DIVERGENCE IN MECHANISM BETWEEN AHR AGONISTS AND ANTAGONISTS IN THE AHR SIGNAL TRANSDUCTION PATHWAY

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Introduction

Halogenated aromatic hydrocarbons (HAHs), including polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/F), and biphenyls (PCB), have been widely identified in every compartment in the environment, wild life and humans[1]. Exposure to these persistent organic pollutants invariably involves complex mixtures rather than individual congeners. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the prototype for HAHs that have a common mechanism of toxicity and biochemical responses mediated by the aryl hydrocarbon receptor (AhR). Less potent HAHs such as PCB 153 antagonize the toxicity of the more highly potent congeners [2, 3]. Our objective is to investigate the mechanism of antagonism on each step of the Ah receptor signal transduction pathway leading to the induction of cytochrome P450 1A1 in primary rat hepatocytes, and to identify the point of divergence in the mechanism of action between the potent and non-potent ligands. Polybrominated diphenyl ethers (PBDEs) are another class of halogenated aromatic hydrocarbons (HAHs) that are now found ubiquitously in the environment, due to their widespread use as flame retardants for soft furnishings, electronic equipment, and automobiles [4]. The interaction of PBDE congeners and TCDD on each step of the Ah receptor signaling system will also be discussed.

Materials and Methods

Electrophoretic mobility shift assay

Two complementary 32-base pair oligonucleotides containing the Ah receptor consensus binding sequence 5'-T-GCGTG-3' were [^{32}P]-end labeled by T4 polynucleotide kinase. Aliquots of ligand activated cytosol were then incubated at 23 ^{o}C with 500 ng poly (dIdC) for 15 min, then 1 µL of [^{32}P]-DRE (~500 000 cpm/mL) was added and the samples were mixed and incubated for a further 15 min at 23 ^{o}C. The protein-DNA complexes were then resolved in a 5% polyacrylamide gel in TBE buffer at 11 V/cm. The formation of DRE complexes was visualized by autoradiography and quantitated by densitometry.

Preparation and culture of primary rat hepatocytes

Primary rat hepatocytes were prepared by a modified protocol of Kreamer [5] followed by EGTA and collagenase two-step perfusion. The living cells were enriched by percoll iso-density centrifugation. The cells were then counted using a hemocytometer. The viability of the cells was > 90 % as assessed by trypan blue exclusion. For immunoblotting experiment, hepatocytes were inoculated (3 x 10^6 cells/ 3.0 mL attachment media) in polystyrene tissue culture dishes (Corning, 60 mm) pre-coated with collagen. After 2 h the medium was changed to 3.0 mL serum-free media. The cells were then incubated for 22 h at 37 ^{o}C (95 % air, 5 % CO_2). After 24-h pre-incubation, the medium was refreshed and the cells were treated with various concentrations of TCDD and PBDE congeners, and incubated for a further 24 h. For EROD assays, cells were plated in 48-well collagen-coated culture plates at a

density of 50 000 cells/well in 0.5 mL attachment media. Cell culture condition and treatment of the cells were the same for tissue culture in 60mm dishes.

Western blotting for CYP 1A1

Monolayer cells were rinsed with cold PBS and harvested by scraping off with PBS, pelleted and resuspended in HEGD buffer. The cells were lysed by sonication and microsomes were prepared by centrifugation. Microsomes with equivalent amount of protein (~20 mg) were used for SDS-polyacrylamide electrophoresis (10%) separation with Tris-glycine buffer system at 100 V for 1.5 h. Separated proteins were transferred onto nitrocellulose membrane at 100 V for 2 h, then the membrane was blotted with 5 % skim milk for 1 h to block non-specific binding sites. Goat anti-rat CYP 1A1 (Gentest, Woburn, MA) polyclonal antibody (1:1000 dilution in 1% gelatin-PBS) was applied to membrane for 1 h. After washing with TTBS, the primary antibody was visualized using alkaline phosphatase conjugated anti-goat Ig G (1:5000 dilution).

Assay of CYP 1A1 activity

CYP 1A1 activity was determined by the 7-ethoxyresorufin-O-deethylase (EROD) assay. After 24 h treatment, cell monolayers were rinsed, and the intact cells were used for the EROD assay [6]. Resorufin was measured with a BioRad Fluoromark fluorometric plate reader. For each congener, data are based on the means of at least two independent experiments, each of which involved 3-5 replicates.

Results and discussion

Ah receptor binding

TCDD binds with high affinity to the Ah receptor; the affinity of other ligands is generally determined by competition with [^{3}H]-TCDD using the HAP assay. Less potent ligands totally displace TCDD at sufficiently high concentration. Ligand binding is initially reversible, but the AhR-HAH complex is then transformed to a form that has an increased binding affinity for the bound ligand. At this stage in the mechanism there is no significant difference in behavior between AhR agonists and antagonists [7].

Formation of AhR-Arnt-DRE complex

Activation of the ligand-Ah receptor complexes was evaluated by the electrophoretic mobility shift assay, using a double stranded 32-mer oligonucleotide, end-labeled with [^{32}P]-ATP. Cytosol treatment of 10^{-8} M TCDD was used as a positive control for 100 % activation. Ah receptor antagonist PCB 153 did not activate the formation of DRE complex, but it inhibited the action of 10 nM TCDD. However, when the TCDD concentration was 1 nM, PCB 153 did not show antagonistic behavior [7]. Dose-response behavior was obtained over the range 10^{-9} to 10^{-5} M for PBDE congeners (Figure 1). Congener 119 exhibited almost full activation compared with 10^{-8} M TCDD, congeners 77 and 126 showed moderate activation, and congeners 100, 156, 153, 183 showed very weak DRE complex formation. The commercial mixtures and the other congeners failed to activate AhR to the DRE binding form.

Dose-dependent interactions of PBDE congeners and TCDD on the step of DRE complex formation were studied. The most potent congener PBDE 119 demonstrated additive behavior with TCDD (Figure 2, left). Congeners 77 and 126, which can not reach the full activation compared with 10 nM TCDD, interacted with TCDD additively at low concentrations. However, at higher concentrations of TCDD, they acted antagonistically (Figure 2, right). Environmentally predominant congeners 47 and 99 and the commercial PBDE mixtures, which did not show DRE activation, all inhibited TCDD-induced AhR activation. These observations are explicable in terms of limited AhR binding capacity, with the strongly activating ligand TCDD competing for a partial agonist (PBDEs 77,

119, 126) or an antagonist (PBDEs 47, 99 etc). The ability to activate the formation of AhR-DRE complex could be the critical step for the activation of gene transcription. The study of interactive effects of CYP 1A1 gene transcription is in process.

Interactive effect on induction of CYP 1A1

PCB congeners, 77, 156 and 153 were selected to study the interaction with TCDD in the induction of P450 1A1 protein. PCBs 77 and 156 showed full induction of P450 1A1 at the high concentration, comparable with TCDD (Figure 4, left), and showed no antagonism in this step, even at very low concentration of TCDD. PCB 153 can only slightly decreased the induction of P450 1A1 by 1 nM TCDD, but only about 40% inhibition at the concentration of 10^{-5} M. The inhibition potency was less than with the P450 1A1 inhibitor a-naphthoflavone (Figure 3, left).

PBDE congeners 77, 126, 119 showed increasing CYP1A1 protein monotonically with dose and the maximum induced levels were comparable to the reference of 10^{-9} M TCDD [8]. The strong induction of CYP 1A1 protein was consistent with their greater AhR activation to the DRE binding form by these congeners. Congeners 66, 100, 153, and 183 were moderate CYP 1A1 inducers; induction only occurred at high concentration. Cells co-treated with PBDE 77 and $5x10^{-11}$ M TCDD showed additive induction of the P450 1A1 protein (Figure 3, right). The pentaBDE commercial mixture and environmentally predominant congener 47 showed only very weak inhibition of 1nM TCDD even at 10^{-4} M.

Discrepancy between the P450 1A1 level and EROD activity

The final endpoint examined was CYP 1A1 formation, as studied through both immunoblotting of the protein itself and through enzyme activity studied by the 7-ethoxyresorufin-O-deethylase (EROD) assay in intact culture cells. Most of the EROD induction curves from cell cultures were bell shaped, however, while the induction of P450 1A1 protein increased monotonically. The phenomena were found in both PCB congeners and PBDE congeners [8]. The low EROD activity could be due to competitive enzyme inhibition [9]. Hence the Western analysis gives a more direct measurement by which to compare agonist strength.

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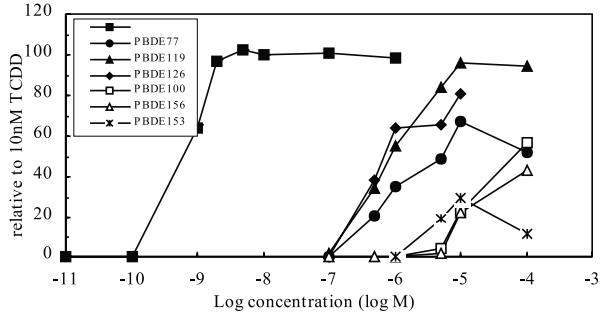

Figure 1. Formation of the AhR-DRE complex by PBDE congeners compared with TCDD

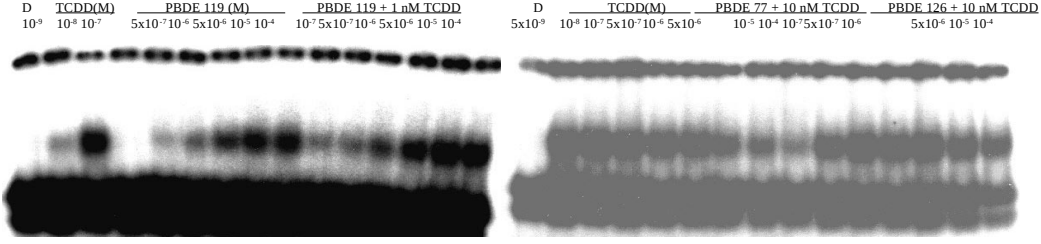

Figure 2. Dose–dependent additive behavior for PBDE congener 119 (left) and antagonistic behavior for PBDE 77 and 126 (right) shown by images from electrophoresis mobility shift assay.

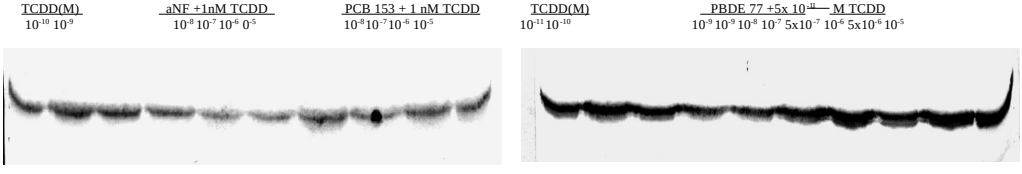

Figure 3. Inhibition of aNF and PCB 153 on CYP 1A1 induction by 1nM TCDD (left) and additive induction of CYP 1A1 in mixture of PBDE 77 and $5x10^{-11}$ M TCDD in primary rat hepatocytes using Western blotting assay

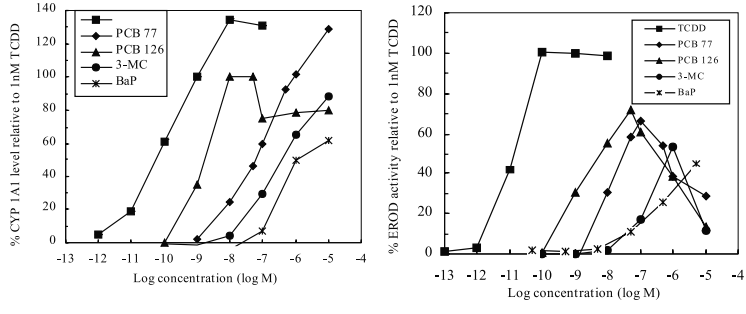

Figure 4. Different induction curves for P450 1A1 induction based on immunodetectable protein assay and enzyme activities in primary rat hepatocytes.