DOES THE BINDING OF STRUCTURALLY DIVERSE CHEMICALS TO THE AH RECEPTOR ALTER ITS NUCLEOTIDE SPECIFICITY OF DNA BINDING?

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Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription that mediates a relatively diverse spectrum of toxic and biological effects produced by halogenated aromatic hydrocarbons (HAHs), including the polyhalogenated dibenzo-p-dioxins, dibenzofurans and biphenyls (of which 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) is the most potent), and by numerous polycyclic aromatic hydrocarbons (PAHs) and PAH-like chemicals, such as 3-methylcholanthrene (3MC) and β-naphthoflavone (BNF)[1-3]. These compounds have been defined as "classical" AhR ligands due to similarities in their physiochemical properties (planar, hydrophobic) and ability to bind to and activate the AhR and AhR signaling pathway[1-3]. Mechanistically, AhR ligands diffuse into the cell and bind to the cytosolic AhR multiprotein complex, stimulating translocation of the liganded AhR complex into the nucleus, wherein it dissociates from its associated protein subunits and binds the aryl hydrocarbon nuclear translocator (ARNT) protein[4,5]. The liganded AhR:ARNT heterodimer binds with high affinity to specific DNA recognition sites known as dioxin responsive elements (DREs) to stimulate transcription of downstream genes[4,5,7]. In contrast to the classic AhR ligands, recent studies have demonstrated that the AhR can actually bind and be activated by a relatively large group of structurally diverse (often non-planar) chemicals that are referred to as nonclassical AhR ligands[2,6,8]. Although many of these compounds were identified by their ability to bind to and/or stimulate AhR-dependent gene expression, their divergent structures and biological effects suggest that there may be multiple mechanisms by which ligands stimulate AhR-dependent biological and/or toxic effects. While some studies have reported ligand-specific effects on AhR functionality, such as the differential recruitment of coactivator proteins to the AhR:ARNT:DRE complex[9,10], others suggest that there are some nonclassical ligands that may relax the nucleotide specificity of DNA binding such that the ligand:AhR:ARNT complex can bind to sequences that are distinctly different from the established DRE DNA consensus sequence[11-14]. Few studies have examined this mechanism in any detail. Given the potential biological and toxicological implications of AhR and AhR:ARNT complex binding to non-DRE responsive elements (i.e. a unique mechanism of AhR-mediated alteration in gene expression and likely toxicity), a systematic evaluation of ligand-specific changes in the nucleotide specificity of DNA binding was warranted. Here we have utilized a DNA binding site selection and amplification approach as an unbiased method to identify all DNA sequences to which the AhR/AhR:ARNT complex can bind when activated by structurally diverse AhR agonists.

Materials and Methods

AhR:ARNT DNA binding site selection

DNA selection and amplification was performed using a modification of the method of Wright et al.[15]. A synthetic 56-mer oligonucleotide was synthesized (Operon Biotechnologies) to contain a 15-base variable region (each base represented at each position) flanked by two invariant sequences containing PCR primers (5'-GGCGCGCCGTAATGCCGGCATTACGATATGCATTATACGN_{15}GCGCCGCGCGTAGCATGCCGTAGCATGCATTACGTAATGCC-3') and made double-stranded by Taq amplification after annealing with a primer (5'-GCTAGATCTCAGCTCAGGGCC-3'). For the first round of AhR DNA binding site selection, the AhR was transformed into its high-affinity DNA binding heterodimer (AhR:ARNT) by incubating guinea pig hepatic cytosol (8 mg/ml) with each test compound for 2 hours at room temperature[16]. An aliquot of this reaction was incubated with poly [dI•dC], 14 fmol (0.5 ng) of the double-stranded, purified randomer and DNA binding allowed to proceed. The AhR:ARNT:DNA protein complexes were immunoprecipitated with an AhR antibody[13] and Dynabeads Protein A, washed and an aliquot amplified by PCR. For subsequent rounds of binding site selection, approximately 250 pg of PCR-amplified DNA was used in binding reaction and a total of four rounds of selection carried out. The resulting oligonucleotides were directly subcloned into pGEM-T and sequenced. Sequence alignment was carried out and sequence logos depicting the prevalence of each base at each position

were generated using WebLogo 3.1[17]. Electrophoretic mobility shift assays (EMSA) were carried out with [^{32}P]-labeled oligonucleotide containing the wild-type DRE or binding site selected sequence as described in detail[16]. The ability of novel DRE-like sequences to confer TCDD-/AhR-responsiveness upon a heterologous promoter and gene was carried out by transient transfection of plasmids containing an oligonucleotide with the desired DNA sequence upstream of the MMTV promoter and firefly luciferase gene into mouse hepatoma (Hepa1c1c7) cells incubation with DMSO or 1 nM TCDD for 24 h.

Results and Discussion

To determine whether binding of structurally diverse chemicals (i.e. agonists) within the ligand binding domain of the AhR could lead to unique conformational changes in AhR structure that alter its nucleotide specificity of DNA binding, selection of appropriate ligands was necessary. 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 were identified for binding site selection studies and included TCDD, YH439, βNF, 3MC, indirubin and L-kynurenine[2,8] (Figure 1). Equipotent concentrations of these compounds stimulate comparable levels of AhR binding to DRE-containing DNA (Figure 2) and they were used in DNA binding site selection analysis studies.

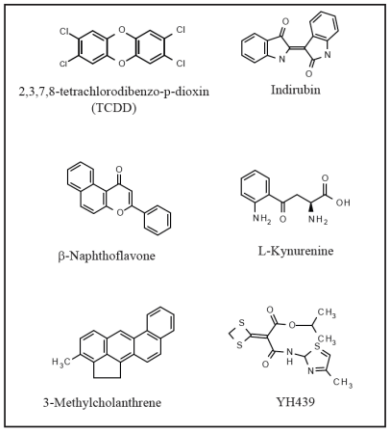

Figure 1. Structures of the compounds for AhR-DNA binding site selection.

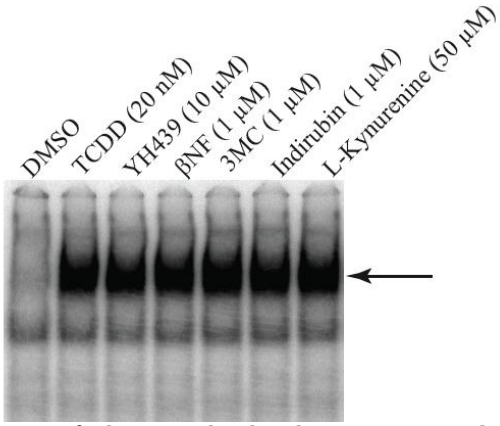

Figure 2. EMSA of AhR DNA binding by test compounds and the arrow indicates the position of the induced AhR:DNA complex.

To determine whether ligand-specific differences in the nucleotide specificity of DNA binding exists, we examined the ability of structurally diverse AhR agonists to stimulate AhR DNA binding and isolated and sequenced all AhR-bound DNA oligonucleotide sequences using DNA selection and amplification procedures. Ligand-activated AhR complexes were incubated with oligonucleotides containing a 15-base variable region (all nucleotides represented at each position), and the resulting ligand:AhR:ARNT:oligonucleotide complexes were isolated by immunoprecipitation with an AhR antibody. The selected/isolated DNA was PCR amplified and used in the next round of binding site selection and this process repeated for a total of four rounds. Oligonucleotides from each round were radiolabeled and analyzed using EMSA to confirm enrichment of the oligonucleotide pool for AhR:ARNT DNA binding sites. An example of the progressive binding site selection enrichment of binding sites for L-kynurenine activated AhR is shown in Figure 3. The resulting four rounds of selected oligonucleotides were subcloned, sequenced, and manually aligned for sequence comparisons. The final alignment results (from between 27- 46 individual DNA sequences for each AhR ligand) were compared and the results demonstrated that AhR activated by all structurally diverse ligands led to its binding to DNA that contained the canonical DRE core consensus sequence (5'-GCGTG-3'), although sequence variations in the nucleotides flanking the core were observed (Figure 4). There were minor variations in some nucleotides previously reported to be contained within the DRE consensus sequence and to determine whether these sequence variations were of any consequence, we examined the ability of ligand-activated AhR complexes (using all of the structurally diverse ligands) to stimulate AhR DNA binding (by EMSA) or reporter gene expression (by transient transfection

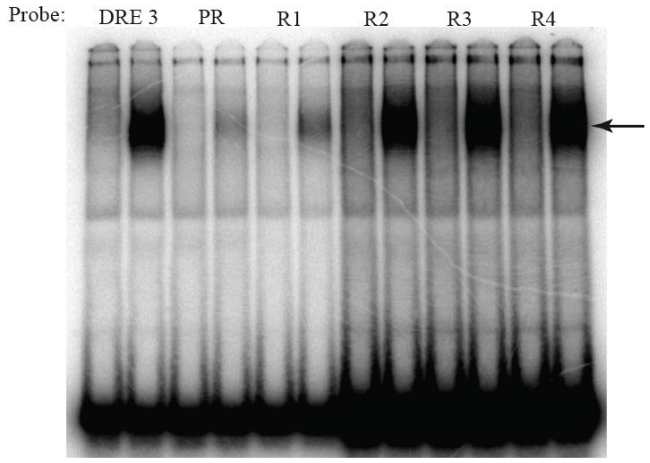

Figure 3. Progressive enrichment for ligand (L-kynurenine)-induced AhR:ARNT DNA binding elements from within the random oligonucleotide pool during DNA binding site selection and amplification procedure.

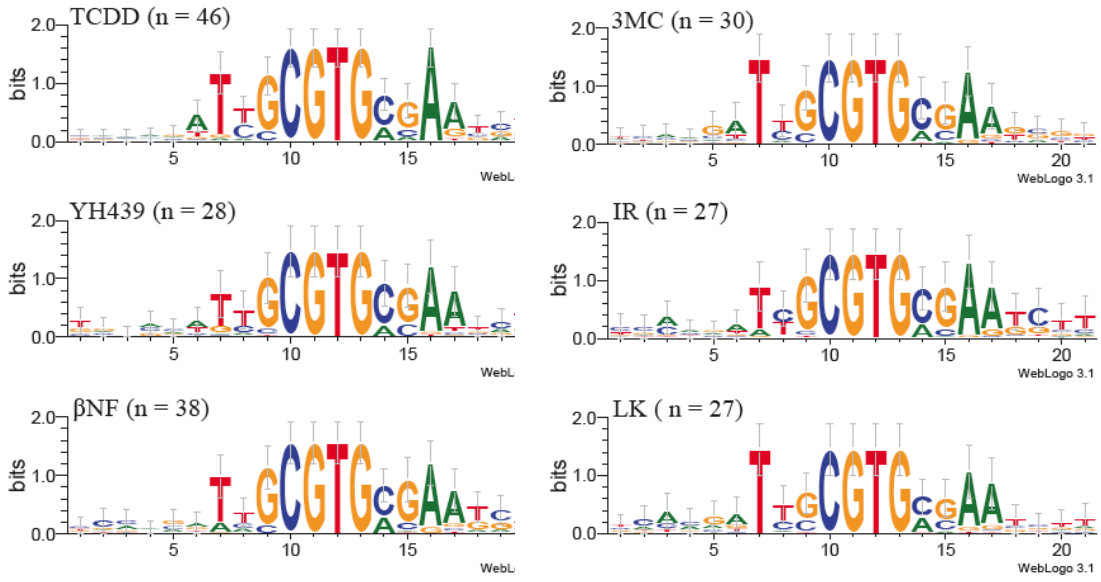

Figure 4. Nucleotide specificity of DNA binding of guinea pig hepatic AhR:ARNT complexes activated by structurally diverse ligands as revealed by DNA binding site selection.

studies). The results of these analyses revealed no significant nucleotide-specific differences in the ability of any of the six ligands to stimulate AhR binding to DNA containing these variant DRE sequences nor the ability of any of these variant DNA sequences to confer ligand-dependent activation of luciferase reporter gene expression. Although the DRE core sequence is necessary for AhR:ARNT-dependent transcriptional activation, it is not sufficient, and nucleotides flanking the core sequence are known to play a key role in determining whether AhR:ARNT DNA binding will induce transcription of downstream genes[7]. The results of our studies help to further define the DRE consensus sequence and indicate that it is more flexible with regards to acceptable nucleotide substitutions than previously reported. An unanswered question that arises from the current study is

whether a combination of mutations in these flanking sequences can mediate AhR:ARNT transcription by ligands other than TCDD. One limitation of our study is that we tested single mutations placed within the wild type DRE 3 rather than using the specific ligand-selective sequences pulled-down in the DNA selection and amplification assay. Although all of the mutants were responsive to TCDD, we did not characterize these sequences with respect to other ligands. Currently, however, there is only limited evidence that suggests that flanking nucleotides may differentially affect AhR:ARNT transcription based on the structure of the bound ligand and the available data is complicated by the use of a metabolically labile AhR agonist and the lack of direct evidence demonstrating AhR:ARNT binding to the degenerate DRE sequences[11]. In conclusion, while we and others have demonstrated that structurally diverse AhR agonists can differentially bind within the AhR LBD, ligand-dependent transformation and nucleotide-specificity of DNA binding of the ligand:AhR:ARNT complex appears to be very highly conserved. Our results strongly suggest that currently identified agonists of the AhR function essentially as an "on/off" switch to stimulate the AhR signal transduction pathway and this activation is mediated through an interaction of the ligand activated AhR with DRE-containing DNA. Although the mechanism of action activation appears to be predominantly mediated by the classical DRE-dependent mechanism of AhR signaling, ligand-specific differences in response are likely mediated by alternative AhR mechanisms, such as ligand-selective interaction of nuclear AhR complexes with coactivators and/or other nuclear regulatory factors. Further studies examining ligand-selective alterations in AhR structure and function as well as in-depth analysis of subsequent interactions of these diverse ligand:AhR:ARNT complexes with coactivators are necessary to gain insights into ligand specific differences in the toxic and biological effects resulting from activation of the AhR and AhR-dependent gene expression.

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