THE PROPOSED INDIRECT ACTIVATION MECHANISM BY THE TRYPTOPHAN PHOTOPRODUCT FICZ DOES NOT ACCOUNT FOR THE STRUCTURAL DIVERSITY OF AH RECEPTOR LIGANDS

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Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor responsible for modulating a diverse spectrum of toxic and biological effects produced halogenated aromatic hydrocarbons (HAHs) such as the polychlorinated dibenzo-p-dioxins, dibenzofurans and biphenyls and polycyclic aromatic hydrocarbons (PAHs) and PAH-like chemicals, such as benzo(a)pyrene, β-naphthoflavone (BNF) and 3-methylcholanthrene.[1-3] The binding and activation of the AhR by these prototypical "classical" AhR ligands (of which 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) is the most potent) have been extensively characterized and they not only have common physiochemical characteristics, but they appear to activate the AhR signaling pathway via a common mechanism of action.[3,4] Mechanistically, AhR ligands passively diffuse into a responsive cell and bind to the cytosolic AhR which exists as a multiprotein complex. Ligand binding is presumed to lead to a conformational change in the AhR that leads to exposure of a nuclear localization sequence, followed by translocation of the liganded AhR protein complex into the nucleus. Once in the nucleus, the AhR is released from its associated protein subunits as a result of its binding to a related protein called the aryl hydrocarbon nuclear translocator (ARNT) protein[3,4]. Formation of the liganded AhR:ARNT heterodimer converts the protein complex into its high affinity DNA binding form and its interactions with its specific DNA recognition site (the dioxin responsive element (DRE)) results in stimulation of transcription of downstream genes.[43-5] In stark contrast to the classic AhR ligands, our laboratory and others have reported that AhR-dependent gene expression can be activated by a relatively large group of structurally diverse (often non-planar) chemicals with little in common with the prototypical HAHs AhR ligands.[2-4,6] These structurally diverse ligands are referred to as nonclassical AhR ligands and their identification has raised questions regarding the specificity and promiscuity of AhR ligand binding. Numerous AhR-based cell bioassays have been developed for use in the detection of dioxin and dioxin-like HAHs and the existence of structurally diverse AhR agonists has complicated rapid analysis of sample extracts for the desired dioxin-like HAHs, as these undesired AhR ligands must be removed from sample extracts via time-consuming and costly clean-up procedures prior to their analysis by CALUX cell bioassay methods.[7] However, there are a variety of validated methods to accomplish this and the results of such comparative analysis have demonstrated a good correlation between the analysis of sample extracts by the CALUX bioassay for bioanalytical equivalent (BEQ) determination and GC/HRMS instrumental analysis methods for toxic equivalency (TEQ) determinations.[7]

Recently, a study by Wincent and coworkers[8] strongly suggested that the apparent structural diversity observed for AhR ligands was an artifact of the experimental assay system and that the AhR was not actually bound and activated by these widely divergent chemicals. These authors suggested that the apparent AhR-dependent induction by the structurally diverse chemicals reported by most investigators was actually due to their ability to inhibit cytochrome P4501A1/1A2/1B1-dependent monooxygenase activity which blocked the metabolism of 6-formylindolo[3,2-b]carbazole (FICZ) and FICZ was the inducer. FICZ is a PAH-like photoproduct of tryptophan that is an exceptionally potent AhR agonist that is rapidly metabolized by cells (i.e. by CYP1A1/1A2/1B1) and it has been shown to be present at very low concentrations in tissue culture media and formed in vivo.[8] Wincent and coworkers[8] concluded that the inhibition of CYP-dependent degradation of FICZ by these presumed/proposed structurally diverse AhR ligands resulted in indirect induction of AhR-dependent gene expression by FICZ and that investigators incorrectly assumed that the observed induction response was by the test chemical. If correct, this has significant implications with regards to the current methods used analysis of sample extracts by AhR-based bioassay/bioanalytical methods, particularly relative to the necessity for

extensive sample cleanup methods. If the AhR is not actually activated by a large variety of structurally diverse chemicals as proposed by Wincent and coworkers[8], but indirectly activated simply by increasing the stability of FICZ, then elimination of FICZ from media and other biological test materials should facilitate sample preparation and result in a dramatic improvement in AhR-based bioassay analysis. However, while the FICZ hypothesis appears to run counter to an extensive published literature that documents the ability of the AhR to be activated by structurally diverse chemicals, the role of FICZ in the response could have been overlooked and the promiscuity of AhR ligand binding may be incorrect. Accordingly, here we have described analysis of the hypothesis that the AhR can directly bind and be activated by a structurally diverse ligands and that FICZ plays little or no significant role in defining the apparent promiscuity of AhR ligand structure.

Materials and Methods

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.[9]

AhR Bioassay Analysis. The ability of the indicated extracts to stimulate AhR-dependent gene expression was carried out using recombinant mouse hepatoma (H1L16.1c2) cell line that contains a stably transfected AhR-responsive firefly luciferase reporter gene (pGudLuc6.1) that responds to AhR agonists with the induction of luciferase in a chemical-, time-, dose- and AhR-dependent manner.[10] Luciferase activity was determined 4 or 24 hours after treatment with TCDD or the indicated chemical using a microplate luminometer as described.[10] Determination of the ability of the indicated chemicals to stimulate AhR-dependent transformation and binding to a [^{32}P]-labeled oligonucleotide containing the wild-type DRE DNA binding in vitro was determined by gel retardation analysis using guinea pig cytosol as previously described.[9] Direct binding of each chemical to the AhR ligand binding domain was determined by examining their ability to compete with [^{3}H]TCDD for binding to the guinea pig hepatic cytosolic AhR using hydroxyapatite or sucrose density centrifugation binding assays.[9]

Results and Discussion

Incubation with mouse hepatoma cells with a variety of structurally diverse chemicals has been previously shown to stimulate AhR-dependent gene expression, however, it has been proposed that induction by these diverse chemicals is indirect and mediated by FICZ, whose metabolic degradation is inhibited by these diverse compounds. To evaluate the importance (or lack of importance) of FICZ in the apparent diversity of AhR ligands, we examined a collection of structurally diverse AhR agonists representing a range of chemicals with reported differences in their ability to bind to and activate the AhR (including TCDD, YH439, βNF, 3-methylcholanthrene, indirubin, L-kynurenine, carbaryl, prostaglandin G2, tryptamine and others).[2-4,11-13] As previously reported, each of these compounds was observed to stimulate AhR-dependent luciferase reporter gene expression in mouse hepatoma (H1L6.1c2) cells in a concentration-dependent manner. These results demonstrate the ability of these chemicals to stimulate AhR-dependent gene expression, but do not test whether FICZ contributes to the induction response since FICZ may be present in the media/serum. Given that we observe little or no change in background luciferase activity following addition of fresh media/serum or media/serum that has been exposed to sunlight for several hours (as some investigators have reported), we hypothesize that FICZ is of little significance. However, although we can envision how FICZ may stimulate AhR-dependent gene expression in cells in culture if a test chemical can block its degradation, it would clearly have no affect on in vitro assays that measure the ability of a chemical to bind to the cytosolic AhR or stimulate cytosolic AhR DNA binding. Accordingly, if a chemical can competitively bind to the AhR and it can stimulate AhR transformation and DNA binding (in addition to its ability to stimulate AhR-dependent gene expression), then it must be an AhR agonist. Even if FICZ was present in the cytosol (which is unlikely), it would simply contribute to an elevated background activity as it would bind to the AhR and activate it and would not affect the ability of the test chemical to bind to the AhR or stimulate AhR DNA binding. Ligand binding analysis was carried out on a series of structurally diverse chemicals and these experiments demonstrated the ability of each to directly compete with [^{3}H]TCDD for binding to the guinea pig hepatic AhR. Examples of ligand binding analysis by carbaryl, tryptamine and indole acetic acid are shown in Figure 1 and these and additional results

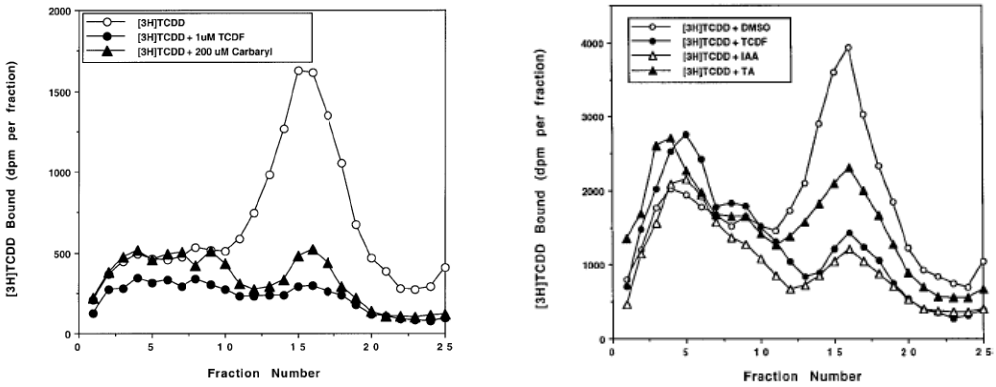

Figure 1. Carbaryl, indole acetic acid (IAA) and tryptamine (TA) directly compete with [^{3}H]TCDD for binding to the guinea pig hepatic cytosolic AhR. Cytosol was incubated with 5 nM [^{3}H]-TCDD in the absence or presence of 1 uM TCDF, 200 uM carbaryl, 100 uM IAA or 100 uM TA for 1 h at 4°C and aliquots analyzed by sucrose density centrifugation.

with the additional compounds demonstrate the ability of these structurally diverse ligands to directly bind to the AhR. To further confirm this, we examined the ability of these ligands to direct stimulate the transformation and DNA binding of the guinea pig hepatic cytosolic AhR in vitro as determined by gel retardation analysis. An example of the results of these analyses using equipotent concentrations of TCDD, YH439, BNF, 3MC, indirubin and L-kynurenine are shown in Figure 2 along with the structures of the individual test chemicals to showcase their structural diversity. The results of these experiments not only further confirm the ability of all of these structurally diverse compounds to directly stimulate AhR transformation and DNA binding, but they are consistent with a lack of involvement of FICZ in this process since FICZ would not be present or have an impact on cytosolic AhR assays.

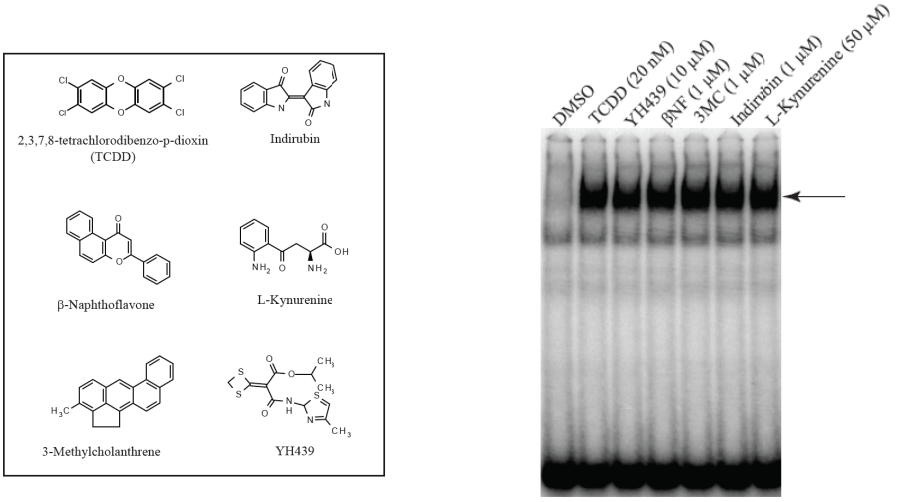

Figure 2. Structurally diverse chemicals stimulate AhR transformation and DNA binding as determined by gel retardation. The arrow indicates the position of the induced AhR:DNA complex. The structures of the specific compounds tested in this analysis are shown.

Similar to our results, several reviews of the literature has identified a large number of other structurally diverse chemicals that not only can stimulate AhR-dependent gene expression but that have also been shown to directly bind to the AhR and/or stimulate AhR transformation and DNA binding.[2-4,6] Taken together, our results and the results of others, remain consistent with the conclusion that the AhR can bind and be activated by a wide variety

of structurally diverse ligands. These results not only support the promiscuity of AhR ligand structure, but they further question the significance and contribution of the indirect activation mechanism to AhR ligand diversity as proposed by Wincent and coworkers.[8]

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