be further translated to the protein expression levels, we examined the effect of co-exposure to Cr^{6+} and TCDD on the protein levels. Our results showed that Cr^{6+} alone did not significantly affect Cyp1a1 protein levels in the kidney (Figs. 3.10.). TCDD alone significantly induced Cyp1a1 protein levels in the kidney by 1.72-fold, compared to control (Fig. 3.10.). On the other hand when animals were co-exposed to Cr^{6+} and TCDD, Cr^{6+} significantly inhibited the TCDD-mediated induction of Cyp1a1 protein levels in the kidney to 0.63-fold, compared to control (Fig. 3.10.).

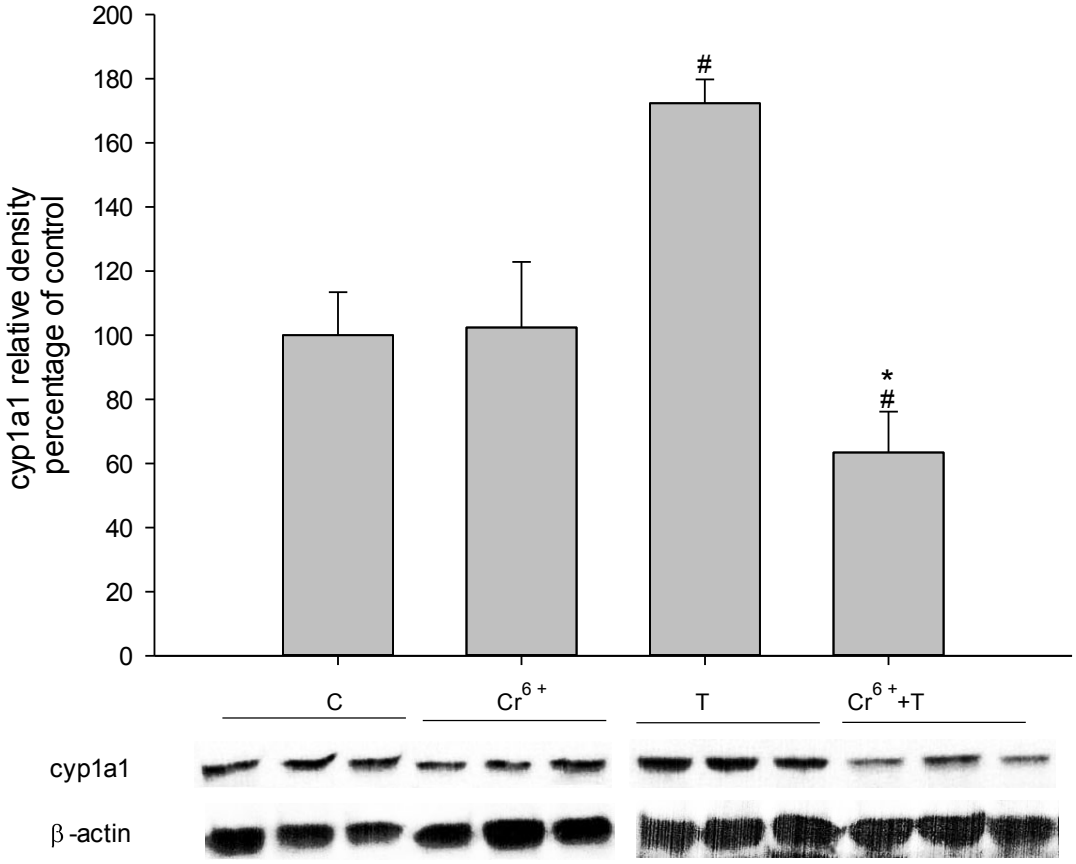

Fig. 3.10. Effect of co-exposure to Cr^{6+} and TCDD on Cyp1a1 protein levels in the kidney of C57BL/6 mice.

Kidney microsomal proteins were isolated after 24 h of treatment. Thirty micrograms of microsomal proteins were separated by 10% SDS–PAGE. Proteins were detected using the enhanced chemiluminescence method. The graph represents the relative amounts of protein normalized to actin signals (mean ± SEM, *n*=6), and the results are expressed as a percentage of the control values taken as 100%. Values are presented as the mean ± SEM (*n*=6). (#)P<0.05, compared to control (C; vehicle treated animals); (*)P<0.05, compared to respective TCDD (T) treatment.

3.1.11. Effect of co-exposure to Cr^{6+} and TCDD on EROD catalytic activity in the kidney of C57BL/6 mice.

At the catalytic activity level, Cr^{6+} alone did not significantly affect EROD activity in the kidney (Fig. 3.11.). TCDD alone significantly induced EROD activity in the kidney by 36.4-fold, compared to control (Fig. 3.11.). When animals were co-exposed to Cr^{6+} and TCDD, Cr^{6+} significantly inhibited the TCDD-mediated induction of EROD activity in the kidney to 19.6-fold, compared to control (Fig. 3.11.).

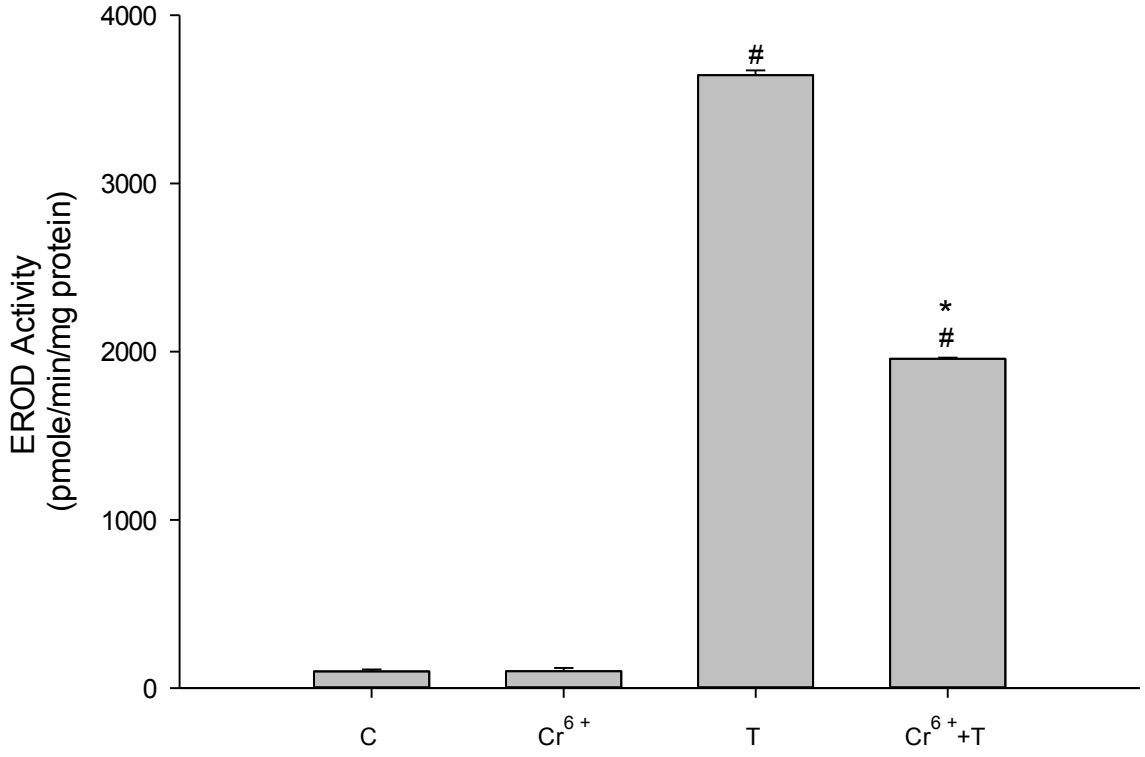

Fig. 3.11. Effect of co-exposure to Cr^{6+} and TCDD on EROD catalytic activity in the kidney of C57BL⁄6 mice.

EROD activity was measured using 7-ethoxyresorufin as a 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 acetonitrile. Values are presented as the mean ± SEM (n=6). (#)P<0.05, compared to control (C; vehicle treated animals); (*)P<0.05, compared to respective TCDD (T) treatment.

3.1.12. Effect of co-exposure to Cr^{6+} and TCDD on Cyp1a1 mRNA in the lung of C57BL/6 mice.

Our results showed that Cr^{6+} alone did not significantly affect the Cyp1a1 mRNA levels in lung compared to the control (Fig. 3.12.). TCDD alone significantly increased Cyp1a1 mRNA in lung by 39.3-fold, compared to control (Fig. 3.12.). When animals were co-exposed to Cr^{6+} and TCDD, Cr^{6+} significantly potentiated the TCDD-mediated induction of Cyp1a1 mRNA levels in the lung to 209.3-fold, compared to control (Fig. 3.12.).

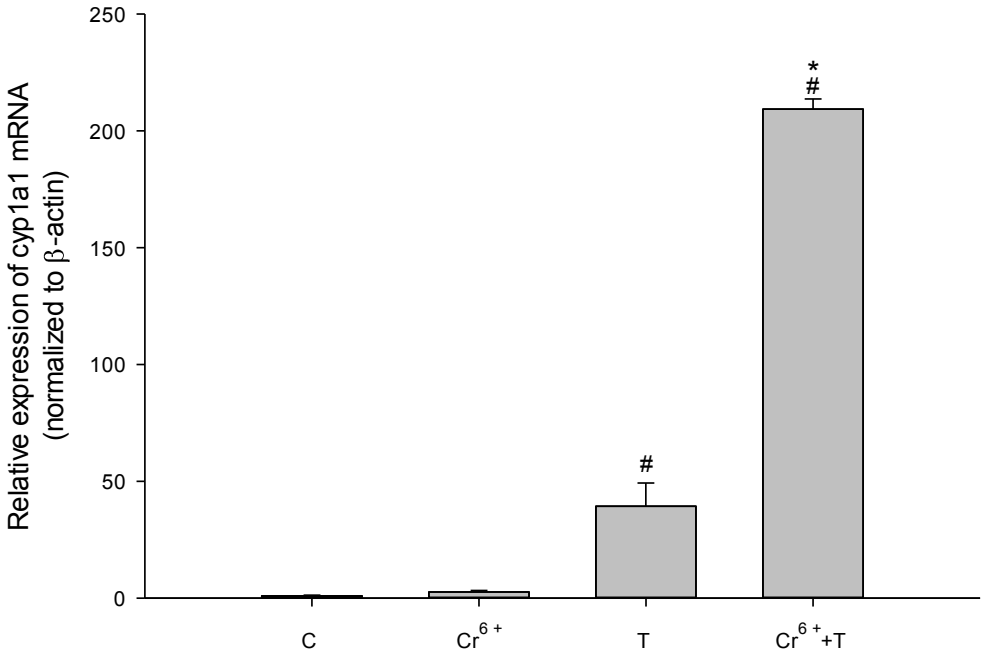

Fig. 3.12. Effect of co-exposure to Cr^{6+} and TCDD on Cyp1a1 mRNA levels in the lung of C57Bl/6 mice.

Animals were injected i.p. with 20 mg/kg Cr^{6+} in the absence and presence of 15 μg/kg TCDD. First-strand cDNA was synthesized from total RNA (1.5 μg) extracted from kidney and lung and the expression of Cyp1a1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. Results were calculated as mean ± S.E.M. (n = 6). (#)$P<0.05$, compared to control (C; vehicle treated animals); (*)$P<0.05$, compared to respective TCDD (T) treatment.

3.1.13. Effect of co-exposure to Cr^{6+} and TCDD on Cyp1a1 protein expression levels in the lung of C57BL/6 mice.

Our results showed that Cr^{6+} alone did not significantly affect Cyp1a1 protein expression levels in the lung (Fig. 3.13.). TCDD alone significantly induced Cyp1a1 protein expression levels in the lung by 1.3-fold, compared to control (Fig. 3.13.). On the other hand when animals were co-exposed to Cr^{6+} and TCDD, Cr^{6+} significantly inhibited the TCDD-mediated induction of Cyp1a1 in the lung to 0.70-fold, compared to control (Fig. 3.13.).

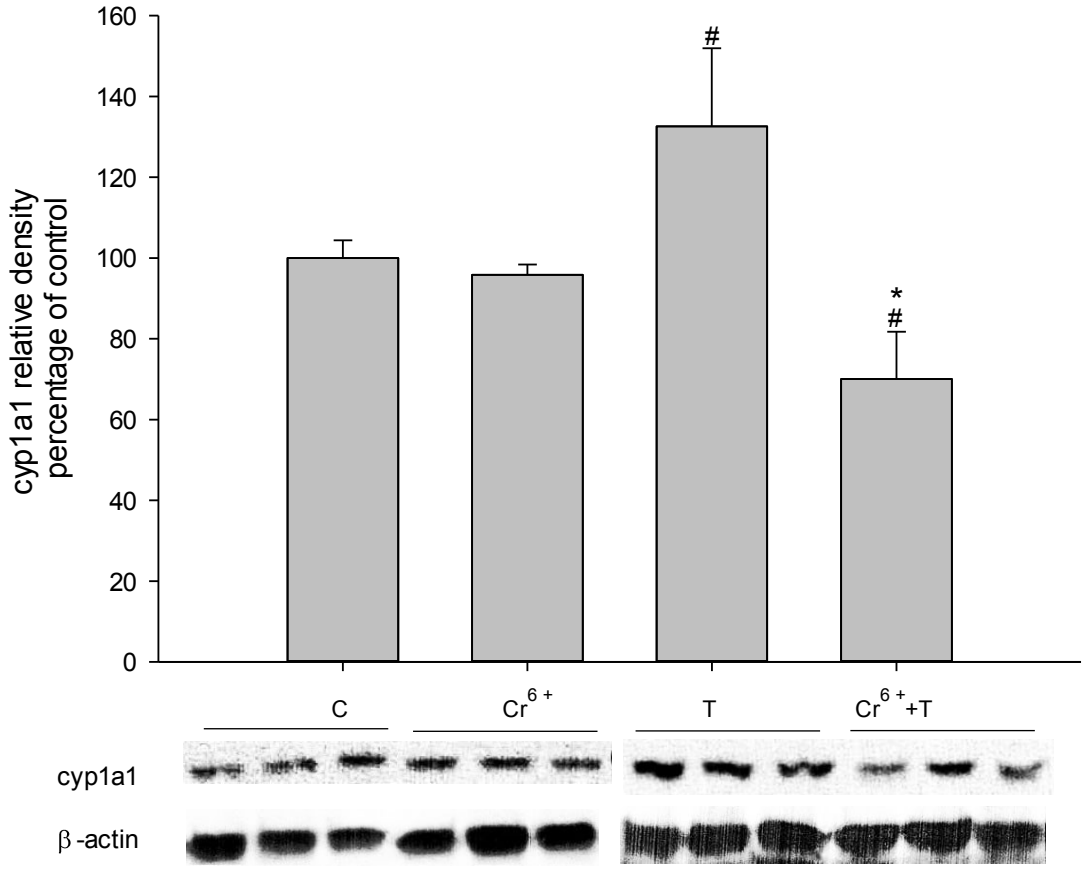

Fig. 3.13. Effect of co-exposure to Cr^{6+} and TCDD on lung Cyp1a1 protein levels in C57BL/6 mice.

Lung microsomal proteins were isolated after 24 h of treatment. Thirty micrograms of microsomal proteins were separated by 10% SDS–PAGE. Proteins were detected using the enhanced chemiluminescence method. The graph represents the relative amounts of protein normalized to actin signals (mean ± SEM, *n*=6), and the results are expressed as a percentage of the control values taken as 100%. Values are presented as the mean ± SEM (*n*=6). (#)P<0.05, compared to control (C; vehicle treated animals); (*)P<0.05, compared to respective TCDD (T) treatment.

3.1.14. Effect of co-exposure to Cr^{6+} and TCDD on EROD catalytic activity in the lung of C57BL/6 mice.

At the catalytic activity level, Cr^{6+} alone did not significantly affect EROD activity in the lung (Fig. 3.14.). TCDD alone significantly induced EROD activity in the lung to 10.7-fold, compared to control (Fig. 3.14.). When animals were co-exposed to Cr^{6+} and TCDD, Cr^{6+} significantly inhibited the TCDD-mediated induction of EROD activity in the lung to 8.6-fold, compared to control (Fig. 3.14.).

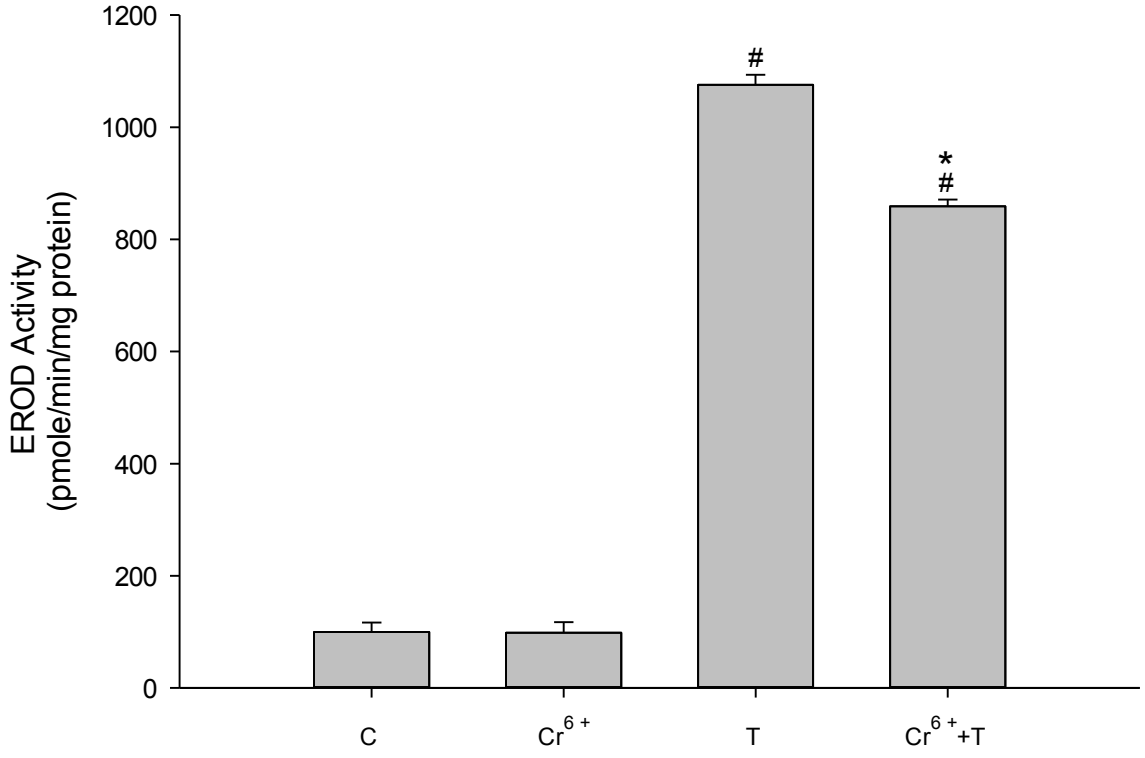

Fig. 3.14. Effect of co-exposure to Cr^{6+} and TCDD on EROD catalytic activity levels in the lung of C57BL/6 mice.

EROD activity was measured using 7-ethoxyresorufin as a 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 acetonitrile. Values are presented as the mean ± SEM (n=6). (#)$P<0.05$, compared to control (C; vehicle treated animals); (*)$P<0.05$, compared to respective TCDD (T) treatment.

3.1.15. Effect of co-exposure to Cr^{6+} and TCDD on Nqo1 mRNA in the liver of C57Bl/6 mice.

The fact that Cr^{6+} was capable of altering the liver phase I AhR-regulated genes prompted us to investigate its possible effect on phase II AhR-regulated genes which are not only regulated by AhR but are also regulated by the Nrf2. Cr^{6+} alone significantly induced Nqo1 mRNA levels in the liver by 2-fold, compared to control (Fig. 3.15.). TCDD alone significantly induced the Nqo1 mRNA levels in the liver by 6-fold, compared to the control (Fig. 3.15.). When animals were co-exposed to Cr^{6+} and TCDD, Cr^{6+} significantly potentiated the TCDD-mediated induction of Nqo1 mRNA levels in the liver to 13.1-fold, compared to control (Fig. 3.15.).

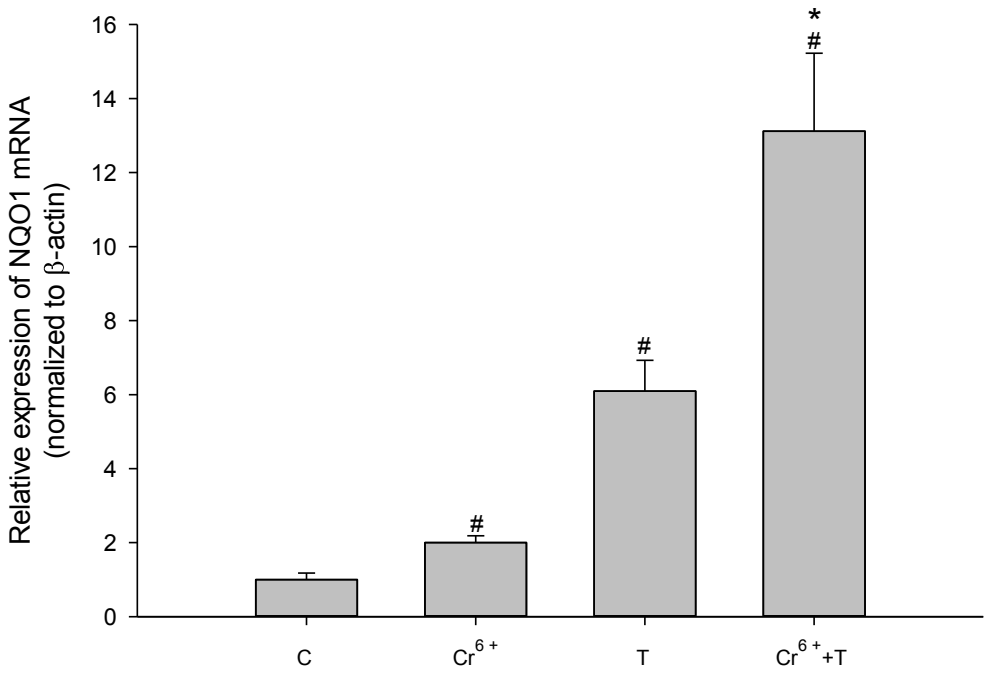

Fig. 3.15. Effect of co-exposure to Cr^{6+} and TCDD on Nqo1 mRNA levels in the liver of C57Bl/6 mice.

Animals were injected i.p. with 20 mg/kg Cr^{6+} in the absence and presence of 15 μg/kg TCDD. First-strand cDNA was synthesized from total RNA (1.5 μg) extracted from the liver and the expression of Nqo1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. Results were calculated as mean ± SEM (n = 6). (#)$P<0.05$, compared to control (C; vehicle treated animals); (*)$P<0.05$, compared to respective TCDD (T) treatment.

3.1.16. Effect of co-exposure to Cr^{6+} and TCDD on NQO1 protein levels in the liver of C57BL/6 mice.

Our results showed that Cr^{6+} alone significantly induced NQO1 protein levels in the liver by 5.6-fold, compared to control (Fig. 3.16.). TCDD alone significantly induced NQO1 protein levels in the liver by 100-fold, compared to control (Fig. 3.16.). On the other hand when animals were co-exposed to Cr^{6+} and TCDD, Cr^{6+} significantly potentiated the TCDD-mediated induction of NQO1 protein levels in the liver to 610-fold, compared to control (Fig. 3.16.).

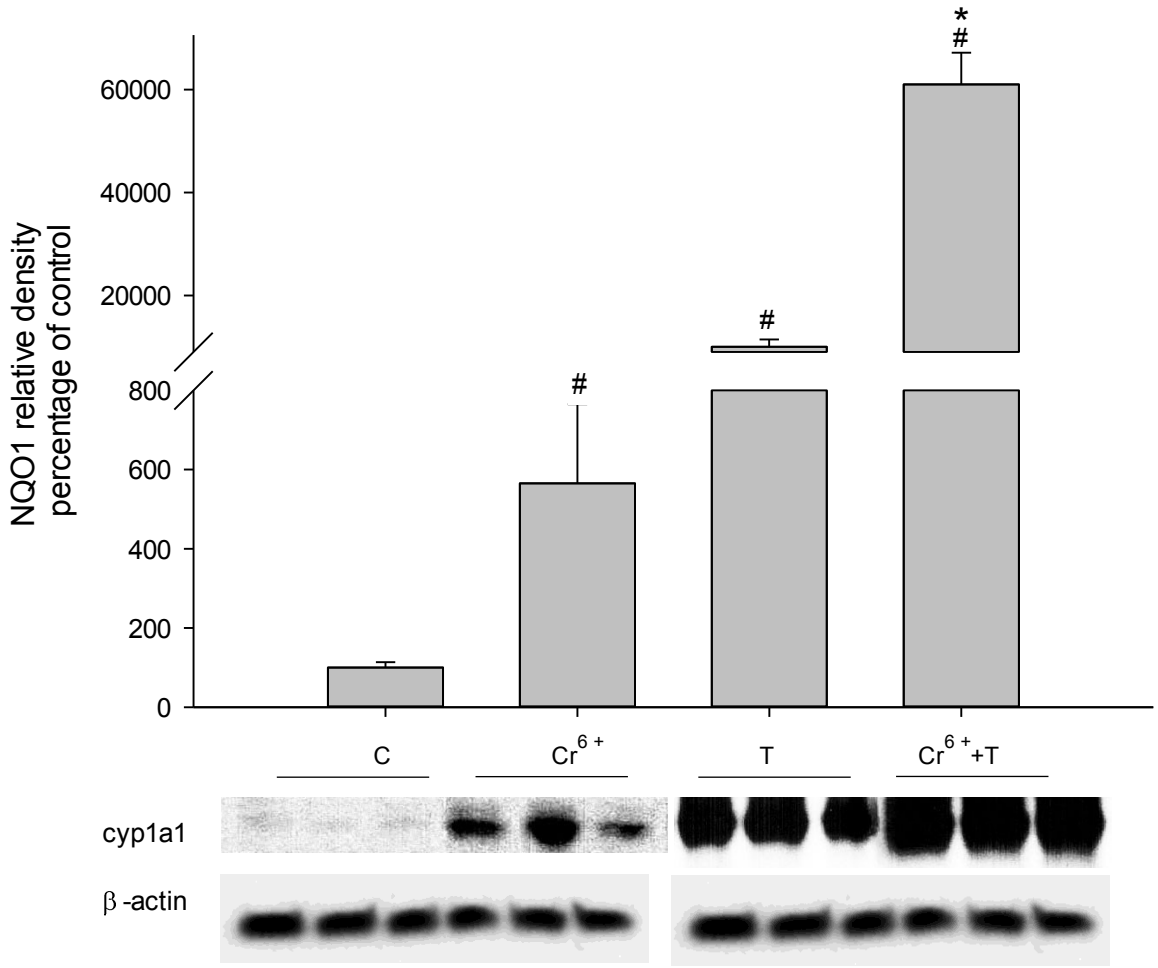

Fig. 3.16. Effect of co-exposure to Cr^{6+} and TCDD on NQO1 protein levels in the liver of C57BL/6 mice.

Liver microsomal proteins were isolated after 24 h of treatment. Thirty micrograms of microsomal proteins was separated by 10% SDS–PAGE. Proteins were detected using the enhanced chemiluminescence method. The graph represents the relative amounts of protein normalized to actin signals (mean ± SEM, *n*=6), and the results are expressed as a percentage of the control values taken as 100%. Values are presented as the mean ± SEM (*n*=6). (#)P<0.05, compared to control (C; vehicle treated animals); (*)P<0.05, compared to respective TCDD (T) treatment.

3.1.17. Effect of co-exposure to Cr^{6+} and TCDD on NQO1 catalytic activity in the liver of C57BL/6 mice.

At the catalytic activity level, Cr^{6+} alone did not significantly affect NQO1 catalytic activity in the liver (Fig. 3.17.). TCDD alone significantly induced NQO1 activity in the liver to 2.2-fold, compared to control (Fig. 3.17.). When animals were co-exposed to Cr^{6+} and TCDD, Cr^{6+} significantly potentiated the TCDD-mediated induction of NQO1 activity in the liver to 4.5-fold, compared to control (Fig. 3.17.).

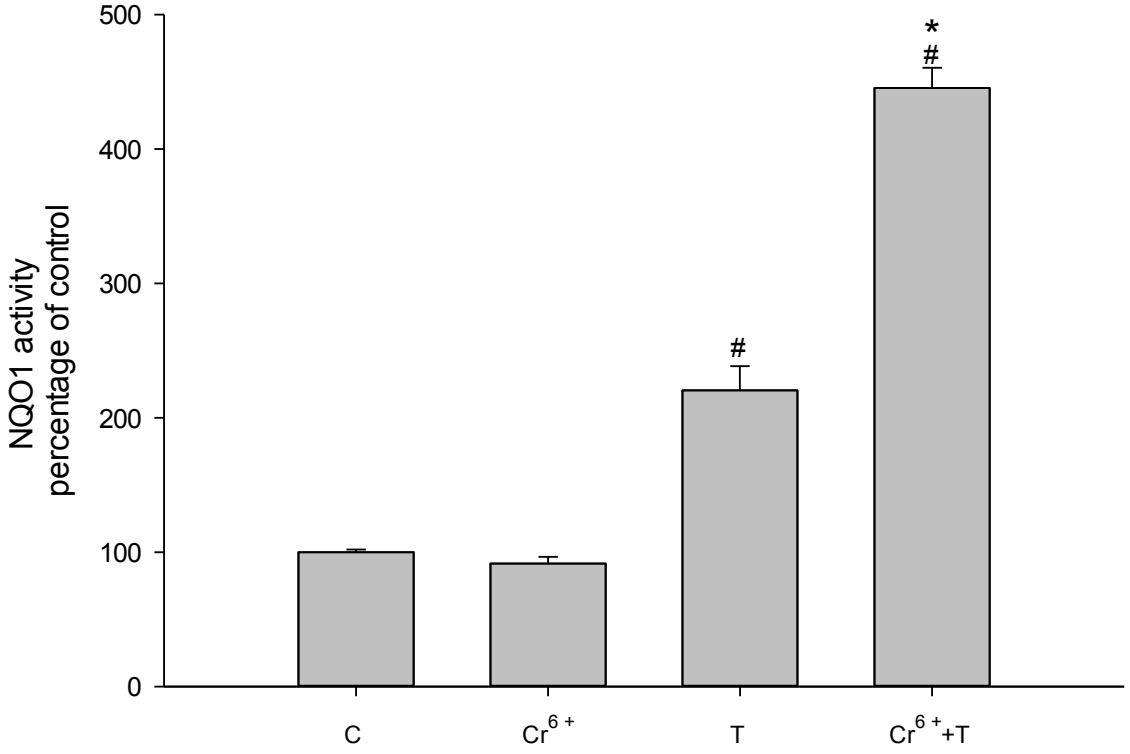

Fig. 3.17. Effect of co-exposure to Cr^{6+} and TCDD on NQO1 catalytic activity in the liver of C57BL/6 mice.

Nqo1 enzyme activity was determined spectrophotometrically using DCPIP as substrate, and dicoumarol as specific Nqo1 inhibitor. Results were calculated as mean ± SEM (n = 6). (#)P<0.05, compared to control (C; vehicle treated animals); (*)P<0.05, compared to respective TCDD (T) treatment.

3.1.18. Effect of co-exposure to Cr^{6+} and TCDD on HO-1 mRNA in the liver of C57Bl/6 mice.

Our results showed that Cr^{6+} alone significantly induced HO-1 mRNA levels in the liver by 1.6-fold, compared to control (Fig. 3.18.). TCDD alone failed to significantly affect the HO-1 mRNA levels in the liver (Fig. 3.18.). Upon co-exposure to Cr^{6+} and TCDD, there was a further potentiation of the Cr^{6+}-mediated induction of HO-1 mRNA levels to 1.7-fold, compared to control (Fig. 3.18.).

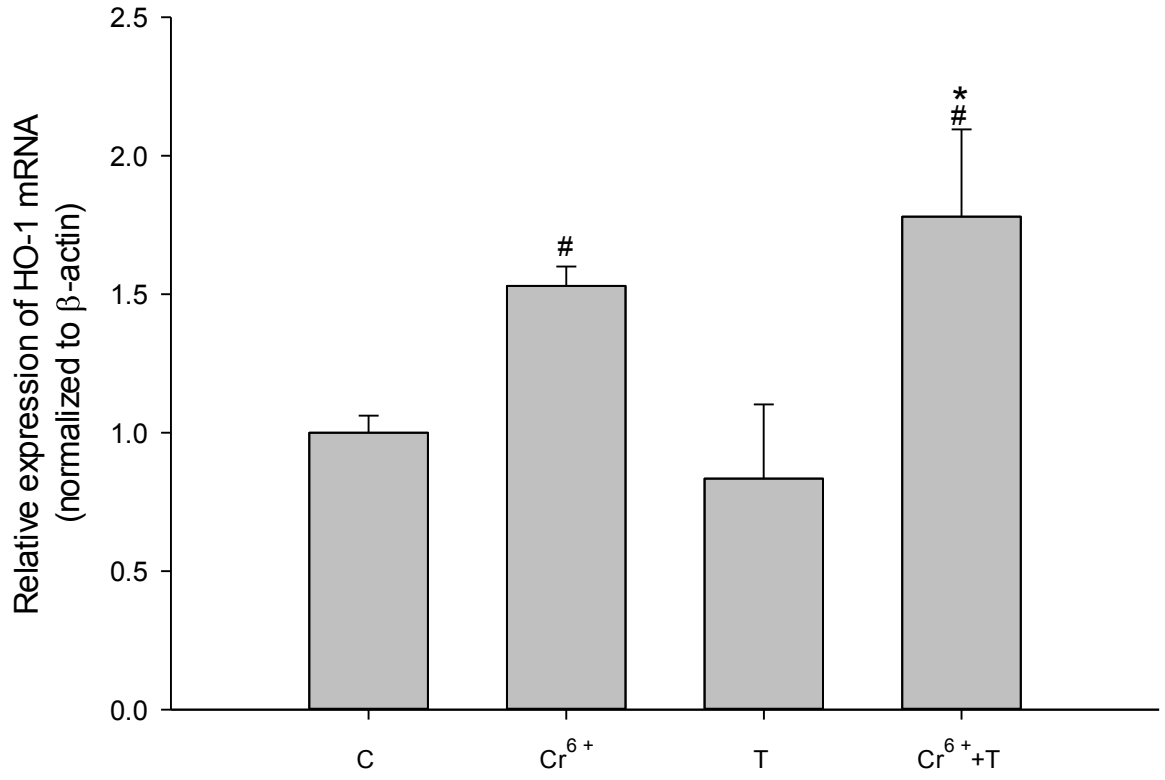

Fig. 3.18. Effect of co-exposure to Cr^{6+} and TCDD on HO-1 mRNA levels in the liver of C57Bl/6 mice.

Animals were injected i.p. with 20 mg/kg Cr^{6+} in the absence and presence of 15 μg/kg TCDD. First-strand cDNA was synthesized from total RNA (1.5 μg) extracted from the liver and the expression of HO-1 was measured using real-time PCR. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of six independent experiments. Results were calculated as mean ± SEM (n = 6). (#)$P<0.05$, compared to control (C; vehicle treated animals); (*)$P<0.05$, compared to respective TCDD (T) treatment.

3.1.19. Effect of co-exposure to Cr^{6+} and TCDD on HO-1 catalytic activity in the liver of C57BL/6 mice.

At the catalytic activity level, Cr^{6+} alone did not significantly affect HO-1 catalytic activity in the liver (Fig. 3.19.). Similarly, TCDD alone did not significantly affect HO-1 catalytic activity in the liver (Fig. 3.19). When animals were co-exposed to Cr^{6+} and TCDD, Cr^{6+} significantly induced HO-1 activity in the liver by 1.2-fold, compared to control (Fig. 3.19.).

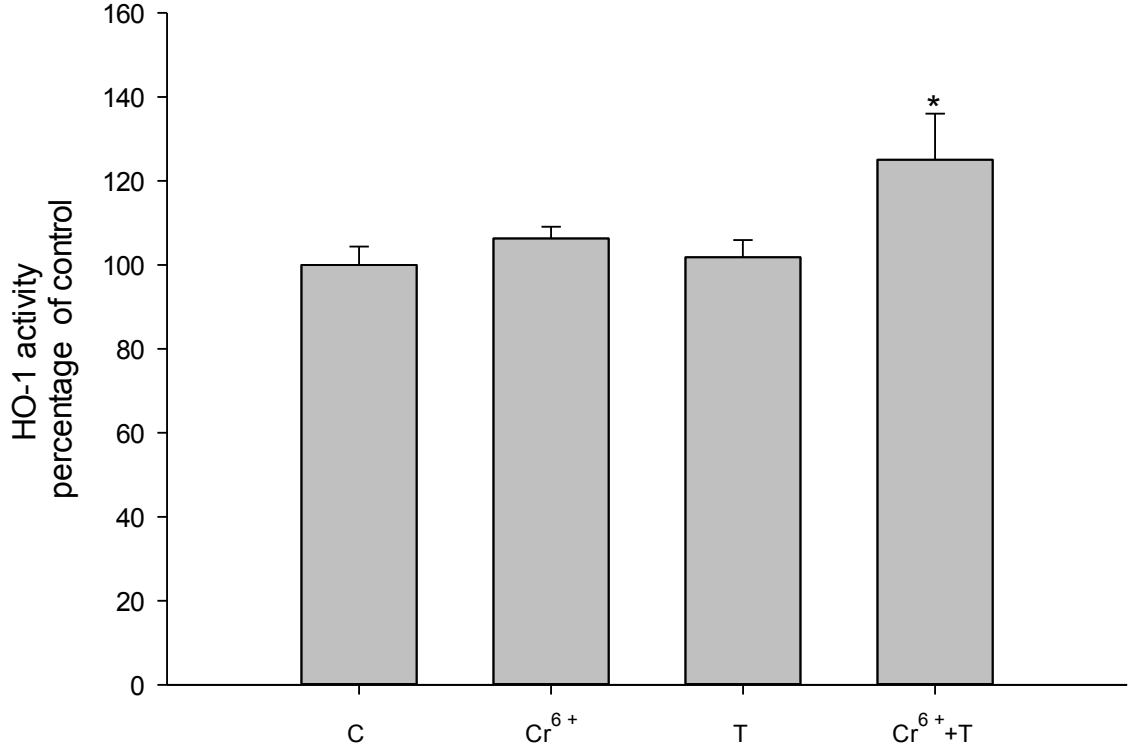

Fig. 3.19. Effect of co-exposure to Cr^{6+} and TCDD on HO-1 catalytic activity in the liver of C57BL⁄6 mice.

HO-1 activity was determined fluorometrically measuring the conversion of heme to protoporphyrin IX by oxalic acid. Results were calculated as mean ± SEM (n = 6). (#)P<0.05, compared to control (C; vehicle treated animals); (*)P<0.05, compared to respective TCDD (T) treatment.

Chapter 4- Discussion

4.1 Discussion

Heavy metals and AhR ligands are common environmental co-contaminants with important toxicological consequences. The persistent exposure to these contaminants causes different biological responses involving the xenobiotic metabolizing system [166]. It has become apparent that the combination of metals and AhR ligands may cause imbalance in the regulation of both phase I and phase II xenobiotic metabolizing enzymes. Thus, there is a persistent need to evaluate the biological response of metal–AhR ligand combinations as it would seem that the combination confers a response that is apparently different than expected based on the toxicological mechanisms of each class evaluated separately. In this study, we have investigated the effect of Cr^{6+} on the AhR ligand -mediated induction of the prototypical AhR regulated phase I enzyme, Cyp1a1, and the prototypical AhR regulated phase II enzyme, Nqo1.

The toxicity of Cr^{6+} has been previously manifested by its ability to induce DNA strand breaks, DNA-DNA and DNA-protein cross-links which cannot take place in cell-free systems in the lack of reducing agents [150]. Based on the concentration and nature of the reducing agent involved, Cr^{6+} is converted eventually to Cr^{3+} either directly or through different intermediates, such as Cr^{5+} or Cr^{4+} before being converted to Cr^{3+} [157]. While Cr^{3+} directly interacts with phosphate groups and nitrogen bases in DNA [158], the formation of ROS during the reduction procedure is believed to mediate the detected genotoxicity associated with Cr^{6+} [159].

In addition, data from our laboratory and others showed that heavy metals other than Cr^{6+} are capable of modifying the carcinogen-metabolizing enzyme, Cyplal at different stages of its

regulatory pathway [167-169]. In the current study, we hypothesize that co-exposure to Cr^{6+} and TCDD disrupts the coordinated balance of AhR-regulated genes *in vivo*. In addition, co-exposure to $Cr^{6+}$and TCDD *in vivo* alters AhR-regulated genes in a time-, tissue-, and AhR-regulated gene-dependent manner. Therefore, the key objective of the current study was to determine the potential effects of co-exposure to Cr^{6+} and TCDD on the expression of Cyp1a1, as a prototypical AhR-regulated phase I xenobiotic metabolizing enzyme and Nqo1, as a prototypical AhR-regulated phase II detoxifying enzyme. To the best of our knowledge, this is the first study to examine the effect of co-exposure to Cr^{6+} and TCDD on the AhR-regulated genes in C57Bl/6 mice.

Humans consume substantial amounts of Cr^{6+} in water and food [170, 171]. The estimated amount of Cr^{6+} absorbed in humans is 20 to 80 μg/day [172]. Also, it has been recommended by the Food and Nutrition Board of the National Research Council (NRC) that the safe and adequate intake of Cr^{6+} is 50-200 μg/day [173]. Depending on the chemical species of Cr^{6+} the absorption may vary, overall the oral absorption of Cr^{6+} is considerably higher than Cr^{3+}. As soon as it gets absorbed, Cr^{6+} is rapidly distributed to all the organs in the body and in the end is excreted in the urine with a half-life of 30 – 40 hours [174]. Additionally, the selection of the Cr^{6+} dose in the current study was based on a previously published study in which animals were administered 20 mg/kg Cr^{6+} by a single (i.p.) injection [175].

In the current study, we have utilized the C57BL/6 mouse strain as an animal model because it contains a responsive AhR allele (AhRb) [176]. With regard to the choice of the TCDD dose,

TCDD is a metabolically stable compound and has an estimated half-life of around 20 days in mice [177]. Furthermore, different TCDD doses have been examined previously for Cyp1a1 induction and for AhR activation in the same mouse strain and it was demonstrated that for a submaximal saturation/activation of the AhR 15 μg/kg TCDD (i.p.) is needed[178].

Despite the fact that TCDD is metabolically stable, the bioactivation of several other PAH and hydrophobic environmental procarcinogens into their ultimate carcinogenic forms occurs through the xenobiotic metabolizing enzyme, Cyp1a1 [103]. As such, DNA adducts are formed from reactive intermediates when Cyp1a1 metabolizes PAH, which leads to mutagenesis and carcinogenesis [103]. The evidence of a strong correlation between the activity of Cyp1a1 and the potentially high risk of diverse human cancers such as lung, colon, and rectal cancers has been reported previously [179, 180]. Hence, Cyp1a1 is considered a biomarker that would help reveal the exposure to several carcinogens. Moreover, the inhibition of AhR activity and its regulated gene, Cyp1a1 may result in the prevention of the toxic effects triggered by the AhR ligands, including carcinogenicity [181].

Our results showed that Cr^{6+} alone did not affect liver Cyp1a1 at the mRNA, protein or catalytic activity levels. Importantly, Cr^{6+} inhibited the TCDD-mediated induction of liver Cyp1a1 mRNA levels at 24 h. To examine whether the Cr^{6+}-mediated inhibition of the TCDD-mediated induction of Cyp1a1 mRNA is further translated to protein and catalytic activity levels, we measured Cyp1a1 protein and catalytic activity using Western blot analysis and EROD activity,

respectively. Of interest, Cr^{6+} inhibited the TCDD-mediated induction of liver Cyp1a1 protein expression levels with a concomitant inhibition in the EROD catalytic activity levels.

In an attempt to examine whether or not the effects of Cr^{6+} are species-specific, we examined the effect of co-exposure to Cr^{6+} and TCDD on CYP1A1 mRNA, protein, and catalytic activity levels, using human HepG2 cells. The human HepG2 cells were used in the current study for the following reasons: first, these cells have proven to be a useful model for studies investigating the regulation of human CYP1A1 [182-186]; second, the human HepG2 cells are one of the most widely used human hepatoma cells and are considered a potentially useful model for several toxicological studies [187]. Our results in human HepG2 cells showed that Cr^{6+} alone did not significantly affect CYP1A1 mRNA, protein, or catalytic activity levels. In contrast, upon co-exposure to Cr^{6+} and TCDD, Cr^{6+} significantly inhibited the TCDD-mediated induction of CYP1A1 at the mRNA, protein, and catalytic activity levels. Furthermore, Cr^{6+} also inhibited the TCDD-mediated induction of the XRE-driven luciferase reporter activity. In agreement with our results it has been previously shown that Cr^{6+} decreases the TCDD-mediated induction of Cyp1a1 mRNA, protein, and activity in a concentration-dependent manner in Hepa-1 cells [188]. Cr^{6+} also inhibited the XRE-dependent luciferase reporter activity but failed to attenuate TCDD-induced nuclear accumulation of the AhR protein. Additionally, it was shown that Cr^{6+} was able to reduce benzo[k]fluoranthene- and TCDD-induced CYP1A1 mRNA in HepG2 and Huh7 cells, respectively [169, 189]. An explanation offered for the inhibition of XRE-driven luciferase reporter activity and subsequently CYP1A1 mRNA expression levels is that Cr^{6+} blocks the recruitment of polymerase II to the CYP1A1 promoter, thus inhibiting its transcription [190]. In contrast, data from our laboratory have shown that Cr^{6+} differentially up-regulates *Cyp1a1* gene

expression and causes further potentiation of the TCDD-mediated induction of Cyp1a1 mRNA in murine hepatoma Hepa 1c1c7 cells [165]. The controversy between the effect of Cr^{6+} on the human HepG2 and mouse Hepa 1c1c7 cells could be attributed to the mechanistic differences in the regulation of CYP1A1 gene expression upon treatment by TCDD [191, 192]. Factors that could be responsible for these species-specific characteristics of AhR functions, and subsequently CYP1A1 inducibility, could be summarized in three major components: nuclear translocation, transcription initiation via remodeling of chromatin, and finally proteasomal degradation of the AhR [193]. For example, it has been shown that in Hepa 1c1c7 cells the co-activator CREB-binding protein (CBP) is recruited to the CYP1A1 promoter region post-treatment with TCDD, reaching its peak at 4 h, and this coincided with the recruitment of AhR and polymerase II, while there was no recruitment of p300 [194]. In contrast, in HepG2, p300 recruitment is increased in response to TCDD to reach its peak between 4 – 12 h, while CBP recruitment is unaffected [194]. Furthermore, The pro-oxidant buthionine-(S,R)-sulfoximine caused further potentiation to the Cr^{6+}-mediated induction of Cyp1a1 mRNA, while *N*-acetylcysteine protected against this induction. Therefore, it was concluded, could be principally attributed to ROS production by Cr^{6+}. Thus, it is possible that the antioxidant capacity of human HepG2 cells is greater than that of murine Hepa 1c1c7 cells, and hence the difference between the two cell lines with regard to CYP1A1 inducibility in response to Cr^{6+} alone.

It is worth mentioning that most of the previous studies focused mainly on one tissue, the liver, without giving comparative information regarding the other tissues. Therefore, we investigated

the effect of Cr^{6+} on TCDD-induced Cyp1a1 in kidney and lung. In the current study Cr^{6+} alone did not significantly affect Cyp1a1 at the mRNA, protein, or catalytic activity levels in the kidney of C57BL/6 mice. On the other hand, Cr^{6+} significantly inhibited the TCDD-mediated induction of Cyp1a1 at the mRNA, protein, and catalytic activity levels in the kidney of C57BL/6 mice. To this end, this is the first study to show the effect of Cr^{6+} on the constitutive and TCDD-induced Cyp1a1 mRNA, protein, and catalytic activity levels in the kidney of C57BL/6 mice. However, it has been previously reported that other heavy metals, precisely pentavalent metals such as vanadium, also inhibited the TCDD mediated induction of *Cyp1a1* gene expression at mRNA, protein and catalytic activity levels in the kidney of C57BL/6 mice [195]. On the other hand, di- and tri-valent metals such as mercury and arsenic potentiated the TCDD-mediated induction of *Cyp1a1* gene expression at mRNA levels in the kidney of C57BL/6 mice [196, 197].

The coordinated regulation of phase I and phase II enzymes is a vital cause of toxicant fate since phase II enzymes defend the cell against electrophiles which are phase I metabolism byproducts of some AhR ligands. Two phase II enzymes, Nqo1 (which is partially under control of AhR), and HO-1, were studied [197]. With regard to Nqo1, Cr^{6+} alone significantly induced Nqo1 at the mRNA and protein expression levels, while this induction was not observed at the catalytic activity level in the liver. Importantly, the co-exposure to Cr^{6+} and TCDD significantly potentiated the induction of Nqo1 at the mRNA, protein, and catalytic activity levels in the liver of C57Bl/6 mice. In the current study it is not clear if Cr^{6+} potentiated the TCDD-mediated effects or TCDD potentiated the Cr^{6+}-mediated effects on the phase II AhR-regulated

genes. Nonetheless, TCDD is considered a bi-functional inducer as it induces both phase I and phase II AhR-regulated genes [198], and a mono-functional inducer as it induces these genes primarily through activating the AhR [198]. Cr^{6+} is not known to be an AhR ligand, yet it has been shown to be an oxidative stress inducer [199]. Keeping that in mind, it is therefore anticipated that Cr^{6+} would significantly induce phase II AhR-regulated genes through activating the redox sensitive transcription factor, Nrf2. In contrast to our results, data from our laboratory has previously shown that Cr^{6+} alone does not significantly affect Nqo1 mRNA, protein, or catalytic activity levels in Hepa 1c1c7 cells [200]. Furthermore, Cr^{6+} significantly inhibited the TCDD-mediated induction of Nqo1 at the mRNA and catalytic activity levels. An explanation for this controversy could be that the effect observed at the *in vitro* level is a direct effect of Cr^{6+} while the effect at the *in vivo* level is an indirect effect due to secondary mediators that can be present only at the *in vivo* level. To better exemplify this phenomenon, previous data from our laboratory demonstrated that arsenic, mercury, and vanadium potentiated the TCDD-mediated induction of Cyp1a1 mRNA, protein, and catalytic activity levels *in vivo* while inhibiting the TCDD-mediated induction of Cyp1a1 mRNA, protein, and catalytic activity *in vitro* [201-203]. It was found that hemoglobin, as an internal mediator, was accountable for the arsenic-, mercury-, and vanadium-mediated potentiation of the TCDD-mediated induction of Cyp1a1 mRNA, protein, and catalytic activity levels *in vivo* [201-203].

Despite the fact that Cr^{6+} significantly inhibited the TCDD-mediated induction of Cyp1a1 at the mRNA, protein, and catalytic activity levels in the liver of C57BL/6 mice, it was important for us to see if the Cr^{6+}-mediated inhibition of the TCDD-mediated induction of Cyp1a1 at the catalytic activity level was partially due Cr^{6+}-mediated induction of HO-1. Gene expression of

HO-1 , a rate-limiting enzyme in heme catabolism, has been shown to modify cellular heme, a prosthetic group of CYP450, content and hence the enzyme activity [135]. In the current study, we have demonstrated that Cr^{6+} alone did not significantly affect HO-1 mRNA or catalytic activity levels in the liver of C57BL/6 mice. Importantly, the co-exposure to Cr^{6+} and TCDD significantly induced HO-1 mRNA and catalytic activity levels in the liver of C57BL/6 mice. In the current study, it is not apparent that Cr^{6+}-mediated induction of HO-1 significantly affected the TCDD-mediated induction of Cyp1a1 at the catalytic activity levels, yet we can not eliminate this possibility despite the low induction of HO-1 at the mRNA and catalytic activity levels.

In conclusion, the present study demonstrates that Cr^{6+} down-regulates the bioactivating enzyme Cyplal through transcriptional and translational mechanisms in a tissue-specific manner. Furthermore, the effect on one AhR-regulated gene could not be generalized to other genes despite the fact that it is an AhR-regulated gene, as there are multiple factors that could act separately or concomitantly to cause differential effects within the same species and organ.

4.2 Future Directions

(1) To determine the effect of acute and chronic co-exposure to Cr^{6+} and its metabolites in addition to AhR ligands on the expression of AhR- and Nrf2- regulated genes *in vivo*,

(2) To characterize the role of NF-kB and AP-1 signaling pathways in the modulation of AhR-regulated genes by Cr^{6+} and its metabolites,

(3) To examine the effect of Cr^{6+} and its metabolites on histone unwinding and the possible role in AhR inhibition, and the possible physical interaction of Cr^{6+} metabolites with Nrf2,

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

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

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

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