SELECTIVE LIGAND-DEPENDENT AH RECEPTOR-COACTIVATOR INTERACTIONS OBSERVED IN A MAMMALIAN TWO-HYBRID ASSAY

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Abstract

Halogenated aromatic hydrocarbons (HAHs) such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PeCDD), 3,3',4,4',5-pentachlorobiphenyl (PCB126), 2,3,7,8- tetrachlorodibenzofuran (TCDF) and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) are aryl hydrocarbon receptor (AhR) agonists and along with many other structurally-diverse AhR agonists including chemoprotective phytochemicals induce CYP1A1. However, induction of many other responses are ligand structure and cell context-dependent and several selective AhR modulators (SAhRMs) have been identified. The ability of HAHs to exhibit SAhRM-like activity was investigated by determining ligand structure-dependent differences in the interaction of AhR with a series of coactivators in mammalian two-hybrid assays. TCDD, PeCDD, TCDF, PeCDF and PCB126 induced CYP1A1 in mouse Hepa1c1c7 and human Panc1 and HEK293 cancer cells. However, we observed structure-dependent activation of luciferase activity in these cells co-transfected with AhR and GAL4-coactivator chimeras (SRC-1, SRC-2, SRC-3, CARM-1, PGC-1 and TRAP220). In Panc1 cells, TCDD and PeCDD induced AhR interactions with TRAP220 and TCDD, but not PeCDD, induced AhR interactions with CARM-1; coactivator AhR interactions were not observed for any other HAH. In HEK293 cells, only TCDD induced AhR interactions with CARM-1 and none of the other HAHs induced AhR interactions with the coactivators. These results suggest that HAHs selectively interact with coactivators and these interactions are dependent on cell-context.

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and structural related polychlorinated dibenzo-*p*-doxin (PCDD), dibenzofuran (PCDF) and biphenyl (PCB) congeners bind the aryl hydrocarbon receptor (AhR) and induced CYP1A1 gene expression in multiple tissues and cells.[1] In addition, 2,3,7,8-TCDD and isosteric 2,3,7,8-substituted and lateral substituted (3-, 4-, and 5-) PCBs induce many of the same biochemical and toxic responses in some cell culture and animal models.[2] Based on these results, it has been assumed that TCDD and structurally-related compounds were identical and differed only on their potency. This led to the development of the toxic equivalency factor (TEF) or relative potency (RP) approach for risk assessment of these compounds where the TEFs for 2,3,7,8-substituted PCDDs/PCDFs and coplanar/mono-ortho PCBs were expressed as fractional values relative to 2,3,7,8-TCDD which was assigned a TEF of 1.0.[2] The development and application of TEF and TEQ approach for risk assessment of PCDDs/PCDFs in the late 1980s and early 1990s was based on a number of assumptions including (i) TEFs are valid only for persistent "TCDD-like" halogenated aromatics; and (ii) the effects of individual compounds are essentially additive with parallel dose response curve. However, during the past 10-15 years, some studies have suggested that the biological basis for application of the TEF/TEQ approach is compromised for the following reasons.[3]

(i) For some congeners, their range of TEF values is considerable for several Ah receptor mediated responses.

(ii) Microarray assay studies show some differences in gene expression induced by TCDD and structurally related compounds.[4]

We hypothesize that even some of the more potent AhR agonists such as TCDD, 1,2,3,7,8-PeCDD, 2,3,7,8-PCDF, 2,3,4,7,8-PeCDF and 2,3',4,4'5-PCBP may be selective AhR modulators (SAhRMs) that exhibit differences in AhR activation.[5] Differences between compounds that activate receptors are due, in part, to the ligand-induced conformational changes in the receptor which then differentially interact with nuclear coregulator/coactivator proteins. The halogenated aromatics used in this study differentially interact with coactivators in different cell lines in a mammalian two-hybrid assay suggesting that these compounds are SAhRMs and thereby do not differ only in

their relative potencies, but would be expected to exhibit some cell context-dependent difference in modulation of genes and/or responses.

MATERIALS AND METHODS

Cell lines, constructs and Antibodies. Panc1 and HEK293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Hepa1c1c7 cells were kindly provided by Dr. Yanan Tian (Texas A&M University, TX). The pDRE$_3$-luciferase reporter plasmid was constructed in this laboratory and contains three tandem consensus dioxin response elements (DRE) (TCT TCT CAC GCA ACT CCG A—a single DRE sequence). The Gal4 reporter containing 5x Gal4DBD (Gal4Luc) was kindly provided by Dr. Marty Mayo (University of North Carolina, Chapel Hill, NC). Gal4-coactivator fusion plasmids pM-SRC-1, pM-AIBI, pM-TIFII, pM-DRIP205, pM-TRAP220, and pM-CARM-1 were kindly provided by Dr. Shigeaki Kato (University of Tokyo, Tokyo, Japan). VP-AhR was kindly provided by Dr. Gary Perdew (Penn State Univerisity, PA). Antibodies for CYP1A1, AhR and Arnt proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for β-actin was obtained from Sigma (St. Louis, MO). *Transfection of the Cells.* Cells were cultured in 12-well plates in 1 ml of DME/F12 medium supplemented with 2.5% fetal bovine serum. After 16-20 h when cells were 30-50% confluent, constructs were transfected using lipfectamine2000 Reagent (Invitrogen, Carlsbad, CA). Cells were harvested 36-44 h after transfection by manual scraping in 1X lysis buffer (Promega). *Western Immunoblot Analysis.* An aliquot of whole cell lysates containing 30 μg protein was diluted with loading buffer, boiled, and loaded on a 10% SDS-polyacrylamide gel. Samples were electrophoresed at 150-180 V for 3-4 h, and separated proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Proteins were detected by incubation with polyclonal primary antibodies CYP1A1, AhR, Arnt and β-actin (1:1000 dilution), respectively, followed by blotting with horseradish peroxidase-conjugated anti-rabbit (for CYP1A1, AhR and Arnt) or anti-mouse (for β-actin) secondary antibody (1:5000 dilution). *Quantitative Real-Time PCR.* Total RNA was isolated using the RNeasy Protect Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. RNA was eluted with 30 µl of RNasefree water and stored at -80°C. RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. cDNA obtained from reverse transcription using a combination of oligodeoxythymidylic acid and dNTP mix (Applied Biosystems, Foster City, CA) and Superscript II (Invitrogen). Each PCR was carried out in triplicate in a 25-µl volume using SYBR Green Master mix (Applied Biosystems) for 15 min at 95°C for initial denaturing, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min in the ABI Prism 7700 sequence detection system (Applied Biosystems). The ABI Dissociation Curves software was used after a brief thermal protocol (95°C 15 s and 60°C 20 s, followed by a slow ramp to 95°C) to control for multiple species in each PCR amplification. Primers were purchased from Integrated DNA Technologies (Coralville, IA). *Statistical Analysis.* Statistical significance was determined by analysis of variance and Scheffe's test, and the levels of probability are noted. The results are expressed as means ± SD for at least three separate (replicate) experiments for each treatment group.

Results and Discussion

1. TCDD and related AhR agonists activate the nuclear AhR complex, which recruits nuclear cofactors to activate gene transcription (data not shown) and previous studies have shown that compounds such as 6-MCDF are SAhRMs.[4]
2. In this study, 5 halogenated aromatics with TEFs≥0.1[1] were investigated in a gene expression assay (CYP1A1 activation) and in a mammalian two-hybrid assay which measures ligand-dependent receptor (VP-AhR) coactivator (GAL4-coactivator) interactions.
3. The 5 congeners induced a concentration-dependent activation of luciferase activity in human HEK293 and Panc1 and mouse Hepa1c1c7 cells transfected with Ah-responsive DRE3-luc construct (Fig. 1); similar results were observed for induction of CYP1A1 mRNA levels and CYP1A1 protein in the same cell lines (data not shown).

4. The 5 AhR agonists induced activity (i.e. AhR-coactivator interactions) in the 3 cell lines co-transfected with GAL4-coactivator chimeras containing SRC-1, TRAP220, CRM-1 and TIFII (SRC-2) but not with PGC-1, AIB1(SRC-3) and SMRT (data not shown).
5. Inspection of the results shows that the 5 AhR agonists differentially induce AhR-coactivator interactions and there are both cell context-dependent differences and similarities (Fig. 2).
   (a) For example, TCDD but not the other 4 congeners induced TRAP220-AhR interactions in HEK293 cells whereas in Hepa1c1c7 cells none of the congeners induced this interaction.
   (b) Only TCDD induced CARM-1-AhR interaction in HEK293 cells and only 3,3',4,4',5-PCBP induced activity in Hepa1c1c7 cells, and the other 4 congeners did not induce these interactions .
   (c) In contrast, ligand-dependent AhR-TIFII interactions were similar in Hepa1c1c7 and Panc1 cells.

Conclusions: TCDD and 4 related congeners (TEF≥0.1) exhibited cell context and structure-dependent differences in their activation of AhR-coactivator interactions suggesting that there compounds are SAhRMs. This suggests that the 5 congeners may induce some different genes and/or responses and this may be due, in part, to differential interactions of the AhR (bound) with coactivators. These possibilities are currently being investigated.

Acknowledgements
Supported by a gift from The Dow Chemical Company.

References

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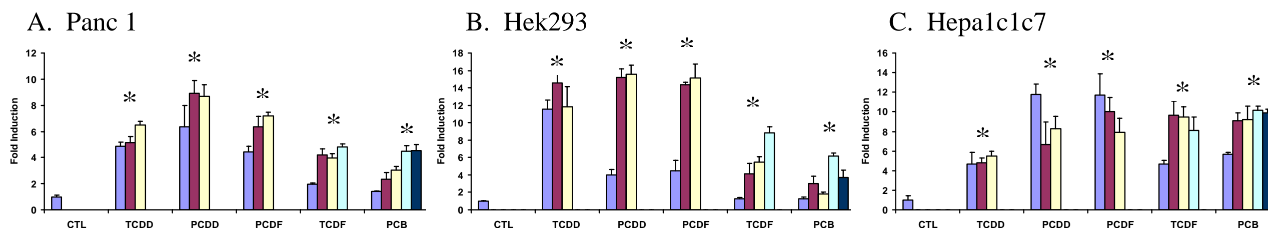
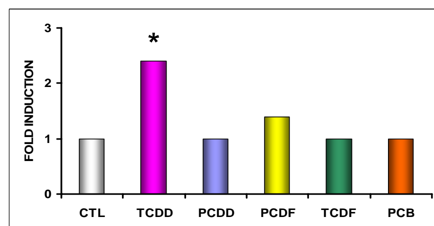


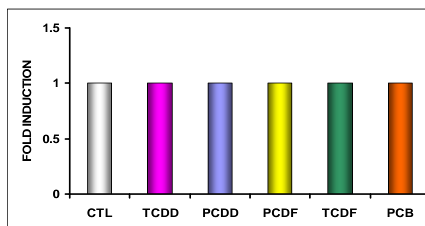
Fig. 1 TCDD, PCDD, PCDF (1, 10, 20 nM), TCDF (1, 10, 20, 50 nM) and PCB126 (1, 10, 20, 50, 100 nM) induce Ah-responsive pDRE3-Luc in Panc1(A), Hek293(B) and Hepa1c1c7 cells (c). Cells were transfected with Ah-responsive pDRE3-Luc construct and treated with Me2SO, TCDD and other four HAHs at different concentration for 24 h, and luciferase activity was determined as described under the Materials and Methods. Results summarized in A, B and C are means ± SD for three replicate determinations for each treatment group, and all of the five compounds can significantly ($p < 0.05$) increased activities (compared to solvent control) and are indicated by an *asterisk*.

A. CELL CONTEXT--DIFFERENCE

TRAP220-HEK293

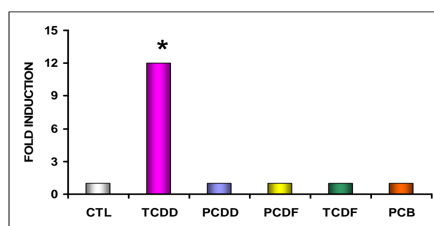


TRAP220-HEPA1C1C7

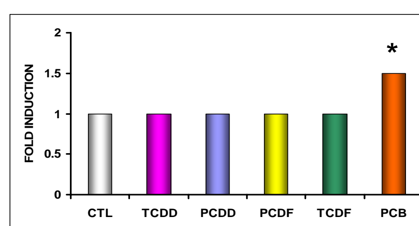


B. CELL CONTEXT--DIFFERENCE

CARM1-HEK293

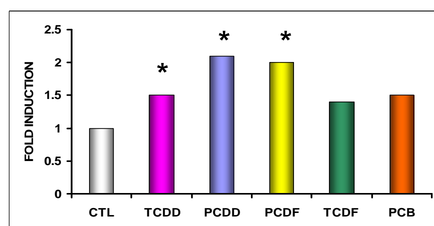


CARM1-HEPA1C1C7



C. CELL CONTEXT--SIMILARITIES

TIFII-Hepa1c1c7



TIFII-PANC1

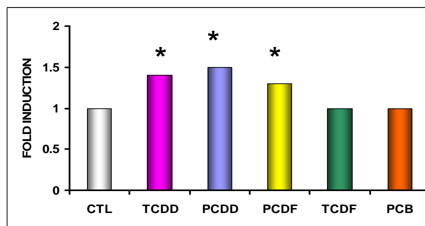


Fig. 2 Examples of cell context dependent differences and similarities. TCDD (10 nM), PCDD (20 nM), PCDF (20 nM), TCDF (20 nM) and PCB126 (50 nM). Significantly ($p < 0.05$) increased activities (compared to solvent control) are indicated by an *asterisk*.