Promiscuous Ligand-Dependent Activation of the Ah Receptor: Chemicals in Crude Extracts from Commercial and Consumer Products Bind to and Activate the Ah Receptor and Ah Receptor-Dependent Gene Expression.

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Introduction

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related halogenated and polycyclic aromatic hydrocarbons (HAHs and PAHs) produce a variety of toxic and biological effects, the majority of which are mediated by their ability to bind to and activate the Ah receptor (AhR) and AhR-dependent gene expression.[1,2] While previous studies suggested that the physiochemical characteristics of AhR ligands (i.e. HAH and PAH agonists) must meet a defined set of criteria, it has recently become abundantly clear that the AhR can be bound and activated by structurally diverse range of synthetic and naturally occurring chemicals.[3,4] Based on the spectrum of AhR ligands identified to date, the structural promiscuity of AhR ligands is significantly more diverse than that observed for other ligand-dependent nuclear receptors.[5] However, a detailed understanding of the structural diversity of AhR ligands and their respective biological and toxicological activities remains to be established and could provide insights into the identity of endogenous ligands. Over the past several years we have developed and utilized several AhR-based in vitro and cell-based bioassay systems to screen pure chemicals and chemical libraries as well as mixtures of chemicals with the goal of defining the spectrum of chemicals that can bind to and activate/inhibit the AhR and AhR-dependent gene expression.[4,6,7] In addition, demonstration of the presence of AhR agonists/antagonists in extracts containing complex mixtures of chemicals from a variety of biological and environmental samples, coupled with AhR bioassay-based fractionation procedures, provides an avenue in which to identify novel AhR ligands.[8,9,10] In previous preliminary screening studies we demonstrated the presence of AhR agonists in extracts of commercial and

consumer products using an in vitro guinea pig hepatic AhR DNA binding and mouse gene induction assays.[11] Here we have extended these studies and have examined the ability of crude DMSO and ethanol extracts of selected commercial and consumer products to not only bind to and stimulate AhR DNA binding, but to also induce AhR-dependent gene expression in a variety of species.

Materials and Methods

Materials. TCDD and 2,3,7,8-tetrachlorodibenzofuran (TCDF) were obtained from Dr. Steve Safe (Texas A&M University, USA). The indicated commercial and consumer products were readily available materials from a variety of commercial sources and/or obtained from retail stores.

Sample Extraction. The indicated commercial and consumer products were diced with scissors and extracted overnight (~16 h) in glass tubes at room temperature in DMSO, ethanol or water using 1.5 volumes of solvent for each sample with the exception of paper products and other absorbent materials which were extracted in 10 volumes. Extracts were stored at room temperature in teflon-capped vials until use.

Preparation of Cytosol. Hepatic cytosol from male Hartley guinea pigs, Sprague-Dawley rats and C57BL/6 mice was prepared in HEDG buffer (25 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM DTT and 10% (v/v) glycerol) and protein concentrations determined as previously described.[12]

AhR Bioassay Analysis. The ability of sample extracts to stimulate AhR-dependent transformation and DNA binding in vitro was determined by gel retardation analysis using guinea pig cytosol incubated with 2 µl of the indicated extract as previously described.[12] The ability of extracts to compete with [^{3}H]TCDD for binding to the hepatic cytosolic AhR from several species was determined using the hydroxyapatite binding assay. The ability of the indicated extracts to stimulate AhR-dependent gene expression was carried out using recombinant mouse, rat and guinea pig cell lines that contain a stably transfected AhR-responsive firefly luciferase reporter gene (pGudLuc1.1) that responds to AhR agonists with the induction of luciferase in a time-, dose- and AhR-dependent manner.[12,13] With these cell lines, luciferase activity is determined 4 hours after treatment with TCDD or the sample extracts.[13,14] To assess the effect of metabolism on AhR-dependent induction by sample extracts, we used mouse hepatoma cells containing the stably transfected luciferase reporter plasmid pGudLuc1.1 (for analysis at 4 hours of incubation) or pGudLuc6.1 (for analysis at 24 hours of incubation). The stability of the luciferase gene product expressed from these two different plasmids is dramatically different with luciferase expressed from the pGudLuc1.1 plasmid significantly more labile.[15] For induction, cells grown in 96 well microplates, were incubated with 1 µl of extract for 4 or 24h hours followed by lysis in the wells and automatic measurement of luciferase activity (using the Promega luciferase assay system) in an Anthos Lucy2 luminescent plate reader. Activity was corrected for the amount of luminescence present in the DMSO-treated sample wells.[13,14]

Results and Discussion

Numerous studies have recently demonstrated that the AhR can be bound and activated by chemicals which are dramatically different in structure from known "classical" HAH and PAH ligands for the AhR. In one initial serendipitous experiment we found that rubber cap inserts for vials and bottles contained a DMSO extractable AhR agonist(s) that could stimulate AhR transformation and DNA binding (data not shown). In subsequent experiments, a wide variety of samples were collected from the laboratory (specific materials are indicated), extracted into DMSO and examined for their ability to stimulate transformation and DNA binding of guinea pig hepatic cytosolic AhR. As can be seen in figure 1, DMSO extracts from a variety of plastic, rubber and paper products can stimulate AhR transformation and DNA binding (the ligand:AhR:Arnt:DRE complex is indicated by the arrow). The highest relative DNA binding activity was observed with DMSO extracts of paper products. In additional experiments, we observed that DMSO extracts of some children's pacifiers and bottle nipples could stimulate AhR DNA binding, with silicon nipples from a variety of companies yielding a response ~25% of that observed with TCDD (data not shown). These results support the presence of AhR agonists in DMSO extracts from the indicated products.

To examine whether more water soluble chemicals could also be extracted from commercial and consumer products, we extracted a series of products with DMSO, ethanol or water and determined whether any of the extracts contained chemicals which could stimulate transformation and DNA binding of the guinea pig hepatic AhR complex. Quantitative results from these DNA binding analysis are shown in figure 2 and they reveal that DMSO, ethanol and water extracts of selected products contain AhR agonists. Based on gel retardation analysis, all extracts of newspaper contained AhR agonists, while only the DMSO and ethanol extracts of other materials exhibited AhR agonist activity, with the greatest activity obtained with extracts of rubber products (figure 2). These results demonstrate the ability of the AhR to be activated by polar and nonpolar compounds.

While DMSO and ethanol extracts of a variety of plastic, rubber and paper products can induce AhR-dependent luciferase expression in stably transfected guinea pig cells, the only water extracts that could activate the AhR and AhR gene expression were from newspapers (figure 3 and induction data not shown). A similar gene induction profile was obtained using the stably transfected mouse and rat cell lines. Thus the AhR from different species responded similarly in these analyses. Comparison of the ability of each extract to stimulate transformation and

DNA binding of guinea pig hepatic cytosolic AhR and to induce luciferase gene expression in stably transfected guinea pig intestinal carcinoma cells is shown in figure 3. These results reveal a good correlation between the ability of DMSO, ethanol and water extracts of selected commercial products to stimulate guinea pig AhR transformation and DNA binding in vitro and their ability to induce luciferase gene expression in pGudLuc1.1 stably transfected guinea pig intestinal adenocarcinoma (GPC16) cells. In addition, the relative ability of the indicated extracts to compete with [^{3}H]TCDD for binding to the guinea pig cytosolic AhR complex was also relatively comparable to the degree of AhR transformation and DNA binding and gene expression (data not shown). Not only do these results demonstrate the presence of AhR agonists in crude extracts from a variety of materials, but the presence of AhR agonists in ethanol and water extracts indicates the existence of polar AhR agonists. Given that these induction experiments were carried out for only 4 hours, it was unknown whether the inducing chemicals were metabolically stable enough to induce AhR-dependent gene expression for longer periods of time. To examine the metabolic stability of the AhR active chemicals present in the DMSO, ethanol and water extracts, we examined the ability of a given sample extract to stimulate AhR-dependent gene expression at 4 hours (using mouse hepatic (H1L1.1c2) cells containing pGudLuc1.1) compared to its ability to stimulate reporter gene expression at 24 hours (using mouse hepatic (H1L6.1c3) cells containing pGudLuc6.1). Reporter gene induction by the extracts was significantly reduced at 24 h as compared to 4 h (data not shown) and this likely results from metabolism and inactivation of the inducing chemical(s) by enzymes present in the cell line, reducing its binding affinity and thus biological potency.

Overall, the results of our experiments demonstrate the presence of unknown polar and nonpolar AhR agonists in extracts from a variety of commercial and consumer products. We are currently employing bioassay-driven chemical fractionation procedures to isolate and identify the active polar AhR agonists from selected materials. While the biological/toxicological significance of these compounds remains to be examined, their identity may provide additional insights into the spectrum of chemicals that can bind to and activate the AhR and AhR signal transduction.

Acknowledgements
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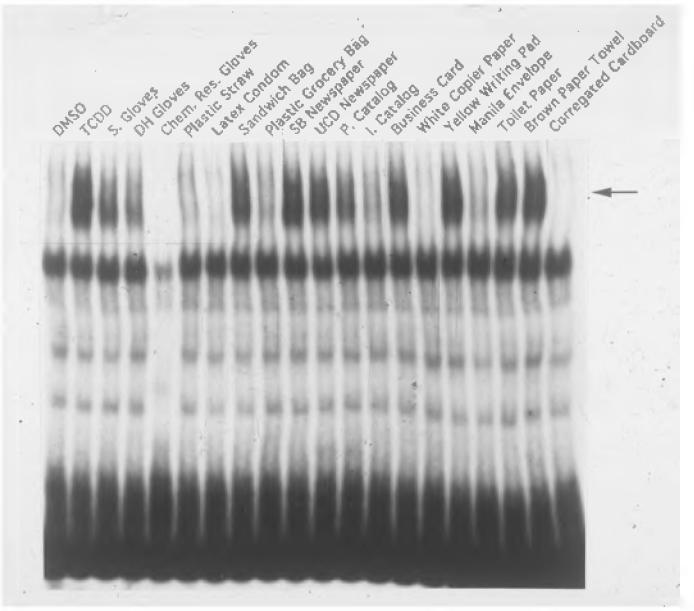

Figure 1. Gel retardation analysis of DMSO extracts of a variety of commercial and consumer products. The arrow indicates the position of the ligand:AhR:Arnt:DRE complex.

Figure 2. Quantitative gel retardation results examining the ability of DMSO, ethanol and water extracts of commercial and consumer products to stimulate guinea pig hepatic AhR DNA binding.

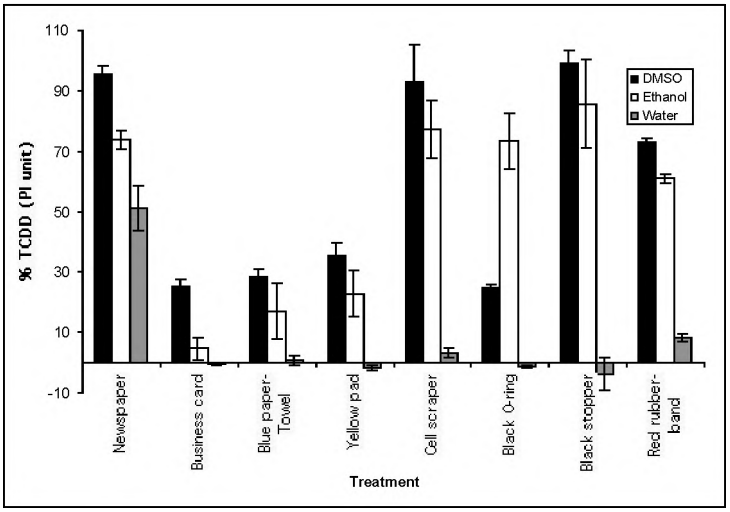

Figure 3. Comparison of the relative potency of DMSO, ethanol and water extracts of commercial and consumer products to stimulate guinea pig cytosol AhR DNA binding in vitro (gel retardation analysis) and induction of reporter gene expression in guinea pig intestinal cells.