Species-Specific Differences in Ah Receptor Ligand Binding and Transcriptional Activation: Implications for Bioassays for the Detection of Dioxin-Like Chemicals

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1. Introduction

Polychlorinated diaromatic hydrocarbons (PCDHs) are a diverse group of widespread environmental contaminants, which include polychlorinated biphenyls (PCBs), dibenzofurans (PCDFs), dibenzo-*p*-dioxins (PCDDs) as well as many other subclasses of PCDHs and polycyclic aromatic hydrocarbons (PAHs).[1)] Some PCDHs are not only lipophilic and persistent in the environment but they are known to bioaccumulate and biomagnify in the food chain.[2)] Exposure to specific PCDHs results in a wide variety of species- and tissue-specific effects, such as tumor promotion, lethality, birth defects, hepatotoxicity, immunotoxicity, dermal toxicity, alterations in endocrine homeostasis and induction of numerous enzymes, including that of cytochrome P4501A1.[1-5)] Induction of cytochrome P450IA1 by PCDHs is mediated by the Ah receptor (AhR) complex to which these chemicals bind with high affinity.[2-5)] Following ligand (PCDH) binding, the AhR complex is transformed into its DNA binding form and PCDH:AhR complexes accumulates within the nucleus.[2,5,6)] The binding of transformed PCDH:AhR complexes to its specific DNA recognition site (the dioxin responsive element (DRE)) adjacent to the P450IA1 gene is necessary for its transcription activation.[5,6)] Since quantitative structure-activity relationship (QSAR) studies also support a role for the AhR in mediating the toxicity of these chemicals[1-4)], the induction of P4501A1 and its monooxygenase activities (7-ethoxyresorufin O-deethylase (EROD)) has been one response that has been used as a bioassay to evaluate the relative biological/toxicological potency of PCDHs and complex mixtures containing these chemicals.[1,3,7,8)]

In the environment, including some hazardous waste sites, PCDHs are present as complex mixtures. Samples from these sites may theoretically contain up to 209 different PCBs, 135 different PCDFs, and 75 different PCDDs isomers and congeners and many other related chemicals with vastly different chemical, physical, and toxicological properties.[1,3)] Currently, the toxicological potency of complex mixtures of contaminants is assessed by the addition of the toxic equivalent factor (TEF) multiplied by the concentration of the individual compounds present in the mixture. TEF values have been determined for many PCDHs by measuring their ability to induce EROD activity in cell culture or a toxic endpoint[1,3,9)] and then expressing the magnitude of the response in terms relative to that observed with the most potent PCDH, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), as toxic equivalents. The specific TEF values used in these analysis vary widely relative to the specific species and endpoint examined.[1,3,9)] Although additive models to assess toxicological potency of mixtures of PCDHs based on TEF values do

take into account the biological effects of the compounds, however, they do not account for interactive effects (antagonism, synergism[1,3,9]) between or among active or inactive PCDHs or other xenobiotics. These points are significant, expecially since species- and tissue-differences in PCDH responsiveness (particularly to that of TCDD) have been observed.[2,3,9] Although the AhR appears to mediate these responses in all species, it is not identical.[10,11] We recently observed species-specific antagonism of TCDD-inducible, AhR-dependent gene expression by several "nonAhR" di-ortho-substituted PCBs (such as 2,2',5,5'-tetrachlorobiphenyl (TCB)) (Aarts et al., submitted) and of hepatic AhR transformation and DNA binding by α-napthoflavone (ANF) an AhR antagonist.[12] These observations are of particular importance, given that PCDHs/PAHs exist in the environment as complex mixtures and that interactions between chemicals within the mixture could not only differentially affect the overall biological/toxicological potency of the mixture to various species, but also measurements using available AhR-dependent bioassays. Here we have examined the apparent species differences in AhR antagonism by examining the effect of TCB and ANF on AhR functionality (AhR ligand binding, transformation, DNA binding and transcriptional activation).

2. Materials and Methods

Preparation of Cytosol: Hepatic cytosol from male Hartley guinea pigs (250-300g, Michigan Department of Public Health, Lansing, MI USA), and male Sprague-Dawley rats (200g, Charles River Laboratories, Wilmington, DE USA) and from male C57BL/6N mice (20g, Charles River Breeding Laboratories) was prepared as described.[8,13]

AhR Ligand and DNA Binding Analysis: AhR ligand binding in hepatic cytosol was determined by sucrose density centrifugation using [3H]TCDD and DNA binding was carried out using gel retardation analysis.[13]

Luciferase Expression Assay: Continuous cell lines (Hepa1 (H1L1.1c2), HepG2 (HG2L1.1c3), GPC16 (G16L1.1c8) and H4IIE (H4L1.1c4)) which have been stably transfected with the HAH-inducible reporter plasmid pGudLuc1.1 (Garrison et al., submitted) were used in these studies. pGudLuc1.1 contains the firefly luciferase gene under PCDH-inducible control of four dioxin responsive elements. Exposure of these cells to TCDD and related AhR agonists results in induction of firefly luciferase activity in a time-, dose- and AhR-dependent manner. Cells were incubated with the indicated chemical for 24 h and, after incubation, cells were lysed and luciferase activity of the cleared lysate was determined using the Promega luciferase assay system.

3. Results

We and others have previously demonstrated that ANF can act as an antagonist to the rat AhR.) However, preliminary studies revealed that ANF was not a very effective antagonist of the guinea pig AhR complex (Denison, unpublished observations). In order to characterize this in greater detail, we incubated rat and guinea pig hepatic cytosol with TCDD (5nM) alone or increasing concentrations of ANF in the absence or presence of TCDD for 2 h at 20°C followed by measurement of the amount of AhR that was transformed into its DNA binding form. These results demonstrate that although ANF is a weak partial agonist in rat (it weakly induces AhR transformation and DNA binding) it antagonized the action of TCDD (i.e. ANF reduced the amount of TCDD-inducible protein-DNA complex formation by 70%) (Fig 1). In contrast, ANF was a significantly better AhR agonist of the guinea pig AhR, inducing complex formation to about 60% of that induced by TCDD, and it appeared to only slightly antagonize TCDD-inducible protein-DNA

complex formation (Fig. 1). These results were consistent with ligand binding analysis (data not shown), which revealed that ANF effectively competed with TCDD for binding to the rat AhR complex; no competition was observed with the guinea pig AhR complex.

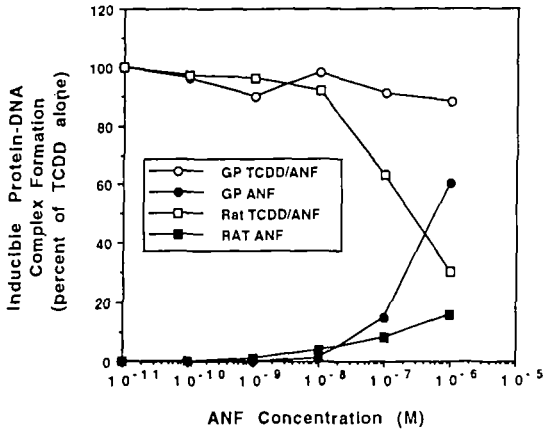

Figure 1. Dose-dependent effect of α-napthoflavone (ANF) on guinea pig and rat cytosolic AhR transformation and DNA binding. Cytosol was incubated with increasing concentrations of ANF alone (O,O) or TCDD and increasing concentrations of ANF (,) and the amount of inducible *protein-DNA complex formed* determined by gel retardation analysis. Values represent the mean of at least three determinations.

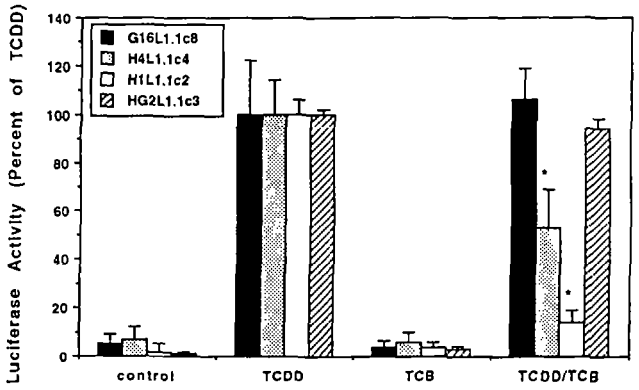

Figure 2. TCB antagonizes TCDD induction of luciferase activity in a species-specific manner. Cells were incubated with DMSO, TCDD (1nM), TCB (5μM) or TCDD and TCB for 24 h. Values represent the mean ± SE of at least three determinations.

In order to characterize the apparent species-specific nature of TCB antagonism, we have examined the effect of TCB on the ability of TCDD to induce luciferase activity in stably-transfected cell lines from various species (guinea pig G16L1.1c8, rat H4L1.1c4, mouse H1L1.1c2 and human HG2L1.1c3 cell lines). Exposure to TCDD (5 nM) alone induced luciferase activity in all cell lines while TCB (5 μM) had no significant effect (Fig. 2). Although induction of luciferase activity by TCDD in guinea pig and human cells was not affected by co-administration of TCDD and TCB (Fig. 5), significantly less luciferase induction was observed in the rat and mouse cell lines (47% and 86%, respectively). Since the DNA regulatory elements controlling expression of the luciferase gene are identical in each of the stably transfected cell lines, the differences in responsiveness of the cell lines are the result of species differences in the AhR, its associated proteins and/or

trans-acting factors present in each cell type. Ligand binding analysis (data not shown) has demonstrated that this species difference in antagonism is due to the differential ability of TCB to competitively inhibit TCDD binding to the mouse and rat AhRs but not to the guinea pig and human AhRs. These results also demonstrate that the ligand binding specificity of the AhR is not identical among species.

4. Conclusions

Our results demonstrate a significant species-specific difference in the ability of an AhR ligand (ANF) and a previously documented "nonAhR" ligand (TCB) to act as an AhR antagonists. Additional experiments have demonstrated that the differential antagonism is correlated with the ability of the TCB or ANF to bind to the AhR. In those species in which these chemicals are not antagonistic, they fails to compete with TCDD binding, and, as in the case of ANF and guinea pig AhR, they can act as an AhR ligand. These results demonstrate distinct species differences in the ligand binding specificity of the AhR. In addition, they raise some interesting questions regarding analysis of the biological effects of complex mixtures using AhR-dependent bioassay systems, in that the presence of high levels of TCB in a complex mixture would affect its biological activity in guinea pig or human cells significantly less than that which would be observed in rat or mouse. The presence of AhR antagonists in sample extracts could be detected utilizing a combination of both in vitro (ligand and DNA binding assays) and in vivo (cell based) bioassays in which a known amount of inducer (TCDD) is mixed with the test mixture and the relative potency of the response compared to the inducer alone. The presence of AhR antagonists in complex chemical mixtures and the observed species differences in AhR ligand binding would complicate detection of dioxin-like chemicals using AhR-based bioassays. Accurate estimation of the potency of unknown sample extracts containing dioxin-like chemicals should involve multiple species bioassay systems (both *in vivo* and *in vitro*).

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