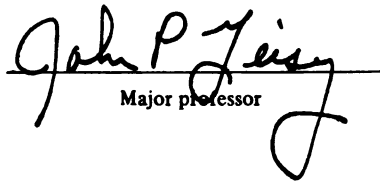This is to certify that the

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INTERACTION BETWEEN ARYL HYDROCARBON RECEPTOR (AHR)
AND HYPOXIA SIGNALING PATHWAYS: A POTENTIAL
MECHANISM FOR TCDD TOXICITY

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INTERACTION BETWEEN ARYL HYDROCARBON RECEPTOR (AHR) AND HYPOXIA SIGNALING PATHWAYS: A POTENTIAL MECHANISM FOR TCDD TOXICITY

By

Minghua Nie

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Zoology

1999

ABSTRACT

INTERACTION BETWEEN ARYL HYDROCARBON RECEPTOR (AHR) AND HYPOXIA SIGNALING PATHWAYS: A POTENTIAL MECHANISM FOR TCDD TOXICITY

By

Minghua Nie

Many of the toxic responses of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) are mediated through the AhR, which requires ARNT to regulate gene expression. ARNT is also required by HIF-1α to enhance the expression of various genes in response to hypoxia. Since both the AhR and hypoxia transcriptional pathways require ARNT, some of the effects of TCDD and similar types of ligands could be explained by interaction between the AhR and hypoxia pathways involving ARNT. The studies on which I report here were conducted to test the hypothesis that there is cross talk between AhR- and HIF-1-mediated transcription pathways. TCDD significantly reduced the hypoxia-mediated reporter gene activity in B-1 cells. Reciprocally, the hypoxia response inducers desferrioxamine or CoCl$_2$ inhibited AhR-mediated CYP1A1 enzyme activity in B-1 and Hepa 1 cells, and the AhR-mediated luciferase reporter gene activity in H1L1.1c2 cells. The inhibition of AhR-mediated transcription by hypoxia inducers, however, was not observed in H4IIE-luc cells. The interaction between AhR- and HIF-1-mediated transcription can be attributed to changes in DNA binding activities. TCDD-induced protein binding to the dioxin responsive element (DRE) was diminished by desferrioxamine, and TCDD reduced the binding activity to the HIF-1 binding site in

desferrioxamine-treated Hepa 1 cells. This mutual repression may provide an underlying mechanism for many TCDD-induced toxic responses. The results reported here indicate that there is cross talk between ARNT-requiring pathways. Since ARNT is possibly required by a number of pathways, this type of interaction may explain some of the pleiotropic effects caused by TCDD.

To my parents Guangmao Nie and Shan Fu for their unconditional love, and to

my mentor Dr. Gordon Ultsch for his understanding and support.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

AHH – Aryl hydrocarbon hydroxylase

AhR – Aryl hydrocarbon receptor

Ah receptor – Aryl hydrocarbon receptor

ARNT (or Arnt) – Ah receptor nuclear translocator

bHLH – Basic-Helix-Loop-Helix

CYP1A1 – Cytochrome p450 1A1

CYP1A2 – Cytochrome p450 1A2

Dfx – Desferrioxamine

DRE – Dioxin response element

EROD – Ethoxyresorufin O-deethylase

HIF-1α -- Hypoxia inducible factor 1 alpha

HIF-1β -- Hypoxia inducible factor 1 beta

HRE – Hypoxia responsive element

Hsp 90 – Heat shock protein 90

PAS – Per-Sim-Arnt

PCB – Polychlorinated biphenyl

PCDD – Polychlorinated dibenzo-*p*-dioxin

PCDF – Polychlorinated dibenzofuran

PCDH – Polychlorinated diaromatic hydrocarbon

TCDD (or 2,3,7,8-TCDD) – 2,3,7,8-tetrachlorodibenzo-p-dioxin

INTRODUCTION

Polychlorinated diaromatic hydrocarbons (PCDHs), such as polychlorinated biphenyls (PCBs), and polychlorinated naphthalenes (PCNs), polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), are industrial compounds or byproducts. PCDHs are widespread environmental contaminants that have been identified in air, sediment, domestic animals, wildlife, and human tissues. Environmental contamination by PCDHs has adversely affected wildlife, and has raised concern of its consequences for human health (Poland and Knutson, 1982; Safe, 1986 and 1990). For instance, some pathological defects observed in the colonial, fish-eating water birds of the North American Great Lakes have been attributed to exposure to PCDHs. These effects include embryonic deformities and lethality, immune suppression, edema, and porphyria, and were caused by eating contaminated fish (Giesy *et al.*, 1994a and 1994b; Ludwig *et al.*,1996).

As the most potent member of PCDHs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been studied more extensively than other structurally related compounds. In animals, TCDD elicits a wide range of biological effects, including alteration in metabolic pathways, immunological changes, cardiac dysfunction, hepatotoxicity, carcinogenicity, endocrine dysfunction, teratogenic effects, and neoplasia (Schmidt and Bradfield, 1996; Okey *et al.*, 1994; Pohjanvirta and Tuomisto, 1994; Poland and Knutson, 1982). Diverse biochemical responses to TCDD have been observed, such as drug metabolizing enzyme induction (Whitlock, 1990 and 1993), modulation of cytokine

levels, receptor function, and ligand binding (Abbott and Birnbaum, 1990; Choi *et al.*, 1991; Clark *et al.*, 1991), and activation of protein kinases (Enan and Matsumura, 1993; Bombick *et al.*, 1985).

The evidence supports the hypothesis that most, if not all, TCDD effects are mediated through the aryl hydrocarbon receptor (Ah receptor, or AhR; see Okey *et al.*, 1994; Hankinson, 1995 for reviews), a cytosolic receptor protein that was first discovered by Poland and coworkers (1976). The AhR signaling transcription pathway is initiated by TCDD diffusion into the cell, where it binds with high affinity to the cytosolic AhR protein complex, which also includes heat shock protein 90 (Hsp90) and a 38-kDa, immunophilin-related protein (Ma and Whitlock, 1997; Carver and Bradfield, 1997). The ligand binding activates AhR and stimulates the dissociation of AhR-associated proteins. The ligand-receptor complex is subsequently translocated into the nucleus, where it dimerizes with AhR nuclear translocator (ARNT) (Hankinson, 1995; Probst *et al.*, 1993). The heterodimers are capable of recognizing and binding DNA at the consensus sequence, GCGTG, of dioxin responsive elements (DREs) (Denison *et al.*, 1989; Dong *et al.*, 1996). This action either increases or decreases the transcription of target genes (Nebert *et al.*, 1993; Schmidt and Bradfield, 1996), including cytochrome P-450 (CYP1A1, CYP1A2) (Whitlock, 1987; Quattrochi and Tukey, 1989), NAD(P)H:quinone reductase (Favreau and Pickett, 1991), class 3 aldehyde dehydrogenase (Asman *et al.*, 1993), and glutathione S-transferase (Paulson *et al.*, 1990).

The ARNT protein, however, does not function uniquely in the AhR signaling pathway, but also pairs with HIF-1α (hypoxia inducible factor 1α) to regulate genes active in response to low oxygen stress (Guillemin and Krasnow, 1997; Semenza, 1994;

Wenger and Gassmann, 1997). HIF-1α is continuously synthesized and degraded under normal oxygen tension. Hypoxic conditions inhibit the degradation of HIF-1α by the ubiquitin proteasome system, and triggers the nuclear localization of HIF-1α (Huang *et al.*, 1998; Kallio *et al.*, 1997; Salceda and Caro., 1997). Transition metals, such as cobalt, and iron chelators, such as desferrioxamine (Dfx), elicit the same response in cells, which suggests that these stimuli work at different stages of a common oxygen sensing pathway (Guillemin and Krasnow, 1997). Inside the nucleus, HIF-1α heterodimerizes with ARNT to form a transcription factor complex HIF-1 (Jiang *et al.*, 1996; Salceda *et al.*, 1996; Wood *et al.*, 1996). The binding of HIF-1 to the core DNA sequence, TACGTG, of the hypoxia response enhancer (HRE) (Jiang *et al.*, 1996; Semenza *et al.*, 1991; Wang and Semenza, 1993) and subsequent transactivation enhence expression of genes such as Epo for erythropoiesis (Semenza, 1994), VEGF for angiogenesis (Forsythe *et al.*, 1996; Goldberg and Schneider, 1994; Maxwell *et al.*, 1997; Shweiki *et al.*, 1992), and GLUT-1 for glucose transport (Semenza *et al.*, 1994; Wenger and Gassmann, 1997) (Figure 1).

In vitro, ARNT can also form a homodimer or heterodimers with a number of PAS (Per-ARNT-Sim) proteins, which include AhR, ARNT, HIF-1α, and transcription factors involved in various gene regulation pathways (Hahn, 1998; Hogenesch *et al.*, 1997). These results suggest ARNT might be a central regulator of many cellular pathways through its formation of functional transcription activators or repressors by dimerizing with other members of the PAS family. In adult animals, ARNT is ubiquitously expressed in all tissues. The essential role of ARNT is supported by studies on ARNT null mice, which are not viable beyond day 10.5 of gestation (Kozak *et al.*, 1997; Maltepe *et al.*, 1997).

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The importance of ARNT during development and the observation that ARNT dimerizes with various PAS proteins suggest that availability of ARNT could be critical for various cellular activities, and competitive recruitment of ARNT may repress the activation of certain genes. TCDD is a potent inducer of AhR-mediated gene activation and can cause prolonged activation of AhR. It is possible that exposure to TCDD and subsequent recruitment of ARNT through AhR may inhibit other signal transduction pathways depending on ARNT. We hypothesize that the pleiotropic effects of TCDD could be due to TCDD-induced ARNT deficiencies in multiple ARNT-requiring pathways. Because the AhR and HIF-1 pathways are two of the most well understood pathways involving ARNT, they were chosen as a model to study the potential effect of TCDD on linked pathways. The interaction between the AhR and hypoxia pathways was investigated at the level of transcription, nuclear protein complex formation, and DNA-binding activities.

Figure 1. **Schematic representation of the involvement of ARNT in AhR- and HIF-1-mediated transduction pathways.** The AhR pathway is initiated when AhR ligands, such as TCDD, diffuse into the cell (1), bind to and activate the cytosolic AhR complex, which also includes Hsp90s and a immunophilin-related protein (IRP) (2). Activation leads to dissociation of the AhR-associated proteins (3) and nuclear translocation of AhR-ligand complex (4). Inside the nucleus, the AhR-ligand complex heterodimerizes with ARNT (5), and binds to DREs to alter gene expression (6). The hypoxia response pathway is initiated by stimuli such as hypoxia, $CoCl_2$, or Dfx (7), which inhibit the degradation of cytosolic HIF-1α by the ubiquitin proteasome system (8), and trigger the nuclear localization of HIF-1α (9). In the nucleus, HIF-1α forms HIF-1 a complex with ARNT (10), then binds to the HIF-1 binding site of hypoxia responsive enhancer (HRE) and enhances the transcription of genes regulated through HRE.
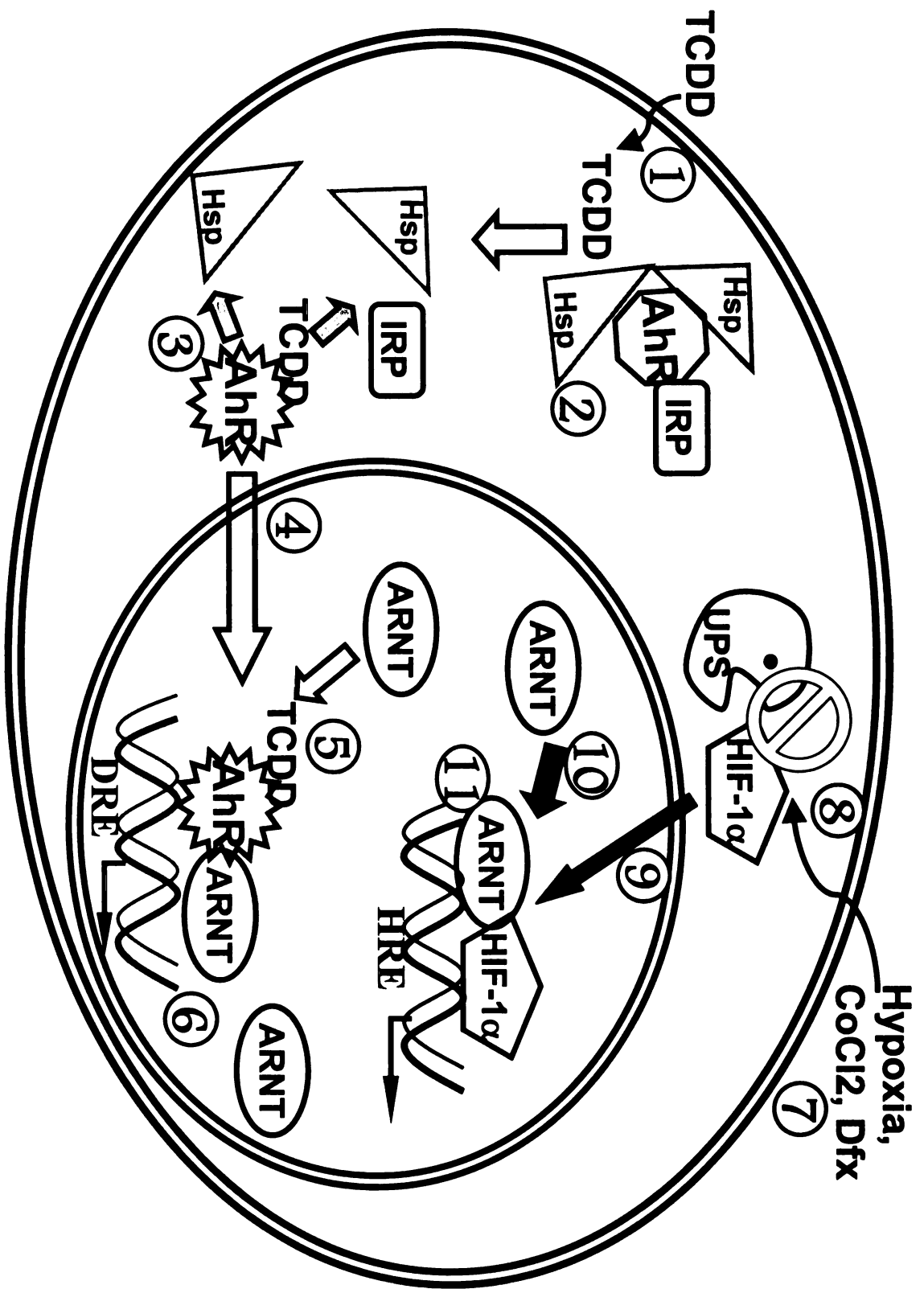
MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions

Four cell lines were selected for study because of particular properties that were useful in investigating cross talk between the two pathways of interest. B-1 cells were derived from the human hepatoma Hep 3B cell line, which has been stably transfected with an expression vector containing luciferase cDNA under the control of a minimal Epo promoter (300-base pair *Sfa*NI-*Xba*III fragment) and the hypoxia responsive enhancer from the human Epo gene (150-base pair *Apa*I/*Pst*I fragment) (Salceda and Caro, 1997). H1L1.1c2 and H4IIE-luc cells are mouse and rat hepatoma cells, respectively, which are stably transfected with a luciferase reporter gene under control of dioxin-responsive elements (DREs) (Garrison *et al.*, 1996). B-1 cells were obtained from Dr. J. Caro (Department of Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA). Wild-type Hepa 1 cells were obtained from Dr. O. Hankinson (Department of Pathology, University of California at Los Angeles, Los Angeles, CA). H1L1.1c2 cells were provided by Dr. M. Denison (Department of Environmental Toxicology, University of California at Davis, Davis, CA). B-1 cells were maintained in minimal essential medium. Hepa 1 and H1L1.1c2 cells were maintained in alpha-modified minimal essential medium. H4IIE-luc cells were grown in Dulbecco's Modified Eagle Medium. All media were supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml).

Cells were grown under sterile conditions at 37 °C in a humidified atmosphere with 5% CO_2.

Chemicals and Treatments

Desferrioxamine (Dfx) or $CoCl_2$ (in Millipore H_2O) was used as inducers of the hypoxia response pathway (Salceda and Caro, 1997), TCDD (in MeOH or DMSO) was used as AhR ligand. The concentrations of each treatment were selected in pilot studies to result in maximal responses in each cell line. The concentration ranges used were non-toxic to cells as determined by microscopic morphological examination, cell viability assays based on esterase activity and DNA integrity, and protein assays.

Luciferase Assay

The luciferase assay was based on the methods of Sanderson *et al.* (1996). In brief, cells were trypsinized from plates containing 80-100% confluent monolayers, resuspended in media, and diluted to a concentration of approximately 10^5 cells/ml and seeded into the 60 interior wells of 96-well culture ViewPlate™ (Packard Instruments, Meriden, CT) at 250 µl per well. The 36 exterior wells of each plate were filled with 250 µl culture media. Cells were incubated overnight, then dosed with 1.25 µl of the appropriate treatment chemical or vehicle solvent (methanol). Blank wells received no dose.

Luciferase and protein assays (Kennedy and Jones, 1994) were conducted after 24 to 30 hr of exposure. Briefly, culture medium was removed by vacuum manifold and the

cells were rinsed with Dulbecco's phosphate-buffered saline (PBS, containing 0.02% (w/w) KCl, 0.02% (w/w) KH$_2$PO$_4$, 0.8% (w/w) NaCl, 10 mM Na$_2$HPO$_4$, pH 7.4). Into each well, 75 µl PBS with Ca^{2+} and Mg^{2+} (0.01% (w/w) of CaCl$_2$ and MgCl$_2$) and 75 µl LucLite reagent (from LucLiteTM Kit; Packard Instruments, Meriden, CT) were added. The plates were incubated for 15 min at 30 °C and then scanned with an ML3000 microplate reading luminometer (Dynatech Laboratories, Chantilly, VA, USA). Following the luminometer scan a 1.08 mM solution of fluorescamine in acetonitrile was added to each well and plates were assayed for protein after a 15 min incubation at room temperature, using a Cytofluor 2300 (Millipore Corp., Bedford, MA) (excitation 400 nm, emission 460 nm).

EROD Assay

Assays for ethoxyresorufin-*O*-deethylase activity (EROD) of CYP1A1 were performed as described by Sanderson *et al.* (1996). Briefly, the cells were trypsinized and seeded as described in the description of the luciferase assay. At the end of the exposure period, medium was aspirated from each well, which was washed twice with PBS before adding 30 µl nanopure water. The plates were frozen at –80 °C for 10 min and thawed at room temperature, and then 100 µl HEPES-dicumoral buffer (50 mM HEPES and 40 µM dicumarol) and 50 µl ethoxyresorufin (20 µM in HEPES-dicumoral buffer) were added to the wells. The EROD reaction was started by adding 20 µl NADPH (1.25 mM in 40 mM HEPES). After a 60-min incubation at 30 °C, the reaction was stopped by adding 50 µl of 1.08 mM fluorescamine (in acetonitrile), followed by an

additional 15-min incubation at room temperature. Fluorescence was then measured on a Cytofluor 2300. Resorufin was measured at excitation/emission wavelengths of 530/590 nm and protein was measured at excitation/emission wavelengths of 400/460 nm. BSA and resorufin standard curves were prepared on the day of assay.

Nuclear Protein Extraction

Hepa 1 cells from four 90% confluent 100 mm petri dishes were harvested into ice-cold PBS using a cell scraper. The cell suspension was centrifuged for 5 min at 500 g. After discarding the supernatant, the pellet was resuspended and incubated in 1 ml HM (10 mM HEPES, 3 mM $MgCl_2$, pH 7.5) on ice for 10 min. The suspension was then homogenized with a tight fitting (B pestle) Dounce Homogenizger. The homogenate was centrifuged at 1000 g for 5 min at 4 °C. The supernatant was removed and the pellet was washed twice with HMK (25 mM HEPES, pH 7.5; 3mM $MgCl_2$, 100 mM KCl) and centrifuged to remove supernatant. The pellet was then resuspended in 2 volumes of HEKG (25 mM HEPES, pH 7.5; 1 mM EDTA, 400 mM KCl, 10% glycerol), vortexed, and incubated on ice for 30 to 40 min. At the end of the incubation, the suspension was centrifuged at 15,000 g for 15 min at 4 °C. The supernatant was collected as nuclear protein extract and stored at –80 °C. The protein content was measured using Bradford protein assay using BSA as a standard. All buffers for extraction were supplemented with 2 µg/ml aproptinin, 2 µg/ml leupeptin, 1 mM DTT, 0.1 mM Na_3VO_4, and 0.5 mM PMSF before each use.

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Binding sequences for electrophoresis mobility shift assays (EMSA) were DRE3 (5'-GAGCTCGGAG<u>TTGCGTG</u>AGAAGAGCCT-3') and W18 (5'-GCC<u>CTACGTGCT</u> GTCTCA-3'). The DNA probes (underlines indicate the protein binding sites) were made by the Macromolecular Facility at Michigan State University. The probes were end-labeled with EasyTide® [γ ^{32}P] ATP (NEN Life Science Products, Inc., Boston, MA) using Ready-To-Go T4 polynucleotide kinase (Pharmacia Biotech) and purified using NucTrap Push Column (Stratagene). Before the assay, 4% acrylamide gel was pre-run for 1 to 2 hr at 110 volts using TAE buffer (6.7 mM Tris, pH 8.0, 3.3 mM sodium acetate, 1 mM EDTA), and was supplied with fresh TAE before loading the samples. The final sample mixture contained 9 μg nuclear proteins, 25 mM HEPES (pH 7.5), 1 mM EDTA, 80 mM KCl, 1mM DTT, 10% glycerol, 2 μg Poly (dI-dC), and 40000 to 60000 cpm radiolabeled oligonucleotides to make up a final volume of 30 μl. For the samples containing cold competitor, 200-fold excess of non-radiolabeled probe was added 5 min before adding ^{32}P-labeled probe. For HRE binding assays, the samples were incubated on ice instead of at room temperature. For the samples used in the supershift assays, 1.5 μg antibody was incubated with the protein mixture (1:5 antibody to protein ratio) on ice for 1 hr before adding the probes. AhR antibody was purchased from Affinity Bioreagents (Golden, CO). HIF-1α antibody was purchased from Novus Biologicals, Inc. (Littleton, CO). After sample loading, the gel was run for 2.5 to 3.25 hr at 110 volts, dried on a gel dryer, and then exposed to Hyperfilm MP in a film cassette with intensifying screen overnight to two days at -80 $^{\circ}$C.

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Statistical Analysis

The data were analyzed using SigmaStat software (Jandel Scientific, Inc., Rafael, CA). Pair-wise t-tests were performed with the probability of type I error (α) set to be less than 0.05.

RESULTS

Effect of TCDD on HIF-1-Mediated Reporter Gene Activity

Effects of TCDD on HIF-1-mediated gene expression were determined by measuring changes in luciferase activity in response to factors ($CoCl_2$ or Dfx) known to induce hypoxia-like responses in the presence or absence of TCDD in B-1 cells. The luciferase activity in B-1 cells was increased 12-fold by 125.0 μM Dfx and 7-fold by 40 μM $CoCl_2$ after 24-hr exposure. To examine the effect of AhR-mediated induction on HIF-1-mediated transcriptional activation, B-1 cells were treated with a range of concentrations of TCDD from 0.125 to 12.5 nM for 6 hr before the administration of hypoxia inducer. Pre-incubation with TCDD caused a concentration-related reduction of luciferase activity (Figure 2). At a concentration of 12.5 nM, TCDD reduced the reduced the maximal induction of the hypoxia response pathway by approximately 35% in both Dfx and $CoCl_2$-treated B-1 cells. Methanol, the carrier solvent of TCDD had no significant effect on HIF-1-mediated luciferase induction.

Effects of Inducers of the Hypoxia Response Pathway on AhR-Mediated CYP1A1
Induction

To investigate whether AhR-mediated responses are affected by the activation of the hypoxia response signaling pathway, CYP1A1 induction was examined through the

Figure 2. **TCDD suppresses the hypoxia-inducible enhancer in B-1 cells.** (A) B-1 cells expressed luciferase activity in response to Dfx. Cells were treated with solvent control (methanol) or 0.0 to 12.5 nM TCDD for 30 hr and 125 μM Dfx for 24 hr before being assayed for luciferase activity. The responses to the treatments with or without TCDD were compared. The probability of type I error for comparison of each treatment with the appropriate control is labeled on the graph if it was less than 0.05. Confidence intervals represent one standard deviation of the mean. (B) In a parallel experiment, 40 μM $CoCl_2$ was used to induce the hypoxia response in B-1 cells.

A

Luciferase Activity (100xRLU)

Solvent or TCDD (nM)	BLK	0	MeOH	0.125	1.25	12.5
Dfx (125.0 µM)	-	+	+	+	+	+

p=0.0009
p<0.0001
p<0.0001

B

Luciferase Activity (100xRLU)

| Solvent or TCDD (nM)	BLK	0	MeOH	0.125	1.25	12.5
CoCl$_2$ (40 µM)	-	+	+	+	+	+

p=0.0006
p<0.0001
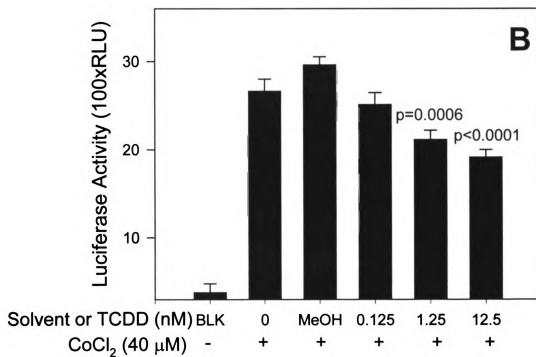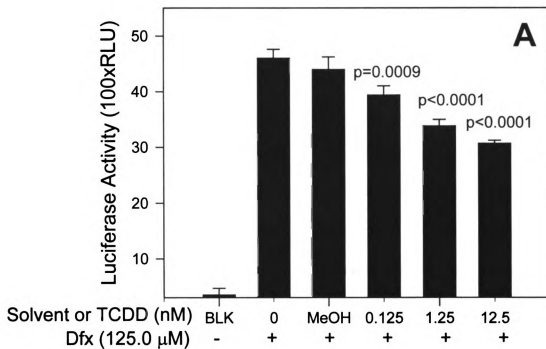
measurement of its EROD activities in B-1 and Hepa 1 cells. EROD activity was maximally induced by exposure to 3.75 nM TCDD in B-1 cells and by exposure to 375 pM TCDD in Hepa 1 cells after 24 hr exposure. To examine the effect of activation of the hypoxia response pathway on induction of AhR-mediated responses, various concentrations of Dfx were added 6 hr before the cells were treated with TCDD. A concentration-dependent reduction of TCDD-induced EROD activity was observed in both cell lines (Figure 3). Exposure to 250 μM Dfx reduced the maximal EROD activity by 67% in Hepa 1 cells. In B-1 cells, 125 μM Dfx reduced the EROD activity induced by 3.75 nM TCDD to background levels. The concentrations of Dfx that caused the reduction of EROD activity in B-1 cells were inversely related to the concentrations that induced luciferase activity as a measure of HIF-1-mediated transcriptional activation.

Effects of Inducers of the Hypoxia Response Pathway on AhR-Mediated Reporter Gene Activity

To confirm that the response of the endogenous reporter system, EROD, was not an artifact caused by the effects of Dfx on iron availability and the synthesis of cytochromes, the effect of HIF-1-mediated induction on the TCDD-inducible enhancer was studied using H1L1.1c2 cells. An increase of greater than 15-fold increase in luciferase activity was observed in H1L1.1c2 cells exposed to 5.0 nM TCDD for 24 hr. Luciferase induction was significantly inhibited by pre-incubation for 6 hr with 5 μM Dfx or by 10 μM $CoCl_2$ (Figure 4).

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Figure 3. **Dfx inhibits the TCDD-induced EROD activity in B-1 and Hepa 1 cells.** (A) B-1 cells expressed EROD activity in response to TCDD. The cells were treated with concentrations of Dfx ranging from 0.0 to 125 μM for 30 hr and solvent (methanol) or 3.75 nM TCDD for 24 hr before being assayed for EROD activity. (B) Hepa 1 cells treated with concentrations of Dfx ranging from 0.0 to 125 μM for 30 hr and methanol or 375 pM TCDD for 24 hr before EROD assays. Pairwise comparisons of the responses to each of the Dfx treatments were compared to cells treated with TCDD alone by use of a one-tailed T-test. The probability of type I error is labeled on the graph if it was less than 0.05. Confidence intervals represent one standard deviation of the mean.
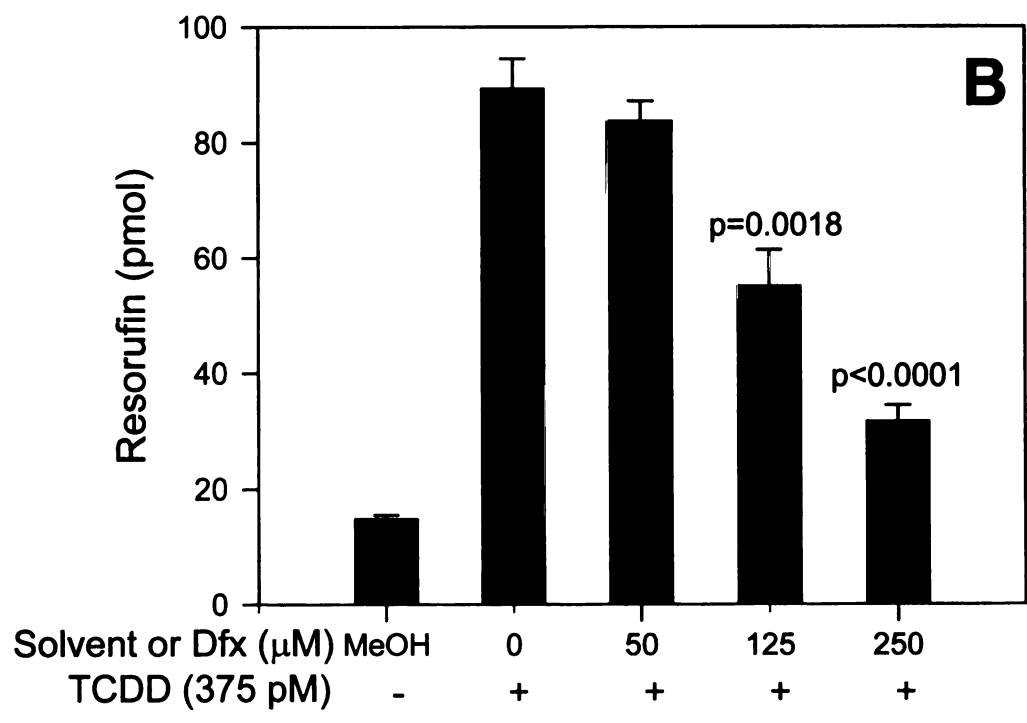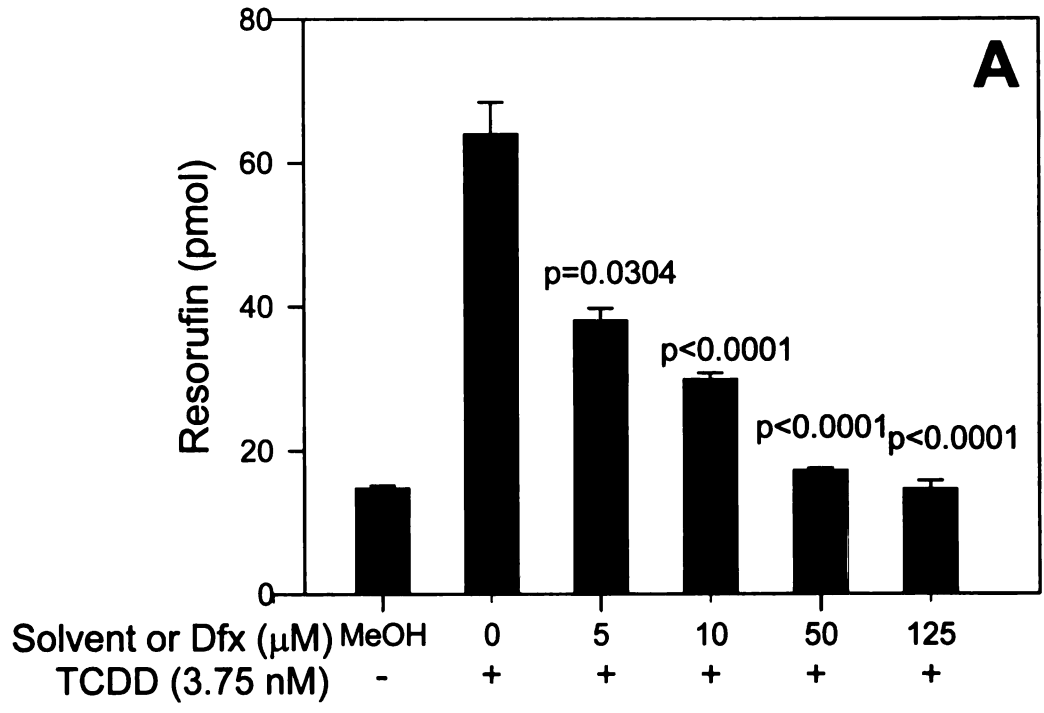
Figure 4. **Dfx and CoCl$_2$ suppress TCDD-inducible CYP1A1 enhancer in H1L1.1c2**

cells. (A) H1L1.1c2 cells expressed luciferase activity in response to TCDD. The cells

were treated with concentrations of Dfx ranging from 0.0 to 150 μM for 30 hr and

methanol or 5 nM TCDD for 24 hr before being assayed for luciferase activity. (B) In a

parallel experiment, luciferase activity induced by TCDD was studied in the presence of

concentrations of CoCl$_2$ ranging from 0.0 to 100 μM. The responses to TCDD with

hypoxia response inducers were compared to the response to TCDD alone by use of a

one-tailed T-test. The probability of type I error is labeled on the graph if it was less than

0.05. Confidence intervals represent one standard deviation of the mean.
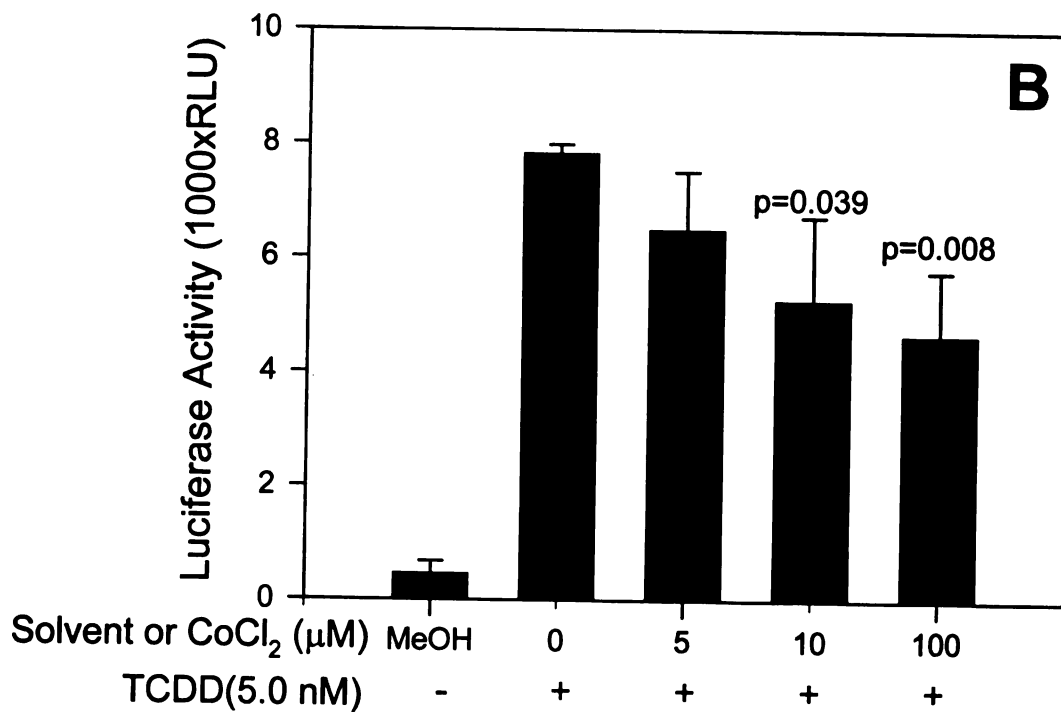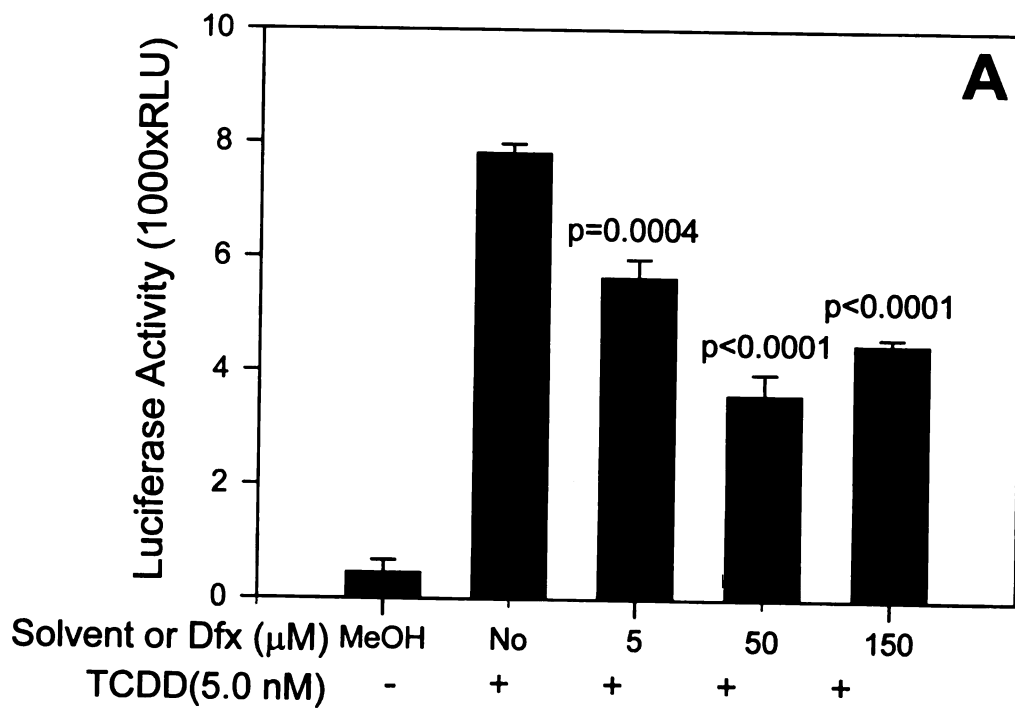
Cell Line Dependency of Interaction between the AhR and Hypoxia Pathways

Responses of both endogenous and exogenous gene activities mediated through AhR in the presence of inducers of the hypoxia response pathway observed in B-1, Hepa 1, and H1L1.1c2 indicated that there is an interaction between AhR and hypoxia signaling pathways in these cell lines. However, the inhibitory effect of hypoxia response inducers on AhR-mediated gene expression was not observed in rat hepatoma H4IIE-luc cells. AhR-mediated transcriptional activation after treatment with TCDD can be measured either as endogenous enzyme activity (EROD) or via an exogenous reporter system (luciferase), which has been introduced into H4IIE-luc cells. When H4IIE-luc cells were exposed to Dfx or $CoCl_2$ before being treated with TCDD, no reduction of either luciferase activity or EROD activity was observed (Figure 5).

Interaction of TCDD and Dfx on Protein Complex Binding Activities to DRE3 and HIF-1

Nuclear protein binding activity to DRE3 was examined in Hepa 1cells (Figure 6A). Treating the cells for 1 hr with 5 nM TCDD increased the binding activity (*lane 4*), compared to that of untreated cells (*lane 2*) or carrier solvent control (DMSO) (*lane3*). There were two bands that are inducible by TCDD treatment, which is possible due to the presence of two isoforms of the AhR. The constitutive band is due to binding of the nuclear protein to single-stranded DRE (Denison and Yao, 1991). The DRE3 binding activity of TCDD-treated cells was reduced by 1 hr pre-treatment with 125 µM Dfx (*lane 5*). The specificity of DRE3 binding was examined using a 200-fold molar excess of

unlabelled DRE3 (*lane 6*). The presence of AhR in the DRE-binding complex was investigated using an AhR antibody (Figure 6B). The antibody to AhR caused an up-shift of the bands (*lane 3*), which demonstrates the presence of the AhR in the protein complex when it binds to DRE3

The DNA-binding activity to the HIF-1 binding site was induced in Hepa 1 cells treated for 4 hr with 125 μM Dfx (Figure 7A, *lane 3*), relative to untreated cells (*lane 2*). One hour pre-incubation with 5 nM TCDD before Hepa 1 cells were treated with Dfx caused marked reduction of the Dfx-induced DNA-binding (*lane 5* and *6*). Carrier solvent (DMSO) alone and TCDD in DMSO had no effect on background HIF-1 binding activity (*lane 7* and *8*). The specificity of nuclear protein binding to the HIF-1 binding site was examined by using a 200-fold excess of unlabelled probe to competitively displace the labeled probe (*lane 4*). This demonstrates that the protein binding to the oligonucleotide was specific to the sequence of the probe used. To examine the identity of the DNA-binding protein complex, antibody to HIF-1α was used. The antibody to HIF-1α caused the reduction of the protein complex binding to the probe (Figure 7B, *lane 3*), compared to the mouse IgG control (*lane 4*). This result demonstrates that HIF-1α is necessary for binding of the nuclear protein complex with the HIF-1 binding site. Furthermore, this demonstrates that the inducible bands contain the HIF-1α proteins.

Figure 5. **Effect of hypoxia inducers on the AhR-mediated gene expression in H4IIE-luc cells.** (A) H4IIE-luc cells increase DRE-mediated luciferase and EROD activity in response to TCDD. The cells were treated with concentrations of Dfx ranging from 0.0 to 125 μM for 30 hr and solvent control (methanol) or 1.25 nM TCDD for 24 hr before being assayed for luciferase or EROD activities. (B) H4IIE-luc cells were treated with concentrations of $CoCl_2$ ranging from 0.0 to 100.0 μM instead of Dfx. Confidence intervals represent one standard deviation of the mean.
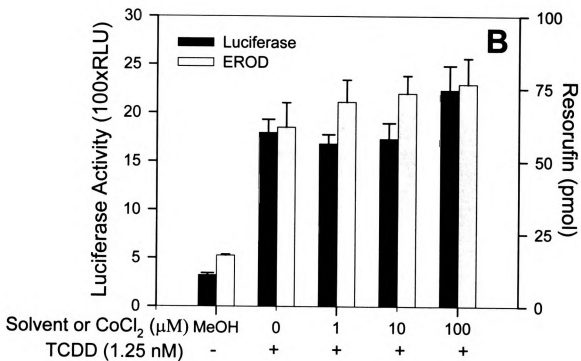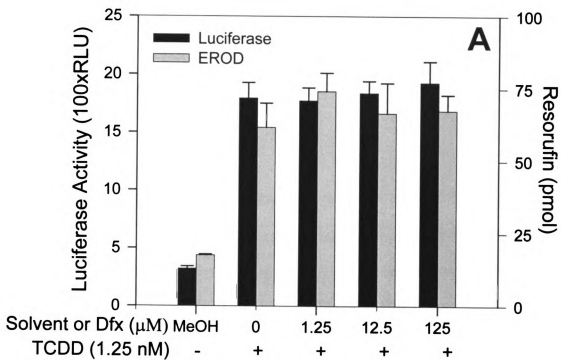
Figure 6A. **Effect of Dfx on AhR:ARNT binding activity to DRE3.** The electrophoresis mobility shift assay shows DRE probe alone (*lane 1*), nuclear protein from Hepa 1 cells with no treatment (*lane 2*), 1 hr carrier solvent (DMSO) treatment (*Lane 3*), and 1 hr 5 nM TCDD (*lane 4*). The effect of Dfx was examined in cells treated with 125 µM Dfx for 1 hr (*lane 5*). The specificity of DRE-binding was evaluated using 200-fold molar excess of unlabelled DRE probe (*lane 6*). **IN** ➔: induced binding activity, **C** ➔: constitutive binding, **F** ➔: free probe. This figure is representative of more than three independent experiments.

Cold Competitor   -   -   -   -   +
125 µM Dfx   -   -   -   1h   -
5 nM TCDD   -   -   1h   1h   1h
Solvent (DMSO)   -   +   +   +   +

Probe only

IN →
IN →
C →

F →

Lane   1   2   3   4   5   6
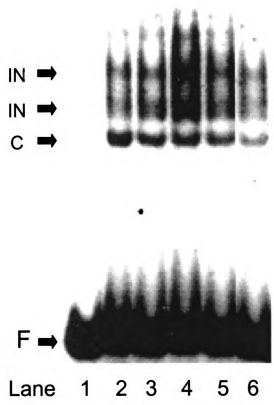
Figure 6B. **Presence of AhR in protein complexes binding to DRE3**. Hepa 1 cells were either untreated (*lane 1*) or treated with 5 nM TCDD for 1 hr (*lane 2* to *4*). AhR antibody (*lane 3*) was used and compared to mouse IgG controls (*lane 4*).
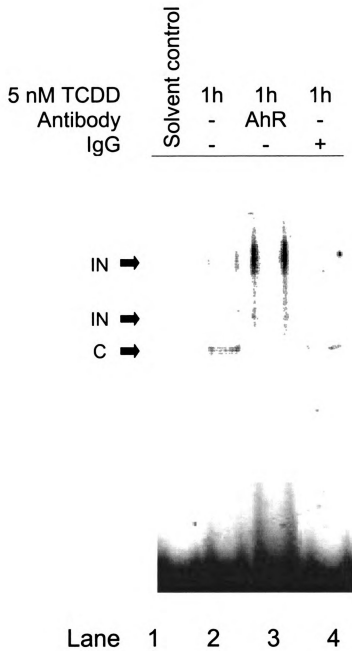
Figure 7A. **Effect of TCDD on DNA-binding activity of HIF-1.** Electrophoresis mobility shift assay with W18 probe, which contains the HIF-1 binding site, alone (*lane 1*), with nuclear protein from untreated Hepa 1 cells (*lane 2*), or 4 hr 125 µM Dfx (*lane 3*). To study the effect of TCDD, cells were treated with 5 nM TCDD for 5hr (*lane 5*) or 8 hr (*lane 6*) and with 125 µM Dfx for 4 hr. DNA-binding activity in the cells treated with solvent (DMSO) alone (*lane 7*) was also examined. The specificity of HIF-1-binding was demonstrated using a 200-fold excess of unlabelled W18 probe (*lane 4*). **IN** ➔: induced binding activity, **NS** ➔: non-specific binding; **F** ➔: free probe. This figure is representative of more than three independent experiments.

Cold Competitor		-	-	+	-	-	-
125 µM Dfx		-	4h	4h	4h	4h	-
5 nM TCDD		-	-	-	5h	8h	-
Solvent (DMSO)		-	-	-	+	+	+

IN ➡

IN ➡

NS ➡

F ➡

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
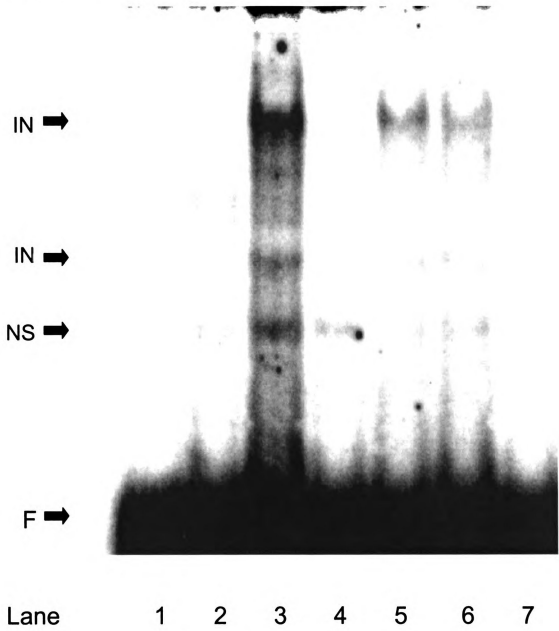
Figure 7B. **Presence of HIF-1α in the protein complex that binds to HIF-1.** Hepa 1 cells were untreated (*lane 1*) or exposed to 125 μM Dfx for 4 hr (*lane 2* to *4*). HIF-1α antibody was used (*lane 3*) and compared to mouse IgG control (*lanes 4*).

125 μM Dfx	Blank	4h	4h	4h
Antibody		-	HIF-1α	-
IgG		-	-	+

IN ➡

IN ➡

NS ➡

F ➡

Lane   1   2   3   4
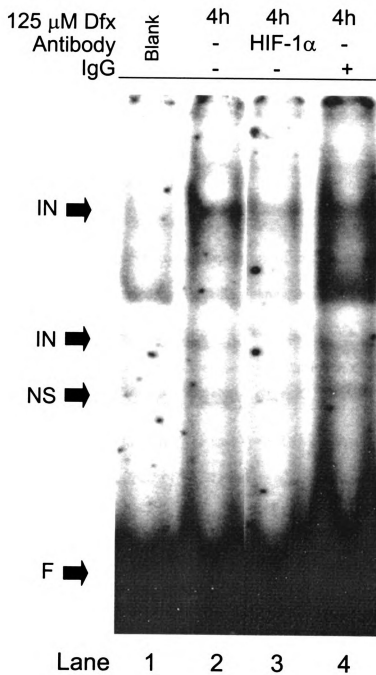
DISCUSSION

Induction of the TCDD-activated AhR signaling pathway, which is represented by the induction of CYP1A1, alters gene expression controlled by the enhancer sequence containing DREs. One explanation of the pleiotropic responses of animals to exposure to TCDD is that there are multiple DRE-controlled genes which are regulated by AhR-ligand complex. This mechanism of action confines the effect of the AhR ligands to the regulation of genes specified by the DRE enhancer sequences. This limited scope of action, however, can not explain the wide spectrum of toxic responses, ranging from cell proliferation to apoptosis, caused by the AhR ligands, such as PCDHs. The correlation between CYP1A1 induction and species sensitivity to TCDD is poor. For example, guinea pigs, which are the most sensitive species to the lethal effects of TCDD, do not show induction of liver metabolizing enzymes (Poland and Knutson, 1982). It is, however, unlikely that all of the effects caused by TCDD are due to the direct response of DREs. The fact that many effects of TCDD can be described as alteration of cell growth and differentiation (Okey *et al.*, 1994) suggests an interaction among multiple signaling pathways. Therefore, an alternative hypothesis that could explain the pleiotropic responses of animals to TCDD is cross talk among pathways with common mechanisms of signaling transduction. In this study, it has been demonstrated that the AhR and hypoxia pathways interact at the level of gene expression and such interaction is correlated with interference of DNA binding activity and nuclear complex formation. Reciprocal repression of gene expression of the two pathways was observed in B-1 cells.

Inhibitory effects of the inducers of the hypoxia response pathway on AhR-mediated gene expression were also observed in Hepa 1 and H1L1.1c2 cells. This suggests that such an interaction is not unique to B-1 cells. Although cytochrome p450, such as CYP1A1, are heme proteins whose production requires iron, repression of EROD activity in B 1 and Hepa 1 cells by Dfx was not caused by direct inhibition of protein synthesis because the luciferase reporter enzyme whose production is independent of iron was also reduced by Dfx in H1L1.1c2. Furthermore, the H4IIE-luc cells did not exhibit effect of Dfx on either EROD or luciferase activity when cells were treated with same concentrations of Dfx. These results suggest that the concentrations of Dfx used are sufficient to activate the hypoxia response pathway, yet did not affect CYP1A1 protein synthesis.

AhR, ARNT, and HIF-1α belong to bHLH (basic-helix-loop-helix)/PAS protein family. The bHLH motif is characteristic of a family of proteins that function as modulators of cell proliferation and differentiation. PAS proteins are found in representative organisms of all five kingdoms and may play a role in determining target gene specificity (Hahn, 1998). PAS proteins are involved in development and differentiation (Sim group, trachealess) (Nambu *et al.*, 1991; Isaac and Andrew, 1996), regulation of circadian clocks (Per, CLOCK) (Huang *et al.*, 1995; King *et al.* 1997), sensing and responding to oxygen tension (HIF-1α, EPAS-1/HLF) (Wenger and Gassmann, 1997; Tian *et al.*, 1997), and steroid receptor signaling (SRC-1) (Yao *et al.*, 1996). The myogenic bHLH proteins autoregulate their own expression and cross-regulate the expression of other family members (Olson, 1990). Since the secondary dimerization surface, PAS domain can provide the specificity for dimerization among

PAS/bHLH proteins (Pongratz *et al.*, 1998). PAS proteins, therefore, may behave in a way similar to the myogenic bHLH proteins and interact with each other through the PAS domain. Since ARNT has been found to dimerize with many PAS proteins such as AhR, Sim1, Sim2, HIF-1α, and EPAS-1 (Hogenesch *et al.*, 1997; Jiang *et al.*, 1996; Moffett *et al.*, 1997; Sogawa *et al.*, 1995; Tian *et al.*, 1997), it may act as a central regulator of PAS protein-dependent pathways.

Eukaryotic gene regulation can be viewed as an interplay between opposing activating and repressing influences. Transactivation of genes is controlled at many levels, but typically involves binding by transcription factors to specific regulatory *cis*-elements. While the *cis*-elements can be specific to a particular set of genes, the transcription factors may be less diverse and shared by several pathways. Sharing of a few common transcription factors by many transcription regulation pathways also provides the advantage of network regulation and more sophisticated, fine-tuned controls. This sharing also leads to the observed phenomenon known as "squelching", which refers to alteration of transcription by sequestering limiting components required for transcriptional activation or repression away from the promoter in the affected gene (Cahill *et al.*, 1994). For instance, the estrogen hormone receptor has been shown to inhibit the transcriptional activation mediated by the progesterone and glucocorticoid receptors (Meyer *et al.*, 1989). Squelching could be a common mechanism employed by transcription factors to down-regulate promoters whose activity is governed by coactivators of low abundance.

Reciprocal repression of the AhR- and HIF-1-mediated pathways was observed in B-1 cells, but the degrees of interaction were different. Inhibition of AhR-mediated gene

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expression by induction of the hypoxia response pathway was greater than the inhibition of the hypoxia response pathway by TCDD. HIF-1α has been found to bind to ARNT with greater affinity than does AhR (Gradin *et al.*, 1996). This differential binding affinity to ARNT may explain the stronger inhibition by hypoxia response inducers on the AhR pathway, and may serve as a regulatory mechanism in addition to dimerization specificity.

Results of electrophoresis mobility shift assays provided further elucidation of the mechanism of interaction between the two pathways. The reduction of hypoxia-induced DNA-binding activity by TCDD (Figure 7A) and TCDD-induced DNA-binding activity by Dfx (Figure 6A) offers support for the hypothesis that interaction between the AhR- and HIF-1-mediated pathways is due to recruitment of ARNT (Hogenesch *et al.*, 1997). Therefore, ARNT may serve as a nuclear integrator and modulator of various signal transduction pathways. Alternatively, these data do not exclude the possibility that other commonly involved transcription activators and coactivators are also recruited. For instance, the coactivator CBP/p300 has been found to be associated with both the AhR- and HIF-1-mediated pathways, acting synergistically with the transcription factors and basal transcription machinery (Bunn *et al.*, 1998; Ebert and Bunn, 1998; Kallio *et al.*, 1998; Kobayashi *et al.*, 1997). Therefore, besides potential competition for ARNT, the AhR- and HIF-1-mediated pathways may also interact through squelching of CBP or other shared coactivators.

Unlike in B-1, Hepa 1, and H1L1.1c2 cells, AhR-mediated gene expression in H4IIE-luc cells was not affected by the induction of hypoxia response pathway. This suggests that the interaction between the AhR and hypoxia pathways might be different

among cell lines, tissues, or species. The degree of interaction among ARNT-dependent pathways may depend on the abundance of ARNT in the cells. In H4IIE cells, the ratio of AhR to ARNT is 0.3, compared to 10 in Hepa 1 cells (Holmes and Pollenz, 1997), which indicates that ARNT exists in excess in H4IIE cells. However, to elucidate this hypothesis, more needs to be known about the abundance of HIF-1α in cells. Another explanation for the lack of effect of hypoxia inducers on the induction by TCDD is that H4IIE-luc cells are defective in the hypoxia pathway, or insensitive to hypoxia inducers.

In conclusion, our study provided evidence for a potential mechanism of action for TCDD toxicity. In this model, TCDD acts as a disrupter of multiple gene regulation pathways through its recruitment and sequestering of ARNT by activating AhR. TCDD, by altering the transcription regulation network, therefore can cause diverse toxicological effects by changing the relative and absolute rates of transcription of a variety of genes expressed under normal physiological conditions (Figure 9). This model offers a plausible explanation for the wide range of toxic effects of TCDD. For example, VEGF, a growth factor that regulates angiogenesis, is regulated by HIF-1α and ARNT in response to oxygen availability. ARNT- and HIF-1α-null mice can not survive gestation due to the defects in vasculature development (Maltepe *et al.*, 1997; Iyer *et al.*, 1998). TCDD has been reported to cause reduced vasculature in developing chicks and medaka (*Orizias latipes*) (Walker *et al.*, 1997; Cantrell *et al.*, 1996). TCDD is also known to elicit a wasting syndrome, which is partially attributed to its down regulation of glucose transporters (Matsumura, 1995). At least one of the glucose transporters, GLUT-1, has been found to be regulated through HIF-α and ARNT (Ebert *et al.*, 1995). Furthermore, this hypothesis could also explain the wide range of sensitivities among tissues and

species. As more genes regulated by ARNT to be discovered and more ARNT-interacting transcription factors to be characterized, it should be possible to offer better and more complete explanations of TCDD toxicity.

Figure 8. **A potential mechanism of action for TCDD.** In this model, TCDD acts through AhR to sequester ARNT and reduce the abundance of free ARNT that can be used by other ARNT-dependent pathways, such as the hypoxia pathway and other ones possibly involving the members of the PAS protein family. The activation of the AhR pathway by ligands like TCDD can also reduce the availability of common cofactors such as CBP, and therefore affect gene regulations involving non-PAS proteins. This disturbance of multiple gene regulation pathways at the cellular level may be manifested *in vivo* as the pleiotropic effects of TCDD.

MOP: Member of PAS
CBP: CREB-binding protein

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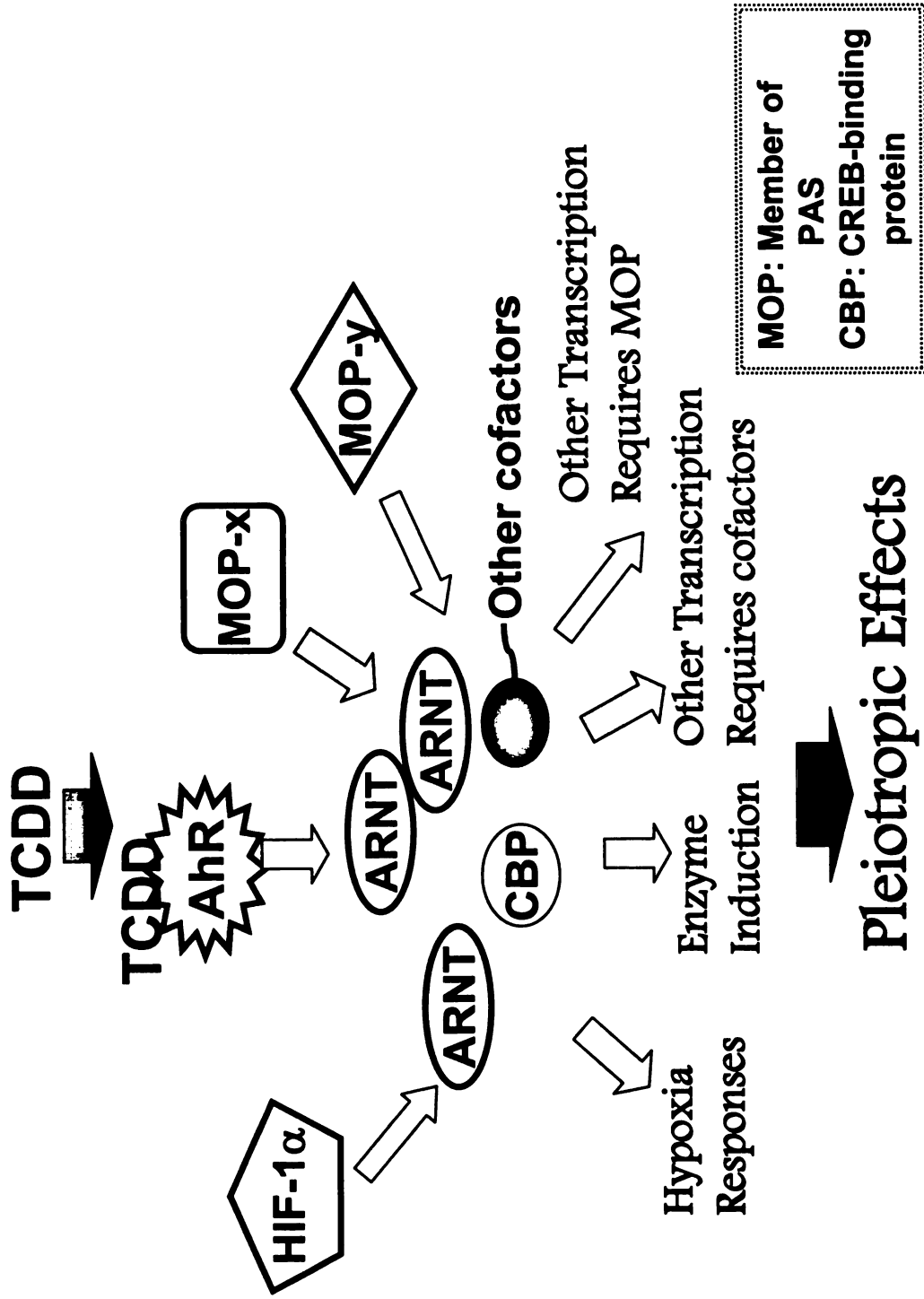
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