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And Now for Something Completely Different: Diversity in Ligand-Dependent Activation of Ah Receptor Responses

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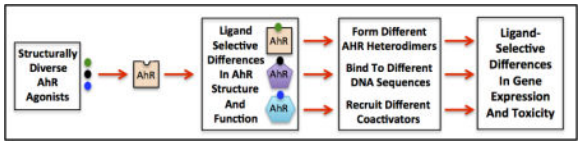
Abstract

Ligand-dependent activation of the Ah receptor (AhR) can result in an extremely diverse spectrum of biological and toxic effects that occur in a ligand-, species- and tissue-specific manner. While the classical mechanism of AhR-dependent signal transduction is directly related to its ability to modulate gene expression, the dramatic diversity in responses observed following AhR activation or inhibition is inconsistent with a single molecular mechanism of AhR action. Recent studies have revealed that key molecular events underlying the AhR signaling pathway are significantly more varied and complex than previously established, and the specificity and diversity in AhR response can be selectively modulated by a variety of factors. Here we describe new insights into the mechanistic diversity in AhR signal transduction that can contribute to ligand-, species- and tissue-specific differences in AhR reponse.

Graphical abstract



Keywords

Ah Receptor; AhR; TCDD; Ligand Binding; Ligand Specificity; Toxicity

1. Introduction

The Ah receptor (AhR) is a ligand-dependent basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS)-containing transcription factor that responds to exogenous and endogenous chemicals by inducing or repressing the expression of a number of genes and mediating a diverse spectrum of biological and toxic effects in a wide range of species and tissues [1–7]. Additionally, the AhR has been shown to pay a key modulatory role in the regulation of a variety of physiological responses including developmental and immune processes [6–9]. While the AhR signal transduction pathway has similarities to that of nuclear receptors (e.g.

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steroid hormone receptors), the AhR is unique in that it can differentially respond to structurally diverse chemicals to produce a variety of ligand-selective toxic and/or biological effects, which in turn can be mediated by several different AhR-dependent mechanisms. This review highlights our current understanding of the diversity in ligand-dependent mechanisms of AhR signal transduction and response.

2. Diversity in AhR-Dependent Mechanisms of Gene Expression

Initiation of the classical or cannonical AhR signal transduction mechanism requires binding of the inducing ligand to the PASB domain of the AhR, which is part of a cytosolic multiprotein complex containing heat shock protein 90 (hsp90), XAP2 and p23 [3,4,6], and a subsequent ligand-dependent conformational change in the AhR leading to its nuclear translocation [3,4,6]. Dimerization of the AhR with ARNT (AhR nuclear translocator), a structurally and functionally related bHLH-PAS protein, displaces the AhR from its associated proteins and transforms the AhR into its high affinity DNA binding form [4,10,11]. Binding of the transformed ligand:AhR:ARNT complex to its specific DNA recognition site, the dioxin responsive element (DRE; also referred to as a xenobiotic responsive element (XRE) or Ah responsive element (AHRE)), present in or adjacent to AhR-responsive genes, leads to coactivator recruitment, chromatin rearrangement, and increased gene transcription [4,6,10,12].

While numerous gene products have been identified that are consistently altered in different species and tissues (e.g. CYP1A1) in response to a given AhR ligand, gene expression array and chromatin immunoprecipitation (ChIP) analysis has also revealed significant differences in gene expression profiles [13–15]. The diversity in AhR-dependent gene expression responses observed between cell types can be attributed to a variety of factors, including, but not limited to: AhR/ARNT expression, the presence/absence of specific co-activators/co-repressors and/or transcription factors that can compete with the AhR for ARNT (e.g. Hypoxia Inducible Factor 1α (HIF1α) or AhR repressor (AHRR)), and differences in chromatin structure and epigenetic modifications of AhR target genes [4,12,16–19]. AhR-dependent alterations in the expression of genes that lack an apparent AhR DNA (DRE) binding site, coupled with established cross-talk between the AhR and cellular signaling pathways and other transcription factors, suggests that the AhR participates in several novel noncannonical pathways by which the AhR can stimulate gene expression [6,20–23]. Ligand-activated AhR can dimerize with nuclear proteins other than ARNT (e.g., Kruppel-like factor 6 (KLF6) and RelB), and these unique heterodimers stimulate gene expression via their interaction with DNA binding sites that are significantly different from that of a DRE [24–28] to regulate a unique set of genes (Figure 1). While little is known about the specific protein:protein interactions that occur between the AhR and RelB [24], deletion and functional analysis studies revealed that the mode of AhR:KLF6 dimerization is distinctly different from that of the AhR:ARNT dimer [27]. In addition to these unique AhR heterodimers, ligand-activated AhR can enhance gene expression via its ability to function as a coactivator for other nuclear transcription factors such as the estrogen receptor and E2F1 [29,30]. More recently, it has been observed that binding of the AhR by selective AhR modulator ligands can repress the expression of a unique battery of genes and although the mechanism remains to be determined, it does not appear to require the AhR DNA binding

domain [31,32]. While the canonical AhR:ARNT:DRE-dependent mechanism appears to be the principal AhR signaling pathway, ligand-dependent activation and nuclear localization of the AhR can regulate expression of diverse genes via multiple mechanisms, and others may still be identified (Figure 1).

3. Diversity in AhR Ligand Structure

The best-characterized high affinity ligands for the AhR include a variety of toxic halogenated aromatic hydrocarbons (HAHs), such as the polychlorinated dibenzo-p-dioxins (e.g., 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin)), dibenzofurans, and biphenyls, and numerous polycyclic aromatic hydrocarbons (PAHs) and PAH-like chemicals, such as 3-methylcholanthrene (3MC) and beta-naphthoflavone (BNF) [2,6,33]. It is now well established, by our laboratory and others, that the AhR can bind and be activated/inhibited by a relatively large number of natural, endogenous and synthetic AhR agonists/antagonists whose structure and physicochemical characteristics are dramatically different from the prototypical HAH and PAH ligands [6,33–38]. The lack of a 3D crystal/NMR structure of the AhR ligand binding domain (LBD) has hampered a detailed mechanistic understanding of AhR binding by structurally diverse ligands. However, site-directed mutagenesis and structure-function analysis, including those based on a homology model of the AhR LBD originally developed in collaboration with Dr. Laura Bonati [39–41], have not only provided further evidence for differential binding of structurally diverse ligands within the ligand binding pocket of a single AhR or between AhRs from different species [39–46]. These studies have also provided new insights into the mechanisms contributing to significant differences in the binding affinity of structurally different AhR ligands.

The extreme structural diversity of ligands for the AhR is very similar to the well-established ligand promiscuity reported for the pregnane X receptor (PXR), a member of the nuclear receptor superfamily [47–49]. Crystal and NMR structural analysis of PXR revealed that it has a very large and flexible ligand binding pocket and ligands reportedly can bind in different orientations and with different residues within the pocket [47,48]. The structural diversity of ligands and promiscuity of ligand binding demonstrated by both AhR and PXR ligands suggested some similarities in the ligand binding pockets and ligand-selective activation by these two different receptors. Gene expression analysis of a chemical library of >300,000 compounds allowed direct comparison of ligand-dependent activation of both AhR and PXR reporter gene responses by structurally diverse chemicals [50]. In these studies, a collection of 2281 structurally diverse chemicals, selected from 7790 AhR active compounds identified in the first screen of the library, were tested for their ability to simulate both AhR- and PXR-dependent induction of gene expression in stably transfected human hepatoma (HepG2) cells. These analyses revealed for the first time a striking overlap of AhR and PXR agonists, with 1982 of the 2281 structurally diverse schemicals stimulating AhR-dependent gene expression and 2017 of the 2281 chemicals stimulating PXR-dependent gene expression; 126 chemicals were shown to be selective for the AhR. The ability of the most potent AhR-selective agonists to competitively bind to and/or stimulate AhR DNA binding in vitro was demonstrated in subsequent analysis. The identification of potent and high affinity structurally diverse AhR-selective ligands/agonists that did not activate PXR indicated that although these receptors demonstrate innate similarities in ligand promiscuity,

the molecular mechanism(s) of AhR and PXR activation by structurally diverse ligands is not identical [50].

AhR ligand diversity is commonly determined by measuring the ability of a chemical(s) to simulate AhR-dependent gene expression in cells in culture. However, Rannug and coworkers recently challenged this concept and proposed that the apparent structural diversity observed for AhR ligands is actually an artifact of cell culture-based gene expression assay systems [8,9,51,52]. These authors suggest that the AhR has a limited range of acceptable ligands, and that the apparent AhR-dependent gene induction observed with structurally diverse chemicals was actually an indirect response resulting from the ability of these diverse chemicals to inhibit cytochrome P4501-dependent degradation of 6-formylindolo[3,2-b]carbazole (FICZ), a high affinity AhR agonist reportedly present in cell growth media, and that FICZ was the actual inducing chemical [8,9,51,52]. While FICZ could play a role in AhR-dependent gene induction in certain experimental conditions with certain chemicals as these authors proposed, this “indirect activation” hypothesis failed to consider the extensive amount of in vitro binding results available in the published literature that provide strong support for AhR ligand promiscuity. Such studies have already clearly demonstrated the ability of structurally diverse chemicals to not only directly bind to the AhR in vitro (using cytosolic and/or in vitro expressed AhR in competitive radiolabeled ligand binding assays), but also to stimulate AhR DNA binding in vitro (using cytsolic and/or in vitro expressed AhR/ARNT in gel retardation assays) [6,33–37,44,46,53]. In these experimental methods, FICZ, even if present, would not affect the ability of the test chemical to bind to the available unliganded AhR and/or to stimulate AhR DNA binding. Thus, the extensive amount of published literature provides strong support the conclusion that the AhR can directly bind and be activated by structurally diverse chemicals.

4. Diversity in AhR Ligand-Dependent Gene Induction

Given that it has already been established that the AhR can heterodimerize with factors other than ARNT to produce distinct complexes that can bind to distinctly different DNA sequences [24,26–28], the idea that diverse ligands simply activate the AhR to produce an identical AhR:ARNT complex that binds to the same DNA binding sites (i.e. the DRE) and yet produce diverse gene expression responses is clearly not correct. Studies by our laboratory and others have shown that the structural promiscuity of AhR ligands results from differences in the interactions of these diverse ligands with residues within the AhR binding pocket, however, whether these ligand-selective interactions produce AhRs with altered structure and/or functional activity remains an open question. The idea that the structure and functional activity of the AhR may be differentially altered depending on the specific ligand to which it is bound has been suggested by gene expression studies, where equipotent concentrations of diverse AhR ligands not only produce distinctly different magnitudes of induction of the same gene, but also induce a ligand-specific set of AhR-dependent gene products in the same cells [6,13,14,54–56].

It is possible that specific AhR heterodimers or heterodimer combinations can be formed in a ligand-selective manner, and this could contribute to ligand diversity in response, but this has not been examined. Alternatively, it has been suggested that ligand-specific differences

produced in the overall structure of the AhR and/or ARNT could alter the nucleotide specificity of AhR:ARNT DNA binding (or perhaps DNA binding of other AhR heterodimers), leading to ligand-specific differences in gene induction responses. While this was an attractive hypothesis and several novel ligand-selective DNA binding sites were proposed [54–57], subsequent PCR-based binding site analysis revealed that AhR:ARNT complexes activated by structurally diverse agonists only bound to DRE-containing DNA [53] and the proposed novel ligand-selective DNA binding sites could not be confirmed [58].

Alternatively, by analogy with steroid hormone receptor mechanisms [59–62], ligand-selective modulation of AhR signaling pathways and the magnitude of response in a given cell may result from ligand-specific changes in the structure of the AhR, AhR:ARNT, and/or other AhR:protein complexes, which could allow interactions with different subsets of transcriptional modulators (e.g. coactivators, corepressors), thereby producing different gene expression responses. This mechanism (Figure 2) is consistent with results from a two-hybrid analysis study that demonstrated that the binding of different HAH ligands to the AhR resulted in distinct differences in coactivator recruitment and were suggestive of ligand-selective differences in AhR structure [63]. However, since that study used only a small fragment of the AhR as part of a protein chimera and did not include ARNT, it remains to be determined whether the ligand-selective differences in coactivator recruitment occurs with the full-length AhR and/or ARNT proteins. To date, although ligand (TCDD)-dependent alterations in AhR structure have been observed using in vitro synthesized [^{35}S]-labeled AhR and proteolysis approaches [64], ligand-specific differences in overall AhR and/or ARNT structure have not yet been reported. However, a recent study was one of the first to clearly demonstrate AhR ligand-specific differential gene induction in a single cell type [65]. Stannocalcin 2 (Stc2) is a gene whose promoter contains numerous DREs [56,65], however, while classical AhR agonists (TCDD, 3MC and BNF) stimulate AhR binding to DREs upstream of CYP1A1 (measured using ChIP analysis) and induced CYP1A1 gene expression in primary hepatocytes, they failed to stimulate Stc2 gene expression or ligand-dependent binding of AhR to Stc2 promoter DREs [56,65]. In contrast, cinnabarinic acid, a newly identified tryptophan-derived AhR agonist [66], stimulated both AhR binding to the Stc2 promoter DREs and Stc2 gene expression, but failed to stimulate AhR binding to CYP1A1 DREs or induce CYP1A1 gene expression [65]. While details of the mechanism(s) responsible for the differential ligand responses of CYP1A1 and Stc2 genes remain to be elucidated, these results are consistent with ligand-selective differences in AhR gene expression and are suggestive of ligand-selective differences in AhR structure/function.

5. Diversity in AhR Ligand-Dependent Toxicity

While structurally diverse ligands can stimulate AhR-dependent gene expression and produce biological responses like TCDD and TCDD-like HAHs, they do not produce the major toxic effects observed with these compounds (i.e., lethality, wasting, birth defects, chloracne, etc) [1,2,6,33,37,67]. This suggests differences in the overall mechanism of action of “toxic” and “nontoxic” AhR ligands. Current evidence suggests that the persistence of AhR-dependent gene expression produced by metabolically stable TCDD-like HAHs is responsible for the prototypical spectrum of AhR-dependent toxicity [1,2,28,67]. In contrast, metabolically labile AhR ligands (e.g. BNF, 3MC and most structurally diverse ligands) only

transiently activate AhR-dependent gene expression, which is suggested to be insufficient to produce the prototypical spectrum of dioxin-like toxicity. If persistent AhR activation is responsible for the observed toxic effects of TCDD-like HAHs, it could be postulated that chronic daily exposure to high doses of a “nontoxic” AhR agonists would be expected to result in persistent AhR activation and produce AhR-dependent dioxin-like toxicity. This has only been indirectly examined in one study in which C57 mice were chronically fed high doses (150 mg/kg) of the relatively potent AhR agonist BNF, 5 days a week for 6 weeks [68]. The lack of any reported AhR-dependent toxic effects suggests that additional factors may contribute to toxicity beyond simply persistence of AhR activation. One hypothesis is that there is an additional molecular target that is selectively affected by toxic TCDD-like HAHs and not by nontoxic AhR ligands, and the combined activation of these distinct targets is required for the observed AhR-dependent toxicity to be manifested. While several AhR-independent effects of TCDD have been previously reported [6,15,69–73], their role in the prototypical spectrum of TCDD toxic responses is unknown. Alternatively, ligand-selective modulation of AhR:ARNT structure and function has also been proposed to at least partially explain the differential ability of ligands to produce the prototypical spectrum of AhR-dependent toxic effects (i.e., lethality, wasting, birth defects, chloracne, etc) and selective TCDD-like HAH gene expression responses [1,2,33,46,67]. Site-directed mutagenesis and functional analysis has revealed that the binding of TCDD-like HAHs within the AhR LBD was distinctly different from that of structurally diverse nontoxic AhR ligands [46], suggesting that AhRs bound by TCDD-like HAHs could have a distinctly different structure/function. Whether HAH-specific structural/functional differences in the AhR in combination with the metabolic persistence of HAHs contribute to AhR-dependent toxicity remains to be examined.

6. Concluding Remarks

Early insights into the molecular mechanism of AhR signal transduction were primarily the result of research into the effects of TCDD and TCDD-like HAHs on CYP1A1 gene expression, and these studies provided new avenues to understand the mechanism by which these widespread environmental contaminants produced toxicity. However, these studies provided few insights into how this relatively simple mechanism could produce the diverse spectrum of toxic and biological effects of these compounds. What was apparent was that a wide variety of structurally diverse dioxin-like and non-dioxin-like ligands (agonists) could stimulate expression of the same spectrum of classical AhR-dependent genes that are induced by TCDD (e.g. CYP1A1, CYP1B1, etc.). Thus, simply demonstrating the ability of a chemical or mixture to stimulate expression of a given AhR-dependent gene provides no useful information as to its ability or potential to produce dioxin-like toxicity, only that it is an AhR agonist. For an AhR ligand to actually be considered dioxin-like, it must be able to produce TCDD-like toxicity in vivo. Understanding the diversity in AhR signaling and response is a complex process. The AhR is known to be a key regulatory factor in a wide variety of endogenous physiological processes, adaptive gene responses and adverse health effects, and that the specificity and magnitude of individual AhR responses vary in a ligand-, cell-, tissue- and species-specific manner. Recent demonstration that the AhR can form heterodimers with nuclear factors other than ARNT to stimulate expression of a distinctly

different subset of genes, coupled with the ability of the AhR to bind and be activated by structurally diverse ligands and produce differential gene responses, has expanded the complexity of the AhR signaling pathway and provided new avenues in which to begin to understand AhR diversity in response (Figure 3). The recent identification of a role of the AhR in human disease has not only made it a new and significant target for the development of human therapeutic drugs, but demonstration of both the structural diversity and species selectivity of AhR ligands has provided pharmaceutical companies with numerous lead compounds for AhR drug development. However, an increased understanding of the biochemical and molecular mechanisms by which toxic and nontoxic ligands can differentially regulate AhR functionality and downstream responses is now even more important for the continued development and ultimate approval of such drugs for human use. Overall, even though a significant amount of information has been generated on the AhR signaling pathway, it remains an exciting area for continued research and many significant open questions still remain.

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Abbreviations

AhR	Aryl hydrocarbon receptor
AhRE	Ah responsive element
AHRR	Ah receptor repressor
ARNT	Ah receptor nuclear translocator
bHLH-PAS	Basic Helix-Loop-Helix-Per-ARNT-Sim
BNF	β-Naphthoflavone
ChIP	Chromatin immunoprecipitation
DRE	Dioxin responsive element
FICZ	6-formylindolo[3,2-b]carbazole
HAHs	Halogenated aromatic hydrocarbons
HIF1a	Hypoxia inducible factor 1α
hsp90	Heat shock protein 90
KLF6	Kruppel-Like Factor 6
LBD	Ligand binding domain
3MC	3-Methylcholanthrene

PAHs	Polycyclic aromatic hydrocarbons
PXR	Pregnane X receptor
Stc2	Stannocalcin 2
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
XRE	Xenobiotic responsive element

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Highlights

- The AhR can bind and be activated by structurally diverse ligands and species differences in ligand selectivity have been observed
- The AhR can stimulate gene expression by a combination of cannonical and noncannonical mechanisms
- AhR-dependent gene expression can vary in a ligand-, mechanism-, cell-, species-, and tissue-specific manner
- Mechanisms responsible for the toxicity of select AhR ligands still remains to be determined

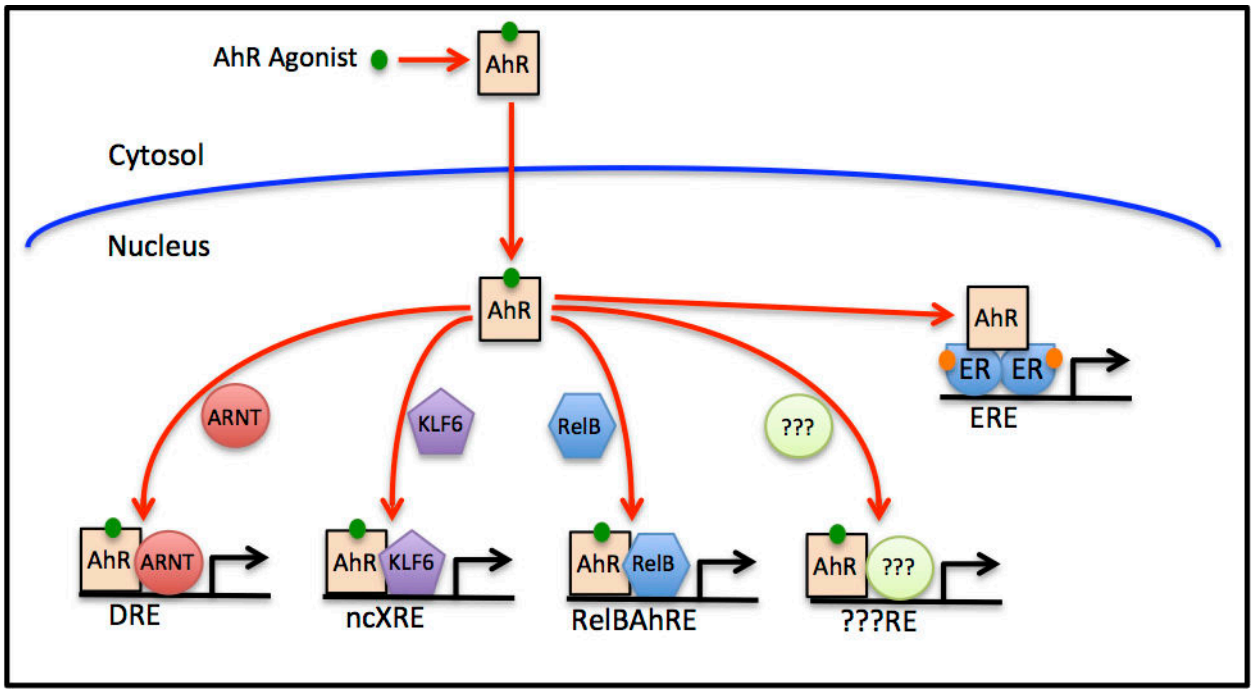

Figure 1.

Multiple mechanisms by which a specific ligand-activated AhR can stimulate gene expression. In the classical mechanism of AhR action, ligand binding stimulates AhR nuclear translocation, dimerization with the ARNT protein and the binding of the ligand:AhR:ARNT complex to its DNA binding site (the DRE) stimulates gene expression. However, the dimerization of liganded AhR with other proteins (e.g., KLF6 or RelB) results in the formation of unique protein complexes that bind to distinctly different DNA recognition sites (e.g., a ncXRE or RelBAhRE, respectively) to regulate subsets of genes not regulated by the AhR:ARNT complex. Whether ligand bound AhRs can interact with additional DNA binding partners remains to be determined, but is a possibility. In addition to multiple heterodimers, the AhR has also been observed to bind to other nuclear protein complexes (e.g., estrogen receptor (ER) dimers) and function as a coactivator, enhancing gene expression by these transcription factors.

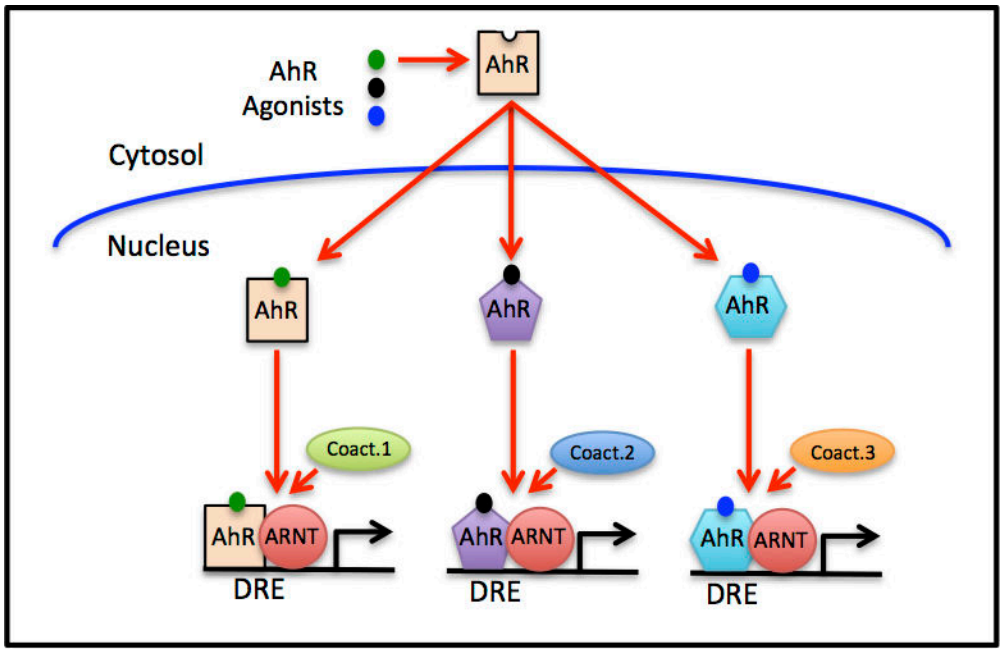

Figure 2.

Ligand-selective differences in AhR-dependent gene expression. Binding and activation of the AhR by structurally diverse AhR ligands could produce significant differences in the overall structure of the AhR and/or its dimerization partner that result in recruitment of distinctly different coactivators to the DNA bound AhR complex and differential gene expression. Although this figure only depicts an alteration in the structure of the AhR, ligand-selective structural changes could also occur in the ARNT protein, or in any other protein(s) to which the AhR is bound (such as KLF6 or RelB (see Figure 1)), facilitating differential coactivator recruitment by a greater diversity of ligand-activated AhR complexes and even a greater diversity in gene expression responses.

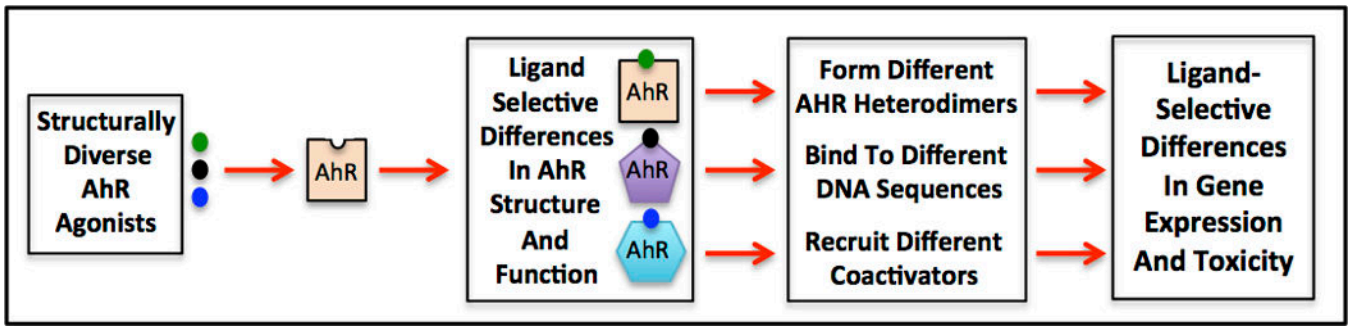

Figure 3.

Overall mechanisms by which structurally diverse AhR ligands can contribute to ligand-selective differences in AhR-dependent gene expression and toxicity. See text for details.