UNIVERSITY OF ALBERTA

MODULATION OF ARYL HYDROCARBON RECEPTOR-REGULATED GENES BY VANADIUM

By

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This work is dedicated to my parents,

Gamal Abd El-Naser Anwar Mohamed

&

Badria Nagy Sabra Hammam

My Brother and Sister, Mohamed and Aya

Thank you

ABSTRACT

Vanadium (V^{5+}), a heavy metal contaminant with important toxicological consequences, has received attention as an anticancer agent, although the mechanisms remain unknown. Therefore, we examined the effect of V^{5+} on the expression of the aryl hydrocarbon receptor (AhR)-regulated genes cytochrome P450 1a1 (Cyp1a1), and NAD(P)H: quinone oxidoreductase (Nqo1) in Hepa 1c1c7 cells. Our results showed that V^{5+} inhibited TCDD-mediated induction of Cyp1a1 and Nqo1 at mRNA, protein, and activity levels in a dose-dependent manner. In addition, V^{5+} inhibited the SUL-mediated induction of Nqo1 at mRNA, protein, and catalytic activity levels in both wild-type and AhR-deficient cells. Importantly, V^{5+}-inhibited the AhR/Arnt/XRE and Nrf2/ARE complex formation. Looking at the post-transcriptional and post-translational levels, V^{5+} did not affect the Cyp1a1 mRNA or protein stability. This study provides the first evidence that V^{5+} down-regulates the expression of Cyp1a1 and Nqo1 through a transcriptional mechanism.

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LIST OF ABBREVIATIONS

3MC	3-Methylcholanthrene
7ER	7-Ethoxyresorufin
Act-D	Actinomycin D
AHH	Aryl hydrocarbon hydoxylase
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
βNF	β-Naphthoflavone
C12	AhR-deficient Hepa 1c1c7 C12 cells
c-AMP	Cyclic adenosine monophosphate
CHX	Cycloheximide
CYP or Cyp	Cyptochrome P450
CYP1A1 or Cyp1a1	Cytochrome P4501A1
CYP1A2 or Cyp1a2	Cytochrome P4501A2
CYP1B1 or Cyp1b1	Cytochrome P4501B1
CYP2S1 or Cyp2s1	Cytochrome P4502S1
DCF	2′,7′-Dichlorofluorescein
DCF-DA	2′,7′-Dichlorofluorescein diacetate
DCPIP	2,6-Dichlorophenolindophenol
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
Ecto-ATPase	Ecto adenosine triphosphatase
EMSA	Gel electrophoretic mobility shift assay
EROD	7-Ethoxyresorufin *O*-deethylation

Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
GSTA1	Glutathione transferase A1
H_2O_2	Hydrogen peroxide
HAH	Halogenated aromatic hydrocarbon
Hepa 1c1c7	Murine hepatoma Hepa 1c1c7
HO-1	Heme oxygenase 1
HSP90	90 kDa heat-shock proteins
Keap1	Kelch-like ECH associating protein 1
MG-132	Carbobenzoxy-L-leucyl-L-leucyl-leucinal
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NES	Nuclear export signals
NH_4VO_3	Ammonium metavanadate
NLS	Nuclear localization signals
NQO1 or Nqo1	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
pGudLuc1.1	XRE-luciferase reporter plasmid
PKA	Protein kinase A
PKC	Protein kinase C
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SUL	Isothiocyanate sulforaphane
$t_{1/2}$	Half-life
*t*BHQ	*tert*-Butyl hydroquinone
TBS	Tris-buffered saline
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

UGT1A6	Uridine diphosphate glucuronosyltransferases 1A6
V^{5+}	Vanadate
WT	Wild-type murine hepatoma Hepa 1c1c7 cells
XAP2	Hepatitis B virus X-associated protein 2
XRE	Xenobiotic responsive element
ε	Extinction coefficient

CHAPTER 1

1. INTRODUCTION

1.1 AhR historical background

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

1.2. AhR signaling pathway

The AhR is a member of basic-helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) family of transcription proteins. Inactive AhR resides in the cytoplasm bound to two 90-kDa heat-shock proteins (HSP90), the 23-kDa heat shock protein (p23), and hepatitis B virus X-associated protein 2 (XAP2). Upon ligand binding, the AhR-ligand complex dissociates from the cytoplasmic complex and translocates to the nucleus where it associates with the

aryl hydrocarbon nuclear translocator (ARNT) (Nebert and Duffy, 1997). The whole complex then acts as a transcription factor that binds to a specific DNA recognition sequence, termed the xenobiotic responsive element (XRE), located in the promoter region of a number of AhR-regulated genes. Among these genes are those encoding a number of drug metabolizing enzymes, including four phase I enzymes [cytochrome P450 1A1 (CYP1A1), CYP1A2, CYP1B1, and CYP2S1] and four phase II enzymes [NAD(P)H: quinone oxidoreductase-1 (NQO1), glutathione-*S*-transferase A1 (GSTA1), cytosolic aldehyde dehydrogenase-3 and UDP-glucuronosyltransferase 1A6 (UGT1A6) (Nebert and Duffy, 1997; Rivera et al., 2002) (Figure 1.1)].

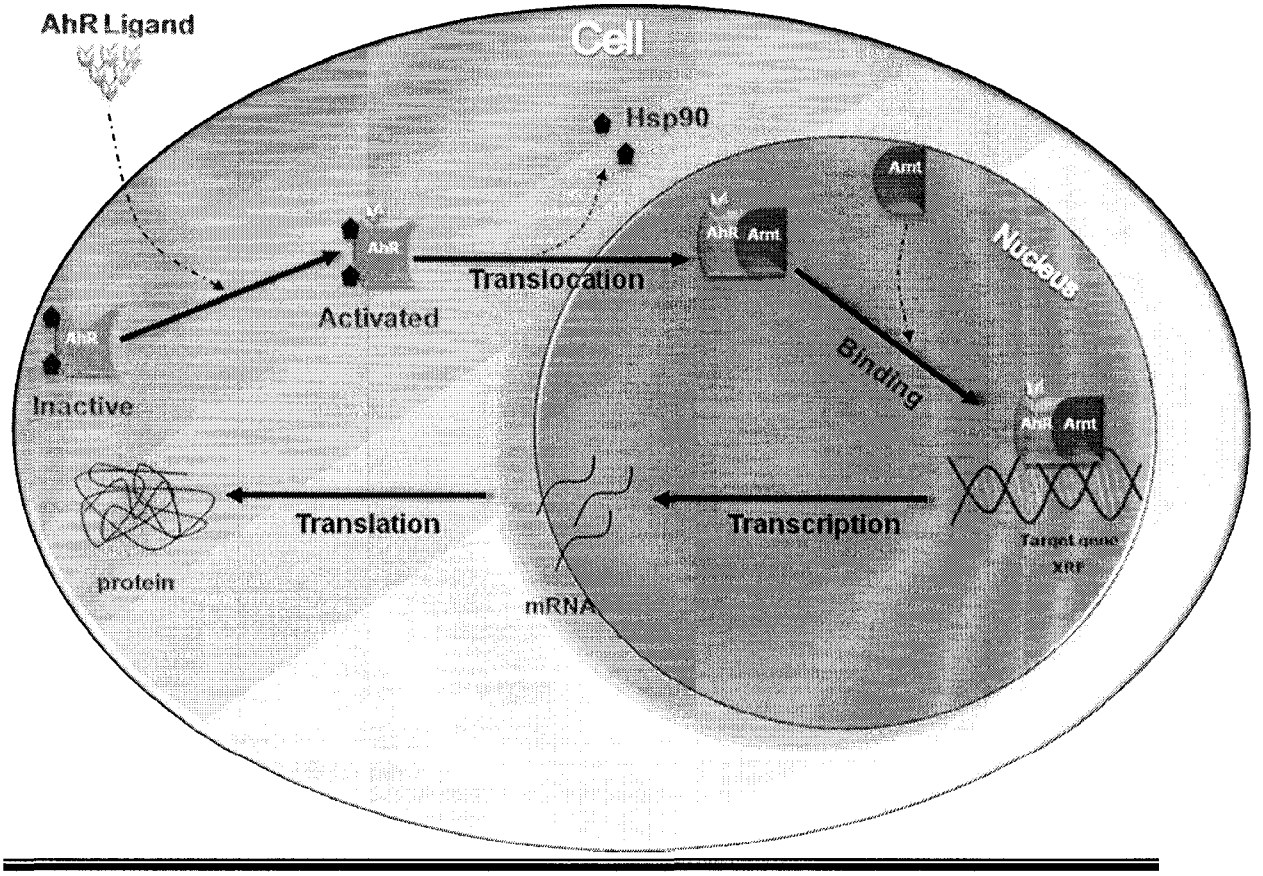

Figure 1.1. A working model for AhR signaling pathway. Adapted from (Hankinson, 1995).

The AhR protein contains several domains critical for its function. The bHLH motif located in the N-terminal of the protein contains two functionally distinctive and highly conserved domains (Fukunaga et al., 1995). The first is the basic-region which is mainly involved in the binding of AhR to DNA. The second is the helix-loop-helix domain which is embroiled in protein-protein interactions. Members of the bHLH family also include Drosophila circadian rhythm protein period (Per), the AhR nuclear translocator (ARNT), and the Drosophila neurogenic protein single minded (Sim) (Schmidt et al., 1993; Schmidt and Bradfield, 1996; Schmidt et al., 1996). The AhR protein contains two Per-ARNT-Sim (PAS) domains, PAS-A and PAS-B. These PAS domains are involved in secondary interactions with other PAS containing proteins, for example AhR and ARNT (Schmidt et al., 1993; Schmidt et al., 1996). Additionally, the PAS-B domain contains the AhR ligand binding site (Kazlauskas et al., 2001). Finally, a glutamine (Q) rich region allocated within the C-terminal was found to be responsible for the transcriptional activation upon AhR binding to the DNA (Fukunaga et al., 1995).

While the HSP90 interacts with the PAS-B and bHLH domains of the AhR, mounting evidence supports the role of HSP90 in maintaining the high affinity ligand binding conformation of the AhR, and repressing its intrinsic DNA-binding activity (Dolwick et al., 1993). The prostaglandin E synthase 3 (p23) is thought to stabilize the interaction between HSP90 and AhR (Kazlauskas et al., 1999), and further protect the receptor from being degraded through proteolysis, in addition to preventing the premature binding of AhR to ARNT. On the other hand, XAP2 interacts with the C-terminal of

HSP90 and the nuclear localization sequence (NLS) of the AhR, preventing inappropriate trafficking of the receptor to the nucleus (Ikuta et al., 1998; Kazlauskas et al., 2000).

1.3. AhR-regulated genes

The importance of the toxicological sequences of AhR ligands arise from the ability of these ligands to induce a battery of genes, in hepatic and extra-hepatic tissues, through activating the AhR (Spink et al., 2002). In addition to their ability to activate the AhR, several PAHs and HAHs are also substrates for phase I AhR regulated enzymes such as CYP1A1, CYP1A2, and CYP1B1 (Spink et al., 2002). Such an interaction would result in the formation of diol-epoxides capable of forming covalent adducts when these genotoxic metabolites interact with guanines in critical genes, thus initiating tumorigenesis and other toxicological consequences (Spink et al., 2002). In contrast, activation of phase II AhR-regulated enzymes such as NQO1 counteracts this process through detoxifying the mutagenic and carcinogenic metabolites formed by phase I enzymes (Spink et al., 2002).

1.3.1. The Phase I AhR-regulated genes

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

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

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

The CYP1A subfamily is comprised of only two members, CYP1A1 and CYP1A2. CYP1A1 is primarily an extra-hepatic enzyme since its constitutive expression is low (Nebert et al., 2004). On the other hand, CYP1A2 is primarily a hepatic enzyme that is constitutively expressed. Nonetheless, the CYP1A1 may be induced 8-16 fold in

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

1.3.2. CYP1A1 regulation

Among the AhR-regulated CYPs, CYP1A1 has received considerable attention since it is highly induced by a broad range of xenobiotics such as PAHs and HAHs via the AhR-XRE transcription pathway (Denison and Nagy, 2003). CYP1A1 is capable of producing polar, toxic, or even carcinogenic metabolites from various AhR ligands, including PAHs and HAHs. Dissection of the mammalian *CYP1A1* gene revealed multiple regulatory elements which may modulate its expression. The first being the XRE, in which several XRE sequences were localized approximately 1 kb upstream of the transcription start site in all mammalian *CYP1A1* genes (Hines et al., 1988). Secondly, the presence of three glucocorticoid responsive elements (GREs) in the human, rat, and mouse C*yp1a1* gene, which explains the modulation of PAHs-induced CYP1A1 by glucocorticoids (Monostory et al., 2005). Thirdly, the presence of a negative regulatory element (NRE), located between -560 and -831, which inhibits the constitutive expression of CYP1A1, due to an interaction with the nuclear transcription factor Oct-1 (Hines et al., 1988).

1.3.3. The phase II AhR regulated genes

Phase II drug metabolizing enzymes including GSTA1, UGT1A6, and the cytosolic ALDH3 but not NQO1 catalyze drug-conjugation reactions (Nioi and Hayes, 2004; Swinney et al., 2006). Such an effect helps in detoxifying xenobiotics and carcinogenic metabolites formed by phase I drug metabolizing enzymes. Numerous studies have shown that these enzymes are regulated by the antioxidant responsive element (ARE) in addition to the XRE (Friling et al., 1990; Chen and Kunsch, 2004; Miao et al., 2005; Xu et al., 2005).

1.3.4. NQO1

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

Being a reductase enzyme it was first classified as a phase I drug metabolizing enzyme according to classical toxicology definitions as it serves to introduce functional groups to xenobiotics which is a typical behavior of this class of drug metabolizing enzymes (Nioi et al., 2003). In spite of being unable to catalyze conjugation reactions which is a unique feature of phase II drug metabolizing enzymes, NQO1 has been

included in this class because it cannot introduce new functional groups into inert lipophilic xenobiotics (Prochaska et al., 1985). Moreover, it has been shown to be potentially induced by cancer chemopreventive agents along with other members of phase II drug-conjugating enzymes including glutathione *S*-transferase (GST) and UDP-glucuronosyl transferase (UGT) isoenzymes (Prochaska et al., 1985). However, in this specific occasion it might be ultimately incorrect to categorize NQO1 as a phase II drug metabolizing enzyme, because NQO1 has also been shown to be induced by certain inducers of the phase I CYPs such as TCDD (Prochaska and Talalay, 1988). Perhaps the most noticeable feature of this enzyme that earned it phase II membership is its ability to catalyze a two-electron reduction of several environmental xenobiotics and endogenous compounds (Ross, 2004; Korashy et al., 2007a).

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

tumorigenic cells (Dinkova-Kostova and Talalay, 2000; Ross et al., 2000; Talalay and Dinkova-Kostova, 2004).

1.3.5. NQO1 regulation

Constitutive NQO1 expression is tissue type-specific, in which maximum induction of the NQO1 mRNA was observed in liver and kidneys followed by lung and the heart (Jaiswal, 2000; Joseph et al., 2000). *NQO1* gene expression can be induced through two separate regulatory elements associated with its 5`-flanking region. The first pathway includes activation of a cytosolic transcription factor, the AhR as discussed previously. The second pathway involves activation of the ARE, which does not require functional AhR. In fact, the increased expression of *NQO1* gene expression in response to oxidative stress caused by agents such as isothiocyanate sulforaphane (SUL), *tert*-butylhydroquinone (t-BHQ) and H_2O_2 occurs primarily through the ARE signaling pathway (Venugopal and Jaiswal, 1996; Itoh et al., 1997). Perturbation in the redox status of the cell activates the nuclear factor erythroid 2-related factor-2 (Nrf2), a redox-sensitive member of the cap 'n' collar basic leucine zipper (CNC bZip) family of transcription factors (Itoh et al., 1997). Subsequently, Nrf2 dissociates from its cytoplasmic tethering polypeptide, Kelch-like ECH associating protein 1 (Keap1), and then translocates into the nucleus, dimerizes with a musculoaponeurotic fibrosarcoma (MAF) protein, and thereafter binds to and activate ARE (Ma et al., 2004; Korashy et al., 2007a) (Figure 1.2.).

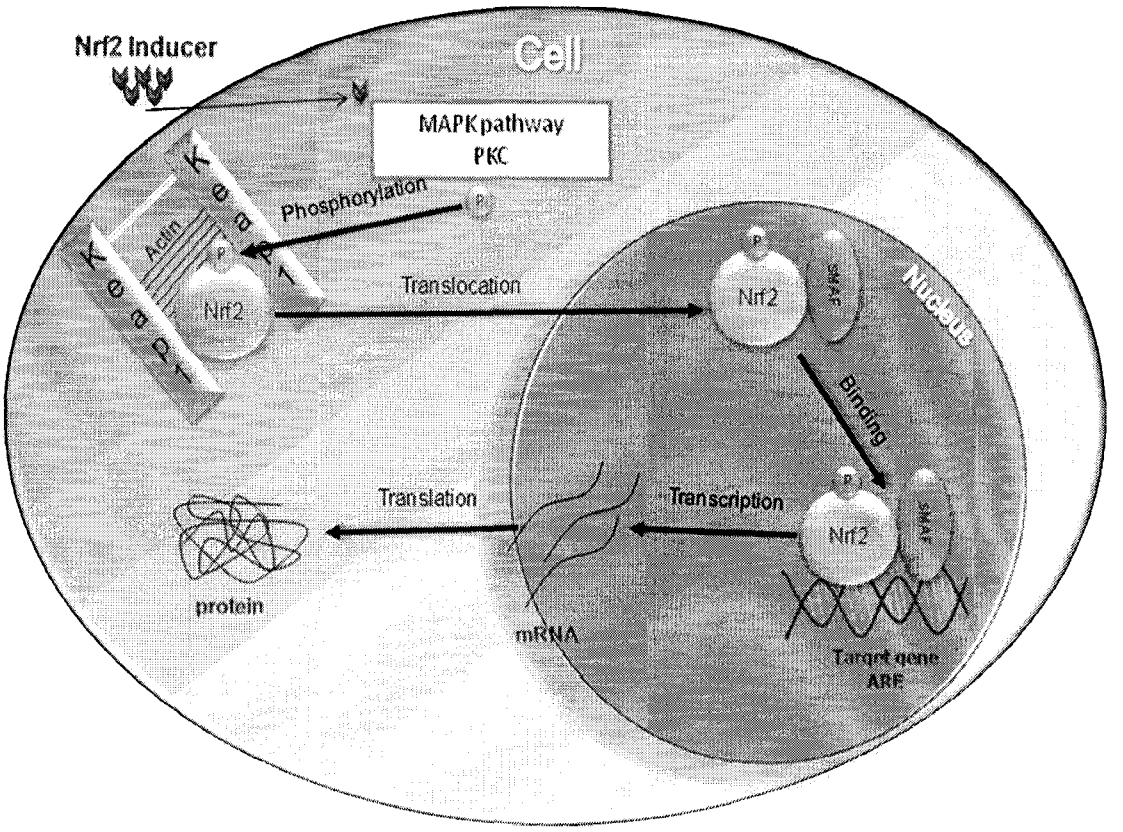

Figure 1.2. A working model for Nrf2 signaling pathway. Adapted from (Jaiswal, 2004; Nioi and Hayes, 2004).

Previous reports have shown that Keap1 negatively regulates the transcription of ARE-reporter and endogenous detoxication genes by Nrf2 (Itoh et al., 1999; McMahon et al., 2003). Keap1 is a cytoplasmic protein that interacts with cellular actin, and is thought to control the subcellular distribution of Nrf2 (Itoh et al., 2003; McMahon et al., 2003). This interaction between Keap1 and Nrf2 increases the rate of ubiquitination Nrf2 prior to its degradation by the proteasome. Current studies indicate that under oxidative stress conditions, cysteine residues 273 and 288 in Keap1 are modified, thus abolishing the Keap1-Nrf2 interaction (Itoh et al., 2003; McMahon et al., 2003). Breaking this interaction benefits Nrf2 two ways, first by increasing its sub-cellular levels and the

second is its translocation to the nucleus (Nioi et al., 2003). In addition, certain signaling pathways may contribute to this process. Protein kinase C (PKC)-mediated phosphorylation of Nrf2 has been identified as an important step in the nuclear translocation of Nrf2 during oxidative stress (Huang et al., 2002). Furthermore, activation of mitogen activated protein kinase (MAPK) pathways activate ARE reporter genes by an Nrf2-dependent mechanism (Yu et al., 2000) while phosphotidylinositol 3-kinase (PI3K) is involved in the rearrangement of actin microfilaments and the subsequent nuclear translocation of Nrf2 (Kang et al., 2002).

XRE- and ARE-driven regulations of the NQO1 gene were generally thought to function independently. However, the proximity of the two sequence sites suggests a possible cross-talk and functional overlap. Recent reports suggest that bifunctional inducers which activate both XRE and ARE signaling pathways, such as TCDD, require direct cross-talk between the XRE- and ARE-mediated pathways for the induction of *NQO1* (Ma et al., 2004; Marchand et al., 2004; Miao et al., 2005). Furthermore, it has been reported that the induction of *NQO1* by ARE inducers requires the presence of AhR, suggesting a more direct cross-talk between the XRE- and ARE-mediated pathways (Ma et al., 2004; Marchand et al., 2004; Miao et al., 2005). In addition, studies using the electrophoretic mobility shift assay (EMSA) have demonstrated that AhR can bind to both XRE and ARE consensus sequences in the promoter region of phase II genes such as *NQO1* (Vasiliou et al., 1995).

1.4. Ecto-ATPase

Ecto-ATPase, a transmembrane enzyme that catalyzes hydrolysis of extracellular ATP and to ADP and inorganic phosphate (Burnstock, 1971). Furthermore, ecto-ATPase may also synchronize with other ecto5'-nucleotidases to convert extracellular AMP to adenosine (Bruns, 1990). It was also shown that ecto-ATPase participates in the recycling of nucleosides of purine/pyrimidine biosynthesis and general cell metabolism (Bruns, 1990). Interestingly, it has been found that ecto-ATPase gene expression is induced in response to TCDD in Hepa 1c1c7 cells (Gao et al., 1998). Moreover, this induction was shown to be at the transcriptional level. In contrast to *CYP1A1*, the ecto-ATPase gene exhibits constitutive expression, which is ARNT-independent (Gao et al., 1998). Thus, induction of ecto-ATPase activity by TCDD may cause perturbation to purinergic signaling and cellular metabolic pathways.

It has been previously reported that an inhibition of cellular ATP levels results in a decreased TCDD binding to AhR (Gudas and Hankinson, 1986). This inhibition was correlated with the inhibition of glucose metabolism which resulted in the decrease in cellular ATP levels (Gudas and Hankinson, 1986). Moreover, the inhibition of TCDD binding to AhR was not due to any effect on the receptor affinity to the ligand, but rather due to failing to maintain AhR in its active ligand binding form (Gudas and Hankinson, 1986). Intriguingly, the study concluded that nuclear AhR cannot be inhibited from binding to XRE, yet the cytosolic form is the one that can be inhibited. More specifically, it was shown that nuclear AhR is capable of binding to ATP in a highly specific manner,

than the cytosolic AhR (Perdew, 1991). An explanation offered to explain this phenomenon is that after dissociation from HSP90, the AhR binds directly to ATP while the cytosolic form does not (Perdew, 1991). This hypothesis was further confirmed by another study that showed that V^{5+} inhibits the nuclear accumulation of AhR which did not coincide with an inhibition of AhR transformation (Wang and Safe, 1994). In addition, when ATP was added to Hepa 1c1c7 cells it significantly increased the formation of nuclear AhR complex as evident by nuclear EMSA (Wang and Safe, 1994).

Previous studies have demonstrated an important role of protein kinase C (PKC) and the subsequent phosphorylation of AhR and ARNT proteins at the transformation step (Berghard et al., 1993; Schafer et al., 1993). Of interest, it was shown that ATP was unable to affect the transformation step in the AhR signaling pathway (Wang and Safe, 1994). This result is in consistence with other studies showing that PKC is not required for the transformation step (Schafer et al., 1993).

1.5. AhR Ligands

AhR ligands can be classified according to their origin into synthetic and natural (Denison and Nagy, 2003). The majority of the AhR ligands identified to date, fall in the first class which includes planar, hydrophobic HAHs and PAHs (Denison and Nagy, 2003). HAHs are metabolically more stable than PAHs, and they further represent the most potent class of AhR ligands. In contrast, the metabolically labile PAHs bind with relatively lower affinity to the AhR, making it less potent than HAHs. Furthermore, there has been another classification for the AhR ligands based on their planar configuration into classical and non-classical ligands.

Theoretically, there are two hypotheses for AhR interaction with its ligands (Mhin et al., 2002). The first is electrostatic interaction, in which effective interaction of the ligand with the receptor depends on the molecular electrostatic potential around the ligand (Mhin et al., 2002). For example, it has been demonstrated that all dioxin compounds that were able to activate the AhR share a unique molecular charge distribution pattern, which was dramatically changed with chlorination (Mhin et al., 2002). Secondly, structure activity relationship studies have proposed that AhR ligand binding pocket can bind planar ligands with maximal dimensions of 14 Å x 12 Å x 5 Å (Denison and Nagy, 2003). Furthermore, it was revealed that binding affinity was dependent on the electronic and thermodynamic properties of the ligand (Kafafi et al., 1993, Waller and McKinney, 1995).

Ligand bound AhR dissociates from the HSP90 and AIP, and this step will further cause the liganded AhR to translocate to the nucleus (Hankinson, 1995). Nuclear AhR heterodimerizes with an 87 kda nuclear transcriptional factor protein, ARNT (Hankinson, 1995). Although ARNT and AhR share ~20% of their amino acid sequence, ARNT does not have any ligand binding domain, and thus appears to be out of the repressive scope of HSP90 (Whitelaw et al., 1994; Hankinson, 1995). The Ligand/AhR/ARNT complex then binds to a specific DNA consensus sequence, GCGTG, within a responsive element known as XRE (Moore et al., 1993). The XRE is located in the promoter region of a number of genes known as the *AhR* gene battery, which includes *CYP1A1*, *CYP1A2*, *CYP1B1*, *CYP2S1*, *NQO1*, *GSTA1*, *ALDH3*, and *UGT1A6*. The Ligand/AhR/ARNT/XRE

complex will then be able to initiate the regulatory pathways controlling the positive or negative expression of these genes (Whitlock, 1999; Pollenz, 2002).

HAHs and PAHs have been classified as bifunctional, i.e. induce both phase I and II xenobiotic metabolizing enzymes, and monofunctional, i.e. induce phase II only, inducers (Prochaska and Talalay, 1988). One mechanism that has been offered to explain this phenomenon is that metabolically resistant inducers, typified by TCDD, activate the AhR, thus inducing both phase I and II drug metabolizing enzymes. On the other hand metabolizable inducers, such as benzo[a]pyrene (BaP), activate phase I enzymes which in turn metabolize the parent compound into monofunctional phase II inducers (Prochaska and Talalay, 1988). For example, 1,6-, 3,6-, and 6,12-quinones, which are end products of BaP metabolism through a series of reactions involving AhR controlled CYPs (Shimada, 2006), are very potent inducers of the phase II drug metabolizing enzyme NQO1.

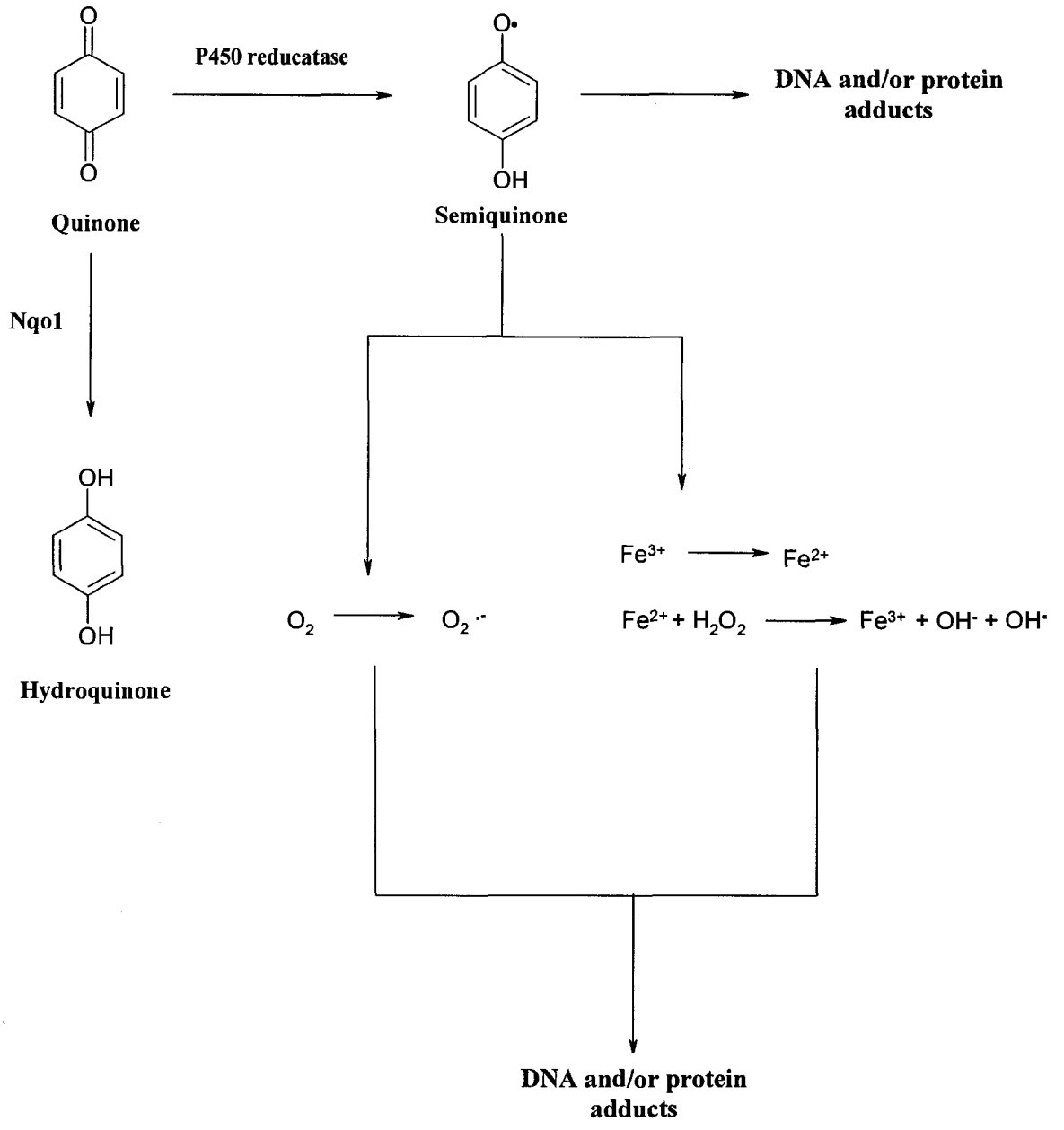

Figure 1.3. Consequences of quinone metabolism. Quinones will induce *Nqo1* gene expression through the Nrf2 signaling pathway. Once induced, the Nqo1 will start detoxifying these quinones to the corresponding hydroquinone. Due to their electrophilicity, semiquinones are capable of direct reaction with cellular macromolecules including protein and DNA, and this may ultimately lead to neoplasia. Redox-cycling may also occur; the unpaired electron from the semiquinone can be used to reduce Fe^{3+} to Fe^{2+}, which in turn drives the Fenton reaction leading to hydroxyl radical production. Alternatively, a semiquinone may reduce molecular oxygen, which can lead to generation of singlet oxygen and superoxide-driven Fenton reactions. Adapted from (Nioi and Hayes, 2004).

1.5.1. Non-Classical AhR Ligands

Non-classical AhR ligands have been previously defined to be those ligands whose structures and physiochemical properties significantly differ from those of PAHs and HAHs (Seidel et al., 2000; Gharavi and El-Kadi, 2005). The existence of endogenous AhR ligands received notoriety in response to the hypothesis that AhR can be activated in the absence of exogenous ligands. Pools of evidences now support the presence of endogenous AhR ligands. For example, it has been shown that hypoxia induces CYP1A1 in rat lungs and liver *in vivo*; this induction coincided with the formation of endogenous AhR ligand (Carlson and Perdew, 2002; Fradette and Du Souich, 2004; Sekine et al., 2006). Furthermore, it has been demonstrated that physiological compound(s) contained in serum induce *CYP1A1* gene expression in human HepG2 cells (Guigal et al., 2000; Guigal et al., 2001). Recently, a variety of endogenous AhR ligands have been identified *in vitro*. Those ligands include bilirubin (Sinal and Bend, 1997), lipoxin (Schaldach et al., 1999), tryptophan (Heath-Pagliuso et al., 1998; Oberg et al., 2005), and arachidonic acid metabolites (Denison and Nagy, 2003; Korashy and El-Kadi, 2006a). Although the majority of these non-classical AhR ligands are weak CYP1A1 inducers, this list has expanded to include a number of widely prescribed drugs such as omeprazole (Lemaire et al., 2004), primaquine (Werlinder et al., 2001), sulindac (Ciolino et al., 2006), ketoconazole, itraconazole, and clotrimazole (Navas et al., 2004; Korashy et al., 2007b) and the food flavoring agent maltol (Anwar-Mohamed and El-Kadi, 2007). In spite of identifying these AhR ligands *in vitro*, their *in vivo* activities remain to be identified.

1.6. Mechanisms involved in the modulation of AhR-regulated genes

1.6.1. Transcriptional and post-transcriptional mechanisms

The AhR is a transcription factor that controls the expression of several genes encoding the XRE recognition sequence. MRNA transcripts are labile units, whose level is determined by the rate of synthesis and the rate of degradation. The inhibition of mRNA synthesis using actinomycin D (Act-D) suggests transcriptional regulation of *CYP1A1* gene expression. In addition, recent studies have demonstrated that the superinduction of the *CYP1A1* gene by cycloheximide (CHX) or carbobenzoxy-l-leucyl-l-leucyl-leucinal (MG-132), a 26S proteasome inhibitor, is a transcriptional mechanism and reflects a change in the synthesis, rather than stabilization, of CYP1A1 mRNA (Ma and Baldwin, 2000; Ma and Baldwin, 2002; Joiakim et al., 2004). However, alteration of gene expression may occur not only at the transcriptional level, but at any stage up to the expression of protein activity. Interestingly, mRNA degradation is regulated by the exonucleases that catalyze mRNA, but protected by a specific terminal structure poly(A) tail at the 3' end of the mRNA transcript (Meyer et al., 2004). In addition, the half-lives of certain mRNAs are known to change in response to extracellular signals (Wilusz et al., 2001). Furthermore, the stability of certain mRNAs representing proteins of the same family may vary greatly within and across species. For example, rodent CYP1A1 and CYP1A2 mRNA half-lives are quite different (Lekas et al., 2000). Furthermore, human CYP1A1 and CYP1A2 mRNAs also have very different decay behavior, with the CYP1A2 being much more long-lived than the CYP1A1 mRNA (Lekas et al., 2000). The

rapid decay of CYP1A1 coincided with a rapid loss in poly(A) tail length, suggesting that deadenylation is the first step in the decay pathway (Lekas et al., 2000). Intriguingly, the CYP1A1 mRNA half life is in sharp contrast to most of the other CYP mRNAs. The difference in CYP mRNA decay times is still unclear and needs further work to better understand this process.

The degradation of NQO1 mRNA has not been elucidated; however, a few studies have examined the half life of NQO1 mRNA transcripts. It has been shown that NQO1 mRNA decay is regulated through the AU rich region in its untranslated 3' end (Gingerich et al., 2004). This region is targeted by AU binding proteins (AUBPs), which in turn bind to the mRNA transcripts and govern their stability (Gingerich et al., 2004). Activation of AUBPs may occur through different mechanisms and the most studied ones by far are the phosphorylation and ubiquitination processes that occur subcellularly in response to different extracellular and intracellular signals (Gingerich et al., 2004). Studies examining the half life of NQO1 mRNA in different species have found NQO1 transcripts are long lived with half-life of more than 17 h (Korashy and El-Kadi, 2006b; Elbekai and El-Kadi, 2008).

1.6.2. Translational and post-translational mechanisms

Post-translational modification can be defined as any functional modification of a translated protein (Han and Martinage, 1992). The majority of these modifications occur after the release of the polypeptide from the ribosome after being translated from its original mRNA transcripts (Han and Martinage, 1992). The post-translational

modification of amino acids alters protein functionality by attaching to it other biochemical functional groups such as acetate, phosphate, and various lipids and carbohydrates. Protein phosphorylation is a common mechanism for controlling its behavior, i.e. activating or inactivating the protein (Aguiar et al., 2005).

1.6.2.1. Phosphorylation

Protein phosphorylation is one of the post-translational modifications, in which phosphate is transferred to the protein from adenosine triphosphate (ATP) via different phosphatases, for cleaving the phosphate, and kinases, for docking the phosphate (Han and Martinage, 1992). Generally, phosphorylation occurs if the exposed amino acid happens to be a serine (Ser), therionine (Thr), or tyrosine (Tyr) (Han and Martinage, 1992). Thus it was proposed that CYPs could be modified by phosphorylation. It was first discovered by Pyerin et al. and co-workers that protein kinase A (PKA) phosphorylated purified rabbit CYP2B4 on its Ser128 residue. Thus, it was proposed that CYP2B4 is a PKA substrate (Pyerin et al., 1983; Pyerin et al., 1987).

The fact that CYPs and PKA both reside in the same cellular endomembrane fractions further supported this theory (Oesch-Bartlomowicz and Oesch, 2005). Interestingly, induced CYP2B1/2 in phenobarbital-treated rats has been shown to be phosphorylated by PKA which was induced by glucagon (Bartlomowicz et al., 1989). In spite of being phosphorylated, CYP2B1 protein, but not catalytic activity, levels remained unaffected (Oesch-Bartlomowicz et al., 2001). In addition there was no decrease in total cellular heme content, suggesting that PKA-mediated loss of activity

does not occur through the phosphorylation dependent degradation of the protein (Oesch-Bartlomowicz et al., 2001). Unexpectedly, it was found that c-AMP dependent phosphorylation of CYP2E1, coincided with a rapid decrease in its half-life and catalytic activity levels (Menez et al., 1993). In contrast, PKA-dependent phosphorylation of CYP2E1 decreased its catalytic activity without affecting its decay behavior (Menez et al., 1993).

Further studies on the effect of phosphorylation on mutagenicity revealed that CYP1A1/2-mediated formation of metabolites in rat liver hepatocytes was significantly reduced by the Ser/Thr protein phosphatase inhibitor, sodium orthovanadate (Pyerin et al., 1983; Oesch-Bartlomowicz et al., 2005). In addition, studies on the regulation of CYP1A1 showed that AhR/ARNT heterodimerization requires phosphorylation of ARNT only, whereas binding of AhR/ARNT to XRE requires phosphorylation of both the AhR and the ARNT (Pongratz et al., 1991; Berghard et al., 1993).

1.6.2.2. Total Heme and Heme Oxygenase

The heme group in all CYPs is essential for their enzymatic activity. Recent studies have shown that CYPs' induction is in tight correlation with that of δ-aminolevulinate synthase, a rate limiting step in the biosynthesis of heme (Lavrovsky et al., 1993). In addition, heme oxygenase-1 (HO-1), an enzyme of 32 kDa, catalyzes the oxidative conversion of heme into biliverdin which serves an important role in protecting cells from oxidative damage, such as free radicals (Marilena, 1997; Kikuchi et al., 2005).

The HO-1 isozyme, the major isoform, is ubiquitously expressed in a wide range of mammalian tissues (Lavrovsky et al., 1993; Kikuchi et al., 2005). HO-1 is induced by oxidative stress stimuli, such as hypoxia, inflammation, heavy metals, and hydrogen peroxide. Furthermore, HO-1 anchors to the endoplasmic reticulum membrane via a stretch of hydrophobic residues at the C-terminus (Schuller et al., 1998). Thus, it is expected to interact with CYPs which are also endoplasmic reticulum-bound enzymes.

1.6.2.3. 26s Proteasome degradation

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

The 26s proteasome is a very large (2500 kda), dynamic holoenzyme that consists of at least 32 different subunits in two copies (Glickman and Ciechanover, 2002; Cohen et al., 2008). Proteasomes are present in the nucleus and cytoplasm of almost all organisms. Intriguingly, treatment of Hepa 1c1c7 cells with TCDD shortened the AhR $t_{1/2}$ from 28 h to 3 h (Ma et al., 2000; Fujii-Kuriyama and Mimura, 2005). The down-

regulation of AhR in response to its ligands is attributed to the ubiquitination of AhR protein prior to its degradation through the 26S proteasome pathway (Eltom et al., 1999). This is supported by the observations that inhibition of the 26S proteasome pathway in Hepa 1c1c7 cells, using MG-132, increased the AhR and ARNT protein levels and hence enhanced the induction of Cyp1a1 gene expression (Davarinos and Pollenz, 1999).

1.7. Heavy metals

The toxic effects of individual AhR ligands have been extensively studied, yet the combined toxic effects of these AhR ligands and other environmental co-contaminants remain to be examined. Heavy metals, a common co-contaminant for AhR ligands have received considerable attention during the past decade. This is because such compounds can be found in air, water, soil, and food side by side with AhR ligands (Evangelou, 2002).

1.7.1. Vanadium (V^{5+})

The Spanish mineralogist del Rio first discovered vanadium (V^{5+}) in 1813, and gave it the name panchromium because of the color changes that occur during the transition to various oxidation states (Sjoberg, 1950). Nils Sëfstrom purified vanadium oxide in 1831 and named the metal after the Norse goddess Vanadis (Tsiani and Fantus, 1997). Vanadium occurs in oxidation states ranging from -1 to +5 (Zhang et al., 2001). Metallic vanadium does not occur in nature, yet it occurs in over 65 known naturally occurring

mineral salts and is a major trace element in fossil fuels (Tsiani and Fantus, 1997). The most prominent forms in biological systems are the pentavalent V^{5+} and tetravalent V^{4+} (Evangelou, 2002). Pure vanadium is a bright white-silver metal, and is the 22nd most abundant element in the earth crust (Mukherjee et al., 2004).

V^{5+} is hard and able to form alloys, and thus V^{5+}-containing alloys became popular components of hard steel alloys used in machines and tools (Barceloux, 1999). V^{5+} exists in water, rocks, and soil in low concentrations, and in coal and oil deposits in relatively high concentrations (Evangelou, 2002). Humans and other species may be exposed to V^{5+} through the atmosphere, food, and water. Foods contain V^{5+} in the concentration of few tens of μg (Evangelou, 2002). In drinking water supplies in the U.S.A., 91% of samples had below 10 μg V^{5+}/L, with the maximum concentration reaching 70 μg/L and an average of 4.3 μg/L (Craun et al., 1981). According to data provided by the manufacturers of vitamin and mineral supplements sold in the U.K. which in turn is representative of at least 70% of the U.K. market, the highest level of V^{5+} in multivitamin products reached 25 μg/ tablet or capsule. Furthermore, weight training athletes are reported to use up to 18.6 mg V^{5+} per day (Barceloux, 1999).

Recent studies have demonstrated that V^{5+} compounds exert protective effects against chemical-induced carcinogenesis, mainly through modifying various xenobiotic metabolizing enzymes (Evangelou, 2002). In addition, there is increasing evidences that V^{5+} accumulates more in cancer cells and tissues than in normal cells or tissues

(Evangelou, 2002). However, the exact mechanism by which this metal may prevent carcinogenesis is still unclear.

1.7.2. V^{5+} toxicity

V^{5+} toxicity in animals is characterized by marked nervous disturbance, hemorrhagic enteritis and a fall of temperature. Death is preceded by paralysis of hind legs, labored respiration and convulsions (Browning, 1969). In rats of unknown strain, vanadium pentoxide and ammonium metavanadate have been reported to have LD 50 of 5.8 and 8.0 mg/kg body weight, respectively (Massmann, 1956). In male Sprague-Dawley rats, sodium metavanadate and vanadyl sulfate pentahydrate were less toxic with oral LD 50 of 41.0 and 90.3 mg/kg body weight, respectively. In male Swiss mice, the LD 50 vlaues of these compounds were 31.0 and 94.0 mg/kg body weight, respectively (Llobet and Domingo, 1984). In humans, Dutton was the first to report the development of dry cough and irritation of the eyes following occupational exposure to the fumes and dusts of V^{5+} (Barceloux, 1999). Furthermore, twelve coronary heart disease patients treated with V^{5+} suffered from persistent abdominal pain, anorexia, nausea, and loss of weight. Five of these patients developed green tongue while other five reported pharyngitis with marginal ulceration of the tongue (Somerville and Davies, 1962). Patients with non-insulin dependent diabetes mellitus treated with V^{5+} experienced gastrointestinal problems, including nausea, diarrhea, abdominal cramps and flatulence (Cohen et al., 1995; Boden et al., 1996; Halberstam et al., 1996).

1.7.3. Environmental co-exposure to heavy metals and AhR ligands

Epidemiological studies reported environmental co-contamination of metropolitan residents with metals and AhR-ligands. For example, PAHs and heavy metals were found in airborne particulates in an industrial north Italian town, certain areas of Czech Republic where coal is used for heating and power (Monarca et al., 1997, Sram et al., 1996), and in tissues of wild city pigeons exposed to air pollution in the Netherlands (Schilderman et al., 1997). Moreover, PAHs and heavy metals were found in various aquatic sites such as the river Meuse in western Europe (Schilderman et al., 1999), the Dniester River in the former Soviet republic of Moldova (Sapozhnikova et al., 2005), the New Bedford Harbor in Massachusetts (Ho et al., 1997), and in the Sheboygan River system in east-central Wisconsin (Schrank et al., 1997).

With the growing human exposure to AhR ligands and heavy metals, emerging evidence suggests that AhR ligands and metal co-exposure generates biological responses different from what is expected based on the toxicological mechanisms of each class separately. It has been shown that BaP-induced leiomyosarcomas in male Wistar rats gradually disappeared between the 20^{th} and 30^{th} day of treatment with V^{5+} (Evangelou et al., 1997); however, the exact mechanism for this antitumor effect remains unclear (Evangelou et al., 1997).

During Gulf War II (Desert storm) oil slicks in some Gulf States polluted the water and coastlines of these countries killing thousands of sea birds (Small, 1991). Moreover, during this war more than 700 oil wells were burnt in Kuwait, forming a very dense black cloud that covered some Gulf States (Browning, 1991). The burning wells

emitted oxides of carbon, nitrogen, sulfur, and hydrocarbon particulates (Browning, 1991). Interestingly, heavy metals such as V^{5+} which is present in Kuwait crude oil in the order of 10-30 mg/kg, was also observed (Bakan, 1991; Browning, 1991). A study conducted soon after this war in Bahrain examined the presence of PAHs and V^{5+} in air expressed as air particulate matter (APM) (Madany and Raveendran, 1992). The study concluded that the concentrations of PAH and V^{5+} were 5.3 and 26 ng/m^3, respectively (Madany and Raveendran, 1992). However, the effect of heavy metals on the metabolism of AhR-ligands was never determined. Any influence of metals on the capacity of AhR ligands to induce AhR-regulated genes, and hence alter their metabolism, will influence the carcinogenicity and mutagenicity of the AhR ligands. Thus, an important determinant of the role of heavy metals in modulating the toxicity of AhR ligands is their effect on the enzymes responsible for the metabolism of AhR ligands.

1.8. Rationale, Hypotheses, and Objectives

1.8.1. Rationale

Classical AhR ligands typified by HAHs and PAHs produce a wide array of toxic effects. Most of the toxic effects produced by these hazardous contaminants have been shown to be mediated by the AhR, a cytosolic receptor to which these contaminants bind. 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. Therefore, the toxicity of these AhR ligands cannot be assessed by measuring CYP induction alone,

because it has been shown that these AhR ligands will also induce the phase II AhR regulated genes as a counterproductive mechanism to this process. Although numerous studies have examined the toxic effects of individual AhR ligands, there are relatively few reports of the combined toxic effects of AhR ligands and other environmental contaminants, typified by heavy metals. Therefore, it was of great importance to evaluate the combined toxic effects, in particular the AhR-driven carcinogenicity and mutagenicity of these AhR ligands typified by TCDD and V^{5+} a common co-contaminant of TCDD.

1.8.2. Hypotheses

Hypothesis 1: V^{5+} down-regulates Cyp1a1 through an AhR-dependent mechanism

Hypothesis 2: V^{5+} down-regulates Nqo1 by an AhR- and Nrf2- dependent mechanisms.

1.8.3. Specific Objectives

1. To determine the effect of V^{5+} on the AhR signaling pathway and the subsequent effect on Cyp1a1.

2. To determine the effect of V^{5+} on the Nrf2 signaling pathway and the subsequent effect on Nqo1.

1.8.4. Experimental plan

To test the hypotheses, *in vitro* studies were carried out in the murine Hepa 1c1c7 cell line. These cell lines offer the principal model for the investigation of the regulatory mechanisms of AhR-regulated genes for two reasons; the first is the availability of mutant Hepa 1c1c7 cell lines deficient in AhR, C12 cells. The second reason is the high

inducible gene expression of *Cyp1a1* and *Nqo1* in these cell lines. The expression of Cyp1a1 and Nqo1 in response to V^{5+} was determined at the mRNA, protein, and activity levels using TCDD as inducer for Cyp1a1, and TCDD or SUL, as bifunctional and monofunctional inducers, respectively, for Nqo1. To determine the level at which V^{5+} exerts its effect, studies were carried out at the transcriptional, post-transcriptional, and post-translational levels using inhibitors of transcription and translation.

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CHAPTER 2

2. Down-regulation of the carcinogen metabolizing enzyme cytochrome P450 1a1 by vanadium

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2.1. Introduction

The Aryl hydrocarbon Receptor (AhR) is a ligand-activated cytoplasmic transcription factor that belongs to the basic-helix-loop-helix protein family. The inactive form of AhR is attached to a complex of two heat shock proteins 90 (HSP90), hepatitis B virus X-associated protein (XAP2), and the chaperone protein p23 (Hankinson, 1995; Meyer et al., 1998). Upon ligand binding, the AhR-ligand complex dissociates from the cytoplasmic compex and translocates to the nucleus where it associates with the aryl hydrocarbon nuclear translocator (ARNT) (Whitelaw et al., 1994). The whole complex then acts as a transcription factor that binds to a specific DNA recognition sequence, termed the xenobiotic responsive element (XRE), located in the promoter region of a number of AhR-regulated genes (Denison et al., 1989; Nebert et al., 2004). Among these genes are those encoding a number of drug metabolizing enzymes, including four phase I enzymes [cytochrome P450 1a1 (Cyp1a1), Cyp1a2, Cyp1b1, and Cyp2s1] and four phase II enzymes [NAD(P)H: quinone oxidoreductase-1, glutathione-*S*-transferase a1, cytosolic aldehyde dehydrogenase-3 and UDP-glucuronosyltransferase 1a6] (Nebert and Duffy, 1997).

Experimental and epidemiological data have shown that various environmental pollutants such as halogenated aromatic hydrocarbons (HAHs) are capable of producing a variety of toxic effects in exposed organisms; some of the most common toxicities include neurotoxicity, immune dysfunction, reproductive and developmental effects, and cancer (Schrenk, 1998; Elbekai and El-Kadi, 2004). Mounting evidence suggests that

most of the toxic manifestations induced by, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic HAH with the greatest affinity for AhR, are occurring through the activation of the AhR (Kransler et al., 2007). As a result of the tight correlation between AhR binding affinity and Cyp1a1 induction, Cyp1a1 has been used as a biomarker for evaluating hazards and risk assessments of environmental pollutants (Behnisch et al., 2001).Itself, Cyp1a1 is capable of producing polar, toxic, or even carcinogenic metabolites from various AhR ligands, including aromatic and halogenated hydrocarbons (Anwar-Mohamed and El-Kadi, 2007a).

Numerous studies have examined the toxicity of individual AhR ligands, yet the combined toxic effects of these ligands and other environmental co-contaminants typified by heavy metals such as vanadium (V^{5+}), are still unclear. V^{5+}, a co-contaminant with AhR ligands, is widely distributed in nature. The oxidation state of V (+4 or +5) seems to be important for its actions on enzymes (Cantley and Aisen, 1979). V^{5+} compounds have been found in various types of food, such as black pepper, mushrooms, dill seed, parsley and shellfish (Rojas et al., 1999). Interestingly, previous studies have demonstrated that V^{5+} compounds exert protective effects against chemical-induced carcinogenesis mainly by modifying various xenobiotic metabolizing enzymes; however the exact mechanism(s) remain unknown (Evangelou, 2002).

The objective of this study was to determine the effect of co-exposure to V^{5+} and TCDD on Cyp1a1 and to investigate the molecular mechanisms involved. As a first step to investigate these mechanisms, we examined the effect of co-exposure to V^{5+} and

TCDD on Cyp1a1 mRNA, protein, and activity levels in Hepa 1c1c7 cells. In order to address whether the observed effects of co-exposure to V^{5+} and TCDD occurred through an AhR-dependent mechanism, we examined the effect of the co-exposure on luciferase activity in Hepa 1c1c7 cells transiently transfected with the XRE-driven luciferase plasmid pGudLuc1.1. Looking at the involvement of post-transcriptional mechanisms, we tested the effect of V^{5+} on Cyp1a1 mRNA and protein stability. We also examined the ability of V^{5+} to modulate AhR/ARNT/XRE binding using the electrophoretic mobility shift assay (EMSA) and the role of Ecto alkaline phosphatase (Ecto-ATPase) in this modulation.

We provide here the first evidence that V^{5+} down-regulates the expression of Cyp1a1 through a transcriptional mechanism.

2.2. Materials and methods

2.2.1. Materials

Ammonium metavanadate (NH_4VO_3), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cycloheximide (CHX), *p*-nitrophenyl phosphate (P-NPP), *p*-nitrophenol (PNP), 2,6-dichlorophenolindophenol, 7-ethoxyresorufin, fluorescamine, anti-goat IgG peroxidase secondary antibody, and protease inhibitor cocktail were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). TRIzol reagent and Lipofectamine 2000 reagents were

purchased from Invitrogen (San Diego, CA). High-Capacity cDNA Reverse Transcription Kit and SYBR® Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA). Actinomycin-D (Act-D) was purchased from Calbiochem (San Diego, CA). Chemiluminescence Western blotting detection reagents were from GE Healthcare Life Sciences (Piscataway, NJ). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA). Cyp1a1 goat polyclonal primary antibody, AhR rabbit polyclonal primary antibody, and anti-rabbit IgG peroxidase secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Luciferase assay reagents were obtained from Promega (Madison, WI). [γ^{32}P]ATP was supplied by the DNA Core Services Laboratory, University of Alberta. All other chemicals were purchased from Fisher Scientific (Toronto, ON).

2.2.2. Cell culture

Hepa 1c1c7 cell line, ATCC number CRL-2026 (Manassas, VA), were maintained in Dulbecco's modified Eagle's medium (DMEM), without 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.

2.2.3. Chemical treatments

Cells were treated in serum-free medium with various concentrations of V^{5+}, in the form of ammonium metavanadate, (25 - 1000 μM) in the absence and presence of 1 nM TCDD as described in figure legends. TCDD was dissolved in dimethylsulfoxide (DMSO) and maintained in DMSO at −20 °C until use. V^{5+} was prepared freshly in double de-ionized water. In all treatments, the DMSO concentration did not exceed 0.05% (v/v).

2.2.4. Effect of V^{5+} on cell viability

The effect of V^{5+} on cell viability was determined using the MTT and ATP-based luminescent assays as described previously (Maniratanachote et al., 2005; Korashy and El-Kadi, 2008). MTT assay measures the conversion of MTT to formazan in living cells via mitochondrial enzymes of viable cells. In brief, Hepa 1c1c7 cells were seeded into 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 V^{5+} (25-1000 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).

The ATP-based luminescent assay measures the quantity of the ATP produced by metabolically active cells; the ATP was determined using CellTiter-Glo Luminescent

assay kit according to manufacturer's instructions (Promega). Briefly, Hepa 1c1c7 cells were seeded into 24-well cell culture plates and incubated for 24 h at 37 °C in a 5% CO_2 humidified incubator. Cells were then treated with various concentrations of V^{5+} (25-1000 μM) in the absence and presence of 1 nM TCDD for another 24 h. At the end of treatment period, 200 μl CellTiter-Glo reagent was added to each well. The generated luminescent signal was monitored on a TD-20/20 luminometer (Turner BioSystems, Sunnyvale CA).

2.2.5. RNA extraction and Quantitative real-time PCR of Cyp1a1

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 mouse Cyp1a1 were: Forward primer 5`- GGT TAA CCA TGA CCG GGA ACT -3`, reverse primer 5`- TGC CCA AAC CAA AGA GAG TGA -3`. Heme oxygenase-1(HO-1): forward primer 5`- GTG ATG GAG CGT CCA CAG C -3`, reverse primer 5`- TGG TGG CCT CCT TCA AGG -3` and for β-actin: forward primer 5`- TAT TGG CAA CGA GCG GTT CC -3`, reverse primer 5`- GGC ATA GAG GTC TTT ACG GAT GTC -3`. These primers and probes were purchased from Integrated DNA technologies (IDT, Coralville, IA). The

fold change in the level of Cyp1a1 (target gene) between treated and untreated cells, corrected by the level of ß-actin, was determined using the following equation: Fold change = $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{(target)} - Ct_{(ß\text{-}actin)}$ and $\Delta(\Delta Ct) = \Delta Ct_{(treated)} - \Delta Ct_{(untreated)}$.

2.2.6. 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 (25 µg) were resolved by denaturing electrophoresis, as described previously (Elbekai and El-Kadi, 2004). 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-mouse Cyp1a1 antibody for 2 h at room temperature, or primary polyclonal rabbit anti-mouse AhR antibody for overnight at 4 °C in TBS containing 0.05% (v/v) Tween-20 and 0.02 % sodium azide. Incubations with a peroxidase-conjugated rabbit anti-goat IgG secondary antibody for Cyp1a1 and Gapdh or goat anti-rabbit IgG for AhR was carried out in blocking buffer for 1 h at room temperature. The bands were visualized with the enhanced chemiluminescence method

according to the manufacturer's instructions (Amersham, Arlington Heights, IL). The intensity of Cyp1a1 protein bands was quantified, relative to the signals obtained for Gapdh protein, using ImageJ software.

2.2.7. Determination of Cyp1a1 enzymatic activity

Cyp1a1-dependent 7-ethoxyresorufin *O*-deethylase (EROD) activity was performed on intact, living cells using 7-ethoxyresorufin as a substrate, as previously described (Elbekai and El-Kadi, 2004). Enzymatic activity was normalized for cellular protein content, which was determined using a modified fluorescent assay (Lorenzen and Kennedy, 1993).

2.2.8. Transient transfection and luciferase assay

Hepa 1c1c7 cells were plated onto 6-well cell culture plates. Each well of cells was transfected with 4 μg of XRE-driven luciferase reporter plasmid pGudLuc1.1 using lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). Luciferase assay was performed according to the manufacturer's instructions (Promega) as described previously (Elbekai and El-Kadi, 2007). Briefly, after incubation with test compounds for 24 h, cells were washed with PBS and a 200 μl of 1x lysis buffer was 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. 5x cell lysis buffer is supplied in the Promega luciferase assay kit (1x = 25 mM Tris-phosphate pH 7.8, 2 mM DTT, 2 mM CDTA, 10% glycerol, and 1% Triton x-100). 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).

2.2.9. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from Hepa 1c1c7 cells, treated for 2 h with vehicle, 5 nM TCDD, in the absence and presence of 50 μM V^{5+} using the method of Denison (Denison et al., 2002). Because of low efficiency of transformation of the mouse AhR due to the extreme resistance of HSP90 to dissociation from the mouse AhR, and the greater degree of transformation of the guinea pig AhR in response to AhR ligand (Bohonowych and Denison, 2007), we used guinea pig cytosol as a model. Therefore, hepatic cytosol of untreated guinea pig, generously provided by Dr. M.S. Denison at University of California (Davies, CA), was incubated with the test compounds at a final concentration of 20 nM TCDD, in the absence and presence of 100 or 250 μM V^{5+} for 2 h at 20 °C. Protein concentrations for the nuclear and cytosolic extracts were determined using the method of Lowry (Lowry et al., 1951). To visualize the ability of V^{5+} to alter the transformation and subsequent DNA binding of the AhR, a complementary pair of synthetic oligonucleotides containing the sequence 5′- GAT CTG GCT CTT CTC ACG CAA CTC CG -3′ and 5′- GAT CCG GAG TTG CGT GAG AAG AGC CA -3′, corresponding to the XRE binding site, were synthesized and radiolabeled with [γ^{32}P]ATP as previously described (Denison et al., 2002) and used as a DNA probe in all experiments. Binding reactions using aliquots of 120 μg cytosolic or 20 μg nuclear extracts and excess radiolabeled oligonucleotides were allowed to proceed for 15 min at 20 °C in a buffer containing 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 25 mM

HEPES, 400 ng to 2 μg poly(dI–dC), and 0.4–0.8 mM KCl. To determine the specificity of binding to the oligonucleotide, a 100-fold M excess of unlabeled XRE probe was added to the binding reaction prior to addition of the γ^{32}P-labeled probe. Protein-DNA complexes were separated under non-denaturing conditions on a 4% polyacrylamide gel using 1× TBE (90 mM of Tris borate, 90 mM of boric acid, 4 mM of EDTA) as a running buffer. The gels were dried and the protein–DNA complexes were visualized by autoradiography.

2.2.10. Cyp1a1 mRNA stability

The half-life of Cyp1a1 mRNA was analyzed by an Act-D-chase assay. Cells were pre-treated with 1 nM TCDD for 12 h. Cells were then washed and incubated with 5 μg/ml Act-D, to inhibit further RNA synthesis, immediately before treatment with (50 μM) V^{5+}. Total RNA was extracted at 0, 1, 3, 6, and 12 h after incubation with the metal. Real-time PCR reactions were performed using SYBR® Green PCR Master Mix (Applied Biosystems). The fold change in the level of Cyp1a1 (target gene) between treated and untreated cells, corrected by the level of ß-actin, was determined using the following equation: Fold change = $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{(target)} - Ct_{(ß\text{-}actin)}$ and $\Delta(\Delta Ct) = \Delta Ct_{(treated)} - \Delta Ct_{(untreated)}$.

2.2.11. Cyp1a1 protein stability

The half-life of Cyp1a1 protein was analyzed by the CHX-chase assay. Cells were pre-treated with 1 nM TCDD for 24 h. Cells were then washed and incubated with 10 μg/ml CHX, to inhibit further protein synthesis, immediately before treatment with V^{5+} (50

μM). Cell homogenates were extracted at 0, 1, 3, 6 and 12 h after incubation with the metal. Cyp1a1 protein was measured by Western blot analysis. The intensity of Cyp1a1 protein bands was quantified, relative to the signals obtained for Gapdh protein, using the ImageJ software. The protein half-life values were determined from semilog plots of integrated densities versus time.

2.2.12. Determination of total cellular heme content

Cellular heme content was determined by a modification of the method of Ward (Ward et al., 1984). After a 24 h incubation period with 50 μM V^{5+} in the absence and presence of 1 nM TCDD, cells were pelleted and boiled in 2.0 M oxalic acid for 30 min followed by resuspension in cold PBS and centrifugation at 14,000 × *g* for 15 min. The supernatant was then removed and the fluorescence of protoporphyrin IX was assayed using the Eclipse fluorescence spectrophotometer (Varian Australia PTY LTD., Australia) using an excitation wavelength of 405 nm and an emission wavelength of 600 nm. Background was determined by measuring the fluorescence in the absence of cells. Cellular heme content was calculated as a percent of serum-free medium-treated cells following normalization of cellular heme content with cellular protein, which was determined using the method of Lowry (Lowry et al., 1951).

2.2.13. Determination of Ecto-ATPase enzymatic activity

To examine the effect of V^{5+} on Ecto-ATPase activity, a colorimetric method utilizing the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol was used as described previously (Anagnostou et al., 1996). In brief, cells were seeded in 12-well plates for 48 h.

Thereafter, the cells were treated with vehicle, V^{5+}, TCDD, or V^{5+} plus TCDD for 6 h. Cells were then washed with phosphate-free buffer (15 mM Tris, 134 mM NaCl, 3 mM $CaCl_2$, 3 mM $MgCl_2$), pH 8.0, and the medium was replaced with 1 ml of 0.5 mM *p*-nitrophenyl phosphate and incubated for 2 h at 37 °C. To stop the reaction, 1 ml of 0.2 M NaOH was added to each well. Thereafter, the cells were harvested and centrifuged at 14,000 g for 5 min. *p*-Nitrophenol in the supernatant was quantified by measuring the absorbance at 405 nm. Enzymatic activity was normalized for cellular protein content, which was determined using the Lowry assay (Lowry et al., 1951).

2.2.14. Statistical analysis

The comparative analysis of the results from various experimental groups with their corresponding controls was performed using SigmaStat for Windows (Systat Software, Inc, CA). A one-way analysis of variance (ANOVA) followed by Student–Newman–Keul's test was carried out to assess statistical significance. The differences were considered significant when $p < 0.05$.

2.3. Results

2.3.1. Effect of co-exposure to V^{5+} and TCDD on cell viability

To determine the maximum non-toxic concentrations of V^{5+} to be utilized in the current study, Hepa 1c1c7 cells were exposed for 24 h to increasing concentrations of V^{5+} (25 – 1000 μM) in the absence and presence of 1 nM TCDD. Thereafter, cytotoxicity was assessed using MTT and CellTiter-Glo Luminescent assays.

Figure 2.1.A shows that V^{5+} alone at concentrations of (25 – 250 μM) did not affect cell viability; however, the highest concentration, 1000 μM, decreased cell viability to approximately 67%. Similarly co-exposure to V^{5+} and TCDD produced a significant decrease in cell viability, at the highest concentration tested (1000 μM), to approximately 70%. On the other hand, concentration-dependent effect of exposure to V^{5+} in the absence and presence of 1 nM TCDD using CellTiter-Glo Luminescent assay exhibited a relatively similar pattern to that observed with the MTT experiment (Figure 2.1.B). Therefore, all subsequent studies were conducted using concentrations of 25 – 250 μM in the absence and presence of 1 nM TCDD.

2.3.2. Concentration-dependent effect of co-exposure to V^{5+} and TCDD on inducible Cyp1a1 mRNA

To better understand the kinetics of Cyp1a1 in response to co-exposure to V^{5+} and TCDD, Hepa 1c1c7 cells were treated with various concentrations of V^{5+} (Figure 2.2.). Thereafter, Cyp1a1 mRNA was assessed using real-time PCR. TCDD alone caused 38-fold increase in Cyp1a1 mRNA that was inhibited in a dose-dependent manner by V^{5+}, starting at a concentration of 25 μM, and reaching a maximum inhibition at the concentration of 250 μM (Figure 2.2.).

2.3.3. Concentration-dependent effect of co-exposure to V^{5+} and TCDD on Cyp1a1 protein and catalytic activity

To examine whether the observed inhibition of the TCDD-mediated induction of Cyp1a1 mRNA is reflected at the protein and activity levels, Hepa 1c1c7 cells were treated for 24

h with increasing concentrations of V^{5+} in the absence and presence of 1 nM TCDD. Figure 2.3.A and 2.3.B show that TCDD alone caused 20- and 35-fold increase in Cyp1a1 protein and catalytic activity, respectively.

On the other hand, V^{5+} significantly reduced the TCDD-mediated induction of Cyp1a1 protein and activity levels in a dose-dependent manner. This inhibition pattern was consistent with that observed at mRNA levels, in which the initial significant inhibition took place with the 50 μM V^{5+}, and reaching the maximal inhibition at 250 μM (Figure 2.3.A and 2.3.B).

2.3.4. Transcriptional inhibition of Cyp1a1 gene induction by V^{5+}

To investigate whether the observed effect upon co-exposure to V^{5+} and TCDD on Cyp1a1 is occurring through an AhR-dependent mechanism, Hepa 1c1c7 cells were transiently transfected with the XRE-driven luciferase reporter gene in order to study the effect of V^{5+} on the AhR-dependent transcriptional activation. Luciferase activity results showed that 50 μM V^{5+} alone did not alter the luciferase activity (Figure 2.4.). In contrast, 1 nM TCDD alone was capable of causing a significant induction of the luciferase activity that reached up to 1200 relative light units (RLU), as compared to control. On the other hand, co-treatment with V^{5+} and TCDD significantly decreased the luciferase activity by 3-fold in comparison to that of TCDD alone (Figure 2.4.).

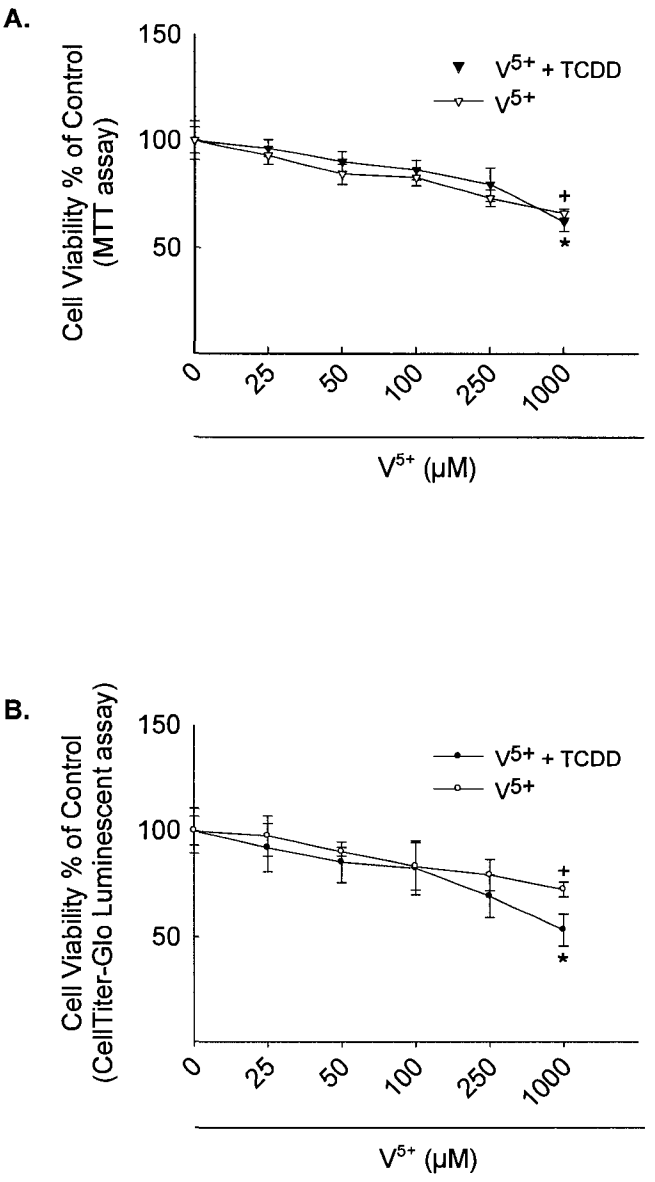

Figure 2.1. Effect of V^{5+} on cell viability. Hepa 1c1c7 cells were treated for 24 h with V^{5+} (0, 25, 50, 100, 250, and 1000 μM) in the absence and presence of 1 nM TCDD. Cell cytotoxicity was determined using MTT (**A**). and CellTiter-Glo Luminescent (**B**). assays. Data are expressed as percentage of untreated control (which is set at 100%) ± SE ($n = 8$). (+) $P < 0.05$, compared to control (concentration = 0 μM); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

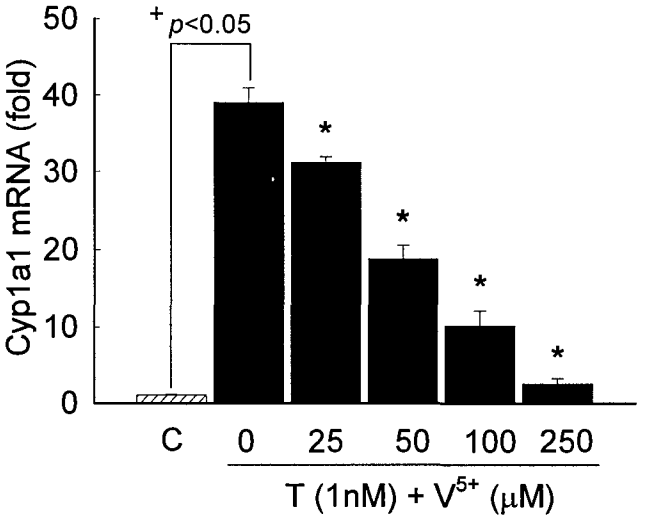

Figure 2.2. Effect of V^{5+} on Cyp1a1 mRNA using real-time PCR. Hepa 1c1c7 cells were treated for 6 h with increasing concentrations of V^{5+} in the presence of 1 nM TCDD. First-strand cDNA was synthesized from total RNA (1 μg) extracted from Hepa1c1c7 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (+) $P < 0.05$, compared to control (C) (concentration = 0 μM); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

In an effort to determine whether V^{5+} interferes with the nuclear binding of the transformed AhR to the XRE, we examined the potential effect of V^{5+} on TCDD-induced translocation of AhR to the nucleus and the subsequent binding to XRE, the promoter sequence of Cyp1a1, by EMSA. For this purpose, Hepa 1c1c7 cells were treated with vehicle, V^{5+}, TCDD, or V^{5+} plus TCDD for 1 h, followed by extraction of nuclear extracts. Extracts from vehicle- and TCDD-treated cells were used as negative and positive controls, respectively.

Figure 2.5.A shows that V^{5+} alone did not induce AhR/ARNT/XRE complex formation, as shown by the intensity of the bands. In contrast, TCDD significantly increased AhR/ARNT/XRE binding. In addition, V^{5+} completely abolished the TCDD-induced nuclear formation of AhR/ARNT/XRE complex (lane 3). The specificity of the TCDD-induced AhR/ARNT heterodimer binding to XRE was confirmed by competition assay using 100-fold M excess of unlabelled XRE.

To examine the ability of V^{5+} to inhibit the direct activation of the cytosolic AhR by TCDD and the subsequent DNA-binding, EMSA was performed on untreated guinea pig hepatic cytosol incubated with vehicle, V^{5+}, TCDD, or V^{5+} plus TCDD for 2 h *in vitro*. Figure 2.5.B shows that V^{5+} alone, at the indicated concentrations, failed to induce the AhR/ARNT/XRE complex transformation, as determined by the shifted band, compared to that of TCDD alone. Furthermore, V^{5+} did not inhibit the TCDD-induced transformation of the AhR/ARNT/XRE complex. Collectively, our data indicate that V^{5+} inhibited the translocation process rather than inhibiting the binding step in the AhR transduction pathway.

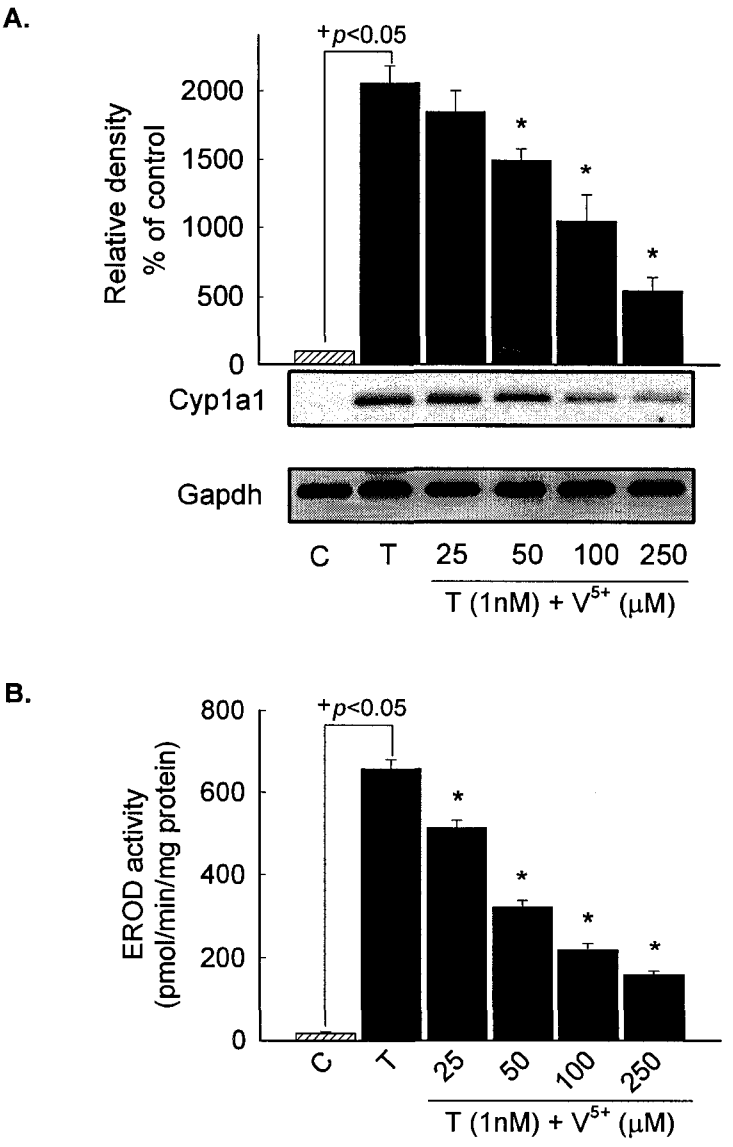

Figure 2.3. Effect of V^{5+} on inducible Cyp1a1 protein and EROD activity. Hepa 1c1c7 cells were treated for 24 h with increasing concentrations of V^{5+} in the presence of 1 nM TCDD. **A.** Protein (25 μg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C and then incubated with a primary Cyp1a1 antibody for 2 h at 4°C, followed by 1 h incubation with secondary antibody at room temperature. Cyp1a1 protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to signals from Gapdh, which was used as loading control. One of three representative experiments is shown. **B.** EROD activity was measured in intact living cells treated with increasing concentrations of V^{5+}, in the absence and presence of 1 nM TCDD for 24 h. Cyp1a1 activity was measured using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SE ($n = 8$). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

2.3.5. Time-dependent effect of co-exposure to V^{5+} and TCDD on AhR protein

To determine whether V^{5+}s' effect on Cyp1a1 is due to increasing AhR protein degradation, we examined the effect of co-exposure to V^{5+} and TCDD on the AhR protein using total cell lysate over a 24 h time-course.

Our results showed that AhR is a short-lived protein with only 15% of AhR protein remained at 6 h following TCDD treatment (Figure 2.6.). On the other hand, co-exposure to V^{5+} and TCDD did not significantly alter the AhR protein level in comparison to that of TCDD alone. These results exclude the possibility that V^{5+} may alter the Cyp1a1 by lowering the cellular levels of AhR protein.

2.3.6. Post-transcriptional modification of Cyp1a1 mRNA by V^{5+}

The level of mRNA expression is not only a function of the transcription rate, but is also dependent on the elimination rate, through processing or degradation. Previous reports from our laboratory have shown that various heavy metals modulate the expression of the Cyp1a1 through transcriptional and post-transcriptional mechanisms (Korashy and El-Kadi, 2005; Elbekai and El-Kadi, 2007).

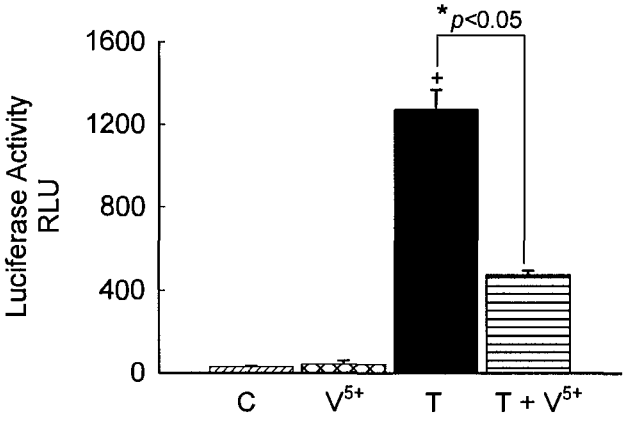

Figure 2.4. Effect of V^{5+} on luciferase activity. Hepa 1c1c7 cells were transiently transfected with the XRE-luciferase transporter plasmid pGudLuc1.1. Cells were treated with vehicle, TCDD (1 nM), V^{5+} (50 μM), or TCDD (1 nM) + V^{5+} (50 μM) for 24 h. Cells were lysed and luciferase activity was measured according to manufacturer's instruction. Luciferase activity is reported as relative light unit. Values are presented as mean ± SE ($n = 6$). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

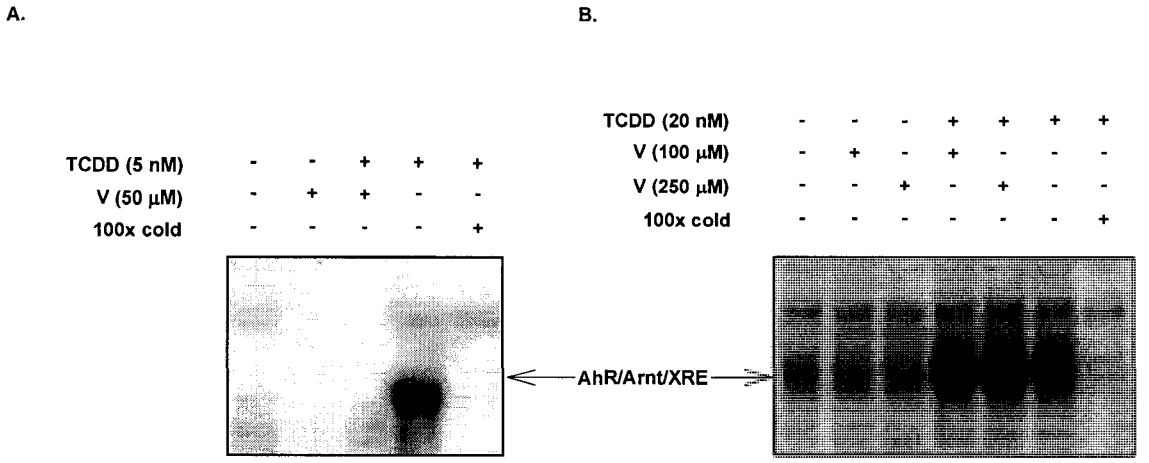

Figure 2.5. Effect of V^{5+} on AhR/ARNT/XRE binding. A. Nuclear extracts (20 μg) from Hepa 1c1c7 cells were treated for 2 h with vehicle, TCDD (5 nM), V^{5+} (50 μM), or TCDD (5 nM) + V^{5+} (50 μM). **B.** Cytosolic extracts (80 μg) from untreated guinea pig liver extracts were incubated for 2 h with vehicle, TCDD (20 nM), V^{5+} (100 μM), V^{5+} (250 μM), TCDD (20 nM) + V^{5+} (100 μM), or TCDD (20 nM) + V^{5+} (250 μM). The cytosolic and nuclear proteins were mixed with [γ^{32}P]-labeled XRE, and the formation of AhR/ARNT/XRE complexes was analyzed by EMSA. The specificity of binding was determined by incubating the protein treated with TCDD with 100-fold molar excess of cold XRE. The arrow indicates the specific shift representing the AhR/ARNT/XRE complex. This pattern of AhR alteration was observed in three separate experiments, and only one is shown.

Therefore, we examined the effect of V^{5+} on the stability of Cyp1a1 mRNA transcripts, using an Act-D chase experiment. If V^{5+} alters Cyp1a1 mRNA stability, a decrease in half-life would be expected to take place. Figure 2.7. shows that Cyp1a1 mRNA decayed with a half-life of 4.73 $\pm$ 0.54 h. Furthermore, V^{5+} did not alter the half-life of Cyp1a1 mRNA, indicating that the decrease of Cyp1a1 mRNA transcripts in response to V^{5+} was not due to increasing its degradation.

2.3.7. Post-translational modification of Cyp1a1 protein by V^{5+}

The fact that V^{5+} inhibited TCDD-mediated induction of Cyp1a1 protein raised the question whether V^{5+} could modify the Cyp1a1 protein stability. Therefore, the effect of V^{5+} on the Cyp1a1 protein half-life was determined using CHX-chase experiments. Figure 2.8. shows that Cyp1a1 protein induced by TCDD degraded with half-life of 8.41 $\pm$ 0.29 h. Interestingly, V^{5+} did not alter the stability of Cyp1a1 protein (Figure 2.8.), implying that V^{5+} did not affect Cyp1a1 protein at the post-transcriptional level.

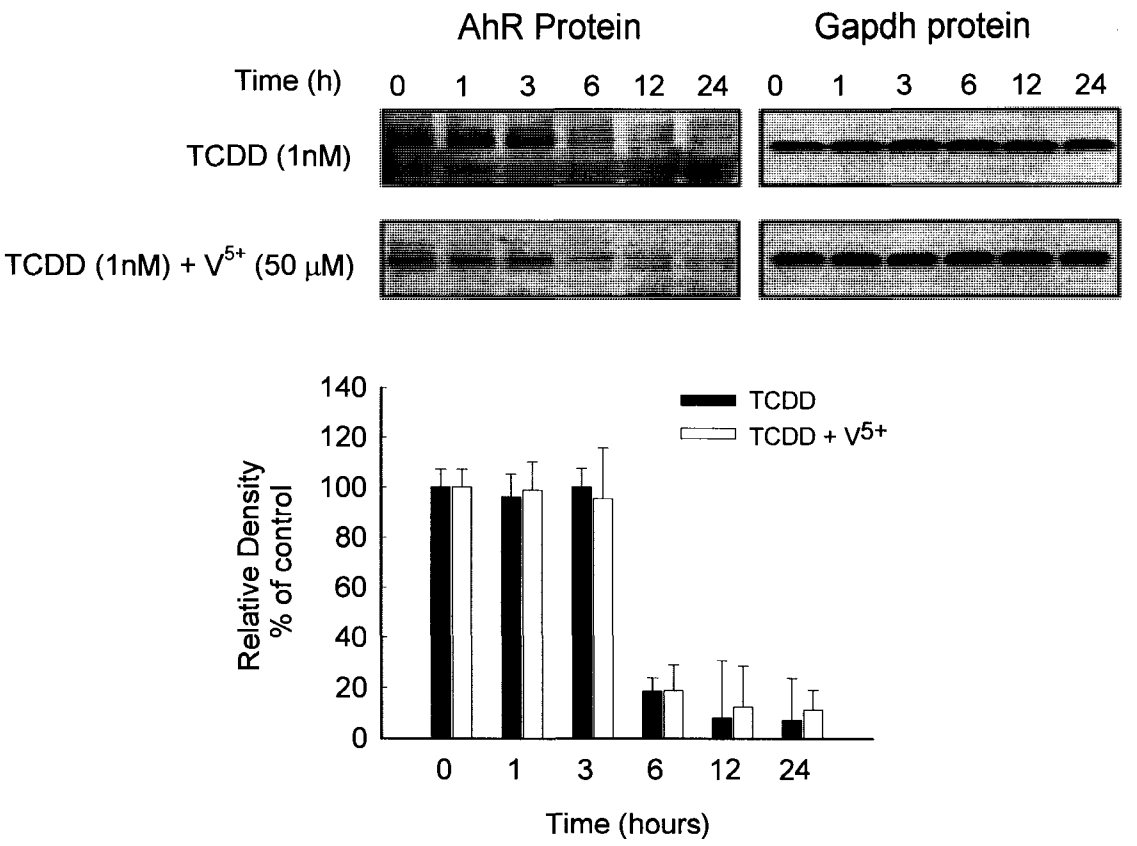

Figure 2.6. Time-dependent effect of V^{5+} on AhR protein. Hepa 1c1c7 cells were treated with 1 nM TCDD for the time points indicated in the absence and presence of 50 μM V^{5+}. Protein (25 μg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C and then incubated with a primary AhR antibody overnight at 4°C, followed by 2 h incubation with secondary antibody at room temperature. AhR protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to signals from Gapdh, which was used as loading control. Graph represents Gapdh-standardized densitometric readings for AhR protein expressed as a percent of AhR protein levels of control (time = 0 h) (which is set at 100%) ± SE (n = 3).

2.3.8. Effect of co-exposure to V^{5+} and TCDD on HO-1 mRNA and total cellular heme content

We have previously reported that heavy metals other than V^{5+} were able to modify Cyp1a1 by affecting its heme content (Korashy and El-Kadi, 2005; Elbekai and El-Kadi, 2007). Therefore, in the current study we examined the effect of V^{5+} on mRNA for HO-1, a rate limiting enzyme of heme degradation, and total cellular heme content. For this purpose, Hepa 1c1c7 cells were treated with 50 μM V^{5+} in the absence and presence of 1 nM TCDD (Figure 2.9.A). Thereafter, HO-1 mRNA was assessed using real-time PCR. Figure 2.9.A shows that V^{5+} and TCDD alone did not alter HO-1 mRNA levels. Similarly, co-exposure to V^{5+} and TCDD did not significantly alter HO-1 mRNA level. In order to confirm the effect of co-exposure to V^{5+} and TCDD on heme degradation, we examined the effect of this co-exposure on total cellular heme content. Total cellular heme content was measured in Hepa 1c1c7 cells 24 h after treatment with 50 μM V^{5+} in the absence and presence of 1 nM TCDD. Our results show that neither V^{5+} nor TCDD was able to decrease total cellular heme content. Furthermore, the co-exposure to V^{5+} and TCDD failed to produce any significant change in the total cellular heme content (Figure 2.9.B). Taken together these results exclude any role for V^{5+} in decreasing Cyp1a1 function through decreasing its heme content.

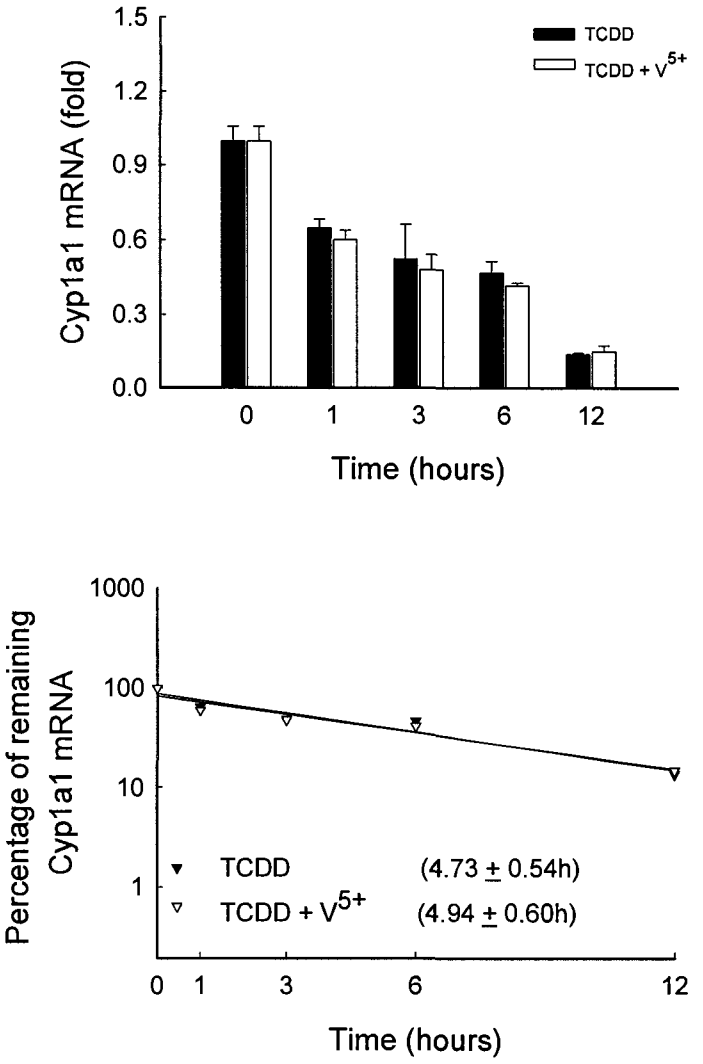

Figure 2.7. Effect of V^{5+} on Cyp1a1 mRNA half-life using real-time PCR. Hepa 1c1c7 cells were grown to 90% confluence in six-well cell culture plates, and then treated with 1 nM TCDD for 12 h. The cells were then washed and incubated in a fresh media containing 50 μM V^{5+} plus 5μg/ml Act-D, a RNA synthesis inhibitor. First-strand cDNA was synthesized from total RNA (1 μg) extracted from Hepa1c1c7 cells. cDNA fragments were amplified and quantitated using an ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. mRNA decay curves were analyzed individually, and the half-life was estimated from the slope of a straight line fitted by linear regression analysis ($r^2 \geq 0.85$) to a semilog plot of mRNA amount, expressed as a percent of treatment at time = 0 h (maximum, 100%) level, versus time. The half-lives obtained from three independent experiments were then used to calculate the mean half-life (mean ± SE, $n = 3$).

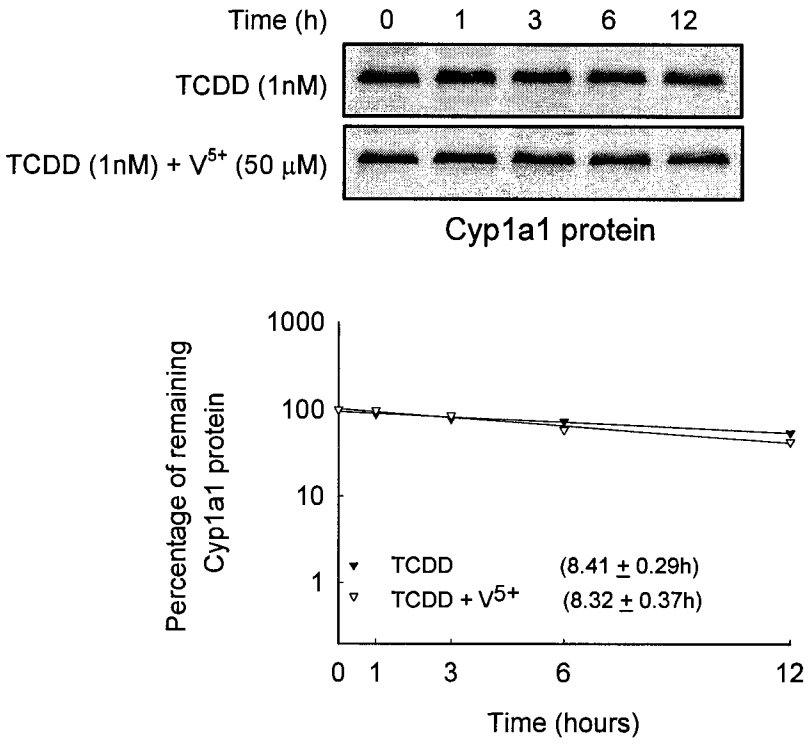

Figure 2.8. Effect of V^{5+} on the Cyp1a1 protein half-life. Hepa 1c1c7 cells were grown to 70% confluence in six-well cell culture plates, and then treated with 1 nM TCDD for 24 h. Thereafter, the cells were washed and incubated in fresh media containing 50 μM V^{5+} plus 10 μg/ml CHX, a protein translation inhibitor. Cyp1a1 protein was extracted at the designated time points after the addition of CHX. Protein (25 μg) was separated on a 10% SDS–PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C and then incubated with a primary polyclonal Cyp1a1 antibody for 2 h at 4°C, followed by 1 h incubation with secondary monoclonal Cyp1a1 antibody at room temperature. Antibody was detected using the enhanced chemiluminescence method. The intensity of Cyp1a1 protein bands were normalized to signals from Gapdh, which was used as loading control (data not shown). All protein decay curves were analyzed individually. The half-life was estimated from the slope of a straight line fitted by linear regression analysis ($r^2 \geq 0.85$) to a semilog plot of protein amount, expressed as a percent of treatment at time = 0 h (maximum, 100%) level, versus time. The half-lives obtained from three independent experiments were then used to calculate the mean half-life (mean ± SE, $n = 3$).

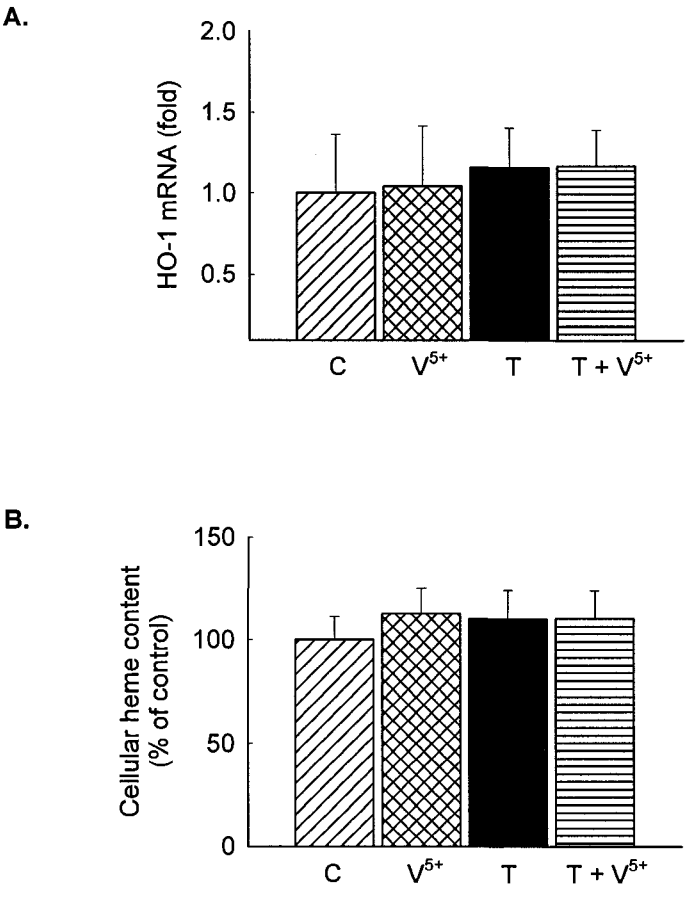

Fig 2.9. Effect of V^{5+} on HO-1 mRNA and total cellular heme content. (A). Hepa 1c1c7 cells were treated for 6 h with 50 μM V^{5+} in the presence and absence of 1 nM TCDD. First-strand cDNA was synthesized from total RNA (1 μg) extracted from Hepa1c1c7 cells. cDNA fragments were amplified and quantitated using an ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (+) $P < 0.05$, compared to control (C) (concentration = 0 μM); (*) $P < 0.05$, compared to respective TCDD (T) treatment. **(B).** Hepa 1c1c7 cells were treated for 24 h with 50 μM V^{5+} in the presence and absence of 1 nM TCDD. Thereafter, cells were pelleted and boiled in oxalic acid, followed by resuspension in PBS. After centrifugation, the fluorescence of protoporphyrin IX was assayed by a spectrophotometric method.

2.3.9. Effect of co-exposure to V^{5+} and TCDD on Ecto-ATPase enzymatic activity

To further investigate if V^{5+} inhibits other AhR regulated genes other than Cyp1a1, we examined the effect of co-exposure to V^{5+} and TCDD on Ecto-ATPase activity, an enzyme responsible for the conversion of extracellular ATP and ADP to AMP. Previous studies have shown that TCDD induces Ecto-ATPase gene expression in Hepa 1c1c7 cells at the transcriptional level (Gao et al., 1998). To examine the effect of V^{5+} on Ecto-ATPase, Hepa 1c1c7 cells were treated with vehicle, V^{5+}, TCDD, or V^{5+} and TCDD for 6 h. Our results show that TCDD alone significantly increased the Ecto-ATPase activity, as compared to control (Figure 2.10.). On the other hand, V^{5+} alone caused a significant decrease in Ecto-ATPase activity by ~ 30% compared to control. Interestingly the co-exposure to V^{5+} and TCDD significantly decreased the Ecto-ATPase activity by ~ 70% in comparison to TCDD alone (Figure 2.10.).

2.4. Discussion

Humans consume appreciable amounts of V^{5+} in food and water (Evangelou, 2002). The estimated daily intake of V^{5+} is 10 - 60 μg (Nechay, 1984). In addition, V^{5+} supplements in the form of 10 mg tablets are available from several commercial sources where V^{5+} is promoted as a body building supplement. An estimate of the total body pool of vanadium in healthy individuals is 100 - 200 μg (Byrne and Kosta, 1978). Taking into consideration that heavy metals such as V^{5+} are significantly deposited in hepatocytes and kidneys (Edel and Sabbioni, 1989), the concentrations used in the current study are of great relevance to those of humans.

During the last few years, V^{5+} compounds have been shown to be effective in inhibiting cancers of the liver (Bishayee and Chatterjee, 1995), lung, breast, and gastrointestinal tract (Kopf-Maier, 1987; Kanna et al., 2003; Kanna et al., 2004). The mechanism for this anticancer effect is not known. However, previous studies have demonstrated that V^{5+} compounds exert protective effects against chemical-induced carcinogenesis mainly by modifying various xenobiotic metabolizing enzymes (Evangelou, 2002). Data from our laboratory and others showed that heavy metals other than V^{5+} are capable of modifying the carcinogen metabolizing enzyme, Cyp1a1 at different stages of its regulatory pathway (Bessette et al., 2005; Korashy and El-Kadi, 2005; Elbekai and El-Kadi, 2007; Khan et al., 2007).

Before performing the current study, we hypothesized that V^{5+} protects against TCDD-mediated toxicity and carcinogenicity by inhibiting *Cyp1a1* gene expression. Hence, the main objective of the current study was to determine the potential effects of co-exposure to V^{5+} and TCDD on the expression of Cyp1a1. We also explored the molecular mechanism(s) by which V^{5+} modulates the expression of Cyp1a1.

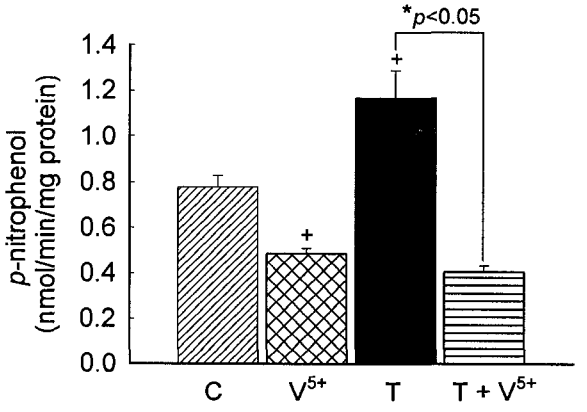

Figure 2.10. Effect of co-exposure to V^{5+} and TCDD on Ecto-ATPase activity. Ecto-ATPase activity was measured in intact living cells treated with increasing 50 μM V^{5+}, in the absence and presence of 1 nM TCDD for 24 h. Ecto-ATPase activity was measured using *p*-nitrophenyl phosphate as a substrate. Values are presented as mean ± SE ($n = 6$). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

Cyp1a1 gene expression involves the activation of a cytosolic transcriptional factor, the AhR, as the first step in a series of molecular events promoting *Cyp1a1* transcription and translation processes (Denison et al., 1989). Initially, we showed that V^{5+} inhibits TCDD-mediated induction of Cyp1a1 mRNA in a concentration-dependent manner and this inhibition was further translated to the protein and catalytic activity levels. Hepa 1c1c7 cells co-exposed to increasing concentrations of V^{5+} and 1 nM TCDD showed a significant dose-dependent inhibition of Cyp1a1 mRNA starting at 25 μM V^{5+} and reaching maximum inhibition at 250 μM.

The transcriptional regulation of *Cyp1a1* gene expression by V^{5+} was supported by a series of evidence. V^{5+} inhibited TCDD-mediated induction of a AhR-dependent luciferase reporter gene expression (Figure 2.4). Thus, V^{5+} either inhibited the transformation of AhR to its DNA-binding form and/or the nuclear accumulation of liganded AhR, thus causing inhibition of AhR/ARNT/XRE complex formation. To determine whether V^{5+} alters Cyp1a1 by interfering with AhR/ARNT/XRE binding, we performed EMSA using nuclear extracts of treated Hepa 1c1c7 cells and untreated guinea pig hepatic cytosol, as described previously (Denison et al., 2002). Our results showed that although V^{5+} failed to inhibit *in vitro* TCDD-mediated transformation of AhR to its DNA-binding in guinea pig cytosol, it completely abolished the nuclear accumulation of the AhR and its subsequent binding *in vivo*.

Previous data have shown that liganded AhR is ubiquitinated prior to its degradation by the 26S proteasomal pathway (Pollenz, 2002). To determine whether or

not the decrease in AhR/ARNT/XRE binding is due to the increase of the degradation of AhR protein by V^{5+}, we examined the effect of V^{5+} on AhR protein levels at different time points. Our results showed that AhR degrades rapidly after exposure to TCDD and at 6 h the remaining AhR was ~ 15% compared to control (time = 0 h). Co-exposure to V^{5+} and TCDD did not significantly alter the AhR protein levels in comparison to TCDD alone. These results suggest that the observed inhibitory effect of V^{5+} on AhR/ARNT/XRE binding is not due to decrease in the cellular level of the AhR protein.

We previously have shown that heavy metals modulate Cyp1a1 through transcriptional, post-transcriptional and post-translational mechanisms (Korashy and El-Kadi, 2005; Elbekai and El-Kadi, 2007). Thus it was of great importance to determine the effect of V^{5+} on the post-transcriptional regulation of Cyp1a1. The cellular mRNA level at any time point is a function of the rate of its production, through transcriptional mechanisms, and the rate of its degradation. Therefore, we examined the effect of V^{5+} on the Cyp1a1 mRNA stability using the Act-D chase experiment. Our results showed that the Cyp1a1 mRNA induced by TCDD is short-lived, with an estimated half-life of 4.73 $\pm$ 0.54 h. Our results are in agreement with previous reports which showed that the half-life of Cyp1a1 mRNA induced by TCDD in Hepa 1c1c7 cells ranges from 3–4.5 h (Miller et al., 1983; Chen et al., 1995). Similarly, V^{5+} did not significantly alter the stability of Cyp1a1 mRNA, suggesting that a post-transcriptional mechanism is not involved in the modulation of Cyp1a1 mRNA by V^{5+}.

In order to examine the effect of co-exposure to V^{5+} and TCDD at the post-translational level, CHX-chase experiment was performed. Our results showed that the Cyp1a1 protein induced by TCDD has an estimated half-life of 8.41 $\pm$ 0.29 h. In contrast, V^{5+} did not significantly alter the stability of Cyp1a1 protein, inferring that a post-translational mechanism is not involved in the modulation of Cyp1a1 protein by V^{5+}.

Our previous studies have shown that heavy metals possess the ability to decrease Cyp1a1 activity through affecting its heme content (Korashy and El-Kadi, 2005; Elbekai and El-Kadi, 2007). These results prompted us to examine the effect of co-exposure to V^{5+} and TCDD on HO-1 mRNA and total cellular heme content. In the current study we showed that V^{5+} did not significantly alter HO-1 mRNA level and total cellular heme content. Thus, these results exclude any possibility that V^{5+} might have decreased Cyp1a1 activity through affecting its heme content.

It has been previously reported that V^{5+} is capable of activating the redox sensitive transcription factor, nuclear factor-κB (NF-κB) (Chen et al., 2001). Of interest, it has been demonstrated that there is a mutual inhibitory interaction between the AhR and the NF-κB signaling pathways (Ke et al., 2001). The possibility that the NF-κB prevents the AhR/ARNT binding to the XRE is excluded, because it has been shown that NF-κB activation does not affect AhR/ARNT binding to XRE (Ke et al., 2001). Therefore, the inhibitory effect of V^{5+} on AhR/ARNT/XRE binding is NF-κB-independent.

Previous studies have demonstrated that Ecto-ATPase is regulated by AhR (Gao et al., 1998). These findings prompted us to investigate the possible effect of V^{5+} on Ecto-ATPase, another AhR-regulated gene. In this study we have shown that V^{5+} decreased Ecto-ATPase enzymatic activity. These results further confirm that V^{5+} inhibits AhR-regulated genes at the transcriptional level.

In conclusion, the present study demonstrates that V^{5+} down-regulates the bioactivating enzyme Cyp1a1 through a transcriptional mechanism. The translocation of the transformed AhR was inhibited by V^{5+}. Thus, these results suggest that V^{5+} may protect against TCDD-mediated toxicity by inhibiting *Cyp1a1* gene expression. However, further studies are needed to investigate the cytoprotective effect of V^{5+} against TCDD-mediated toxicity.

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CHAPTER 3

3. Down-regulation of the phase II drug metabolizing enzyme NAD(P)H: quinone oxidoreductase 1 by vanadium in Hepa 1c1c7 cells

A version of this chapter has been submitted for publication: Anwar-Mohamed, A. and El-Kadi, A.O., 2008. *Drug Metabolism and Disposition.*

3.1. Introduction

The NAD(P)H: quinone oxidoreductase 1 (NQO1) is a cytosolic flavoenzyme that catalyzes the two-electron reduction of a broad range of substrates (Ross, 2004; Ross and Siegel, 2004). NQO1 plays a pivotal role in detoxifying quinones to their corresponding hydroquinone derivatives (Ross, 2004). Such an effect helps in maintaining endogenous antioxidants like ubiquinone and vitamin E in their reduced and active forms, thus protecting tissues from mutagens, carcinogens, and oxidative stress damage (Schlager and Powis, 1990; Winski et al., 1998). In addition to its wide distribution across different human tissues and organs, NQO1 has been found to be exceptionally elevated in a wide variety of human tumors and cell lines (Hankinson, 1995; Meyer et al., 1998).

NQO1 gene expression can be induced through two separate regulatory elements associated with its 5`-flanking region. The first pathway includes activation of a cytosolic transcription factor, the aryl hydrocarbon receptor (AhR). The inactive form of AhR is attached to a complex of two heat shock proteins 90 (HSP90), hepatitis B virus X-associated protein (XAP2), and the chaperone protein p23 (Whitelaw et al., 1994). Upon ligand binding, the AhR-ligand complex dissociates from the cytoplasmic complex and translocates to the nucleus where it associates with the aryl hydrocarbon nuclear translocator (ARNT) (Denison et al., 1989; Nebert et al., 2004). The whole complex then acts as a transcription factor to mediate the induction of *NQO1* through activating the xenobiotic responsive element (XRE) located in its promoter region (Venugopal and Jaiswal, 1996; Itoh et al., 1997). The second pathway involves activation of the

antioxidant responsive element (ARE), which does not require functional AhR. In fact, the increased expression of *NQO1* gene expression in response to oxidative stress caused by agents such as isothiocyanate sulforaphane (SUL), *tert*-butylhydroquinone (t-BHQ) and H_2O_2 occurs primarily through this signaling pathway (Itoh et al., 1997). Perturbation in the redox status of the cell activates the nuclear factor erythroid 2-related factor-2 (Nrf2), a redox-sensitive member of the cap 'n' collar basic leucine zipper (CNC bZip) family of transcription factors (Ma et al., 2004; Korashy et al., 2007a). Subsequently, Nrf2 dissociates from its cytoplasmic tethering polypeptide, Kelch-like ECH associating protein 1 (Keap1), and then translocates into the nucleus, dimerizes with a musculoaponeurotic fibrosarcoma (MAF) protein, and thereafter binds to and activate ARE (Ma et al., 2004; Marchand et al., 2004; Miao et al., 2005).

The XRE- and ARE-driven regulation of *NQO1* gene was generally thought to function independently. However, the proximity of the two sequence sites suggests a possible cross-talk and functional overlap (Ma et al., 2004; Marchand et al., 2004; Miao et al., 2005). Recent reports suggest that bifunctional inducers, which activate both XRE and ARE signaling pathways, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), require direct cross-talk between the XRE- and ARE-mediated pathways for the induction of *NQO1*. Furthermore, it has been reported that the induction of *NQO1* by ARE inducers requires the presence of AhR, suggesting a more direct cross-talk between the XRE- and ARE-mediated pathways (Elbekai et al., 2004).

Halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs) are the most extensively studied AhR ligands due to their ubiquitous nature. HAHs and PAHs are released into the environment through various natural and synthetic processes during incomplete combustion (Kransler et al., 2007). Experimental and epidemiological data have shown that HAHs, typified by TCDD, the most toxic HAH with the greatest affinity for AhR (Schrenk, 1998; Elbekai and El-Kadi, 2004), are capable of producing a variety of toxic effects in exposed organisms; some of the most common toxicities include neurotoxicity, immune dysfunction, reproductive and developmental effects, and cancer (Spink et al., 2002). Several PAHs and HAHs other than TCDD are both AhR ligands and substrates for phase I-inducible enzymes. CYP1A1, CYP1A2, and CYP1B1 are responsible for the conversion of these AhR ligands into toxic diol epoxide compounds capable of forming covalent adducts with guanines in critical genes, thus initiating tumorigenesis and other toxic responses (Evangelou, 2002).

Conglomerates of studies have examined the toxic effects of individual AhR ligands, yet there have been very few studies on the combined toxic effects of AhR ligands and other environmental co-contaminants. Among these, environmental co-contaminants of most concern are heavy metals, typified by vanadium (V^{5+}). V^{5+} is widespread in the environment and is a trace element in several biological systems (Tsiani and Fantus, 1997; Evangelou, 2002). Humans may be exposed to this metal through the atmosphere, food, and water (Tsiani and Fantus, 1997). Many studies have focused on the anti-diabetic effect of V^{5+} which is mediated by inhibiting the activity of

tyrosine phosphatase (Evangelou, 2002). In addition, several studies have reported preventive effects of V^{5+} against chemical-induced carcinogenesis, mainly through modifying various xenobiotic enzymes (Anwar-Mohamed and El-Kadi, 2008).

Recently, we have demonstrated that V^{5+} down-regulates the bioactivating enzyme Cyp1a1 which is solely under the control of XRE, through a transcriptional mechanism (Elbekai and El-Kadi, 2004). In addition, the translocation of the transformed AhR was inhibited by V^{5+} probably through an ATP-dependent mechanism. Therefore, the current study aims to address the possible effect of this metal on the *Nqo1* gene expression in Hepa 1c1c7 cells.

In this study we herein provide the first evidence that V^{5+} down-regulates the inducible expression of Nqo1 in both wild type and AhR-deficient cell lines.

3.2. Materials and methods

3.2.1. Materials

Ammonium metavanadate (NH_4VO_3), cycloheximide (CHX), 2,6-dichlorophenolindophenol, fluoroscamine, anti-goat IgG peroxidase secondary antibody, protease inhibitor cocktail, and isothiocyanate sulforaphane were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). TRIzol reagent was purchased from Invitrogen (Grand Island, NY). High-Capacity cDNA Reverse

Transcription Kit and SYBR® Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA). Actinomycin-D (Act-D) was purchased from Calbiochem (San Diego, CA). Chemiluminescence Western blotting detection reagents were from GE Healthcare Life Sciences (Piscataway, NJ). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA). NAD(P)H: quinone oxidoreductase 1 (Nqo1) rabbit polyclonal primary antibody was generously provided by Dr. David Ross (University of Colorado Health Sciences Center, CO, U.S.A.). Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) primary antibody, anti-goat and anti-rabbit IgG peroxidase secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [γ^{32}P]ATP was supplied by the DNA Core Services Laboratory, University of Alberta. All other chemicals were purchased from Fisher Scientific (Toronto, ON).

3.2.2. Cell culture

Hepa 1c1c7 cell lines, ATCC number CRL-2026, or mutant AhR-deficient Hepa 1c1c7 (C12) cell lines, ATCC number CRL-2710 (Manassas, VA), were maintained in Dulbecco's modified Eagle's medium (DMEM), without 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.

3.2.3. Chemical treatments

Cells were treated in serum-free medium with various concentrations of V^{5+} (25-250 μM) in the presence of 1 nM TCDD or 5 μM SUL. TCDD and SUL were dissolved in dimethylsulfoxide (DMSO) and maintained in DMSO at −20 °C until use. V^{5+} was prepared freshly in double de-ionized water. In all treatments, the DMSO concentration did not exceed 0.05% (v/v).

3.2.4. RNA extraction and quantitative real-time PCR of Nqo1

After incubation with the test compounds for the specified time periods, total cellular RNA was isolated using TRIzol reagent, according to the manufacturer's instructions (Invitrogen Co.), 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 (Applied Biosystems) 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 mouse Nqo1 were: forward primer 5`- GCA GGA TTT GCC TAC ACA ATA TGC -3`, reverse primer 5`- AGT GGT GAT AGA AAG CAA GGT CTT C -3`, and for β-actin: forward primer 5`- TAT TGG CAA CGA GCG GTT CC -3`, reverse primer 5`- GGC ATA GAG GTC TTT ACG GAT GTC -3` were purchased from Integrated DNA technologies (IDT, Coralville, IA). The fold change in the level of Nqo1 (target gene) 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 $\Delta(\Delta Ct) = \Delta Ct_{(treated)} - \Delta Ct_{(untreated)}$.

3.2.5. 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 (25 µg) were resolved by denaturing electrophoresis, as described previously (Korashy and El-Kadi, 2006b). 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-mouse Nqo1 antibody for 2 h at room temperature, or primary polyclonal goat anti-mouse Gapdh antibody for 24 h at 4 °C in TBS containing 0.05% (v/v) Tween-20 and 0.02 % sodium azide. Incubation with a peroxidase-conjugated goat anti-rabbit IgG for Nqo1, and rabbit anti-goat IgG secondary antibody for Gapdh 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 (GE Healthcare Life Sciences, Piscataway, NJ). The

intensities of Nqo1 protein bands were quantified, relative to the signals obtained for Gapdh protein, using ImageJ software.

3.2.6. Determination of Nqo1 enzymatic activity

Nqo1 activity was determined by a continuous spectrophotometric assay to quantitate the reduction of its substrate, 2,6-dichlorophenolindophenol (DCPIP) as described previously (Nioi et al., 2003). Briefly, 20 μg of cell homogenate protein was incubated with 1 ml of the assay buffer [40 μM DCPIP, 0.2 mM NADPH, 5 μM flavin-adenine dinucleotide, 25 mM Tris-HCl, pH 7.8, 0.1% (v/v) Tween 20, and 0.023% bovine serum albumin]. The rate of DCPIP reduction was monitored over 90 sec at 600 nm with an extinction coefficient (ε) of 2.1 mM^{-1} cm^{-1}. The Nqo1 activity was calculated as the decrease in absorbance per min per mg of total protein of the sample.

3.2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from Hepa 1c1c7 cells, treated for 4 h with vehicle, 1 nM TCDD, or 5 μM SUL in the presence and absence of 50 μM V^{5+} using the method of Nioi et al (Lowry et al., 1951). Protein concentrations for the nuclear extracts were determined using the method of Lowry (Nioi et al., 2003). To visualize the ability of V^{5+} to alter the DNA binding of the Nrf2 to ARE, a complementary pair of synthetic oligonucleotides containing the sequence 5′-GAT CTG GCT CTT CTC ACG CAA CTC CG-3′ and 5′-GAT CCG GAG TTG CGT GAG AAG AGC CA-3′, corresponding to the mouse Nqo1 ARE binding site, were synthesized and radiolabeled with [γ^{32}P]ATP as previously described (Anwar-Mohamed and El-Kadi, 2008). Binding reactions using aliquots of

7.5 μg nuclear extracts and excess radiolabeled oligonucleotides were allowed to proceed for 20 min at 20 °C in a buffer containing 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 25 mM HEPES, 3 μg poly(dI–dC), and 0.4 mM KCl. To determine the specificity of binding to the oligonucleotide, a 100-fold M excess of unlabeled ARE probe was added to the binding reaction prior to addition of the γ^{32}P-labeled probe. Protein-DNA complexes were separated under non-denaturing conditions on a 4% polyacrylamide gel using 0.5 × TBE (90 mM of Tris borate, 90 mM of boric acid, 4 mM of EDTA) as a running buffer. The gels were dried and the protein–DNA complexes were visualized by autoradiography after 6 h exposure.

3.2.8. Nqo1 mRNA stability

The half-life of Nqo1 mRNA was analyzed by an Act-D-chase assay. Cells were pre-treated with 1 nM TCDD for 12 h. Cells were then washed and incubated with 5 μg/ml Act-D, to inhibit further RNA synthesis, immediately before treatment with (50 μM) V^{5+}. Total RNA was extracted at 0, 6, 12, and 24 h after incubation with the metal. Real-time PCR reactions were performed using SYBR® Green PCR Master Mix (Applied Biosystems). The fold change in the level of Nqo1 (target gene) between treated and untreated cells, corrected by the level of ß-actin, was determined using the following equation: fold change = $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{(target)} - Ct_{(ß\text{-}actin)}$ and $\Delta(\Delta Ct) = \Delta Ct_{(treated)} - \Delta Ct_{(untreated)}$. The mRNA half-life values were determined from semilog plots of fold-change versus time.

3.2.9. Nqo1 protein stability

The half-life of Nqo1 protein was analyzed by the CHX-chase assay. Cells were pre-treated with 1 nM TCDD for 24 h. Cells were then washed and incubated with 10 μg/ml CHX, to inhibit further protein synthesis, immediately before treatment with V^{5+} (50 μM). Cell homogenates were extracted at 0, 12, 24, 36 and 48 h after incubation with the metal. Thereafter, Nqo1 protein was determined by Western blot analysis. The intensity of Nqo1 protein bands was quantified, relative to the signals obtained for Gapdh protein, using the ImageJ software. The protein half-life values were determined from semilog plots of integrated densities versus time.

3.2.10. Statistical analysis

The comparative analysis of the results from various experimental groups with their corresponding controls was performed using SigmaStat for Windows (Systat Software, Inc, CA). A one-way analysis of variance (ANOVA) followed by Student–Newman–Keul's test was carried out to assess statistical significance. The differences were considered significant when $p < 0.05$.

3.3. Results

3.3.1. Concentration-dependent effect of V^{5+} on TCDD-mediated induction of Nqo1 mRNA

To examine the ability of V^{5+} to modulate *Nqo1* gene expression, Hepa 1c1c7 cells were treated with various concentrations of V^{5+} in the presence of 1nM TCDD (Figure 3.1.).

Thereafter, Nqo1 mRNA was assessed using real-Time PCR. The concentrations of V^{5+} used thereafter were chosen after determining the ability of wide range of concentrations to modulate the *Nqo1* gene expression without significantly affecting cell viability (Venugopal and Jaiswal, 1996; Itoh et al., 1997). Initially, TCDD alone caused 4.7-fold increase in Nqo1 mRNA levels that was inhibited in a dose-dependent manner by V^{5+}, starting at the lowest concentration tested which is 25 μM (3-fold), and reaching the maximum inhibition at the concentration of 250 μM (4-fold) (Figure 3.1.).

3.3.2. Concentration-dependent effect of V^{5+} on TCDD-mediated induction of Nqo1 protein and catalytic activity

To examine whether the observed inhibitory effect of V^{5+} on the Nqo1 mRNA is reflected at the protein and catalytic activity levels, Hepa 1c1c7 cells were treated for 24 h with increasing concentrations of V^{5+} in the presence of 1 nM TCDD. Figures 1B and 1C show that TCDD alone caused 4.5- and 1.7-fold increase in Nqo1 protein and catalytic activity, respectively. On the other hand, V^{5+} significantly reduced the TCDD-mediated induction of Nqo1 at protein and activity levels in a dose-dependent manner (Figure 3.2.A and 3.2.B). This inhibition pattern was consistent with that observed at the mRNA levels, in which the initial significant inhibition took place with the lowest concentration tested, 25 μM V^{5+}. V^{5+} at the concentration of 25 μM inhibited the TCDD-mediated induction of Nqo1 protein and catalytic activity levels by 1.5- and 0.3-fold, respectively. On the other hand, the maximal inhibition took place with the highest concentration tested, 250 μM V^{5+}, in which the TCDD-mediated induction of Nqo1

protein and catalytic activity levels were inhibited by 5- and 0.6-fold in comparison to the TCDD-induced Nqo1 protein and catalytic activity levels (Figure 3.2.A and 3.2.B).

3.3.3. Effect of co-exposure to V^{5+} and SUL on Nqo1 mRNA, protein, and catalytic activity in Hepa 1c1c7 cells

It is well established that TCDD-mediated induction of Nqo1 occurs through the AhR and Nrf2 signaling pathways. The fact that V^{5+} inhibited TCDD-mediated induction of *Nqo1* gene expression raised the question about whether V^{5+} will behave similarly in the presence of SUL which induces *Nqo1* gene expression through the Nrf2 pathway only (Anwar-Mohamed and El-Kadi, 2008). For this purpose, Hepa1c1c7 cells were treated with 5 μM SUL in the presence and absence of 50 μM V^{5+}. If V^{5+} exerts its effect solely through the AhR pathway, we expect to see no effect of V^{5+} on SUL-mediated induction of Nqo1. Figure 3.3. shows that SUL alone significantly increased Nqo1 mRNA levels by 5-fold in comparison to controls. Interestingly, V^{5+} was able to inhibit this induction by 2.5-fold. Furthermore, this inhibition was further translated to the protein and catalytic activity levels, in which SUL alone showed significant induction of Nqo1 protein and catalytic activity levels by 3-fold and 2-fold, respectively (Figure 3.4.A and 3.4.B). On the other hand the co-exposure to V^{5+} and SUL significantly decreased the SUL-induced Nqo1 protein and catalytic activity levels by 0.6- and 0.7-fold, respectively in comparison to SUL alone (Figure 3.4.A and 3.4.B).

3.3.4. Effect of co-exposure to V^{5+} and SUL on Nqo1 mRNA and catalytic activity in AhR-deficient, Hepa 1c1c7 (C12) cells

We took a genetic approach to examine the role of V^{5+} in decreasing *Nqo1* gene expression through the Nrf2 signaling pathway. For this purpose we used the AhR-deficient, Hepa 1c1c7 (C12) cells.

Our results show that SUL (5 μM) significantly induced Nqo1 mRNA and catalytic activity levels by 7-fold and 1.5-fold, respectively (Figure 3.5.A, and 3.5.B). In contrast, V^{5+} alone was able to cause a slight but significant induction of Nqo1 mRNA levels; yet this induction was not adequate to produce a significant induction in the catalytic activity. Noteworthy, we were unable to detect Nqo1 protein levels in these cells. Interestingly, V^{5+} was able to inhibit SUL-mediated induction of Nqo1 at mRNA and catalytic activity levels by 2.5-fold and 0.25-fold, respectively (Figure 3.5.A, and 3.5.B). Taken together, these results reveal that V^{5+} modulates *Nqo1* gene expression through the Nrf2 pathway in addition to its previous reported effect on the AhR signaling pathway (Anwar-Mohamed and El-Kadi, 2008).

3.3.5. Transcriptional inhibition of Nqo1 gene induction by V^{5+}

Our previous data have shown that V^{5+} completely abolished the TCDD-induced nuclear formation of AhR/ARNT/XRE complex (Tsiani and Fantus, 1997). Therefore, it was of great importance to examine whether or not V^{5+} exerts similar effect with respect to the Nrf2/ARE binding complex. In an effort to determine whether V^{5+} interferes with the nuclear binding of Nrf2 to the ARE, we examined the potential effect of V^{5+} on SUL- and

TCDD-induced translocation of Nrf2 to the nucleus and the subsequent binding to ARE by EMSA.

For this purpose, Hepa 1c1c7 cells were treated with vehicle, V^{5+}, SUL, SUL plus V^{5+}, TCDD and TCDD plus V^{5+} for 4 h, followed by extraction of nuclear extracts. Figure 3.6. shows that SUL and TCDD significantly increased the Nrf2/ARE binding, which reflects an increase in the nuclear accumulation of Nrf2 protein. Despite increasing Nrf2 nuclear accumulation, there was a noticeable difference in the magnitude of Nrf2/ARE complex formation between TCDD and SUL as noted by their representative bands, the latter being more potent. Interestingly, V^{5+} was able to inhibit the TCDD- and SUL-mediated induction of nuclear accumulation of Nrf2 and its subsequent binding to the ARE (lanes 4 and 6). The specificity of the Nrf2/ARE band was confirmed by a competition assay using 100-fold M excess of unlabelled ARE (lane 7). Collectively, our data indicate that V^{5+} inhibited the translocation of Nrf2 and/or its subsequent binding to ARE.

3.3.6. Post-transcriptional modification of Nqo1 mRNA by V^{5+}

The level of mRNA expression is not only a function of the transcription rate, but is also dependent on the elimination rate, through processing or degradation. Therefore, we examined the effect of V^{5+} on the stability of TCDD-induced Nqo1 mRNA transcripts, using Act-D chase experiment. If V^{5+} alters Nqo1 mRNA stability, a decrease in half-life would be expected to take place. Figure 3.7. shows that TCDD-induced Nqo1 mRNA decayed with a half-life of 20.38 ± 0.68 h.

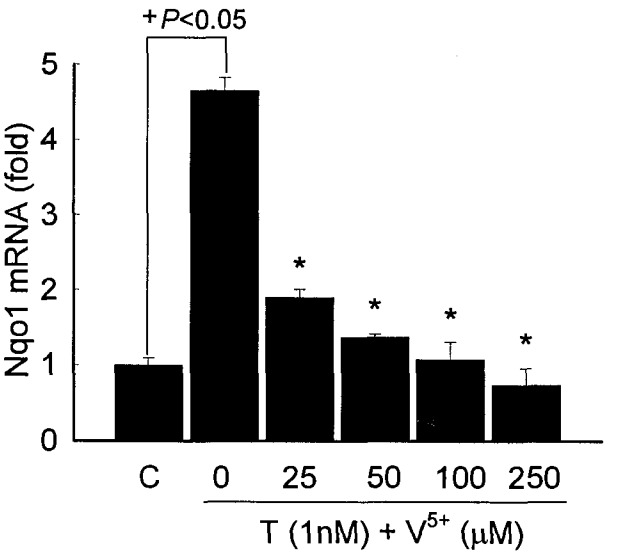

Figure 3.1. Concentration-dependent effect of V^{5+} on TCDD-mediated induction of Nqo1 at mRNA levels in Hepa 1c1c7 cells. Hepa 1c1c7 cells were treated with increasing concentrations of V^{5+} in the presence of 1 nM TCDD for 6 h for mRNA or 24 h for protein and catalytic activity. **A,** First-strand cDNA was synthesized from total RNA (1 μg) extracted from Hepa1c1c7 cells. cDNA fragments were amplified and quantitated using an ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (+) $P < 0.05$, compared to control (C) (concentration = 0 μM); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

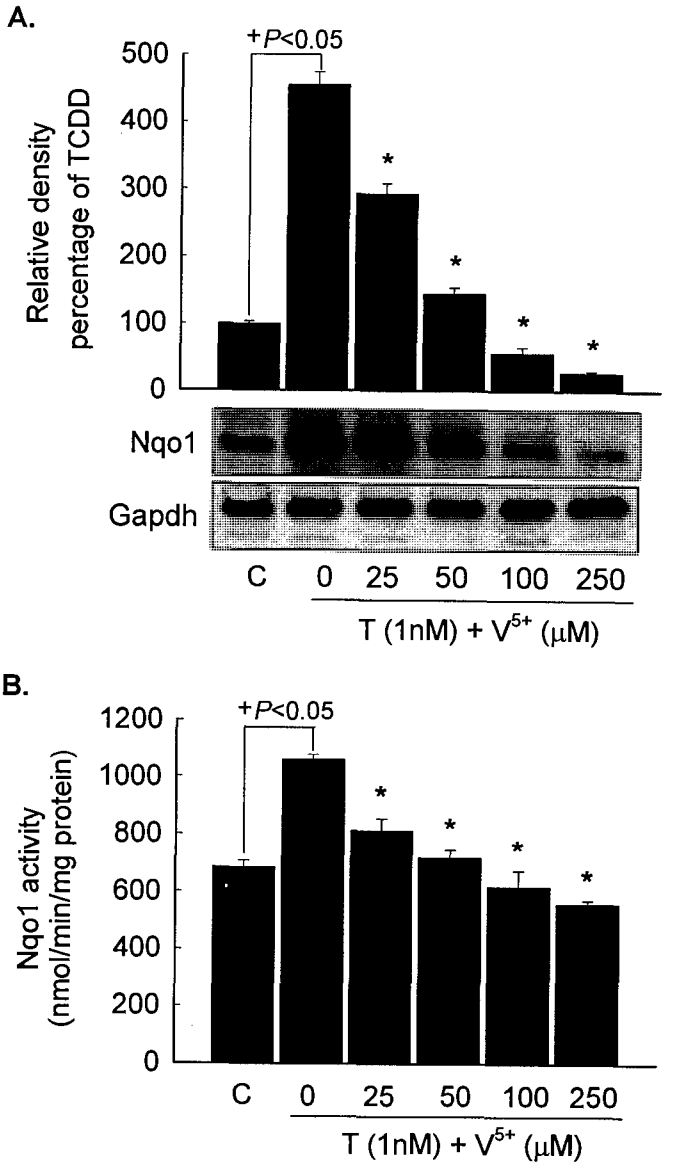

Figure 3.2. Concentration-dependent effect of V^{5+} on TCDD-mediated induction of Nqo1 at protein and catalytic activity levels in Hepa 1c1c7 cells. A, Protein (25 μg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C and then incubated with a primary Nqo1 antibody for 24 h at 4°C, followed by 1 h incubation with secondary antibody at room temperature. Nqo1 protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to Gapdh signals, which was used as loading control. One of three representative experiments is shown. **B,** Nqo1 enzyme activity was determined spectrophotometrically using DCPIP as substrate. Values are presented as mean ± SE ($n = 6$). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

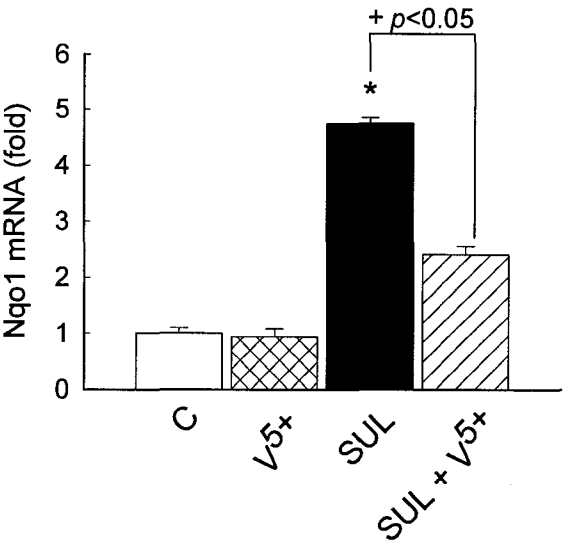

Figure 3.3. Effect of co-exposure to V^{5+} and SUL on Nqo1 at mRNA levels in Hepa 1c1c7 cells. Hepa 1c1c7 cells were treated with 50 μM V^{5+} in the absence and presence of 5 μM SUL for 6 h for mRNA or 24 h for protein and catalytic activity. **A,** First-strand cDNA was synthesized from total RNA (1 μg) extracted from Hepa1c1c7 cells. cDNA fragments were amplified and quantitated using an ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (+) $P < 0.05$, compared to control (C) (concentration = 0 μM); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

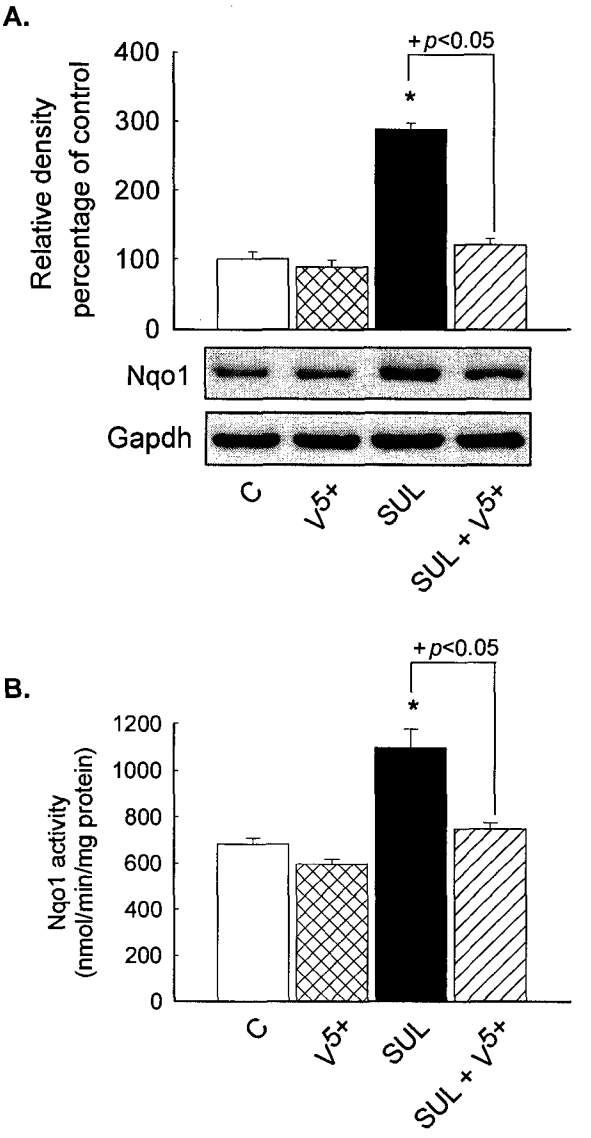

Figure 3.4. Effect of co-exposure to V^{5+} and SUL on Nqo1 at protein and catalytic activity levels in Hepa 1c1c7 cells. A, Protein (25 μg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C and then incubated with a primary Nqo1 antibody for 24 h at 4°C, followed by 1 h incubation with secondary antibody at room temperature. Nqo1 protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to signals from Gapdh, which was used as loading control. One of three representative experiments is shown. **B,** Nqo1 enzyme activity was determined spectrophotometrically using DCPIP as substrate. Values are presented as mean ± SE ($n = 6$). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

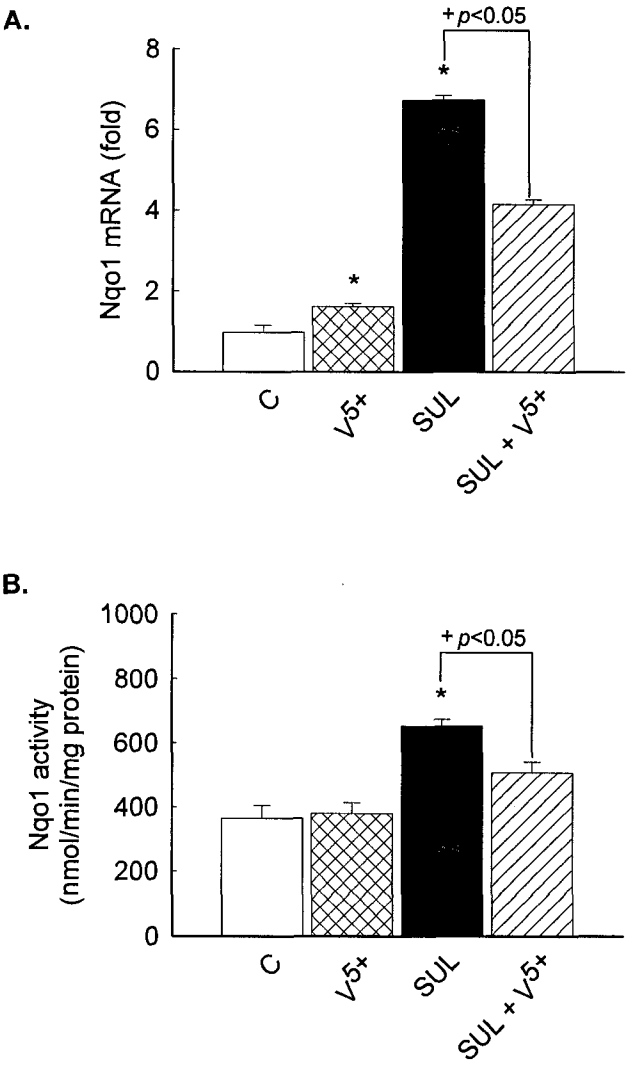

Figure 3.5. Effect of co-exposure to V^{5+} and SUL on Nqo1 at mRNA and catalytic activity in Hepa 1c1c7 (C12) cells. Hepa 1c1c7 (C12) cells were treated with 50 μM V^{5+} in the absence and presence of 5 μM SUL for 6 h for mRNA or 24 h for catalytic activity. **A,** First-strand cDNA was synthesized from total RNA (1 μg) extracted from Hepa 1c1c7 (C12) cells. cDNA fragments were amplified and quantitated using an ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (+) $P < 0.05$, compared to control (C) (concentration = 0 μM); (*) $P < 0.05$, compared to respective TCDD (T) treatment. **B,** Nqo1 enzyme activity was determined spectrophotometrically using DCPIP as substrate. Values are presented as mean ± SE ($n = 6$). (+) $P < 0.05$, compared to control (C); (*) $P < 0.05$, compared to respective TCDD (T) treatment.

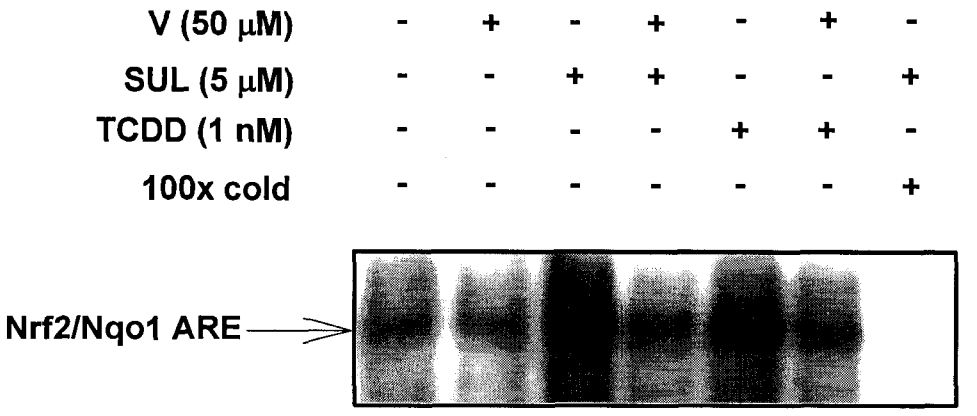

Figure 3.6. Effect of V^{5+} on Nrf2/ARE binding. Nuclear extract (7.5 μg) from Hepa 1c1c7 cells were treated for 4 h with vehicle, V^{5+} (50 μM), SUL (5 μM), SUL (5 μM) plus V^{5+} (50 μM), TCDD (1 nM), or TCDD (1 nM) + V^{5+} (50 μM). The nuclear proteins were mixed with [γ^{32}P]-labeled ARE, and the formation of Nrf2/ARE complexes was analyzed by EMSA. The specificity of binding was determined by incubating the protein treated with SUL with 100-fold molar excess of cold ARE. The arrow indicates the specific shift representing the Nrf2/ARE complex. This pattern of Nrf2 alteration was observed in three separate experiments, and only one is shown.

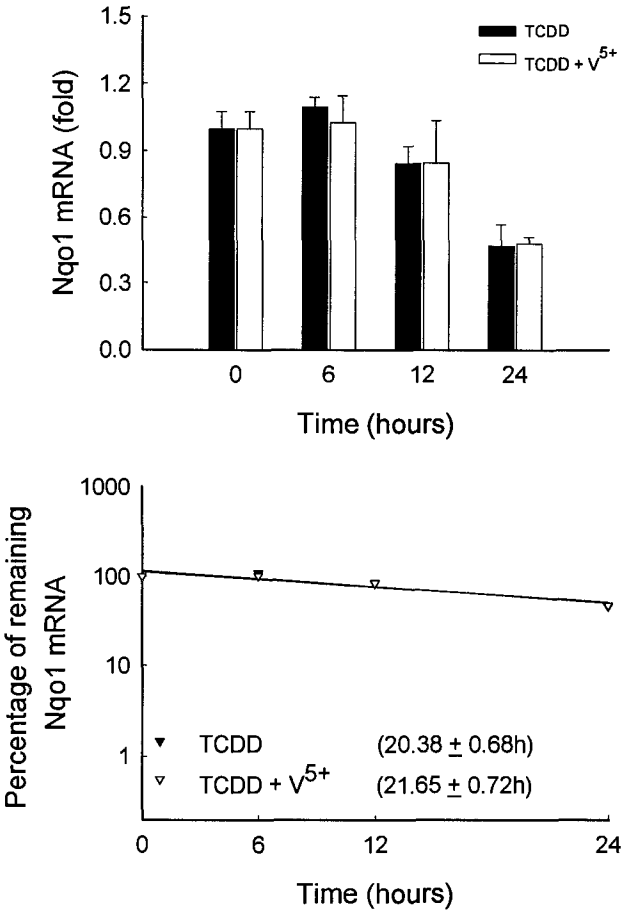

Figure 3.7. Effect of V^{5+} on Nqo1 mRNA half-life. Hepa 1c1c7 cells were grown to 90% confluence in six-well cell culture plates, and then treated with 1 nM TCDD for 12 h. The cells were then washed and incubated in a fresh media containing 50 μM V^{5+} plus 5μg/ml Act-D, a RNA synthesis inhibitor. First-strand cDNA was synthesized from total RNA (1 μg) extracted from Hepa1c1c7 cells. cDNA fragments were amplified and quantitated using an ABI 7500 real-time PCR system as described under Materials and Methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. mRNA decay curves were analyzed individually, and the half-life was estimated from the slope of a straight line fitted by linear regression analysis ($r^2 \geq 0.85$) to a semilog plot of mRNA amount, expressed as a percent of treatment at time = 0 h (maximum, 100%) level, versus time. The half-lives obtained from three independent experiments were then used to calculate the mean half-life (mean ± SE, $n = 3$).

Furthermore, V^{5+} did not alter the half-life of TCDD-induced Nqo1 mRNA significantly as it reached 21.65 $\pm$ 0.72 h, indicating that the decrease of Nqo1 mRNA transcripts in response to V^{5+} was not due to any post-transcriptional decrease in the mRNA stability. These results confirm the transcriptional inhibition of *Nqo1* gene expression by V^{5+}.

3.3.7. Post-translational modification of Nqo1 protein by V^{5+}

The fact that V^{5+} inhibited TCDD-mediated induction of Nqo1 protein raised the question whether V^{5+} could modify the Nqo1 protein stability. Therefore, the effect of V^{5+} on the TCDD-induced Nqo1 protein half-life was determined using CHX-chase experiments. Figure 3.8. shows that Nqo1 protein induced by TCDD degraded with a half-life of 20.44 + 2.14 h. Interestingly, V^{5+} increased the Nqo1 protein half life to 27.17 $\pm$ 1.82 h.

3.4. Discussion

V^{5+} is one of the most abundant elements in the Earth's crust. Metallic V^{5+} does not occur in nature; however, it does occur in over 65 known naturally occurring mineral salts, and is the major metal in fossil fuels (Zhang et al., 2001). V^{5+} can be found in rocks, soil, and to a lesser extent in water (Evangelou, 2002). Previous reports have demonstrated that the daily dietary intake of V^{5+} is in the order of a few tens of μg and may vary widely (Ding et al., 1999; Zhang et al., 2002). Furthermore, V^{5+} is widely used in mining, steel production, chemical industry, and oil-fired boiler electricity generating plants (Capella et al., 2002). Studies measuring V^{5+} concentration in the body estimated a total pool of 100 – 200 μg. Bearing in mind that metals such as V^{5+} are highly deposited in lung, liver, and

kidneys (Dinkova-Kostova and Talalay, 2000; Ross et al., 2000; Talalay and Dinkova-Kostova, 2004), the concentrations used in the current study are of great relevance to those of humans.

Mounting evidence now supports the role of NQO1 in protecting against toxic and neoplastic effects of pro-oxidant chemicals. It is believed that NQO1 achieves that through three different mechanisms. The first is its direct catalytic action and this occurs if the chemical insult happens to be a quinone. The second mechanism would be its indirect antioxidant effect. Thirdly, it stabilizes p53 protein which serves primarily as a transcriptional factor, and plays an important role in preserving genomic integrity, or the elimination of damaged or tumorigenic cells (Evangelou, 2002). Recent data suggest that V^{5+} compounds exert protective effects against chemical-induced carcinogenesis, mainly through modifying various xenobiotic metabolizing enzymes (Evangelou, 2002). In addition, there is increasing evidence that V^{5+} accumulates more in cancer cells and tissues than in normal cells or tissues (Zhang et al., 2006). However, the exact mechanism by which this metal may prevent carcinogenesis is still unclear. Data from our laboratory and others showed that heavy metals other than V^{5+} are capable of modifying the phase II drug metabolizing enzyme, Nqo1 through different stages of its regulatory pathway.

In the current study we hypothesize that V^{5+} down-regulates the inducible *Nqo1* gene expression through inhibiting both the AhR and Nrf2 signaling pathways.

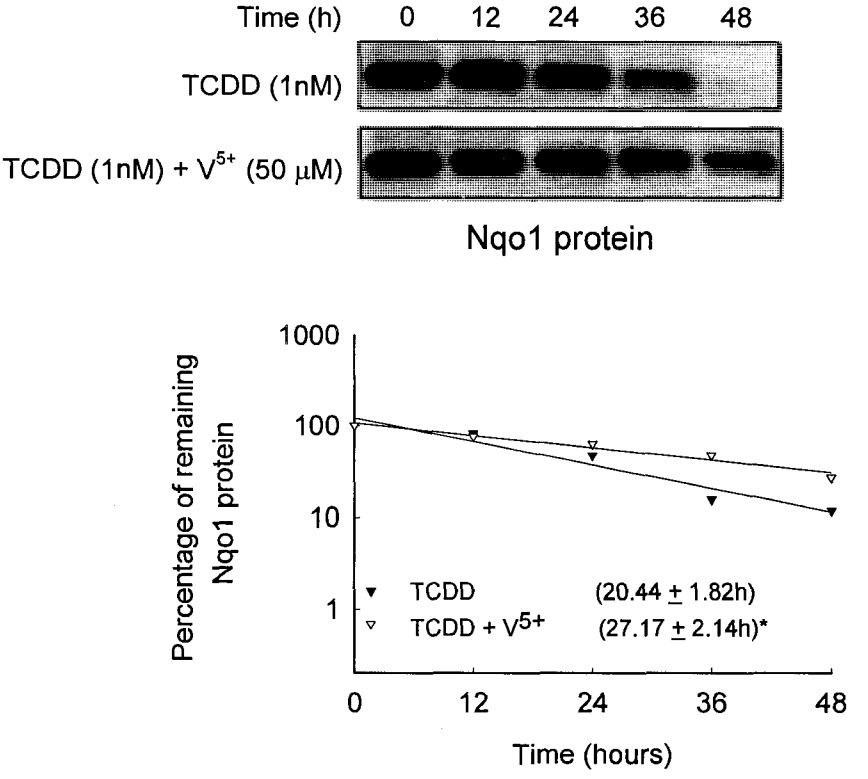

Figure 3.8. Effect of V^{5+} on the Nqo1 protein half-life. Hepa 1c1c7 cells were grown to 70% confluence in six-well cell culture plates, and then treated with 1 nM TCDD for 24 h. Thereafter, the cells were washed and incubated in fresh media containing 50 μM V^{5+} plus 10 μg/ml CHX, a protein translation inhibitor. Nqo1 protein was extracted at the designated time points after the addition of CHX. Protein (25 μg) was separated on a 10% SDS–PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C and then incubated with a primary polyclonal Nqo1 antibody for 24 h at 4°C, followed by 1 h incubation with secondary monoclonal antibody at room temperature. Antibody was detected using the enhanced chemiluminescence method. The intensity of Nqo1 protein bands were normalized to Gapdh signals, which was used as loading control (data not shown). All protein decay curves were analyzed individually. The half-life was estimated from the slope of a straight line fitted by linear regression analysis ($r^2 \geq 0.85$) to a semilog plot of protein amount, expressed as a percent of treatment at time = 0 h (maximum, 100%) level, versus time. The half-lives obtained from three independent experiments were then used to calculate the mean half-life (mean ± SE, $n = 3$). *$p < 0.05$ compared with TCDD.

Hence the main objective of the current study was to determine the potential effect of co-exposure to V^{5+} and TCDD or SUL, as bifunctional and monofunctional inducers, respectively, on *Nqo1* gene expression. Furthermore, we investigated the molecular mechanisms by which V^{5+} modulates the expression of *Nqo1* using wild type and AhR deficient Hepa 1c1c7 (C12) cell lines.

Initially we tested the ability of V^{5+} to modulate the expression of Nqo1 in Hepa 1c1c7 cells. Our results clearly demonstrated that V^{5+} significantly inhibited the TCDD- and SUL-mediated induction of Nqo1 at mRNA, protein, and activity levels in both wild type and AhR deficient cells. These results suggest that V^{5+} inhibits Nqo1 expression through the AhR/XRE and the Nrf2/ARE signaling pathways.

The transcriptional regulation of *Nqo1* gene expression by V^{5+} was supported by several pieces of evidence, the first being its ability to inhibit TCDD- and SUL-mediated induction of Nqo1 mRNA in both wild type and AhR-deficient cell lines (Figure 3.1., 3.3., and 3.5.A). The second piece of evidence was the ability of V^{5+} to inhibit the TCDD- and SUL-induced shuttling of Nrf2 from the cytosol to the nucleus as evident by EMSA (Figure 3.4.). The third piece of evidence was the inability of V^{5+} to significantly alter the Nqo1 mRNA half-life.

We have previously shown that V^{5+} inhibits the *Cyp1a1* gene expression through a transcriptional mechanism. Since one of the two Nqo1 regulatory pathways involves activation and subsequent translocation of the AhR, it is thus expected that V^{5+} by

inhibiting the translocation of AhR would inhibit the *Nqo1* gene expression. In addition, it has been previously reported that the Nrf2 activity is ATPase-dependent (Itoh et al., 1999; McMahon et al., 2003). Bearing in mind that V^{5+} is a potent ATPase inhibitor, it is not surprising to observe a decrease in Nrf2 nuclear accumulation. Therefore we suggest that the inhibitory effect of V^{5+} on *Nqo1* gene expression is occurring primarily through a transcriptional mechanism.

Previous reports have shown that Keap1 negatively regulates the transcription of ARE-reporter and endogenous detoxication genes by Nrf2 (Itoh et al., 2003; McMahon et al., 2003). Keap1 is a cytoplasmic protein that interacts with the cytoskeleton, and is thought to control the subcellular distribution of Nrf2 (Itoh et al., 2003; McMahon et al., 2003). This interaction between Keap1 and Nrf2 increases the rate of Nrf2 degradation by the proteasome. Current studies indicate that under oxidative stress conditions, cysteine residues 273 and 288 in Keap1 are modified, thus abolishing the Keap1-Nrf2 interaction (Nioi et al., 2003). Breaking this interaction benefits Nrf2 in two ways, the first is increasing its sub-cellular levels, and the second is its translocation to the nucleus (Anwar-Mohamed and El-Kadi, 2008). Our previous studies on Hepa 1c1c7 cells demonstrated that AhR protein levels were not altered upon exposure to V^{5+} (Korashy and El-Kadi, 2006b; Anwar-Mohamed and El-Kadi, 2008). Knowing that Nrf2 and AhR proteins share the same degradation pathway, the 26s proteasomal pathway, it is thus less likely that V^{5+} decreases the nuclear accumulation of Nrf2 through increasing its proteasomal degradation. We have previously shown that heavy metals do not affect the Nqo1 mRNA and protein turn-over rates (Ma et al., 2004; Korashy and El-Kadi, 2006b).

Yet it was of great importance to determine the effect of V^{5+} on the Nqo1 post-transcriptional and post-translational levels. The cellular mRNA level at any time point is a function of the rate of its production, through transcriptional mechanisms, and the rate of its degradation. Our results showed that Nqo1 transcripts are long-lived with an estimated half-live of approximately 21 h. These results are in agreement with previous studies that reported a half-life of more than 17 h for Nqo1 mRNA in Hepa 1c1c7 cells (Siegel et al., 2001; Korashy and El-Kadi, 2006b). Furthermore, our results showed that Nqo1 is a long-lived protein with an estimated half-life of 20.44 $\pm$ 2.14 h, which is in agreement with previously published studies (Macara et al., 1980; Legrum, 1986). Interestingly, the stability of Nqo1 protein was increased upon treatment with V^{5+} and reached around 27 h, inferring post-translational modification by V^{5+}.

The discrepancy between the effect of V^{5+} on Nqo1 activity levels and its effect on Nqo1 protein half-life could be explained by the strong binding of V^{5+} to sulfhydryl compounds such as glutathione and cysteine (Crans and Simone, 1991). A pool of data suggest that the effect of V^{5+} on enzymatic activity is most likely due to a non-redox interaction with the enzyme cysteine residues, thus decreasing its catalytic activity .

In conclusion, the present study demonstrates that V^{5+} down-regulates the phase II drug metabolizing enzyme, Nqo1 primarily through a transcriptional mechanism.

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CHAPTER 4

4. General Discussion and Conclusions

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 (Schrenk, 1998). As such, previous studies have suggested that environmental co-contamination by heavy metals and AhR ligands, typified by PAHs and HAHs, could enhance or reduce the potential carcinogenic effect of these ligands through modifying the expression of AhR-regulated genes. Furthermore, previous studies examining the anti-cancer effect of V^{5+} have demonstrated that the chemo-preventive effect of V^{5+} occurs through modifying xenobiotic metabolizing enzymes. However, the exact mechanism(s) by which V^{5+} may exert such effects is still unclear (Evangelou, 2002).

Humans consume appreciable amounts of V^{5+} in their diet. For instance, the adult consumption in the U.S.A is in the order of several tens of μg/day (Nechay, 1984). Furthermore, it has been shown that heavy metals such as V^{5+} are highly deposited in vital organs such as the kidneys, spleen, and liver (Edel and Sabbioni, 1989). Therefore, the V^{5+} concentrations used in the current study are of great relevance to those of humans.

To determine the optimal concentrations to use in our studies, V^{5+} was tested for potential cytotoxicity using Hepa 1c1c7 cells. Firstly we measured the cytotoxicity of V^{5+} using the MTT assay. Although extensively used, the MTT assay is not a sensitive measurement for cytotoxicity. Thus, it was of great importance to measure V^{5+} cytotoxic effects using the ATP-based luminescent assay. According to the literature, this assay

provides the most sensitive measurement for cytotoxicity and viability (Petty et al., 1995). Our results clearly demonstrated that the concentrations of 25- 250 μM did not affect cell viability, in the presence and absence of 1 nM TCDD. Therefore, all subsequent studies were carried out using V^{5+} concentrations of 25 – 250 μM.

In the current study we have demonstrated that V^{5+} decreases the TCDD-mediated induction of Cyp1a1 mRNA in a dose-dependent manner. Furthermore, this effect was further translated to the protein and catalytic activity levels. To decipher the molecular mechanisms involved in the down-regulation of Cyp1a1 expression, we analyzed the effect of V^{5+} on the transcriptional, post-transcriptional, translational, and post-translational levels.

The transcriptional inhibition of Cyp1a1 expression by V^{5+} was supported by several lines of evidence. At first, we examined the effect of V^{5+} on the AhR-dependent luciferase activity. Our results demonstrated that V^{5+} decreases the TCDD-mediated induction of the AhR-dependent luciferase activity. Thus, V^{5+} either inhibited the transformation of the AhR to its DNA binding form and/or inhibited the shuttling of the transformed AhR from the cytosol to the nucleus. Secondly, we examined the ability of V^{5+} to interfere with the nuclear binding of transformed AhR to the XRE by EMSA. Our results were in agreement to that of the luciferase activity, in which V^{5+} completely abolished the nuclear accumulation of the transformed AhR in Hepa 1c1c7 cells nucleus. These results prompted us to test the ability of V^{5+} to inhibit the direct activation of the

cytosolic AhR by TCDD. Interestingly, V^{5+} did not interfere with the activation of AhR by TCDD.

Being unable to affect the direct activation of cytosolic AhR, but inhibiting the formation of nuclear AhR/XRE complex, V^{5+} was suspected to exert this effect through increasing the AhR degradation through the 26s proteasomal pathway. Our results showed that V^{5+} did not cause any further increase in the TCDD-induced degradation of the AhR protein. These results suggest that V^{5+} inhibited the AhR nuclear accumulation without affecting its protein levels.

The subcellular level of mRNA transcripts at any time point is a balance between the synthesis and degradation rate. Data from our laboratory have shown that heavy metals other than V^{5+} are able to modify the Cyp1a1 mRNA half-life. Therefore it was of great importance to measure the Cyp1a1 mRNA decay half-life. Our results demonstrated that Cyp1a1 mRNA is a short-lived mRNA with a half-life of 4.73 ± 0.54 h. V^{5+} did not affect the Cyp1a1 mRNA turn-over rate as the half-life of Cyp1a1 mRNA in V^{5+} treated cells was 4.94 ± 0.60 h. This result eliminates the involvement of any post-transcriptional mechanism in the regulation of Cyp1a1 by V^{5+}, and further confirms the transcriptional regulation.

At the post-translational level, we tested the ability of V^{5+} to affect the Cyp1a1 protein half-life. Our results demonstrated that Cyp1a1 protein decays with a half-life of 8.41 ± 0.29 h. However, V^{5+} was unable to affect the Cyp1a1 protein half-life, inferring

that a post-translational mechanism was not involved in the modulation of Cyp1a1 protein by V^{5+}.

CYPs are heme-containing proteins, the loss of which will result in the formation of a hollow functionless protein. Several studies have demonstrated that heavy metals are able to decrease Cyp1a1 activity through affecting its heme group. Induction of HO-1, a 32 kda protein, is a rate-limiting step in the degradation of heme. Therefore, we measured the HO-1 mRNA and the total cellular heme content. Our results showed that V^{5+} did not significantly alter the HO-1 mRNA level or total cellular heme content. Thus, these results exclude the possibility that V^{5+} decreased the Cyp1a1 activity through affecting its heme content.

Interestingly, we have demonstrated that the V^{5+}-mediated inhibition of Cyp1a1 is not restricted to Cyp1a1, as V^{5+} inhibited other AhR-regulated genes such as Ecto-ATPase by the same manner.

Mounting evidence now supports the role of NQO1 in protecting against several types of chemical-induced carcinogenesis (Dinkova-Kostova and Talalay, 2000; Ross et al., 2000; Talalay and Dinkova-Kostova, 2004). NQO1 is a cytosolic flavoenzyme that catalyzes the two-electron reduction of a broad range of substrates. NQO1 gene expression is controlled by the XRE in addition to the ARE signaling pathways. Bifunctional inducers such as TCDD induce NQO1 gene expression through both the

XRE and ARE signaling pathways. On the other hand, monofunctional inducers such as SUL induce *NQO1* gene expression mainly through the ARE signaling pathway.

Agents producing oxidative stress that is not enough to pose a risk of cancer, such as SUL, induce NQO1 gene expression through causing perturbation in the redox status of the cell (Nioi and Hayes, 2004). In response to this oxidative stress, the redox sensitive transcriptional factor Nrf2 liberates from it tethering polypeptide Keap1 which is responsible for its degradation in the absence of an internal or external stimuli. Once released from Keap1, Nrf2 translocates to the nucleus, dimerizes with small MAF proteins, and thereafter binds to and activates ARE.

It was first thought that the XRE and ARE signalling pathways are independent. However, recent studies have demonstrated that there is a cross-talk and functional overlap between the two signalling pathways. For example, bifunctional inducers such as TCDD have been shown to require a direct cross-talk between the XRE- and ARE-signalling pathways. In addition, inducing NQO1 expression through the ARE signalling pathway has been shown to require the presence of AhR, suggesting a more direct cross-talk between the two signalling pathways (Ma et al., 2004; Marchand et al., 2004; Miao et al., 2005).

In the current study we have demonstrated that V^{5+} down-regulates inducible *Nqo1* gene expression in Hepa 1c1c7 cells through inhibiting both the AhR/XRE and Nrf2/ARE signalling pathways. Our results demonstrated that V^{5+} inhibited the TCDD-

and SUL-mediated induction of Nqo1 mRNA in a dose-dependent manner. This inhibition was also reflected at the protein and catalytic activity levels. These results prompted us to investigate the possible effect of V^{5+} on AhR-deficient C12 cells. Our results showed that V^{5+} inhibited the SUL-mediated induction of Nqo1 in the C12 cells, implying that the inhibition of Nqo1 expression is occurring through the Nrf2/ARE in addition to its previous reported action on the AhR/XRE signaling pathways.

The transcriptional regulation of *Nqo1* gene expression was supported by several pieces of evidences. Firstly, V^{5+} inhibited the TCDD- and SUL-mediated induction of Nqo1 mRNA in both wild type and AhR-deficient cells. Secondly, V^{5+} inhibited the TCDD- and SUL-induced nuclear shuttling of the Nrf2 as evident by EMSA. Thirdly, V^{5+} failed to alter the Nqo1 mRNA half-life.

Previous studies have shown that Nrf2 activity is ATPase-dependent (Zhang et al., 2001). With what is known about the effect of V^{5+} on the ATPase activity, we suggest that the effect of V^{5+} on the Nqo1 expression is occurring primarily through a transcriptional mechanism. Similar to the AhR, Nrf2 is degraded by the 26s proteasomal pathway. Keap1 governs and assures the degradation of the Nrf2 through this pathway. Our studies on the AhR signalling pathway indicated that V^{5+} did not alter AhR protein levels. Thus, it is less likely that V^{5+} decreases the Nrf2/ARE complex through increasing Nrf2 proteasomal degradation.

Unlike the Cyp1a1, the Nqo1 mRNA is less sensitive to degradation. This has been clearly demonstrated by the long half-life of the Nqo1 mRNA compared to that of Cyp1a1. Moreover, V^{5+} was unable to alter the half-life of the Nqo1 mRNA transcripts, excluding the presence of any post-transcriptional mechanism in the modulation of Nqo1 expression by V^{5+}.

Furthermore, our results have shown that Nqo1 protein is also a long-lived protein with an estimated half life of more than 20 h. Interestingly, the stability of Nqo1 protein was increased upon treatment with V^{5+} and the half-life reached a value around 27 h, inferring the presence of post-translational modification by V^{5+}. The controversy between the effect of V^{5+} on the Nqo1 catalytic activity levels and its protein half life could be explained by the possible non-redox interaction between V^{5+} and the enzyme cysteine residues.

In conclusion, we have shown that V^{5+} down-regulates Cyp1a1 expression by an AhR-dependent mechanism. Furthermore, V^{5+} down-regulates Nqo1 expression through AhR- and Nrf2-dependent mechanisms.

4.1. Future objectives:

The results obtained from the current research raise several questions that may be answered by conducting studies:

(1) To determine the effect of co-exposure to V^{5+} and TCDD on the expression of AhR-regulated genes *in vivo*,

(2) To characterize the role of the redox transcription factors NF-kB and AP-1 signaling pathways in the modulation of AhR-regulated genes by V^{5+}.

4.2. References

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