KINETICS OF DNA-BINDING OF POLYCLORINATED DIBENZO-*p*-DIOXIN AND DIBENZOFURAN CONGENERS WITH HEPATIC CYTOSOLIC ARYL (Ah) RECEPTOR

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ABSTRACT

Incubation of rat hepatic cytosol with six polychlorinated dibenzo-*p*-dioxin and dibenzofuran congeners resulted in the interaction of transformed ligand-receptor complexes with the dioxin responsive element (DRE) DNA, as determined by the gel moblity shift assay. Saturation binding data for the transformed ligand:AhR complexes showed that the B_{max} values correlated with their biochemical and toxic potencies. In addition, transformed AhR complexes for all congeners exhibited apparent Kds comparable to those of the estrogen and glucocorticoid receptor complexes with their corresponding hormone response elements. Association/dissociation curves for the liganded AhR:DRE complexes demonstrate that the congeners exhibit kinetic properties which correlate with their corresponding biochemical and toxic potencies.

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) has been extensively used as a prototype for investigating the toxic and biologic effects of the halogenated aryl hydrocarbons, which include the polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), and dibenzofurans (PCDFs).[1] Many of the toxic and biochemical responses to these agents are thought to be mediated through an intracellular protein, the aryl hydrocarbon (Ah) receptor, which binds TCDD and related halogenated aromatic hydrocarbons saturably and with high affinity.[2] Similar to the steroid hormones, TCDD first associates with the cytosolic receptor protein; association is followed by transformation to a DNA-binding form and translocation of the ligand-receptor complex into the nucleus.[3-7] This receptor-ligand complex binds to several dioxin-responsive elements (DREs) located upstream of the CYPIA1 gene and these interactions stimulate transcriptional activation.[8-9] Previously, in this laboratory, we have shown that incubation of rat hepatic cytosol with PCDD/PCDF congeners resulted in the formation of transformed ligand-AhR:DRE complexes.[10] This study utilizes a sensitive gel mobility shift assay to investigate the kinetic properties of DNA-binding of the transformed rat cytosolic Ah receptor.

MATERIALS AND METHODS

Chemicals TCDD, 2,3,7,8-tetrachlorodibenzofuran (TCDF), 1,2,7,8-TCDF, 1,2,3,7,8-pentachlorodibenzodioxin (1,2,3,7,8-PeCDD), 1,2,3,7,8-pentachlorodibenzofuran (1,2,3,7,8-PeCDF), 2,3,7-trichlordibenzodioxin (TrCDD) were synthesized in this laboratory and purified by preparative HPLC (to > 98% purity) as previously described.[11]

Animals Male Long Evans rats (21 days old; ≈ 100 g) were obtained from Harlan Laboratories (Houston, TX) and were housed two per cage in plastic cages with hardwood bedding. They were allowed free access to Purina certified rodent chow, No. 5002, and water and were maintained on a diurnal cycle of 12 h of light and 12 h of darkness.

Preparation of cytosol The animals were terminated by cervical dislocation and the livers were immediately perfused *in situ* by the hepatic portal vein with ice-cold HEGD buffer [25 nM Hepes, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol (v/v), pH 7.6]. The excised livers were rinsed once with 10 ml of the fresh ice-cold HEGD buffer and finely minced. The minced livers were rinsed again with buffer and homogenized in ice-cold HEGD buffer using a Brinkman Homogenizer PT45/80. The homogenate was centrifuged at 10,000 g for 20 min at 2°C and the surface lipids were removed by aspiration. The resulting supernatent was recentrifuged at 105,000 g for 1 h at 2°C and again the surface lipids were removed. Protein concentrations were measured by the method of Bradford and the supernatent (cytosol) was stored at -80° C until use.

Gel retardation analysis Complementary strands of the synthetic oligonucleotide containing a consensus DRE sequence (26 mer) were synthesized, purified by polyacrylamide gel electrophoresis, and annealed. The oligonucleotide was labeled at the 5' end using T4-polynucleotide kinase and γ^{32}-P ATP. Cytosol (16 mg protein/ml) was incubated with DMSO (20 µl/ml) or 20 nM TCDD, 1,2,7,8-TCDF, 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, 1,2,3,7,8-PeCDD, and 2,3,7-TrCDD for 2 h at 20° C prior to gel retardation analysis; 80 µg of cytosol was incubated in HEDGK (HEGD + 0.8 M KCl) buffer with 200 ng poly (dI-C) for 15 min at 20° C to bind nonspecific DNA-binding proteins. Following the addition of ^{32}-P DRE oligonucleotides, the reaction mixtures were incubated for 15 min at 20°C. Protein-DNA complexes were resolved on 5% polyacrylamide gel (acrylamide: bisacrylamide ratio of 30:0.8) at 120 v using a Tris borate-EDTA buffer. Gels were dried and protein-DNA binding was visualized by autoradiography. To determine the amount of protein-DNA complexes formed, the specific radiolabeled band was quantitated using a Betascope 603 Blot Analyzer. The amount of ^{32}-P DRE specifically bound in the ligand-inducible complex was estimated by measuring the amount of radioactivity in the inducible protein DNA complex, isolated from a ligand-treated sample lane, and subtracting the amount of radioactivity present in the same position in a non-ligand treated lane. The difference in radioactivity between these samples represents the ligand-inducible specific binding of ^{32}-P DRE.

Kinetic Binding Assays Saturation binding curves were measured by incubating 80 µg of ligand-treated cytosol with increasing amounts of ^{32}-P DRE ranging from 0-27 nM.[12] The amount of DRE specifically bound in the ligand-inducible complex was determined by mobility shift assay as previously described. The data were analyzed on a Woolf plot.[13] Association curves for transformed ligand:AhR:DRE complexes were measured by incubating 80 µg of transformed ligand:AhR complexes with 5 nM DRE over a 90 min period and subjected to the gel mobility shift assay. The amount of DRE specifically-bound in the ligand-inducible complex was determined as previously described and expressed as a % of the total amount of radioactivity loaded in each lane compared to that observed for the TCDD-treated cytosol. Dissociation rates of ligand:AhR:DRE complexes were measured by the addition of a 200-fold excess of unlabeled DRE after the 15 min incubation with ^{32}-P DRE at specific times over a 2 hr interval.[14] The reactions were subjected to the gel mobility shift assay and quantified as previously descibed.

RESULTS AND DISCUSSION

Figure 1 summarizes the saturation binding curves for the PCDD/PCDF ligands utilizing a constant amount of cytosol containing transformed ligand:AhR complexes and increasing amounts of specific ^{32}-P DRE. Table I shows the B_{max} and apparent Kd values for these ligands using a Woolf plot of the saturation data. The B_{max} values at saturation show that the most active PCDD and PCDF ligands are substituted in the four lateral 2,3,7,8 positions; the removal of one lateral chlorine results in the decrease in the total concentration of transformed AhR bound to the DRE. These values correlate with the biochemical and toxic potencies of these ligands.[15-16] The apparent Kd values of cytosolic transformed ligand:AhR complexes are similar to the values for binding of other steroid hormone receptors to their specific DNA recogntion sites.[17-18]

The association curves shown in Figure 2 compare the ability of the transformed ligand:AhR complexes to bind to the DRE. Cytosol transformed with 1,2,7,8-TCDF and 2,3,7-TrCDD, the congeners with the lowest biochemical and toxic potencies, bind low levels of DRE, while congeners substituted in the lateral positions show maximum binding. However, no discernable differences in the rate of formation of the ligand:AhR:DRE complexes was observed for any of these ligands.

The dissociation rates of DRE complexes formed by AhR bound to different ligands are illustrated in Figure 3. This study shows no discernable difference in the overall dissociation rates of complexes formed by these ligands, which correlates with the dissociation rates for ligand ER:ERE complexes.[14]

Table I. Kinetic Analysis of the Formation of Ligand:AhR:DRE Complexes

Ligand	Kd(nM)	B_{max}(fmol/mg protein)
TCDD	1.86 ± 0.25	38.20 ± 0.98
2378-TCDF	2.93 ± 0.01	37.63 ± 1.61
1278-TCDF	3.23 ± 0.41	13.82 ± 1.05
12378-PeCDD	1.33 ± 0.08	36.91 ± 2.13
12378-PeCDF	3.58 ± 0.27	39.36 ± 1.28
237-TrCDD	0.95 ± 0.09	13.25 ± 1.35

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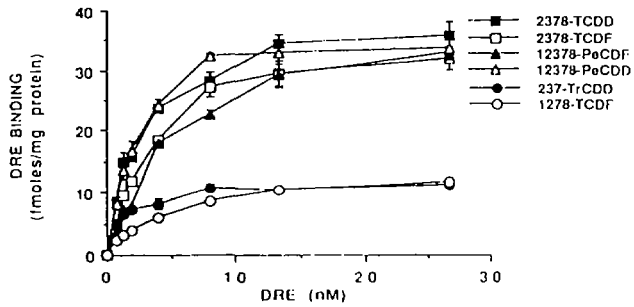

Figure 1. Saturation binding curves of ligand:AhR:DRE complexes

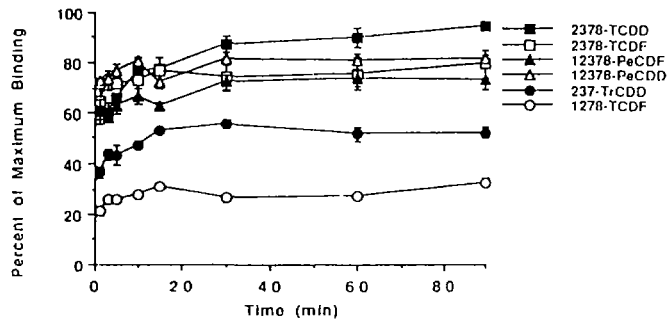

Figure 2. Association Curves for ligand:AhR:DRE complexes

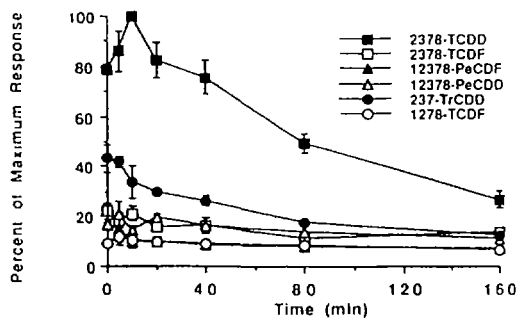

Figure 3. Dissociation Curves for ligand:AhR:DRE complexes