The Search for Endogenous Activators of the Aryl Hydrocarbon Receptor

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The primary design of this perspective is to describe the major ligand classes of the aryl hydrocarbon receptor (AHR). A grander objective is to provide models that may help define the physiological activator or "endogenous ligand" of the AHR. We present evidence supporting a developmental role for the AHR and propose mechanisms by which an endogenous ligand and consequent AHR activation might be important during normal physiology and development. From this vista, we survey the known xenobiotic, endogenous, dietary, and "unconventional" activators of the AHR, including, when possible, information about their induction potency, receptor binding affinity, and potential for exposure. In light of the essential function of the AHR in embryonic development, we discuss the candidacy of each of these compounds as physiologically important activators.

Contents

1. Introduction

It is now clear that the biological response to many environmental pollutants is a direct consequence of their interactions with the aryl hydrocarbon receptor (AHR).[1] An established role for the AHR in the metabolism of benzo[*a*]pyrene as well as in the acute toxicity of halogenated dioxins demonstrate mechanistically how certain classes of hazardous chemicals exert their toxicity and how mammalian organisms adapt to such exposures. In recent years, interest in AHR biology has grown beyond a toxicological perspective as research has uncovered a physiological role for this receptor in normal development. Consequently, AHR-related pharmacology has garnered additional attention as investigators begin to exploit known receptor ligands for insights into the features of the putative endogenous ligand and with an aim to unlock the therapeutic potential that may come from modulating this system.

In this perspective, we first provide a brief description of the underlying AHR signal transduction pathway and summarize the evidence suggesting that this receptor is both a player in chemical toxicity and an important component of normal development. Given the numerous reviews on the role of the AHR in chemical toxicity (*1–3*), we highlight developmental aspects of AHR signaling. Specifically, we present the case for

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[1] Abbreviations: AHR, aryl hydrocarbon receptor; PAHs, polycyclic aromatic hydrocarbons; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; bHLH, basic helix–loop–helix; PAS, PER-ARNT-SIM; ARNT, aryl hydrocarbon receptor nuclear translocator; DREs, dioxin responsive elements; AHRR, aryl hydrocarbon receptor repressor; DV, *ductus venosus*; SAR, structure–activity relationship; HAHs, halogenated aromatic hydrocarbons; PCBs, polychlorinated biphenyls; EROD, ethoxyresorufin O-deethylase; ITE, 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester; TA, tryptamine; IAA, indole acetic acid; FICZ, 6-formylindolo[3,2-*b*]carbazole; dFICZ, 6,12-diformylindolo[3,2-*b*]carbazole; I3C, indole-3-carbinol; ICZ, indolo[3,2-*b*]carbazole; DIM, 3,3′-diindolylmethane; LTr-1,2-(indol-3-yl-methyl)-3,3′-diindolylmethane; LDL, low-density lipoprotein.

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the existence of an endogenous activator. In the second part of this perspective, we discuss known agonists of the AHR, emphasizing those compounds that may be relevant to the putative endogenous ligand and the future pharmacopoeia of the AHR.

2. Background

2.1. Ah Receptor and Adaptive Metabolism. The field of AHR research has its origins in vertebrate toxicology. In the 1960s and 1970s, polycyclic aromatic hydrocarbons (PAHs) were common model compounds used in attempts to understand carcinogen bioactivation and detoxication. In these early studies, the metabolism of PAHs was found to be much more efficient upon secondary exposure (*4–7*). Examination of the underlying mechanism revealed that the primary exposure led to the induction of a battery of cytochrome P450-dependent monooxygenases and conjugating enzymes such as UDP-glucuronosyl transferase and glutathione-*S*-transferase (*8, 9*). Observations that the upregulated enzymes led to increased metabolism of the inducing compounds provided early evidence that this response might serve as a protective mechanism against xenobiotics (*10*).

Experiments designed to describe this metabolic adaptation showed that certain mouse strains differed in their inductive response after PAH exposure (*11, 12*). Analysis of crosses between "responsive" and "nonresponsive" strains demonstrated that a single autosomal dominant locus played a central role in this outcome. This locus was designated *Ah*, for aryl hydrocarbon responsiveness (*13*). Evidence that the *Ah* locus encoded a receptor was drawn from toxicology studies of the CYP1A1 inducer 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Because of its great induction potency, this highly toxic environmental contaminant proved to be a useful probe of the *Ah* system. Through analysis with radiolabeled TCDD, a receptor species was identified from the hepatic cytosol of C57BL/6J mice (*14*). The proof that this site was a "receptor" was two-fold. First, this site bound chlorinated-dioxin congeners with an affinity that was proportional to the individual compound's potency as an inducer of monooxygenases (*15*). Second, the TCDD-binding affinity for this site segregated with the responsive and nonresponsive genotype (*16*). Taken in sum, these experiments provided pharmacological and genetic proof that this binding site was a bona fide receptor and that it was encoded by the *Ah* locus (*13*). We now refer to this PAH- and dioxin-binding protein as the Ah receptor.

3. AHR Signal Transduction Pathway

3.1. Basics of Signaling. Investigations into the role of the AHR in adaptive metabolism resulted in a detailed understanding of the signal transduction pathway that links toxicant-receptor binding to the induction of xenobiotic-metabolizing enzymes. Biochemical studies demonstrate that in the absence of agonist, the AHR exists as an inactive complex with two molecules of the chaperone Hsp90, as well as one molecule each of the cochaperones, ARA9 (also known as AIP1 or XAP2) and p23 (*17–22*). Molecular cloning studies reveal that the AHR contains a basic helix–loop–helix (bHLH) domain similar to that present in many DNA-binding proteins (*23–25*). It also contains a PER-ARNT-SIM (PAS) homology domain similar to that found in other regulators of cellular and organismal responses to the environment (*25–28*). Most notable among the bHLH-PAS superfamily are the hypoxia-inducible factors that regulate

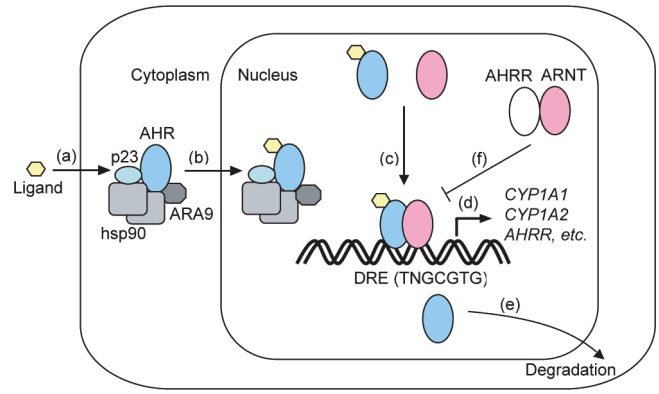


Figure 1. Ligand-activated signal transduction of the AHR. (a) A ligand diffuses into the cell and is bound by the cytosolic AHR complex. (b) The ligand-bound receptor complex translocates into the nucleus. (c) The AHR dimerizes with ARNT, and together, they bind DREs, (d) leading to transcriptional activation of target genes. (e) The AHR is exported to the cytosol and degraded. (f) The dimer of AHRR and ARNT down regulates the transcriptional activity of AHR.

responses to low oxygen tension and the Clock-Mop3 proteins that serve at the core of the mammalian circadian clock (*26*).

In the current model of signaling, agonist binding at the PAS domain of the AHR leads to a conformational change in the receptor (Figure 1). This change alters its associations with chaperones and exposes a nuclear localization signal on the AHR (*18, 29, 30*). As a result, the receptor complex migrates to the nucleus, where the AHR heterodimerizes with another bHLH-PAS protein known as the aryl hydrocarbon receptor nuclear translocator (ARNT) (*31*). The interaction of the AHR with ARNT increases their capacity to bind specific enhancer sequences adjacent to target promoters termed "dioxin responsive elements" (DREs) (*32–35*). An assembly of coactivators and general transcription factors, including p300, SRC-1, p/CIP, and transcription factor IIB, then interacts with gene promoters and potentiates the expression of target loci (*36*). The most well-studied of these responsive genes include the *Cyp1a1*, *Cyp1a2*, and *Cyp1b1* loci that encode the xenobiotic-metabolizing monooxygenases central to the adaptive metabolic response (*10*).

The AHR is also subject to negative regulation. Following ligand-induced activation and nuclear export (*29, 37*), the receptor is degraded via a 26S proteosome pathway (*38–41*). The activity of the AHR-ARNT complex is also attenuated by a second mechanism, the upregulation of a transcriptional repressor known as the aryl hydrocarbon receptor repressor (AHRR) (*42*). A bHLH-PAS protein with high sequence similarity to the AHR, the AHRR represses AHR transcriptional activity by binding ARNT and by the repressive activity derived from the interaction of the ARNT and AHRR complex with DREs (*42*). This attenuation of AHR activity by means of a negative feedback loop and receptor degradation may serve to protect the organism from the consequences of transcriptional hyperstimulation by potent agonists and to provide precise temporal control of this pathway.

4. Physiological/Developmental Role for the AHR

Three independent lines of evidence are in keeping with the idea that the AHR plays an important role in normal physiology. The evolutionary conservation of the AHR, the aberrant phenotypes of *Ahr* mutant mice, and the expression of DRE-responsive genes during development are all consistent with a physiological function of this receptor system. By extension, these observations also support the existence of an endogenous ligand or activator. In the next section, we will review the

evidence that strengthens the idea that the AHR has dual roles in normal biology, that is, one role as a mediator of an adaptive response to xenobiotics and a second role as a mediator of normal embryonic development and adult physiology.

4.1. AHR Has Been Highly Conserved throughout Evolution. If the physiological role of the AHR was limited to the regulation of a metabolic response to foreign chemicals, then this xenobiotic stress would be relatively conserved in most environmental niches. If the xenobiotic or environmental stress was not conserved, it would follow that receptor structure and function would vary significantly in organisms from differing ecosystems. A phylogenetic analysis of the AHR reveals that the primary amino acid sequence of the ligand-binding PAS domain protein is highly conserved across vertebrate species from marine, terrestrial, and avian environments (*43*). The amino acid sequence of the chicken PAS domain shares 81–86% similarity with that of the amphibian and mammalian AHR and 64–69% similarity with those of fish species (*43*). In addition to structural similarities in the PAS domain, the vertebrate AHR orthologues also appear to dimerize with ARNT and drive transcription from DREs (*44*). Moreover, in fish, rodents, and birds, the upregulated target genes include orthologues of the *Cyp1* family of monooxygenases (*45, 46*). We take these observations as an indication that the ligand-binding specificity of the AHR does not differ significantly across species found in diverse environments, signifying that the evolutionary stressor influencing ligand specificity of the AHR is an endogenous rather than exogenous compound.

Additional support for the existence of an endogenous activator can also be garnered from the observation that AHR orthologues found in invertebrate species possess physiological functions yet do not appear to bind xenobiotics. In *Drosophila melanogaster*, the *spineless* and *tango* loci encode bHLH-PAS proteins that bear homology to the mammalian AHR and ARNT, respectively (*47, 48*). Mutations in either *spineless* or *tango* disrupt the lengthening of distal segments in the antennae and legs, demonstrating their essential roles in the organism's development (*47, 48*). In addition, *spineless* expression is also crucial for generating the retinal photoreceptor pattern required for fly color vision (*49*). Proteins with structural similarity to AHR and ARNT have also been identified in *Caenorhabditis elegans*, where deficiency of the AHR orthologue (AHR-1) leads to impaired neuronal differentiation (*50, 51*). Despite sharing protein sequence homology, invertebrate AHRs do not appear to bind xenobiotic agonists recognized by the vertebrate receptor (e.g., β-naphthoflavone, TCDD, or the radioligand, 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin) (*47, 50, 52*). The developmental importance and lack of known xenobiotic agonists of invertebrate AHRs lend support to the idea that the mammalian AHR has a biological role other than being an evolutionary adaptor to environmental pollution (*50, 53, 54*).

4.2. Defects in AHR Null Mice. Perhaps the most compelling argument for the existence of an endogenous activator of the AHR comes from studies of recombinant mouse models in which the *Ahr* and *Arnt* genes have been mutated. Independent laboratories studying the physiology and development of *Ahr* null mice have described developmental aberrations and pathological end points that arise when AHR signaling is compromised by receptor deletion (*55, 56*). A number of allele-specific phenotypes have also been reported. Given these reported differences, we refer to the recombinant animals as either Δ1/Δ1 or Δ2/Δ2 mice, indicating the exon targeted for excision (*57*). For example, a 40–50% neonatal lethality rate, inflammation of the bile ducts, and an 80% depletion of splenic lymphocytes are reported for 3-week old Δ1/Δ1 mice (*55*). As the Δ1/Δ1 mice age, they also exhibit cardiohypertrophy, skin lesions, portal vascular hypertrophy, and pyloric hyperplasia of the gastrointestinal tract (*58*). In contrast, the Δ2/Δ2 mice commonly exhibit normal perinatal survival and normal lymphocyte numbers. While an explanation for the different phenotypes would likely require extensive experimental effort, contributing factors might include the animals' different genetic backgrounds, targeting strategies, or even the housing environment and pathogen status of the mice (*57*).

In our laboratory, the most penetrant and easily observed phenotypes in both Δ1/Δ1 and Δ2/Δ2 mice are vascular in nature. Distinctly, *Ahr* null mice have a 100% frequency of patent *ductus venosus* (DV). The DV is a fetal portocaval shunt of the developing liver that normally closes immediately after birth. In *Ahr* null mice, this shunt fails to close, and the mutants display aberrant hepatovascular blood flow and altered disposition of small molecules requiring hepatic clearance (*59, 60*). Other vascular aberrations noted in the *Ahr* null mouse model include a persistence of the hyaloid artery and an altered limbal vasculature within the developing eye. Like the DV, the hyaloid artery represents a fetal vascular structure that normally resolves postnatally yet persists in the eyes of adult *Ahr* null mice (*59*). These phenotypes provide evidence supporting a function of the AHR in normal development. Upon the basis of these early studies, we conclude that the AHR plays a critical role in the regulation of normal vascular or hematopoietic development.

Studies using AHR hypomorphic animals also establish that receptor activation is a required step in normal mammalian development. When gene targeting was used to generate a mouse with a marked decrease in the level of the AHR (i.e., hypomorphic), a high frequency of patent DV was observed, similar to that seen in the corresponding null animals. Exposure of these hypomorphs to TCDD between embryonic days E12.5 and E18.5 leads to the complete closure of the DV and the maturation of a normal-sized liver by adulthood (*61*). The compensation of a hypomorphic AHR signaling pathway by gestational administration of this potent agonist supports the proposal that an endogenous agonist activates the AHR to regulate vascular development during normal ontogeny.

4.3. Activation of the AHR during Development. If the AHR plays a role in normal ontogeny, one would predict that evidence of receptor activation during development would be detectable. In keeping with this prediction, there are multiple reports to suggest that upregulation of AHR-target genes occurs during embryonic development. In this regard, evidence for *Cyp1a1* expression was observed in mice that carry the *Cyp1a1* promoter driving a *lacZ* transgene (*62*). The detection of β-galactosidase activity in tissue sections of these animals was suggestive of *Cyp1a1* expression in the hindbrain, midbrain, heart, kidney, and tail during embryonic days E8–E14. Transcriptional activity was also noted in the liver at day E13 and in the skin and muscle at days E13 and E14 (*62*). In independent PCR-based studies, pools of cDNA from whole mouse embryos revealed increased levels of *Cyp1a1* transcript at day E7 and *Cyp1b1* transcript beginning at embryonic day E11 (*63*). Analyses of human tissues also showed strong expression of *CYP1A1* in the fetal adrenal, lung, and liver (*64*). Taken in sum, these reports of *Cyp1b1* and *Cyp1a1* expression during embryogenesis indicate that the AHR may signal in response to some tightly regulated developmental cue.

5. Search for Endogenous Activators of the AHR

The appreciation that the AHR is a developmentally important molecule has renewed our interest in the search for the bona fide "endogenous ligand". An analysis of ligand studies carried out over the last ~20 years yields a long list of AHR activators that includes environmental contaminants, therapeutics, naturally occurring chemicals, and small molecules isolated from mammalian tissues (*65, 66*). To sort out this literature, we provide in the next section models that we propose can help define the characteristics of a true endogenous AHR activator. We then proceed to review what is known about the current list of activators and comment on the data suggesting that each candidate compound is an endogenous ligand of the AHR.

5.1. Models of Endogenous Signaling. One model to explain AHR signaling is based on the idea that the AHR has arisen to mediate the adaptive metabolism of harmful chemicals generated endogenously. Such harmful endogenous compounds could be the byproducts of enzymatic reactions and/or normal physiological chemistry. If this model were correct, it would follow that the developmental pathologies observed in the *Ahr* deficient animals result from the unchecked levels of endogenously generated toxins that adversely affect ontogeny. In such a defensive system, the AHR might represent a receptor that recognizes a spectrum of structurally diverse compounds or alternatively, be specifically targeted to upregulate the metabolism of a single highly toxic chemical structure. Ultimately, the AHR would participate in normal development by promoting the clearance of biological molecules that harbor toxic potential.

A second model describes the AHR as part of an essential signaling pathway that is initiated by binding of its endogenous ligand via a mechanism analogous to the activation of the nuclear receptor family of transcription factors (*67, 68*). Contrary to the protective mechanism described above, the endogenous AHR agonist under this system would induce, rather than impede, developmental processes. In this context, the capacity to respond and regulate xenobiotics such as PAHs could have arisen independently and be of secondary importance developmentally.

A third model represents a combination of the two described above. In this model, the foremost physiological role of the AHR is to mediate normal development by transducing the chemical signal of the endogenous agonist to transcriptional events. Similar to the androgen receptors that mediate sexual differentiation, the physiological function of the AHR would likely be highly regulated, such that an endogenous agonist would require clearance at critical time points during embryonic development or normal physiology. Consistent with AHR activation under precise biological conditions, this model would favor the existence of a specific endogenous agonist that, via ligand binding, signals to influence cellular events and upregulates its own metabolism through *CYP1* gene products so that receptor action is not prolonged.

In developing models to explain AHR biology, it is important to consider the possible existence of an endogenous activator that is not a classical ligand (i.e., unlike PAHs or TCDD). This concept is important because, presently, we cannot rule out the possibility of endogenous AHR signaling in the absence of a small-molecule ligand. With ligand-independent signaling, a protein or other macromolecule that catalytically modifies the receptor or influences its shape or localization could activate the receptor. For example, an upstream event that leads to the phosphorylation of the AHR on key residues could transform the receptor and enhance its affinity for ARNT and, consequently, its DRE-regulated targets (*69*).

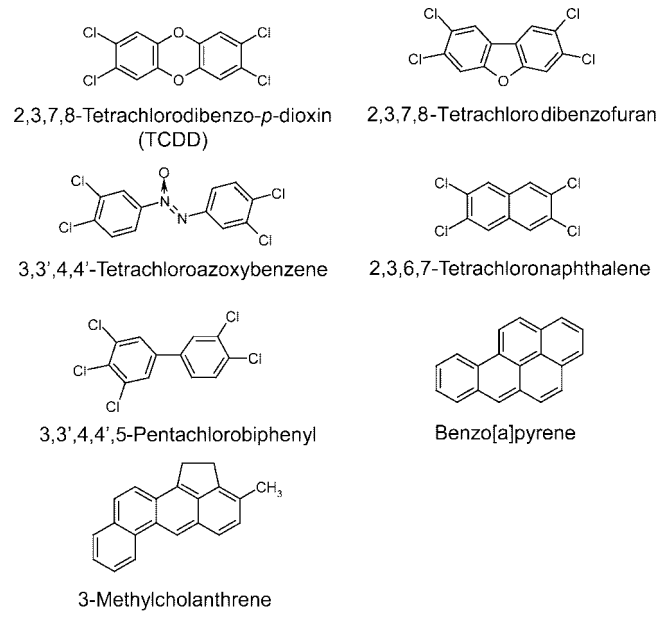


Figure 2. Classic AHR agonists. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; 2,3,7,8-tetrachlorodibenzofuran; 3,3′,4,4′-tetrachloroazoxybenzene; and 2,3,6,7-tetrachloronaphthalene are representative HAHs. 3,3′,4,4′,5-Pentachlorophenyl is a potent PCB. Benzo[*a*]pyrene and 3-methylcholanthrene are PAHs.

6. Known Ligands of the AHR

A search for endogenous ligands of the AHR can benefit from a prediction of what ligands of the AHR typically "look like". To this end, we rely in part on known structure–activity relationships (SAR) for classes of agonists. By relating the binding affinities of known agonists with their structural properties, the physicochemical parameters for a ligand of a given class can be used to predict the likelihood that a similar structure will bind the AHR. Most compounds known to bind and activate the AHR are hydrophobic molecules that commonly fall under two structural classes, PAHs or halogenated aromatic hydrocarbons (HAHs) (*70*). While many AHR agonists are planar compounds, the SAR analysis of polychlorinated biphenyls (PCBs) revealed that absolute planarity is not a requirement for receptor binding; however, coplanarity does influence a ligand's steric fit of the receptor (*71*). This idea is consistent with findings from a comparative molecular field analysis of ~100 analogues of halogenated dibenzo-*p*-dioxin, dibenzofurans, naphthalenes, biphenyls as well as the non-halogenated derivatives of indolo[3,2-*b*]carbazole. Using this data set, it was estimated that an AHR ligand would be between 12.0 and 14.0 Å in length, less than 12 Å in width, and no more than 5.0 Å deep (*72*). For halogenated aromatic ligands, increased receptor affinity and activation are controlled in part by the polarizability of the substituent groups (*71, 73, 74*). A number of recent studies that applied regression analyses to similar sets of test compounds identified electronegativity, hydrophobicity, and hydrogen bonding as properties that could also contribute to receptor interaction (*75, 76*).

7. Xenobiotic Ligands

7.1. Halogenated Dioxins and Related Compounds. The halogenated dibenzo-*p*-dioxins, dibenzofurans, azo(xy)benzenes, and naphthalenes comprise a family of important, structurally related AHR agonists (Figure 2). In general, these chemicals enter the environment through contamination of commercial products, as the result of industrial accident or as products of

waste incineration. When these compounds are extensively halogenated, they are metabolically and environmentally stable. When halogenated at lateral positions of the coplanar rings, they are often found to be potent agonists of the AHR. Although they can vary significantly in their binding affinity for the receptor, certain compounds such as TCDD are among the most potent AHR agonists known (Figure 2). In C57BL/6J mice, TCDD induces hepatic monooxygenase activity with an ED_{50} of 1×10^{-9} mol/kg. This potency is about 1000-fold greater than that of PAHs such as 3-methylcholanthrene or benzanthracene (*11, 77*).

Chlorinated members of this class of agonists elicit a characteristic pattern of toxicity that is mediated via their binding to the AHR. These toxic responses include epithelial hyperplasia, tumor promotion, teratogenesis, thymic involution, and death. In the mouse and rat, the oral LD_{50} of TCDD occurs at doses of 114 and 22–45 μg/kg, respectively (*78–81*). A rich body of pharmacological and genetic evidence supports a role for the AHR in the toxicity of halogenated dibenzo-*p*-dioxins, dibenzofurans, and related compounds (*77, 82–86*). This evidence includes the observation that sensitivity to TCDD toxicity segregates with the Ahr^b and Ahr^d loci (*83, 87–89*). In addition, the toxic potencies of halogenated dibenzo-*p*-dioxin and dibenzofuran congeners correspond to their relative binding affinities to the AHR (*90, 91*). Furthermore, *Ahr* null mice are not susceptible to the toxic effects of TCDD (*86*).

7.2. Polychlorinated Biphenyls (PCBs). There are 209 isomers and congeners of PCBs (*92*), many of which are biologically inactive (*93*). Because of their chemical stability, PCBs are constituents of numerous commercial products, including insulators, flame retardants, and adhesives (*94*). Consequently, the environmental pervasiveness of this chemical family and its trace contamination of wildlife and humans have generated concerns about the subsequent health risks. A pattern of toxicity reminiscent of TCDD poisoning has also been observed in animals and humans who were accidentally exposed to high doses of select PCBs (*95*).

Comparative SAR studies have determined that the presence of halogens on lateral positions of the benzene rings contributes to the potency of PCBs for activating the AHR (*70*), where maximal activity is achieved when halogens exist at both para (4 and 4′) and two or more meta positions (3, 3′, 6, and 6′) (*92*). The degree of substitution at the biphenyl bridge also influences the potency for AHR activation by this compound class. Because the phenyl rings can rotate about the linking bond, the presence of halogens at the ortho positions (e.g., 2 and 2′) is believed to minimize coplanarity and thus reduce their binding to the AHR (*70*). The affinity of PCBs for the AHR, in turn, correlates with their relative potency for AHR activation (*96*).

Among the most potent PCB congeners, 3,3′,4,4′,5′-pentaCB (Figure 2) is about 100 times less potent than TCDD for inducing AHR-regulated enzyme activity in the Wistar rat. In this species, the observed EC_{50} value of 3,3′,4,4′,5′-pentaCB is 0.50 μmol/kg and that of TCDD is 0.004 μmol/kg (*97*). In rat hepatoma H4IIE cells, where ligand metabolism might be less rapid than in the whole organism (*97*), the potency for CYP1A1 induction by 3,3′,4,4′,5′-pentaCB ($EC_{50} = 2.4 \times 10^{-10}$ M) is only ~four-fold lower than that of TCDD ($EC_{50} = 7.2 \times 10^{-11}$ M) (*98*). In this cell system, 3,3′,4,4′,5,5′-hexaCB ($EC_{50} = 6.0 \times 10^{-8}$ M), 3,3′,4,4′-tetraCB (3.5×10^{-8} M), and 2,3,3′,4,4′-pentaCB ($EC_{50} = 8.8 \times 10^{-8}$ M) were shown to be 100–1000-fold less active. Displaying EC_{50} values that were 4–5 orders of magnitude greater than that of TCDD, the mono-ortho analogues are comparable to the PAH, 3-methylcholanthrene, in their ability to induce CYP1A1 activity in this experimental system (*98*).

7.3. PAHs. PAHs represent a large class of AHR agonists that commonly contain four or more conjugated benzene rings (*99*). As products of combustion processes that can be found in chimney soot, charbroiled foods, and smoke exhaust, PAHs have been studied extensively for their induction of AHR-mediated enzymes (*8*). Typified by the compounds benzo[*a*]pyrene and 3-methylcholanthrene (Figure 2), PAHs induce AHR signaling with potencies that are lower than that of TCDD by 3–4 orders of magnitude (*100*). As inducers of the monooxygenases and conjugating enzymes, these agonists normally stimulate their own metabolism. The enzyme-mediated conjugation of O-, N-, and S-containing moieties to the aromatic structures enhances the polarity and elimination of PAHs and their derivatives. However, the same enzymatic activities can also biotransform select PAHs to carcinogens. A well-studied example is the benzo[*a*]pyrene-derived diol epoxides that can covalently bind macromolecules to form genotoxic DNA and protein adducts (*8*). Therefore, while PAHs elicit the metabolic response mediated by the AHR, select compounds of this family can be metabolically activated to toxicants.

8. Endogenous Ligands

In the previous section, we reviewed AHR signaling induced by compounds that come primarily from anthropogenic sources. That is, PAHs, halogenated dioxins, biphenyls, and dibenzofurans, are not commonly synthesized by living organisms. Although PAHs may have been generated through forest fires and other natural combustion processes, significant exposure to the compounds described above is probably a relatively new event from the standpoint of evolutionary pressure on AHR function. Accordingly, it is difficult to think of these pollutants as having provided the evolutionary pressure to explain the emergence and selection of the AHR signaling in vertebrate systems. On this basis, we argue that our search for insights into the natural ligand of the AHR must be extended to additional structural classes. Of particular interest are those that are known to be endogenously synthesized in higher organisms. To this end, we provide a brief review of those candidate endogenous AHR ligands that have been isolated from mammalian tissues.

8.1. Indigoids. Indigo and indirubin have been suggested to be endogenous AHR agonists (Figure 3) (*101*). Indirubin, the more potent of the two, has an EC_{50} for AHR activation of 0.2 nM when measured in a yeast reporter system, where the EC_{50} derived for indigo is 5 nM and that of TCDD is 9 nM (*101*). However, the dose–response curves generated in mammalian cells estimate the potency of indirubin to be ~30-fold lower than that of 2,3,7,8-tetrachlorodibenzofuran (*102*), an HAH with a potency nearing that of TCDD. Because of the CYP1A-mediated metabolism of indirubin in mammalian systems (*102, 103*), the length of ligand exposure and cell type could contribute to the observed range of potency for receptor activation. The EC_{50} value of an indirubin-induced DRE-driven reporter is 100 nM when human hepatoma cells were treated for 24 h (*102*) and 1.26 nM for induction of CYP1A-mediated ethoxyresorufin O-deethylase (EROD) activity when MCF-7 cells were treated for 4 h (*103*). Indigo and indirubin also appear to be specific AHR agonists, as they compete with TCDD for receptor occupancy and upregulate CYP1A1 monooxygenase activity but do not appear to stimulate other xenobiotic sensor systems in rodent models (*102, 104*).

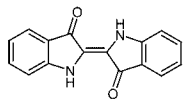
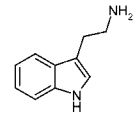
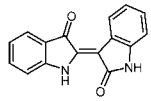
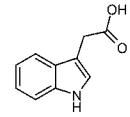
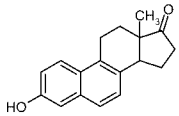
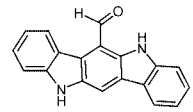
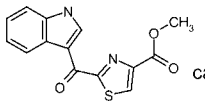
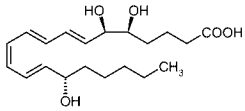
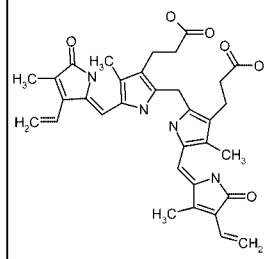
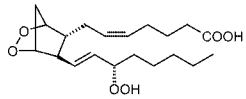
Compound structure	Name	AHR activation (EC_{50})	Compound structure	Name	AHR activation (EC_{50})
	Indigo	5 nM (101) 5 μM (102)		Tryptamine (TA)	0.2 mM (119)
	Indirubin	0.2 nM (101) 1.26 nM (103) 100 nM (102)		Indole Acetic Acid (IAA)	0.5 mM (119)
	Equilenin	10 μM (108)		6-formylindolo-[3,2-b]carbazole (FICZ)	34-830 pM (128)
	2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE)	20 nM (107)			
	Lipoxin 4A	100 nM (114)		Bilirubin	30 μM (117)
	Prostaglandin G2	20 μM (115)			

Figure 3. Endogenous compounds that activate the AHR.

Historically recognized as plant compounds used for textile coloring, indigo and indirubin have been isolated from human urine and bovine serum (*101*). Although the synthesis of these indigoids in higher organisms has not been shown, the cytochrome P450-mediated conversion of indole to the indigo precursor, 3-hydroxyindole, has been demonstrated (*105*). Arguing against their role as biologically relevant endogenous ligands for the AHR is the observation that these indigoids are present in fairly low levels in vertebrate tissues. The average levels of indigo and indirubin in human urine are in the picomolar range, well below the nanomolar concentrations required to significantly upregulate classic DRE-driven responses in mammalian cell systems (*101, 102*). Nevertheless, their candidacy as physiologically relevant ligands cannot be disregarded, since their local concentrations may be higher at certain sites, therefore allowing these compounds to reach physiologically active levels. A better understanding of the spatial and temporal distribution of these compounds as well as additional searches for more active isomers is necessary before we can make firm conclusions regarding the influence of these structures on endogenous AHR signaling.

8.2. 2-(1′H-Indole-3′-carbonyl)-thiazole-4-carboxylic Acid Methyl Ester (ITE). An agonist of the AHR was isolated from porcine lung tissue and identified as 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester or ITE (Figure 3). Three experiments support the conclusion that ITE is an AHR agonist. First, the purified compound was shown to compete with [^{3}H]TCDD for binding to the human, murine, killifish, and zebrafish AHR (*106*). Second, saturation-binding isotherms indicated a high affinity interaction between ITE and the AHR from Hepa cell cytosol (i.e., K_D of 6.5 nM) (*107*). Finally, ITE exposure transformed the AHR to a DRE-binding conformation, augmented CYP1A1 protein expression, and induced DRE-dependent luciferase activity in a concentration- and time-dependent manner (*106, 107*). On the basis of dose–response curves generated in murine hepatoma cells (*107*), ITE has an estimated EC_{50} of ~20 nM or a potency that is approximately 100-fold lower than that of TCDD. Furthermore, the gestational administration of ITE to pregnant mice led to the AHR signaling in fetal tissues without the toxicities related to TCDD exposure, indicating an in vivo bioactivity.

The potency that ITE displayed for AHR activation, coupled with its isolation from tissue, has led to the suggestion that ITE is a physiologically relevant endogenous ligand of the AHR. Although this idea is intriguing, it requires further validation. One issue that should be clarified is whether ITE actually exists in mammalian tissues. In this regard, the method of isolating ITE from lung tissue employed high temperature and acidic conditions, which raises the concern that ITE may be a byproduct of the isolation process. A demonstration of the route of biological synthesis or direct measurement of ITE in biological samples, without such reactive isolation conditions, would provide an important contribution to the body of evidence suggesting a physiological role for ITE.

8.3. Equilenin [3-Hydroxy-1,3,5(10),6,8-estrapentaen-17-one]. Equilenin is an equine estrogen and a constituent of the hormone replacement drug, Premarin (Figure 3). Produced by pregnant mares and excreted in equine urine, equilenin is a reported AHR agonist. A potentially weak ligand, equilenin binds the murine AHR with an estimated affinity of about 1/30000 that of benzo[*a*]pyrene (*108*). In human HepG2 cells, 30 μM equilenin produced a 15-fold increase in *CYP1A1* mRNA level and approximately five-fold induction of DRE-mediated reporter activity. On the basis of dose responses produced in these systems, the EC_{50} of equilenin for AHR activation is estimated to be ~10 μM.

Interestingly, equilenin was also reported to stimulate similar levels of EROD activity in C57BL/6 and DBA/2 mice, strains

that harbor the *Ahr*^{b} and *Ahr*^{d} alleles, respectively. This observation is particularly interesting because the *Ahr*^{d} allele encodes a murine form of the AHR that binds exogenous ligands, such as TCDD, with a 10-fold lower affinity than receptors encoded by the *Ahr*^{b} allele (*16, 109*). The ability of equilenin to similarly induce CYP1A in DBA and C57BL6 mice is an interesting aspect of this compound that is rarely noted about other AHR agonists. However, the weak binding of equilenin to the AHR leaves room for speculation about the mechanism by which it activates the receptor. Future studies addressing the possibility of an equilenin metabolite being the AHR ligand, or AHR activation resulting from the stimulation of another signaling pathway (e.g., the estrogen receptor) by equilenin could help clarify the significance of this compound.

8.4. Arachidonic Acid Metabolites. Arachidonic acid and its related structures have long been considered potential endogenous ligands of the AHR. A number of associations between these pathways provide hints of such a relationship. For example, many cytochrome P450-dependent monooxygenases have metabolic activity toward arachidonic acid and its metabolites (*110, 111*). In addition, the exposure of cultured hepatocytes to TCDD induces the expression of COX2, providing a potential link between AHR activation and prostaglandin synthesis (*112*). Less direct arguments include the observation that changes in cell shape, as induced by shear stress, have been shown to activate AHR signaling (see below). This phenomenon is also associated with prostaglandin release (*113*).

The above arguments led to a screen of endogenous lipophilic substances for AHR agonists, with a focus on arachidonic metabolites. From this screen, lipoxin 4A, a metabolite of arachidonic acid, was shown to be an AHR agonist (*114*). The activation of the AHR by lipoxin 4A was demonstrated by measuring multiple end points, including competitive binding with TCDD for receptor occupancy, induction of a DRE-driven reporter assay, and stimulation of CYP1A1- and CYP1A2-mediated enzyme activity (*114*). Not only does lipoxin 4A induce the CYP1A monooxygenases, but it also appears to be a substrate for these enzymes (*114*). This observation is consistent with a model whereby this compound or related structures regulate their own metabolism in a manner similar to xenobiotics such as PAHs. Interestingly, lipoxin 4A differs structurally from classical AHR agonists, as it contains no rings and bears a negative charge at physiological pH. With an EC_{50} of 100 nM for induction of a DRE-driven reporter in mouse hepatoma cells, it has been suggested that activation of the AHR by nanomolar levels of physiological lipoxin 4A could occur (*114*). The significance of lipoxin 4A in AHR-related biology would be further elucidated if it was demonstrated that its receptor activation regulates the developmental processes that have been attributed to AHR signaling.

In light of a potential relationship between the AHR and arachidonic acid metabolism, a screen employing both a cell-based reporter assay and a gel retardation assay was conducted to investigate the ability of prostaglandins to activate the AHR (*115*). Of the 25 compounds tested, six prostaglandins (prostaglandin B_2, D_2, $F_{3\alpha}$, G_2, H_1, and H_2) induced receptor activity. Dose–response assays conducted in a murine hepatoma cell line indicated that prostaglandins are rather weak inducers, with activity detected at concentrations of 10 μM or higher. The most active molecule among these, prostaglandin G_2 (Figure 3), is active at 1 μM and can induce DRE-dependent transcription with an estimated EC_{50} value of 20 μM. By comparison, lipoxin A4 has an EC_{50} in the nanomolar range. Interestingly, the reporter response induced by 100 μM prostaglandin G_2 exceeded that generated from 1 nM TCDD. Despite deviating structurally from classical AHR agonists, prostaglandin G_2 may be a weak ligand of the AHR, indicated by its partial displacement of [^{3}H]TCDD receptor binding when prostaglandin G_2 was co-incubated at a 20000-fold excess (*115*). On the basis of the low biological potency of these prostaglandins and lipoxins as AHR agonists, it seems unlikely that these particular structures are biologically relevant. However, related structures having higher affinities and potencies that are in range with their biological levels may be important candidates with physiological roles in AHR biology.

8.5. Heme Metabolites. Metabolites of heme are also intriguing candidate as endogenous AHR ligands. Studies of congenitally jaundiced rats link an elevated level of bilirubin with increased CYP1A1 activity (*116*). The biosynthesis of bilirubin begins with the rate-limiting conversion of heme to biliverdin, which in turn is metabolized to bilirubin. Given that CYP1A1 upregulation is a hallmark of AHR activation, these heme derivatives were tested for their regulation of *Cyp1a1* expression and AHR signaling (*117, 118*). The treatment of mouse hepatoma cells with micromolar concentrations of biliverdin, bilirubin, or hemin (the Fe^{3+} oxidation product of heme) led to a time- and dose-dependent expression of *Cyp1a1* mRNA, EROD induction, and DRE-driven reporter activity (*117*). The absences of these activities in cell lines that are deficient in AHR or ARNT expression indicate that these compounds stimulate classical AHR signal transduction.

It has been suggested that bilirubin is the most significant AHR agonist derived from heme metabolism (*117*). In vitro gel shift assays performed using hepatic cytosolic fractions showed AHR transformation by bilirubin but not biliverdin and hemin. These results, coupled with the rank order potency of bilirubin > biliverdin > hemin for CYP1A1 induction, suggest that hemin and biliverdin are precursors, while bilirubin is the ultimate activator of the AHR (*117*). The relative significance of bilirubin is debatable, however, as an independent study has indicated that both bilirubin and biliverdin are active inducers in vivo and in vitro (*118*). These latter studies also provided evidence that both bilirubin and biliverdin can compete with [^{3}H]TCDD for receptor occupancy, indicating that they both may be true ligands of the AHR.

There are arguments for and against bilirubin and related heme metabolites being endogenous ligands of the AHR. Arguing against is the observation that heme mainly exists bound to serum albumin. Therefore, while the normal level of total heme in human plasma is 5–20 μM, a relatively small fraction is accessible to other proteins or to intracellular signaling machinery. However, under pathological conditions where the bilirubin-conjugating enzyme is impaired, the level of free bilirubin might be high enough to activate the AHR. In this regard, patients of Crigler–Najjar syndrome have plasma levels of bilirubin that reach 400–800 μM, a range that is >10 times its estimated EC_{50} of 30 μM for AHR activation in mouse hepatoma cells (*117*).

8.6. Tryptophan Metabolites. The aromaticity of tryptophan has led to the speculation that this amino acid or its metabolites may be endogenous agonists of the AHR. Independent groups have reported AHR activation by tryptamine (TA) and indole acetic acid (IAA) on the basis of observations made in vitro and in cell systems. TA and IAA can stimulate AHR-DRE binding with an EC_{50} in the 0.2−0.5 mM range (*119*). By comparison, the EC_{50} of TCDD is about one million times lower (i.e., approximately 0.1 nM). Both compounds appear to weakly bind the AHR, as they competed with [^{3}H]TCDD for AHR

occupancy when used at a 200000-fold excess (*119*). Furthermore, cellular exposure to high μM levels of TA or IAA leads to AHR-mediated transcriptional activity, reflected by the compounds' induction of DRE-drive reporters in a recombinant yeast system (*120*) and a murine cell line (*119*). Although IAA was found to be inactive, TA competitively EROD activity, an observation suggestive of its metabolism by CYP1A1 and CYP1A2 enzymes (*119*).

The reported physiological level of tryptophan ranges from 70 to 150 μM (*119*). Given their weak potency as inducers of CYP1A1 activity in cell culture, it is unlikely that IAA and TA can affect AHR signaling under physiological conditions. Despite the observation that tissue levels of TA can reach 120 ng/g in the presence of monoamine oxygenase inhibitors (*121*), evidence of endogenous AHR activation by tryptophan metabolites remains to be presented.

8.7. UV Photoproducts of Tryptophan. UV irradiation of tryptophan generates compounds that display high affinity for the AHR and potency for induction of CYP1 monooxygenases (*122, 123*). Chromatographic and structural analyses of the two most active tryptophan photoproducts led to the identification of 6-formylindolo[3,2-*b*]carbazole (FICZ) (Figure 3) and 6,12-diformylindolo[3,2-*b*]carbazole (dFICZ) (*124*). In competitive binding assays, FICZ and dFICZ displaced TCDD with K_D values of 0.07 and 0.44 nM, respectively, suggesting an affinity for the AHR that is in the range of that observed for TCDD (e.g., K_D of 0.48 nM in these experiments) (*122*). Because of its metabolism by the upregulated CYP1A1 and CYP1B1 enzymes, the AHR activity induced by FICZ is transient, peaking at 3 h (*125–127*). Exposure of MH1C1 rat hepatoma cells to increasing doses of FICZ for 3 h yielded an EC_{50} of 34 pM, as compared with 830 pM when cells were incubated for 24 h (*128*).

A compelling body of data provides indirect evidence that the synthesis of FICZ could occur in vivo. The exposure of human skin to UV-B was shown to induce CYP1A1 and CYP1B1 mRNAs and proteins in the epidermis and dermis (*129*). In addition, UV irradiation of rats and hairless mice increased hepatic CYP1A-dependent monooxygenase activity (*130–132*). A recent report also showed that FICZ could be formed from the prolonged exposure of tryptophan to sunlight (*133*). On the basis of such findings, AHR has been suggested to play a role in mediating the cellular response to oxidative stress caused by UV light, with FICZ as the endogenous agonist that triggers the AHR-mediated biological response (*134*). Although the photooxidation of tryptophan can produce FICZ, it remains to be shown that UV exposure leads to the formation of this compound in vivo and that cellular levels of tryptophan can support this biosynthesis.

9. Dietary Compounds

In searching for physiologically relevant ligand(s) that might relate to the phenotypes seen in *Ahr* null mice, the diet is an obvious potential source. The basis for this reasoning includes the observation that lipophilic extracts of numerous plant foods can activate the AHR (*135*). If they are physiologically relevant, we could think of these dietary ligands as toxicants, which when inefficiently cleared lead to patent DV. Alternatively, they could also be likened to an unknown vitamin that is required for normal vascular development. If *Ahr* null phenotypes such as patent DV are the result of an inefficiently metabolized toxicant, it seems most likely that chemistry or fermentative processes within the gastrointestinal tract should generate such a toxicant. This conclusion is based upon the observation that animals raised on purified diets have never been reported to harbor the phenotypes of *Ahr* null mice. If the molecule is more like a vitamin, the same argument holds; it must be present in purified diets or it must be generated from simple constituents in vivo. Below is a short review of those dietary compounds that have been found to activate the AHR and the arguments before and against their role as the physiologically relevant activator of this receptor.

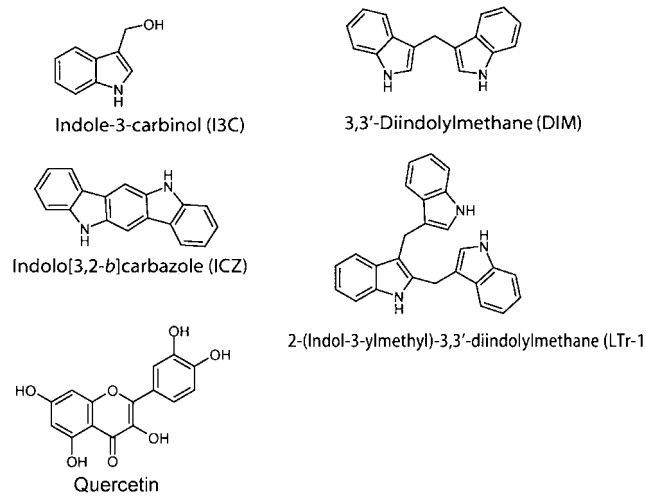


Figure 4. Agonists derived from dietary sources. Indolo[3,2-*b*]carbazole (ICZ), 3,3′-diindolylmethane (DIM) and 2-(indol-3-ylmethyl)-3,3′-diindolylmethane (LTr-1) are acid condensation products of indole-3-carbinol (I3C). Quercetin is a representative flavonoid.

9.1. Indole-3-carbinol Derivatives. A phytochemical found in *Cruciferous* plants including broccoli and Brussels sprouts, indole-3-carbinol (I3C) has been studied for its anticancer properties (Figure 4). Reports describing the therapeutic potential of IC3 derivatives in cancer treatments cite the compounds' ability to modify the metabolism of chemical carcinogens, inhibit the growth tumors, or induce apoptosis of cancer cells (*136–139*). Although 13C derivatives upregulate classical AHR-mediated monooxygenases (*141*), it is unknown as to how much of their activity can be attributed to their activation of the AHR (*140*).

I3C is a secondary metabolite of glucobrassicin that can be converted to a family of AHR agonists. The observation that an oral—but not intraperitoneal—administration of I3C increased hepatic monooxygenase activity led to the conclusion that I3C is converted to higher order AHR agonists in the acidic environment of the stomach (*142*). Chemical analysis of the I3C acid-condensation products revealed numerous di- and trimeric condensation derivatives including the agonists indolo[3,2-*b*]carbazole (ICZ), 3,3′-diindolylmethane (DIM), and 2-(indol-3-ylmethyl)-3,3′-diindolylmethane (LTr-1) (Figure 4) (*143*). The detection of these compounds in the stomach and intestinal contents of rats dosed orally with I3C confirmed the synthesis of these compounds in vivo (*143*).

Among the 13C derivatives that display greatest potency for AHR activation is ICZ. Competitive binding analysis indicates that the AHR binds ICZ with a surprisingly high affinity. The K_D value for ICZ is 1.9×10^{-10} and that of TCDD is 7.1×10^{-12} M (*143*). In addition to being a strong ligand, ICZ is a potent agonist whose in vivo activity is moderated by metabolic clearance. When Hepa1c1c7 cells were incubated with ICZ for 4 h, ICZ induced *Cyp1a1* mRNA expression with a potency that reflects its binding affinity, approximately two orders of magnitude lower than that of TCDD (*144*). However, when treatment time was lengthened to 48 h, ICZ is ~four orders of magnitude less potent than TCDD at inducing EROD activity,

reflected by the EC_{50} of 36 pM for TCDD and 260 nM for ICZ (*143*). Indicative of its metabolism, the preincubation of ICZ in rat hepatic microsomes produced a time-dependent decrease of ICZ detection by HPLC-based analyses. In contrast, no loss of the compound occurred when the control sample was incubated in buffer or heat-inactivated microsomes (*144*). Therefore, while ICZ appears to be a potent agonist that is generated in vivo, it is readily metabolized to compounds without AHR activity. It was estimated that as much as 1–5 nmol of ICZ could be generated from the consumption of a 100 g portion of Brussels sprouts. Other examples of 13C derivatives that bind and activate the AHR include LTr-1 and DIM. Both compounds induce AHR transformation and CYP1A enzyme activities, but with lower potencies than that of ICZ (*143, 145–147*).

9.2. Natural Flavonoids. Ubiquitously found in fruits and vegetables, naturally occurring flavonoids encompass the most abundant class of plant polyphenols (*148*). Because plant foods are rich in flavonoids, it is estimated that their dietary intake can be as high as 1 g per day (*149*). Numerous reports have suggested links of flavonoid consumption with health benefits (or risks) because of their antioxidant, antiproliferative, estrogenic, or antiestrogenic properties (*148*). Interest in their effect on AHR signaling thus stems from the investigation of phytocompounds as dietary modulators of chemical carcinogenesis. Of the six major subgroups of flavonoids, some flavones, flavonols, and isoflavones have been identified as activators of the AHR.

From independent screens of phytochemicals, numerous flavonoids were found to activate the AHR, including chrysin, galangin, baicalein, genistein, daidzein, and apigenin (*150, 151*). Despite their induction of reporter genes in various mammalian cell lines, it remains to be shown whether these natural flavonoids can bind the receptor or whether they are metabolized to agonists. Another natural flavonoid, quercetin (Figure 4), also activated the AHR, as evidenced by multiple measures, including induction of EROD activity and the de novo induction of *CYP1A1* mRNA transcription in a time- and dose-dependent fashion in MCF-7 cells (*152*). By means of semiquantitative RT-PCR analysis, an approximately 22-fold increase of *CYP1A1* expression was detected when cells were treated with a 10 μM dose of quercetin. Furthermore, quercetin was demonstrated to compete with TCDD for receptor binding. While these flavonoids are relatively weak inducers, their prevalence in plant foods warrants consideration of their effect on AHR signaling and xenobiotic metabolism in general. Furthermore, the activation of the AHR by naturally occurring flavonoids can be interpreted to mean that the AHR may have a role in detoxicating and/or metabolizing compounds from dietary sources.

10. Nonligand Activators

There are compelling reasons to consider the possibility that the endogenous activator of the AHR does not bind the receptor as xenobiotic ligands do. As noted above, mouse strains harboring Ahr^b or Ahr^d alleles differ in their binding affinities for the same xenobiotic ligands by an order of magnitude. Yet mice harboring the low-affinity binding allele (Ahr^d) do not display developmental defects reminiscent of the null allele (i.e., patent DV). By comparison, mouse models of the testicular feminization mutation, an amino acid substitution on the androgen receptor that reduces testosterone binding affinity by nearly 10-fold, exhibit external female sexual characteristics while being genetically male (*153–155*). This suggests that either the binding pocket of the Ahr^d and Ahr^b alleles recognizes the endogenous ligand with the same affinity, or developmental activation of the receptor occurs through a distinct mechanism, one that is independent of the ligand-binding pocket (i.e., via post-translational modification or protein–protein interaction).

While the AHR is commonly known as a ligand-induced transcription factor, numerous reports have also documented AHR activation in the absence of the addition of small molecule ligands. In this regard, it has been observed that culturing techniques can alter AHR-regulated transcriptional activity in cells. Among the earliest examples of this relationship is the observation that suspension of human keratinocytes and murine hepatoma cells with methylcellulose enhances CYP1A1 transcription in an AHR- and ARNT-dependent manner (*156–158*). More recently, it has been observed that the disruption of cell–cell contact can activate DRE-driven reporter activity in fibroblast cell culture and that sparsely seeded cells are more likely than confluent cells to display nuclear localization of the AHR and drive the transcription of DRE-driven reporter genes in the absence of ligand (*159, 160*). The relationship between the AHR and the cell shape is also supported by the demonstration that the absence of AHR expression in a mutant mouse hepatoma cell line leads to spindle-shaped cell morphology (*161*).

Modulation of AHR activity by the second messenger cAMP represents another potential mechanism of ligand-independent AHR signaling. The exposure of murine hepatoma cells with a membrane permeable cAMP derivative or the cAMP inducer, forskolin, was shown to induce nuclear localization and DNA binding (*162*). This study has led to the suggestion that cAMP-regulated signaling, occurring through a mechanism that appears distinct from that induced by TCDD, may be central to the endogenous function of the AHR (*162*).

A recent effort to explore the role of the AHR in vascular development has led to the finding that low-density lipoprotein (LDL) can activate the AHR (*163*). This conclusion was derived from the initial observation that exposure of sera to hydrodynamic shear stress can stimulate *CYP1A1* mRNA expression and DRE-dependent transcription of reporter genes in human and rodent hepatoma cells (*163–166*). Following a series of fractionation steps, sheared LDL was identified as the AHR-activating agent. The modification of LDL by sodium hypochlorite, an oxidizing reagent, also induced AHR activity. Interestingly, the sheared sera of AHR null, but not heterozygous mice induced DRE-mediated transcriptional activity, indicating that the AHR may play a role in mediating the metabolism of LDL (*163*).

Collectively, these reports provide ample evidence of xenobiotic-independent AHR activation. Furthermore, these results suggest a role of the AHR in responding to cell–cell contact or cell morphology. Although no xenobiotic ligand was added to the cell cultures in these studies, one cannot exclude the possibility that the loss of cell–cell contact or the suspension and shearing of cells can mobilize an endogenous agonist.

11. Proagonists, Weak Agonists, or Nonspecific Ligands

A number of AHR-inducing compounds that differ from the classical description of an agonist have also been described. Below, we will review examples of small molecules that may activate the receptor through a mechanism that lies outside their binding to the same pocket that recognizes TCDD and other xenobiotics. For more extensive discussions on these nonclassical AHR agonists, the reader is referred to earlier literature reviews (*65, 66*). Because of their structural deviation from

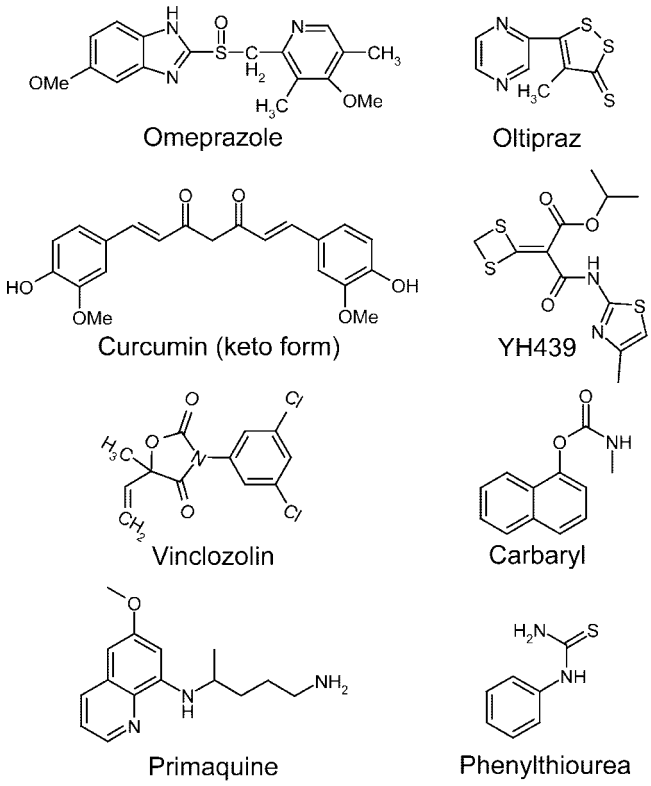


Figure 5. Proagonists/AHR agonists with nonclassical structures.

classic agonists or their lack of receptor binding, multiple proposals have been raised regarding the mechanism of receptor activation by nonclassical ligands. While their modes of action remain to be clarified, analyses of these compounds may provide insights into the endogenous signaling of the AHR.

11.1. Benzimidazoles. Unlike most xenobiotic ligands of the AHR, benzimidazoles do not exhibit extended planarity and do not bind the AHR with high affinity. Recognition of this new group of AHR activators began with the early studies of omeprazole (Figure 5), a proton pump inhibitor whose pharmaceutical formulations are widely used in the treatment of gastric ulcers or dyspepsia (*167*). Other benzimidazoles that have been demonstrated to activate the AHR include lansoprazole as well as the compounds albendazole, mebendazole, thiabendazole, fenbendazole, and oxfendazole that are used as antihelminthics.

Because of their broad clinical and veterinary applications, extensive research efforts have been made to examine their secondary effects, such as their action on xenobiotic-metabolizing enzymes and the influence on the metabolic half-life of drugs taken concurrently. Evidence that benzimidazoles activate the AHR includes their ability to induce DRE-driven reporter systems, *CYP1A1* and *CYP1A2* gene expression, or related monooxygenase activity (*168–173*).

Two interesting aspects of benzimidazoles distinguish them from other known AHR agonists. First, benzimidazole-stimulated AHR activity appears to be species-specific. A comparative study showed that omeprazole, thiabendazole, and lansoprazole were unable to activate the AHR in the mouse cell line, Hepa1c1c7, while they induce receptor activity in the human HepG2 cells (*174, 175*). Second, in contrast to classical AHR agonists, competitive binding analyses of oxfendazole, omeprazole, thiabendazole, or lansoprazole revealed that benzimidazoles were incapable of displacing [^{3}H]TCDD from the AHR (*168, 173, 176–178*). As an exception to this trend, a recent study found that by preincubating thiabendazole with guinea pig cytosol, competitive displacement of [^{3}H]TCDD can occur (*179*). In general, the benzimidazoles possess weak to moderate AHR activation potency, as a significant receptor response was achieved by the treatment of cultured cells with compound concentrations in the micromolar range.

These characteristics of the benzimidazoles form the basis of two hypotheses that attempt to explain the compounds' mechanism of AHR activation. One proposal is that omeprazole-induced AHR signaling is ligand-dependent but stimulated by a metabolic derivative of omeprazole that can bind and activate the receptor (*180*). A second proposal is that this class of compound induces AHR activity via a ligand-independent mechanism. Support of this view comes from the demonstrations that the protein kinase inhibitors daidzein, herbimycin A, and tyrphostins decreased the expression of CYP1A1 induced by omeprazole but not TCDD or B[*a*]P (*69, 178, 181*). In addition, the small-molecule antagonist, PD98059, which binds the AHR, did not affect receptor activation by omeprazole (*182*). It was therefore suggested that omeprazole activates the AHR indirectly, possibly by inducing a signaling cascade that involves the phosphorylation of the AHR (*69, 181*).

11.2. Unusual Structures. A number of compounds that bear little structural resemblance to classic agonists have also been reported to activate the AHR. As shown in Figure 5, compounds such as primaquine, vinclozolin, YH439, phenylthiourea, curcumin, and oltipraz lack the conjugated aromatic rings of the PAHs (*183–188*). In general, these compounds are weak activators of the AHR that require doses in the mid to high micromolar range to elicit receptor activity.

Regarding their specific activation of the AHR, little is known or conflicting results are reported about the ability of these nonaromatic compounds to bind the receptor. For example, opposing conclusions have been drawn concerning antimalarial medication, primaquine. While one study indicated that this molecule did not compete with [^{3}H]TCDD for receptor binding (*183*), another showed that by reducing the concentration of radioligand, competitive binding was observed (*191*). Altered experimental conditions might also explain the discrepant findings on receptor binding by Carbaryl, a widely used carbamate insecticide. Contrasting the suggestion that this compound induces *CYP1A1* gene transcription without direct receptor interaction (*190–192*), a 40000-fold excess of Carbaryl (*193*), or the preincubation of hepatic cytosol with Carbaryl prior to the addition of [^{3}H]TCDD (*179*), did lead to competitive displacement of the radioligand.

Taken in sum, the inconsistent competitive binding data and relatively weak potency for AHR activation by the benzimidazoles and other compounds reviewed in this section imply that they might be proagonists, weak ligands, or nonspecific AHR agonists. Support of the latter conclusion comes from the observation that vinclozolin, in addition to inducing CYP1A1, also increased the expression of CYP3A and CYP4A (*187*), target enzymes of the pregnane X receptor and the peroxisome proliferator-activated receptor, respectively.

12. Conclusion

Advances in recent years have affirmed a role for the AHR as an important regulator of normal development. Future understanding of the receptor's physiological function will ultimately be linked to identification of its endogenous activator. While the identity of the physiologically relevant endogenous agonist remains elusive, the search has yielded numerous candidates. Although these candidates are often of biological interest, none has yet been proven to be an agonist with relevance to AHR-associated physiology. Future efforts to

identify this compound should address these following points: (i) activation of the AHR at biologically relevant concentrations, (ii) a similar potency for activation of the polymorphic murine receptors, and (iii) the ability of the compound to stimulate the developmental closure of the DV.

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