Examination of *In Vitro* Interactive Effects Between TCDD and Other Dioxin-Like Compounds (DLCs) Using the Gel Mobility Shift Assay

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Abstract

The activation of Sprague-Dawley rat aryl hydrocarbon (Ah) receptor as determined by binding to an oligonucleotide containing a ^{32}P-DRE (dioxin response element) in the electrophoretic gel mobility shift assay was examined for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), various DLCs (PCB 153, PCB 169 and 3-methylcholanthrene (3-MC)), and binary mixtures of these DLCs with TCDD in molar ratios spanning 1:1 to 10 000:1. Saturation binding experiments demonstrated that the maximum response for TCDD was achieved at a concentration of 10 nM. In the case of the other DLCs, at no concentration, even those beyond the EC_{50} relative to 10 nM TCDD did the intensity of the shifted band approach that produced by TCDD. Analysis of binary mixtures of DLCs with TCDD revealed that previously reported interactive effects *in vivo* seem to be also observed *in vitro* at the level of receptor activation/DRE binding. When cytosol was incubated with a non-saturating concentration of TCDD (1 nM) and an excess of PCB 153, additive binding was observed. In contrast, if the concentration of TCDD was sufficient to saturate the receptor (10 nM), then an excess of a DLC caused effects that were antagonistic, and these effects occurred at concentrations equal to and greater than the EC_{50} of the competing compound.

Introduction

The Ah receptor is a ligand-activated transcription factor that is thought to mediate many of the toxic responses to dioxin-like compounds (DLCs). The toxicity of DLCs is usually referenced to the most potent Ah receptor agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Most other DLCs have lower binding affinity for the Ah receptor than TCDD and elicit less intense effects both *in vitro* and *in vivo*. The literature also contains several reports of interactive effects that are seen when TCDD is administered simultaneously with various other DLCs. Many of these studies have examined the incidence of cleft palate in mouse pups following maternal exposure during gestation [1,2], but other species and endpoints have also been examined [3,4,5]. These investigators have reported additive [2,4,5], synergistic [2,4], and antagonistic [1,3,4,] effects caused by mixtures of TCDD and various other DLCs.

The present work is part of a larger project in which the behaviour of TCDD/DLC mixtures is being examined *in vitro*, at various stages in the mechanism of action. The results

presented here refer to the behaviour of TCDD/DLC mixtures at the stage in the mechanism of action of receptor activation and DRE binding, as assessed by the binding of activated Ah receptor to an oligonucleotide containing a ^{32}P-labelled DRE, followed by electrophoretic separation and detection via autoradiography (gel shift assay).

Experimental Methods

Chemicals: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin was a generous gift from Brock Chittim, Wellington Labs, Guelph, Ontario. Oligonucleotides were custom synthesized by University of Calgary Core DNA Services, Calgary, Alberta. T4 Polynucleotidekinase was purchased from New England Bio-Labs, Beverly, MA; acrylamide (99.9%) and N',N'-methylene-bis-acrylamide from Bio-Rad, Hercules CA, and X-OMAT AR X-ray film from Eastman Kodak Company, Rochester, NY.

Cytosol Preparation: Cytosol was prepared from immature male Sprague Dawley rats which were sacrificed by CO_2 exposure followed by cervical dislocation. The livers were immediately perfused with ice-cold HEGD buffer (20 mM HEPES, 1 mM EDTA, 10 % (v/v) glycerol, 1 mM DTE, pH 7.6). The rinsed livers were excised, minced, rinsed with HEGD buffer and spun at 9000 x g_{max} at 4 °C for 20 minutes. The supernatant was then spun at 100 000 x g_{max} for 68 min. at 4 °C, following which the surface lipid was removed by aspiration and the cytosol stored in 1 mL aliquots at -70 °C.

Gel Shift Assay: Two complementary 32 base pair oligonucleotides containing the Ah receptor consensus binding sequence 5'-T-GCGTG-3' were ^{32}P-endlabeled with T4-polynucleotide kinase. Cytosol (16 mg protein/mL) was treated with DMSO (control) or TCDD/DLC mixture. The treated cytosol was incubated for 2 h at 23 °C (unless indicated), following which an aliquot was incubated with 500 ng poly (dI•dC) for 15 minutes at 23 °C and then with 200 000 cpm of ^{32}P-DRE for 15 minutes at 23 °C. The mixtures were then loaded onto a 5% non-denaturing polyacrylamide gel and electrophoresed at 11 V/cm. Gels were removed, sealed in plastic wrap and placed onto X-ray film in an autoradiography cassette and allowed to expose the film overnight at -20 °C. Once developed, autoradiograms were analyzed by densitometry using a Bio-Rad model 620 densitometer. Results are reported as % specific binding (% SB), using the optical density (OD) obtained from densitometry analysis.

$$\%SB=[(OD_{SAMPLE}-OD_{DMSO})/(OD_{TCDD}-OD_{DMSO})] \times 100$$

Results and Discussion

Figure 1 shows that the intensity of the retarded oligonucleotide band was maximal at concentration of TCDD > 10 nM, implying that this concentration of TCDD was sufficient to saturate the Ah receptor in the Sprague-Dawley rat cytosol preparation. At 23°C, transformation of the bound TCDD-Ah receptor complex is facile [6], enabling subsequent binding of the transformed complex to the DRE. No other DLC tested produced a response as large as that of TCDD alone, even when present at concentrations greater than the EC_{50} (concentration in a competitive Ah receptor binding assay that would reduce the specific binding of [^{3}H]-TCDD

by 50%). This indicates that not all DLCs that bind to the Ah receptor have equal ability to undergo further steps in the mechanism of action of TCDD. This observation provides experimental justification for this proposal which we made earlier on the basis of competitive binding studies in which the order of addition of ligands was varied [7].

For subsequent analysis, the intensity of the shifted electrophoretic band at [TCDD] > 10 nM was assigned the value 100% specific binding, while control samples incubated with DMSO were defined as 0%. Figures 2 through 4 show the shifted AhR:DRE band in autoradiograms from gel shift assays involving TCDD with mixtures of other Ah receptor binding substances, namely 3-methylcholanthrene (MC: Figure 2), 3,3′,4,4′,5,5′-hecachlorobiphenyl (PCB 169: Figure 3) and 2,2′,4,4′,5,5′-hecachlorobiphenyl (PCB 153: Figure 4). Each of these DLCs alone showed minimal or no intensity of the gel shifted band, even at concentrations of 100 μM, suggesting that these ligands cause very little activation of Ah receptor relative to TCDD. In this context, the term "activation of Ah receptor" is intended to mean transformation of the Ah receptor-ligand complex and/or ability of the transformed complex to associate with the DRE.

Figures 2-4 also show the results of using mixtures of TCDD and MC, PCB 169 or PCB 153 in ratios spanning 1:1 to 1:10 000. In each case, the other ligand reduced the total specific binding of Ah receptor to the DRE relative to TCDD alone, effectively antagonizing the formation of DRE-Ah receptor complex. A dose-response behaviour was observed which also reflected the relative binding affinity of the ligand for the Ah receptor. Of the ligands studied, MC has highest affinity for the Ah receptor (EC_{50} = 20 nM vs. 10 nM for TCDD) [8]; MC showed antagonism in the range of 10 to 100 nM (Figure 2). The relatively high affinity, coplanar PCB 169 has $EC_{50} \approx 1$ μM, and antagonized TCDD-induced activation at concentrations of 1 μM and greater (Figure 3). Correspondingly, the low affinity, di-ortho chlorinated PCB 153, which has EC_{50} = 80 μM [9], showed antagonism only at the highest concentration tested, 100 μM (Figure 4). The six right-hand lanes of Figure 4 also demonstrate that the response to the gel shift assay is more nearly additive when the concentration of TCDD (1 nM) is insufficient to saturate all available Ah receptor. In this case, the % specific binding increases when the concentration of PCB 153 is increased and then decreases again once there is enough PCB 153 present to begin competing with TCDD for the Ah receptor. Similar antagonism of the activation of the Ah receptor by TCDD to its DRE binding form in the gel shift assay has been previously reported for the compounds 1,2,7,8-tetrachlorodibenzofuran and 6-methyl-1,3,8-trichlorodibenzofuran [10]. In these experiments, the concentration of TCDD was 5 nM. This was likely enough to saturate the available receptor and the competitors, which alone produced a lower response than TCDD, displaced TCDD from the receptor, thereby reducing the overall intensity of the shifted AhR:DRE bands in the gel shift assay.

As noted in the Introduction, several studies have revealed interactive effects when TCDD is combined with other DLCs [1-5]. These effects have been observed in intact rats [5] and mice [1,2,4], and in cell cultures [3]; endpoints monitored include cleft palate in mouse pups, immunotoxicity in mice [11], AHH and EROD induction and the production of CYP 1A1 mRNA in various cell lines including H-4-II-E, HEPA 1c1c7, and Hep G2 [3]. Antagonism is the most commonly observed interactive effect [1,3,4,5,10,11], i.e., the intensity of response induced to a fixed dose of TCDD is reduced by simultaneous treatment with a second compound such as a

PCB. Additive responses have been reported in a number of studies [2,4,5], as well the occurrence of synergistic effects [2] in which the response to a mixture is considerably greater than the sum of the responses from either compound alone (i.e. "2 + 2 = 10").

The data presented here demonstrate that the antagonism of the response induced by TCDD by other DLCs is present at the level of receptor activation and DRE binding. The correlation between the concentration of DLC at which the antagonism occurs and the EC_{50} for competitive binding to the Ah receptor shows that the effect operates at the level of these compounds' interaction with the Ah receptor. It is readily observable in our experimental protocol because of the characteristic of these compounds that each of them produces a weaker response than TCDD in the gel shift assay in the absence of TCDD as a competing ligand (Figure 1).

The inability of DLCs to activate the Ah receptor as efficiently as TCDD may explain the frequently reported *in vivo* interactive effects. According to this explanation, in the context of our *in vitro* system, antagonism occurs when the intensity of the response to (TCDD + another DLC) is less than that produced by TCDD alone. This phenomenon was only observed when the concentration of TCDD was high enough by itself to saturate all available receptor molecules, and in addition, the concentration of the second DLC was sufficient to outcompete TCDD from a majority of the Ah receptor binding sites, but was not able to activate the receptor towards DRE binding.

Additive response in the gel shift assay can be observed only if the concentration of TCDD is insufficient to saturate all the Ah receptor sites. Under these conditions, Ah receptor sites are still available for occupation by a second DLC; even if this compound was weaker in terms of DRE activation, the overall effect would be a modest increase in the total amount of activated receptor. If the concentration of the second DLC becomes large enough to compete significantly with TCDD for the Ah receptor sites, then antagonism ultimately becomes observable, as shown in Figure 4.

The least likely outcome of these competitions is synergism, and this was not observed in the present experiments. Synergism would require that the second DLC yielded a stronger DRE activation than TCDD, so that when the second DLC outcompeted TCDD from available Ah receptor binding sites, the overall response intensity increased.

In summary, TCDD activated the Ah receptor to its DRE binding form much more efficiently than the other DLCs that we examined. We are studying the possibility of a relationship between a ligand's Ah receptor binding strength and its ability to promote activation of the receptor to the DRE binding form. The smaller activation of the receptor towards DRE binding found for DLCs other than TCDD may explain why interactive effects, usually antagonisms) are often seen with mixtures of these compounds. In addition, the size of the TCDD dose also plays a role. With a high amount of TCDD saturating the receptor, antagonism is seen, while lower amounts that do not saturate the receptor show more nearly additive responses.

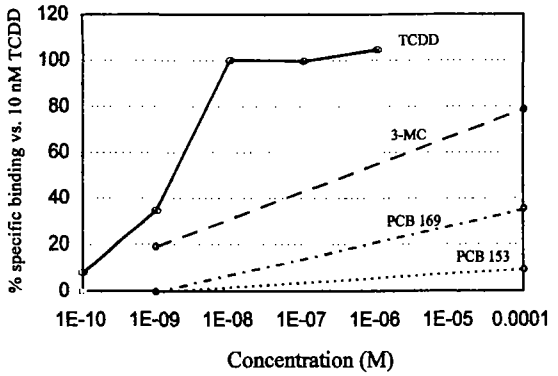


Figure 1: Graph showing the % specific binding of activated AhR to its DRE, relative to 10 nM TCDD. The response for TCDD is maximal at concentrations of 10 nM and greater, while the responses for the other DLCs are lower, even at concentrations equal to and greater than their EC_{50} relative to 10 nM TCDD.

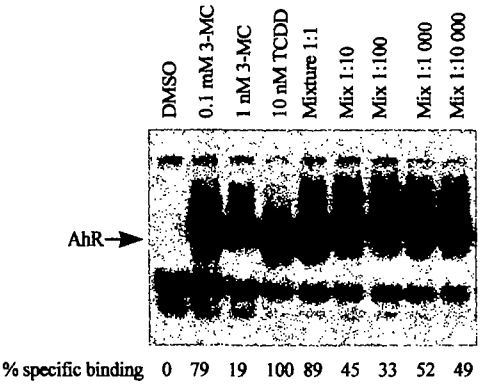
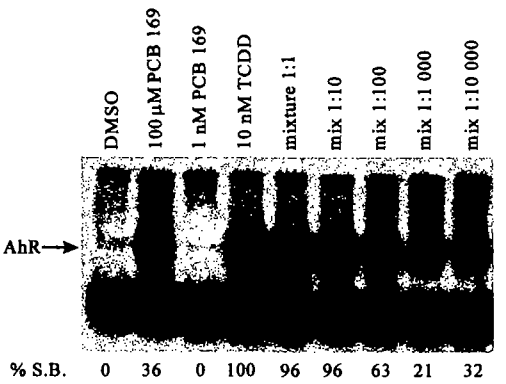


Figure 2: Autoradiogram of a Gel shift assay of DMSO, 10 nM TCDD, 3-methylcholanthrene (3-MC) alone and mixtures of 10 nM TCDD with various molar excesses of 3-MC

Figure 3: Gel shift assay of 10 nM TCDD, 3,3',4,4',5,5'-HCB (PCB 169).alone and mixtures of 10 nM TCDD with various molar excesses of PCB 169



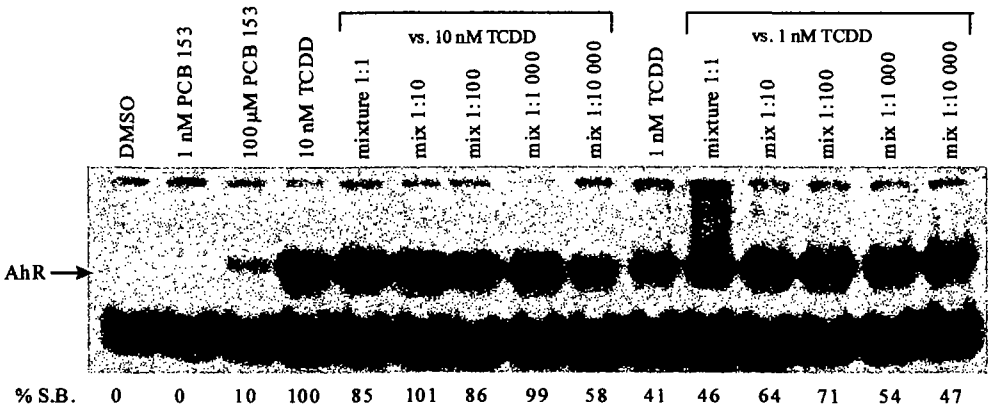


Figure 4: Autoradiogram of gel shift assay showing TCDD, 2,2',4,4',5,5'-HCB (PCB 153) and various mixtures of either 1 nM or 10 nM TCDD with PCB 153

Acknowledgments

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