Binding of 2,3,7,8-Tetrachlorodibenzo-p-dioxin to the AhR from Various Species is Essentially Irreversible

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Introduction

Halogenated aromatic hydrocarbons (HAHs) are a diverse group of widespread, persistent and toxic environmental contaminants that includes the polychlorinated dibenzo-p-dioxins and related chemicals. Exposure to these compounds results in a variety of biochemical and toxic effects, the majority of which are mediated by the aryl hydrocarbon receptor (AhR). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most potent activator of the AhR and AhR-dependent effects.[1-4] Interestingly, while a related class of compounds, the polycyclic aromatic hydrocarbons (PAHs), can bind to and activate the AhR, and produce many of the same biological effects as HAHs, they do not cause HAH-like toxicity. This can be due to differences between these two classes of compounds with respect to their AhR binding affinity, metabolic stability, and/or gene expression. PAHs have a lower affinity for the AhR[5] and, unlike TCDD, can be readily metabolized by cytochrome P450 enzymes[6, 7]. In addition to its high affinity for the AhR, TCDD has been shown to stabilize the rat AhR receptor against thermal inactivation[8, 9] and to persistently bind the rat receptor[10]. This persistent occupancy may also contribute to the differential toxicity of HAHs and PAHs. In addition to its biological and toxicological implications, the apparent lack of significant dissociation of TCDD from the AhR also impacts the design and interpretation of competitive binding experiments which assume traditional receptor-ligand equilibrium binding kinetics where binding is reversible and equilibrium of ligand:receptor complex is reached when rates of association and dissociation are equal. In this study we have further examined whether this persistent occupancy is a characteristic of the AhR among different species.

Materials and Methods

Materials. [^{3}H]TCDD and 2,3,7,8-tetrachlorodibenzofuran (TCDF) were generously provided by Steve Safe (Texas A&M University, USA). HEPES was purchased from Research Organics (Cleveland, OH), ethylenediaminetetraacetic acid (EDTA) and glycerol from Fisher (Fair Lawn, NJ), dithothreitol (DTT) and dextran from Sigma (St. Louis, MO), Norit A from JT Baker (Phillipsburg, NJ), and hydroxyapatite (HAP) from BioRad (Hercules, CA).

Preparation of Cytosol.

Hepatic cytosol from male Hartley guinea pigs, Sprague-Dawley rats, C57BL/6 mice and Golden Syrian hamsters was prepared in HEDG buffer (25 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% glycerol) and protein concentrations determined as previously described[11].

Thermal Stability. Rat hepatic cytosol (2 mg/ml) was incubated at 4°C or 20°C for the times indicated and then incubated with 2 nM [^{3}H]TCDD in the presence or absence of 200 nM TCDF for 2 hours. Aliquots (200 µL) were taken and [^{3}H]TCDD specific binding determined using the HAP assay as previously described[11].

Dissociation of [^{3}H]TCDD. Hepatic cytosol (2 mg/ml) was incubated with 2 nM [^{3}H]TCDD in the presence or absence of 200 nM TCDF for two hours. Samples (1.25 ml) were transferred onto dextran-coated charcoal pellets (0.75 mg:7.5 mg) and incubated at room temperature for 10 minutes with periodic vortexing. The "stripped" supernatant was collected following centrifugation and incubated with TCDF (200 nM final concentration) at the temperatures indicated. The time of re-addition of TCDF was defined for these studies as the time zero point. Aliquots (200 µL) were taken at the indicated times and [^{3}H]TCDD specific binding was determined using the HAP assay[11].

Results and Discussion

To determine the degree of thermal inactivation of unoccupied AhR, rat hepatic cytosol was incubated at 4°C or 20°C for the indicated times prior to the addition of [^{3}H]TCDD and determination of specific binding by HAP analysis (Figure 1). Unoccupied receptor was susceptible to thermal inactivation and almost complete loss of binding was observed by 12 hours at 20°C (9% of T=0). In contrast, less unoccupied receptor was inactivated if it was incubated with 16% [^{3}H]TCDD specific binding remaining at 60 hours. These results are consistent with previous results obtained for the rat AhR receptor[8, 9] and have implications with regards to experimental conditions for ligand binding analysis as described below.

To measure the rate of dissociation of [^{3}H]TCDD from the AhR receptor, cytosol was incubated with [^{3}H]TCDD for two hours and charcoal stripped followed by the addition of unlabeled TCDF (200 nM final concentration). Interestingly, at 60 hours, [^{3}H]TCDD specific binding was decreased by only 17% and 28% of time zero at 4°C and 20°C, respectively. These data not only indicate that TCDD binding confers both stability and resistance to thermal inactivation to the rat AhR, but the minimal decrease in [^{3}H]TCDD specific binding over time also demonstrates that [^{3}H]TCDD does not readily dissociate from the rat AhR at either 4°C or 20°C. To determine whether TCDD can also persistently occupy the AhR at physiological temperature (37°C) and if a similar persistence is observed with another species, we repeated the dissociation experiment at 37°C and 20°C using guinea pig hepatic cytosol (Figure 2). While these results revealed slightly more [^{3}H]TCDD dissociation from the AhR at 37°C as compared to 20°C, the amount of [^{3}H]TCDD specific binding remaining at 68 hours was still 81% of time zero.

The time course of dissociation at 20°C was repeated using hepatic cytosol from mice, hamsters, and rats to determine if the slow rate of [^{3}H]TCDD dissociation from the AhR was also a feature of the AhR from other (Table 1). We observed that the loss of [^{3}H]TCDD specific binding (i.e. dissociation) was slowest for the guinea pig AhR (14% of T=0 at 48h) and fastest for the hamster AhR (44% of T=0 at 48h). In addition, other ligand binding experiments (data not shown) confirm that these decreases are due to displacement of [^{3}H]TCDD from the AhR and not to receptor degradation.

The results of our studies indicate that unoccupied AhR is thermally labile and that binding ligand confers stability to the AhR receptor. In practical terms, since TCDD does not appear to readily

dissociate from the AhR in any of the four species tested, our results question the appropriateness of using equilibrium binding kinetic approaches for analysis of AhR ligand binding affinity. Expression of AhR ligand binding affinity as a dissociation constant (Kd) is incorrect since our results indicate that little [^{3}H]TCDD binding is dissociated during the typical equilibrium binding experiment (2 hours). Determination of accurate equilibrium dissociation constants (i.e. affinity measurements) require that the incubation time for ligand binding be at least 4-5 half lives of ligand dissociation, something that is clearly not feasible. In addition, these results also directly impact the experimental design for competitive binding experiments and possibly provide some explanation for the difficulty in demonstrating the ability of weak ligands such as omeprazole, carbaryl and others to competitively bind to the AhR[12-14]. Studies are currently underway using a wide variety of AhR agonists and antagonists to determine whether this persistent occupancy is a characteristic of all AhR ligands. If lack of dissociation is a feature of all AhR ligands, then demonstrating AhR binding by low affinity ligands and compounds that have been reported to be non-ligand activators of the AhR should be able to be achieved by pre-incubating cytosol with the compound of interest prior to adding [^{3}H]TCDD. These studies also raise interesting questions regarding the mechanism by which compounds bind to and activate the AhR, and regarding AhR-dependent toxic and biological effects.

Acknowledgements

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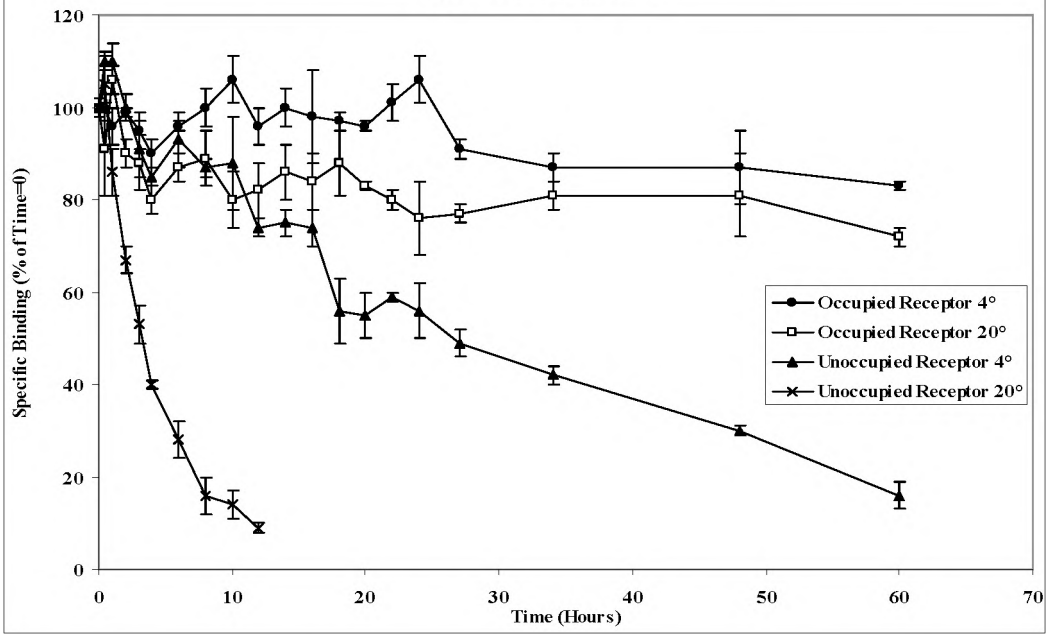
Figure 1: Specific binding of [^{3}H]TCDD to Occupied and Unoccupied Rat Hepatic Cytosol at 4°C and 20°C



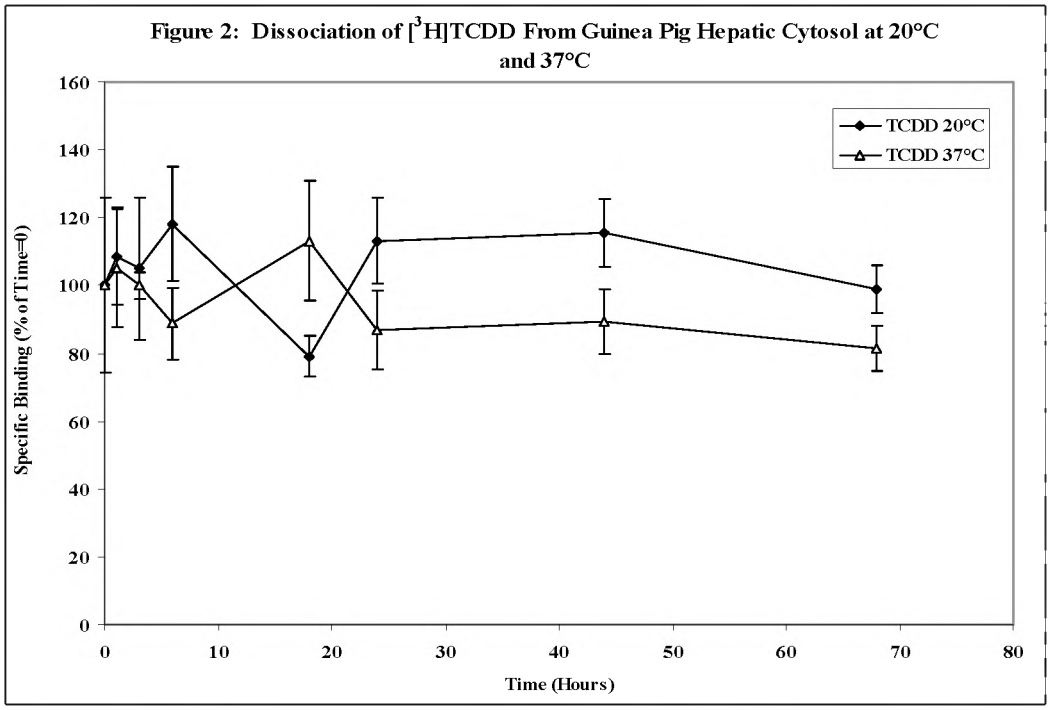


Table 1: Time course of [^{3}H]TCDD dissociation from hepatic cytosol of various species.

Species	Percent Specific Binding of T=0 hours						
	0h	1h	3h	6h	12h	33h	48h
Guinea Pig	100±9	104±9	100±5	92±7	86±4	88±5	86±5
C57 Mouse	100±5	82±3	86±7	82±10	86±10	70±4	73±9
Hamster	100±5	89±9	82±1	71±5	61±2	58±4	56±5
Rat	100±3	84±3	92±7	86±6	76±6	78±5	72±11