Exactly the Same but Different: Promiscuity and Diversity in the Molecular Mechanisms of Action of the Aryl Hydrocarbon (Dioxin) Receptor

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The Ah receptor (AhR) is a ligand-dependent transcription factor that mediates a wide range of biological and toxicological effects that result from exposure to a structurally diverse variety of synthetic and naturally occurring chemicals. Although the overall mechanism of action of the AhR has been extensively studied and involves a classical nuclear receptor mechanism of action (i.e., ligand-dependent nuclear localization, protein heterodimerization, binding of liganded receptor as a protein complex to its specific DNA recognition sequence and activation of gene expression), details of the exact molecular events that result in most AhR-dependent biochemical, physiological, and toxicological effects are generally lacking. Ongoing research efforts continue to describe an ever-expanding list of ligand-, species-, and tissue-specific spectrum of AhR-dependent biological and toxicological effects that seemingly add even more complexity to the mechanism. However, at the same time, these studies are also identifying and characterizing new pathways and molecular mechanisms by which the AhR exerts its actions and plays key modulatory roles in both endogenous developmental and physiological pathways and response to exogenous chemicals. Here we provide an overview of the classical and nonclassical mechanisms that can contribute to the differential sensitivity and diversity in responses observed in humans and other species following ligand-dependent activation of the AhR signal transduction pathway.

Key Words: Ah receptor; AhR; dioxin; mechanism; promiscuity; HAHs; PAHs.

The Ah receptor (AhR) is a ligand-dependent basic helix-loop-helix-Per-ARNT-Sim (PAS)-containing transcription factor that responds to exogenous and endogenous chemicals with the induction/repression of expression of a large battery of genes and production of a diverse spectrum of biological and toxic effects in a wide range of species and tissues (Beischlag et al., 2008; Furness and Whelan, 2009; Hankinson, 2005; Humblet et al., 2008; Marinković et al., 2010; Poland and Knutson, 1982; Safe, 1990; White and Birnbaum, 2009). The best-characterized high-affinity ligands for the AhR include a wide variety of ubiquitous hydrophobic environmental contaminants such as the halogenated aromatic hydrocarbons (HAHs) and nonhalogenated polycyclic aromatic hydrocarbons (PAHs). HAHs, such as the polychlorinated dibenzo-p-dioxins, dibenzofurans and biphenyls, and related compounds, represent a diverse group of contaminants, many of which are highly toxic and both environmentally and biologically persistent (Poland and Knutson, 1982; Safe, 1990). Exposure to and bioaccumulation of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin), the prototypical and most potent HAH, and other dioxin-like HAHs can produce diverse species-specific toxic and biological effects, including (but not limited to) tumor promotion, teratogenicity, immuno-, hepato-, cardio-, and dermal toxicity, wasting, lethality, modulation of cell growth, proliferation and differentiation, alterations in endocrine homeostasis, reduction in steroid hormone-dependent responses, and induction and repression of expression of a large number of genes (Table 1). Although other structurally similar chemicals and classes of chemicals have also been identified that can produce the spectrum of dioxin-like toxic and biological effects and are collectively referred to as dioxin-like chemicals (DLCs), recent evidence has also demonstrated that the AhR can bind and be activated by structurally diverse chemicals (Denison and Nagy, 2003; Denison et al., 1998a; Nguyen and Bradfield, 2008). The dramatic variety of effects produced by TCDD, DLCs, and other AhR ligands suggests significant mechanistic diversity in the action of these chemicals, and numerous biochemical,
genetic, and transgenic animal studies have demonstrated that most, if not all, of these effects are mediated by the AhR and for the most part have been linked to alterations in gene expression.

A tremendous amount of research on dioxin and the AhR has been carried out because the AhR was first reported 35 years ago (Poland et al., 1976), and although these studies have dramatically increased our understanding of the basic mechanisms by which dioxin and the AhR produces many of these effects, including its involvement in endogenous processes, the molecular mechanisms and events responsible for many of the primary biological and toxicological effects of TCDD and other AhR ligands have remained elusive. Although this might at first seem somewhat surprising given the extensive research efforts in this area, substantial progress has been made in our understanding of many aspects of the AhR and the toxic and biological effects of its ligands, and these are highlighted in numerous excellent recent reviews (Abel and Haarmann-Stemmann, 2010; Beislag et al., 2008; Chopra and Schrenk, 2011; Denison and Nagy, 2003; Kerkvliet, 2009; Linden et al., 2010; Marlowe and Puga, 2010; Matsumura, 2009; McMillan and Bradfield, 2007a; Stevens et al., 2009). However, attempts to delineate the molecular mechanisms of the toxic action of AhR ligands have been complicated by the promiscuity and diversity of the AhR and its functional activities and the variations and delay in the development of toxicity. TCDD-induced changes in gene expression and their resulting biochemical effects occur relatively rapidly, and they are amenable to study and manipulation in numerous in vitro systems, and this is where the most progress has been made. However, the major toxic effects of TCDD and related DLCs (lethality, wasting, hepatotoxicity, immunotoxicity, and others) are more complicated to study as they typically require several weeks to occur, and these effects generally can only be produced in intact animals in vivo. Additional complicating factors not only include the species and tissue specificity of many biochemical and toxic effects of TCDD and DLCs but also species- and ligand-specific differences in the structure, function, and mechanism of action of the AhR itself.

Although early studies were focused on the AhR as mediating the biochemical response to xenobiotics (an adaptive role with respect to the induction of xenobiotic metabolism) and the toxic effects of selected xenobiotics (i.e., dioxin-like HAHs), recent studies have also demonstrated key endogenous regulatory roles for the AhR in normal physiology and development (Furness and Whelan, 2009; Linden et al., 2010; McMillan and Bradfield, 2007a; Stevens et al., 2009). Additionally, the ability of the AhR to bind and be activated by structurally diverse chemicals, coupled with significant differences in ligand binding and ligand-specific AhR functionality and responsiveness, suggest a greater degree of complexity in the mechanisms of AhR action. Thus, the biochemical and molecular mechanisms contributing to the diverse AhR-dependent biochemical and toxic effects likely involve a complex interplay of multiple biochemical and molecular mechanisms, and an appreciation of the complexity of these mechanisms is essential to be able to understand the diversity in AhR responses. In this overview, we will describe recent findings highlighting the molecular mechanisms that have been identified that contribute to the complexity and diversity of AhR ligand-dependent signaling events and differences in sensitivity and response in humans and animals to these chemicals.

**CLASSICAL MECHANISM OF AhR ACTION**

Historically, details of the molecular mechanism of action of the AhR were primarily determined in studies examining the ability of TCDD and other chemicals to bind to and activate the AhR and AhR-dependent cytochrome P4501A1 (CYP1A1) gene expression (reviewed in Okey, 2007). The current model of this classical mechanism of AhR action is presented in Figure 1. TCDD and related AhR ligands (agonists) enter the responsive cell by diffusion (as most are very hydrophobic) and...
bind with high affinity to the cytosolic AhR which exists in an inactive state as a multiprotein complex containing heat shock protein 90 (hsp90), HBV X–associated protein 2 (XAP2), and the co-chaperone protein p23 (Beischlag et al., 2008; Hankinson, 1995; Marlowe and Puga, 2010). Following ligand binding, the AhR is presumed to undergo a conformation change that exposes its N-terminal nuclear localization sequence, facilitating translocation of the liganded AhR protein complex into the nucleus. Once in the nucleus, chaperone proteins (hsp90 and p23) are displaced from the AhR by ARNT, and the resulting AhR:ARNT dimers bind to and activate transcription from the DRE-containing promoters such as those for various CYPs, SOS1, AhRR, and TriPARP affecting indicated cellular pathways. The AhRR lacks a transactivation domain and exerts negative feedback regulation on the AhR pathway through its competition for ARNT and formation of inactive AhRR:ARNT transcriptional complexes on DREs. Following transcription, AhR is exported and degraded by the proteosome in the cytoplasm. Activation of CYPs can result in metabolism of exogenous and endogenous AhR ligands.

The presence of an AhR complex in a wide variety of species and tissues and its ability to act as a ligand-dependent transcription factor has suggested that many of the toxic and biological effects of AhR ligands result from differential alteration of gene expression in susceptible cells. Additionally, the ability of metabolically stable AhR agonists (i.e., TCDD and DLCs) but not metabolically labile AhR agonists (i.e., PAHs and related compounds) to produce the spectrum of AhR-dependent toxic effects (Table 1) also has suggested that the overt toxicity of selective AhR ligands results from their ability to persistently activate/repress expression of key AhR-responsive genes. However, the responsible genes and gene products for most of these overt toxic effects have yet to be identified. Consistent with this hypothesis are the results of studies using transgenic mice in which the AhR, or selected functions of the AhR, or ARNT have been disrupted (Bunger et al., 2003, 2008; Gonzalez and Fernandez-Salguero, 1998). These and other studies convincingly demonstrate an absolute requirement of the AhR and ARNT in the ability of TCDD to produce its major toxic and biological effects (Bunger et al., 2003, 2008; Gonzalez and Fernandez-Salguero, 1998) as well as a role for AhR-associated factors (i.e., XAP2) in selected adverse effects (Nukaya et al., 2010). However, the ability of TCDD to produce effects in an AhR-independent manner and/or by nonclassical AhR mechanisms (i.e., mechanisms that do not involve ligand:AhR:ARNT:DRE complex-dependent induction

**FIG. 1.** The classical mechanism of AhR-dependent gene activation. Ligand diffuses into the cell and binds to the cytosolic AhR complex resulting in the exposure of its nuclear localization sequence (NLS), likely dissociation of XAP2 from the complex and nuclear translocation of the activated AhR complex. Once in the nucleus, chaperone proteins (hsp90 and p23) are displaced from the AhR by ARNT, and the resulting AhR:ARNT dimers bind to and activate transcription from the DRE-containing promoters such as those for various CYPs, SOS1, AhRR, and TriPARP affecting indicated cellular pathways. The AhRR lacks a transactivation domain and exerts negative feedback regulation on the AhR pathway through its competition for ARNT and formation of inactive AhRR:ARNT transcriptional complexes on DREs. Following transcription, AhR is exported and degraded by the proteosome in the cytoplasm. Activation of CYPs can result in metabolism of exogenous and endogenous AhR ligands.
of gene expression) has also been observed and likely contributes to the overall adverse effects of these compounds (Table 1).

TCDD- AND AhR-DEPENDENT AND INDEPENDENT GENE EXPRESSION

Although the best-studied AhR-responsive genes produce enzymes involved in drug and chemical metabolism (i.e., CYP1A1 and others), gene microarray studies have identified a large number of gene products that are induced or repressed in an AhR- and ligand-dependent manner (Boutros et al., 2009; Dere et al., 2011a; Hayes et al., 2007; Tijet et al., 2005). Genome-wide analysis of DRE sequences in several species has also identified extensive arrays of gene clusters of likely AhR-responsive genes, many of which have already been shown to be TCDD and AhR responsive (Dere et al., 2011a,b) and have provided novel avenues for the identification of AhR-responsive genes. Interestingly, microarray analysis has also revealed dramatic species- and ligand-specific differences in AhR-dependent gene expression profiles (Dere et al., 2011b; Kopec et al., 2010). The conserved nature of AhR DNA-binding specificity (Bank et al., 1992; Denison et al., 1998b; Swanson et al., 1995) suggests additional mechanisms must exist by which the AhR can modulate gene expression. Consistent with the hypothesis are the results of Perdew and coworkers (Flaveny et al., 2010) comparing DNA microarray analysis of TCDD-treated hepatocytes from wild-type (WT) C57BL/6J mice with those of transgenic C57BL/6J mice, in which the AhR had been selectively replaced with the human AhR. Their results revealed dramatic species-specific AhR-related differences in hepatocyte gene expression, with 1752 genes induced by TCDD in WT mice compared with 1186 genes in humanized mice, with only 265 genes activated by both receptors. In contrast, 1100 and 779 genes were repressed in WT and humanized mouse hepatocytes, respectively, with 462 genes significantly repressed by both receptors in response to TCDD. Although the observed differences in the overall gene induction and repression likely result from variations in interactions between the mouse and human AhRs with resident mouse-specific coactivators/corepressors, the dramatic difference in response still suggests significant differences in the mechanisms by which the human and mouse receptor can modulate gene expression. In fact, consistent with this, Flaveny et al. (2008) reported a significant difference in the ability of mouse and human AhR to bind differentially to LXXLL-containing coactivator proteins. Additional differences in response of the human and mouse AhRs are suggested from TCDD toxicity studies in humanized mice (Moriguchi et al., 2003), wherein mice expressing the human AhR were less susceptible to TCDD-induced cleft palate than mice expressing either the “high-affinity” AhR<sup>H</sup> or “low-affinity” AhR<sup>L</sup> alleles. Taken together, these mechanistic differences in AhR function could contribute to the variation observed in TCDD responses between human and mouse. Comparisons of gene microarray analysis results from livers of TCDD-treated WT AhR (+/+<sup>−</sup>/−<sup>−</sup>) and AhR knockout (-/-<sup>−</sup>/−<sup>−</sup>) mice not only revealed 456 gene products induced and repressed in AhR (+/+<sup>−</sup>/−<sup>−</sup>) mice but they also identified TCDD-dependent alterations in the expression of 32 genes in AhR (+/-<sup>−</sup>/−<sup>−</sup>) mice, demonstrating the ability of TCDD to alter gene expression in an AhR-independent manner (Tijet et al., 2005). In addition, analysis of basal gene expression in AhR (+/+<sup>−</sup>/−<sup>−</sup>) and (-/-<sup>−</sup>/−<sup>−</sup>) mice in this study identified 392 gene products whose expression was dependent on the presence of the AhR, suggesting a role for the AhR in normal liver physiology. These results, taken together with a subsequent study by these investigators (Boutros et al., 2009), support the involvement of the AhR in basal gene expression, whereas differences in the level of endogenous AhR ligands in AhR (+/-<sup>−</sup>/−<sup>−</sup>) and (-/-<sup>−</sup>/−<sup>−</sup>) mice may play a role in these differential responses. In fact, McMillan and Bradfield (2007a) demonstrated the presence of elevated levels of an endogenous AhR ligands (presumably oxidized low-density lipoproteins) in the serum of (-/-<sup>−</sup>/−<sup>−</sup>) mice compared with (+/-<sup>−</sup>/−<sup>−</sup>) mice that could stimulate AhR-dependent gene expression in a mouse hepatoma cell line. The loss of CYP1A1 and related metabolic activity in AhR (-/-<sup>−</sup>/−<sup>−</sup>) mice has been suggested to result in elevated levels of endogenous AhR ligands that normally would be degraded by these enzymes (Chiao et al., 2007; McMillan and Bradfield, 2007b). This is supported by studies demonstrating elevated constitutive activity in cells with low or chemically inhibited CYP1A1 activity (Chang and Puga, 1998; Chiao et al., 2007; Wincent et al., 2009). The above results, when combined with recent studies demonstrating endogenous developmental activities of the AhR and AhR signaling pathway, strongly support a role for the AhR in basal endogenous functions (Furness and Whelan, 2009; Linden et al., 2010; McMillan and Bradfield, 2007b; Stevens et al., 2009) and suggests that these endogenous activities could represent a target for disruption by TCDD, DLCs, and other AhR agonists and antagonist, contributing to the overall diversity in AhR response.

LIGAND-DEPENDENT EFFECTS MEDIATED BY THE CLASSICAL MECHANISM OF AhR ACTION

Many of the major toxic effects produced by TCDD and other AhR ligands likely result from the complex interplay of multiple signaling pathways and cellular regulatory factors and involve combination of both classical and nonclassical AhR-dependent mechanisms. However, recent studies have demonstrated that some adverse effects of TCDD and related DLCs are primarily driven by the classical AhR:ARNT:DRE–dependent gene expression signaling pathway. For example, it is well known that expression of persistently high levels of CYP1A1 and other related CYPs by TCDD and other AhR ligands results in increased metabolism of exogenous and
endogenous chemicals and generation of reactive oxygen species (ROS) and oxidative stress (Biswas et al., 2008; Kopf et al., 2010; Park et al., 1996). Not only can the increase in CYP1A1-dependent ROS result in an increase in oxidative DNA damage (Park et al., 1996) but it can directly activate intracellular kinase signaling pathways (i.e., c-Jun kinase, activator protein 1, nuclear factor-κB [NF-κB], nuclear factor erythroid 2-related factor 2, and others), thus indirectly altering gene expression and cellular responses (Antelmann and Helmann, 2011; Diry et al., 2006; Haarmann-Stemmann et al., 2009; Kim et al., 2009; Puga et al., 2009). Chronic exposure of mice to TCDD not only induces cardiovascular ROS, endothelial dysfunction, and hypertension (Table 1), similar to that suggested from epidemiology studies of dioxin-exposed humans (Everett et al., 2008; Humblet et al., 2008; Lee et al., 2007; Uemura et al., 2009), but it was demonstrated that AhR-dependent CYP1A1 was required for this effect (Kopf et al., 2010). The classical mechanism of AhR action has also been shown to induce expression of several other gene products that play direct regulatory roles in the adverse effects of TCDD. Suppression of hepatic gluconeogenesis is one AhR-dependent effect associated with the wasting syndrome produced by TCDD (Hsia and Kreamer, 1985; Viluksela et al., 1999) and the AhR-responsive gene TiPARP (TCDD-inducible poly(ADP-ribose) polymerase) was recently identified to be a mediator of this suppression (Diani-Moore et al., 2010). Similarly, TCDD induction of Neddl/Hefl/Cas-L gene expression occurs in an AhR- and DRE-dependent manner, and this protein is involved in integrin-based signaling and appears to mediate TCDD-stimulated changes in cell plasticity (i.e., alterations in cell adhesion and shape, cytoskeleton reorganization, and increased cell migration) (Bui et al., 2009). Another recent example is the ability of AhR ligands to induce DRE-dependent expression of son of sevenless (SOS1), the primary mediator of Ras activation, leading to an activated Ras-GTP state, extracellular signal-related kinase activation, and accelerated cell proliferation of human hepatoma (HepG2) cells (Pierre et al., 2011). These effects are mediated at least in part by induction of SOS1. Taken together, these studies clearly demonstrate that some aspects of the adverse effects of TCDD can be mediated by gene products whose expression is directly regulated via the classical AhR- and DRE-dependent mechanism (Tables 1 and 2).

NONCLASSICAL MECHANISMS OF AhR ACTION

The classical genomic mechanism of AhR signal transduction (i.e., ligand-dependent formation of a ligand:AhR:ARNT:DRE complex leading to stimulation of transcription of the adjacent gene) has been long considered the avenue by which TCDD, DLCs, and other AhR agonists produce their biological and toxicological effects. Although the exact mechanisms by which AhR activation leads to the majority of its effects are still being elucidated, detailed mechanistic studies have identified and/or characterized ligand- and/or AhR-dependent alterations in diverse cell signaling pathways and protein regulatory factors (Table 2). Analysis of ligand-dependent alterations in functional activity of many of these new targets and regulatory pathways has revealed that the AhR can exert its action via diverse nonclassical genomic and nongenomic mechanisms. The majority of these nonclassical AhR mechanisms are the direct result of its activity as a nuclear protein- and DNA-binding transcription factor and thus are directly related to its ability to interact and cross talk with other nuclear proteins and signaling factors. An overview of the established classical and nonclassical mechanisms of AhR action is presented in Figure 2.

Although some of these nonclassical AhR pathways have been proposed to play critical roles in or be responsible for some of the toxic effects of TCDD, DLCs, and other AhR ligands, the responsible mechanisms have been elucidated for very few of these responses. To directly determine whether the classical and/or nonclassical AhR signaling pathways play the major role in AhR-dependent toxicity, the toxic and biological effects of TCDD have been examined in transgenic mice in which the AhR had been knocked out (AhR (--/--)) or that contained a mutant form of the AhR that was either unable to translocate into the nucleus and/or to bind to DRE-containing DNA. These studies convincingly demonstrated that the primary toxic and biological effects of TCDD have an absolute requirement for the classical AhR signaling pathway, including AhR nuclear translocation and DRE binding (Bunger et al., 2003, 2008; Gonzalez and Fernandez-Salguero, 1998). Although these studies showed that the nonclassical pathways were not solely responsible for the observed adverse effects of TCDD, they do not preclude involvement of, or a requirement for, these alternative mechanisms. Final confirmation of their involvement awaits the impact of their knockdown or knockout on AhR-dependent effects. However, given the diversity of response to AhR ligands, it is very likely that a majority of ligand- and AhR-dependent toxic and biological effects (Tables 1 and 2) are modulated by a combination of both classical and nonclassical AhR-dependent mechanisms of action, and this could explain why it has been difficult to identify a single responsible mechanism for most AhR-dependent effects.

To best illustrate the complexity of AhR action beyond its classical signaling mechanism, the remainder of this review will describe ligand- and AhR-dependent effects on selected biological responses, signal transduction mechanisms, and species-specific responses where nonclassical AhR mechanisms are best understood. Although the following selected mechanistic descriptions will highlight direct actions of the AhR on cellular signaling and biological pathways, many of these same signaling pathways can modulate AhR responsiveness; however, these reciprocal effects will not be discussed here. The reader is directed toward several excellent reviews for a detailed discussion of the effects of these pathways on
TABLE 2

Diversity in Biochemical and Intracellular Signal Transduction Pathways and Cellular Functions Shown to Be Altered Following Exposure to TCDD and Other AhR Ligands

<table>
<thead>
<tr>
<th>Alteration in levels and/or function of nuclear receptors and transcription factors</th>
<th>Nuclear hormone receptors and receptor signaling pathways</th>
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<tbody>
<tr>
<td>Estrogen, androgen, glucocorticoid, progesterone, thyroid hormone, and retinoid</td>
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<tr>
<td>HIF</td>
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<tr>
<td>Wnt/β-catenin</td>
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<td>NF-κB (RelA, RelB)</td>
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<tr>
<th>Alterations in normal cell biology and signaling</th>
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<tr>
<td>Cell cycle progression and cell proliferation</td>
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<td>Cell adhesion and migration</td>
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<td>Circadian clock rhythm and signaling</td>
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<th>Alterations in intracellular kinases</th>
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<tr>
<td>Receptor tyrosine kinases</td>
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<td>Phosphoinositol 3 kinase</td>
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<td>MAPK</td>
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<td>c-Jun kinase</td>
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<tr>
<td>Protein kinase A and C</td>
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<tr>
<td>Calcium/calmodulin-dependent protein kinase</td>
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<th>Alterations in intracellular signaling pathways</th>
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<tr>
<td>Growth factors and growth factor receptors</td>
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<tr>
<td>Intracellular calcium levels and calcium-dependent signaling pathways</td>
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<td>Intracellular and mitochondrial ROS-responsive signaling factors</td>
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<tr>
<th>AhR-dependent induction of transcription of cellular regulator factors</th>
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<tr>
<td>Vav3 proto-oncogene affects cell adhesion and migration</td>
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<tr>
<td>Nrfr oxidative stress-responsive factor</td>
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<td>p73αβ-:represor of cell cycle progression</td>
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<tr>
<td>HEF1/NEDD9/CAS-L–involved in integrin-based signaling</td>
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<tr>
<td>TIPARP modulates hepatic gluconeogenesis</td>
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<tr>
<td>Amphiregulin—an epidermal growth factor receptor ligand</td>
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Note. Nrfr2, nuclear factor erythroid 2-related factor; TIPARP, TCDD-inducible poly (ADP-ribose) polymerase.

of their ability to competitively sequester away key coactivators or DNA-binding partners used by both receptors (Fig. 2). The AhR has been shown to directly bind to a large number of coactivators and nuclear proteins (p300, cyclic AMP [cAMP] response element-binding [CREB]-binding protein [CBP], steroid receptor coactivators 1/2 [SRC1/2], receptor-interacting protein 140 [RIP140], and others [Beischlag et al., 2008; Hankinson, 2005; Marlowe and Puga, 2010]), and because many of these are also used by steroid receptors, they represent possible targets for AhR-dependent squelching effects. In these instances, in order for the AhR to repress ER signaling, the AhR must have a higher affinity for the factor (which must be in limited concentrations) and/or exist in a higher concentration than the receptor or transcription factor whose activity is being squelched. Alternatively, if coactivator binding affinities and specificities for the AhR and ER are similar, one can envision that activation of the AhR immediately prior to ER activation could reduce the pool of available coactivators needed for ER transcriptional activity. This mechanism of AhR-dependent inhibition would be expected to be very highly cell-type dependent. A clear example of ligand- and AhR-dependent squelching of ER signaling activity by competitive binding of a common factor has been recently reported. ARNT, the dimerization partner of the AhR, has also been shown to be a potent coactivator that can bind to and activate ERs. Other AhR ligands can stimulate calcium influx and calcium-dependent cell signaling events. Details of these nonclassical mechanisms are described in the text.
estrogen-dependent ERβ signaling (Ruegg et al., 2008). Similarly, a reduction in estrogen-activated ERβ signaling was observed following activation of the hypoxia-inducible factor (HIF-1α), which also binds ARNT as its nuclear dimerization partner (Ruegg et al., 2008), or by expression of the ARNT-interacting protein 2 (Aip2) which was shown to block ARNT:ER interactions (Li et al., 2010b). These studies demonstrate the commonality of this type of repressive mechanism. Direct binding between ligand-activated AhR and ERs can also lead to repression of ER-dependent signaling. In this instance, TCDD- and AhR-dependent enhancement of proteosomal degradation of ERα and ERβ has been reported and appears to result from direct AhR:ER protein-protein interactions that leads to polyubiquitination of ERs by a E3 ubiquitin ligase activity found associated with the AhR (Beischlag et al., 2008; Ohtake et al., 2003). Direct binding of AhR and ERs also occurs at both AhR- and ER-responsive genes (Ahmed et al., 2009) where it has been suggested that the recruitment (i.e., “hijacking”) of ERs away from their responsive gene to AhR-responsive genes can contribute to repression of ER signaling, especially when coupled with AhR-dependent ubiquitination and degradation of the ER. The above results clearly demonstrate that activation of the AhR can result in antiestrogenic effects via a combination of classical and nonclassical AhR-dependent mechanisms. However, in contrast to these results, a relatively recent study reported that AhR ligands could actually stimulate ER-dependent gene expression. In this study, the AhR ligand 3-methylcholanthrene (3MC) reportedly could produce opposing effects on ER action, stimulating gene expression by ligand-free ERs while attenuating that regulated by estrogen-bound ERs (Ohtake et al., 2003). It was concluded that activation of unliganded ER-dependent gene expression was modulated by a co-regulatory–like function of activated AhR:ARNT complexes, whereas the antiestrogenic effects on liganded ERs were mediated by one or more of the multiple mechanisms described above. However, several subsequent studies demonstrated that 3MC was actually an ER agonist (Abdelrahim et al., 2006; Liu et al., 2006; Shipley and Waxman, 2006), and its ability to directly bind to and activate unliganded ERs likely accounted for the stimulatory effects reported by Ohtake et al. (2003). The ability of other AhR agonists to also directly bind to and activate ERα was observed in these studies and indicates an additional level of apparent cross talk between these pathways (Abdelrahim et al., 2006; Liu et al., 2006). Taken together, the results of extensive analysis of AhR:ER cross talk described above have revealed multiple nonclassical mechanisms by which the AhR can modulate signal transduction in a manner distinct from the classical AhR:ARNT:DRE–dependent mechanism. Accordingly, given the diversity in AhR response, it is likely that similar types of cross talk between the AhR and other nuclear receptors and transcription factors will be identified that will contribute to its specificity of toxic and biological responses. In fact, as described in the following sections, similar classical and nonclassical AhR mechanisms are involved in mediating the effects of TCDD and other AhR ligands on other cell signaling and biochemical pathways.

AhR-DEPENDENT MODULATION OF CELL PROLIFERATION AND CELL CYCLE

Cell proliferation, like many of the biological and toxicological responses observed following ligand-dependent activation of the AhR, can be differentially affected in a tissue- and cell-dependent manner. The cell cycle is a progressive series of five distinct phases of cell replication that are controlled by a collection of protein and transcription factors including the E2 promoter–binding factor (E2F), the retinoblastoma protein (Rb), cyclins, and cyclin-dependent kinases (CDKs) (Johnson and Walker, 1999), with numerous regulatory feedback checkpoints. One critical control point for entry of cells into the synthesis (S) phase of the cell cycle is CDK-mediated hyperphosphorylation of Rb, which is then released from E2F, leading to E2F derepression and increased transcription of E2F-dependent S-phase–specific genes. Although TCDD- and AhR-dependent inhibition of cell proliferation and cell cycle arrest have been reported in several different cell systems, the ability of TCDD to stimulate cell proliferation has also been reported (Marlowe and Puga, 2010; Puga et al., 2009; Watabe et al., 2010). Repression of cell cycle appears to involve a combination of classical and nonclassical AhR-dependent mechanisms. Classical AhR- and DRE-dependent mechanisms of induction of expression of the genes for the CDK inhibitory factors p27kip1 and p21Waf1/Cip1 lead to inhibition of the activity of several key CDKs and ultimately inactivation of Rb, the key regulator of E2F activity (Kolluri et al., 1999; Pang et al., 2008). Ligand-activated AhR was also shown to repress cell cycle progression through its ability to directly bind to hypophosphorylated Rb, preventing its phosphorylation by CDKs and release from E2F, repressing transcription of E2F-dependent genes needed for cell cycle progression (Barnes-Ellerbe et al., 2004; Huang and Elferink, 2005). Additionally, direct interactions between the AhR and E2F are also reported to recruit AhR to E2F-regulated gene promoters, with a concomitant loss of the histone acetyl transferase p300 (Marlowe et al., 2004), providing an additional avenue in which the ligand-activated AhR can repress E2F-dependent gene expression. In contrast, TCDD has been shown to stimulate proliferation of human lung cancer (A549) cells and while this positive regulatory effect was also mediated by an interaction of the AhR with E2F, in these cells, the AhR functioned as a potent coactivator that recruits positive regulatory factors (Watabe et al., 2010). Although the mechanisms contributing to these opposing responses are unknown, they demonstrate that the ultimate effect of TCDD and the AhR on cell proliferation is cell-type dependent. The results of several studies support an endogenous role for the AhR in modulating cell cycle in the
absence of exogenous ligands but in a cell-type–dependent manner that suggests that the activation of the AhR is more complicated. Although fibroblasts from AhR (−/−) mice that expressed an exogenously added AhR could proliferate significantly faster than cells that lacked AhR, the addition of TCDD or deletion of the AhR ligand-binding domain (LBD) of the transfected AhR did not alter their increased proliferation rates, indicating that the proliferation response was AhR dependent but ligand independent (Chang et al., 2007). Interestingly, although knock down of the AhR could stimulate cell cycle and proliferation in human breast cancer (MCF-7) cells, knock down of the AhR in human hepatoma (HepG2) cells blocked cell cycle progression and inhibited cell proliferation (Abdelrahim et al., 2003). Taken together, the above results not only suggest an endogenous role for the AhR in regulating cell cycle and cell proliferation but also the effect of AhR ligands appears to be mediated through a combination of both classical and nonclassical mechanisms, with the ultimate response and responsible mechanism(s) being dependent upon the specific phenotype of the cell.

**LIGAND AND AhR-DEPENDENT MODULATION OF INTRACELLULAR SIGNALING PATHWAYS**

There have been an extensive number of studies demonstrating interactions and cross talk between the AhR and various intracellular signaling pathways, including protein kinases, receptor tyrosine kinases, mitogen-activated protein kinases (MAPKs), c-Src kinase, phosphodiesterase 2A, calcium signaling pathways, NF-κB signaling, and many others (de Oliveira and Smolenski, 2009; Haarman-Stemmann et al., 2009; Matsumura, 2009; Park et al., 2007; Puga et al., 2009). In contrast to the better understood interactions between the AhR and the ER and cell cycle signaling pathways described above, the mechanisms of cross talk between the AhR and many of these intracellular signaling pathways remain to be elucidated. The NF-κB pathway is one cell signaling pathway that has received considerable attention due to its ability to be activated by a variety of stimuli. Its diverse physiological functions, many of which are affected by TCDD and other AhR ligands, include cell cycle, differentiation, and immune and inflammatory responses. In mammals, five NF-κB transcription factors including V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog A and B (RelA [p65] and RelB, respectively), c-Rel, p50 and p52 together with the inhibitory κB (IκB) proteins, IκB kinases, and cytokine receptors, comprise a complex regulatory cascade activated by a variety of cytokines and whose transcription is dependent upon NF-κB–responsive promoters (Hayden and Ghosh, 2008). Once released from their inhibitory IκB subunits, homo- or heterodimers of the NF-κB transcription factors can translocate into the nucleus and regulate target gene expression. The immunosuppressive effects of TCDD and related compounds prompted studies of possible cross talk between the AhR and NF-κB pathways and revealed mutual transrepression of DRE- and NF-κB–driven reporter genes in transient transfections (Tian, 2009). Subsequent studies demonstrated direct protein binding between AhR and RelA (no RelA:ARNT interactions were observed) and it was proposed that the transrepressive effects were mediated by formation of transcriptionally inactive AhR:RelA dimers that also reduced the concentration of available nuclear AhR and RelA necessary for normal AhR- and NF-κB–mediated gene expression (Tian, 2009). Additionally, because activated nuclear AhR:ARNT and NF-κB complexes can utilize common coactivators (i.e., SRC1 and p300/CBP) for maximal transcriptional activation, competition for the coactivators could result in “squelching” of gene expression by one or both complexes, similar to that which occurs between the AhR and ER or the glucocorticoid receptor and NF-κB (Sheppard et al., 1998). In contrast to RelA, direct interactions between the TCDD-activated AhR and RelB also occur and were reported to produce an AhR:RelB complex that could bind to a specific AhR:RelB DNA recognition site (Fig. 2) and stimulate transcription of inflammatory genes such as interleuken 8 (Vogel et al., 2007). Interestingly, in these studies, AhR:RelB complexes could bind to and stimulate expression from DRE and NF-κB–binding sites, although whether this occurred as part of a larger protein complex with ARNT and/or other NF-κB transcription factors remains to be determined. Together, these results reveal another novel nonclassical mechanism by which the AhR can produce biological responses through cell signaling pathways.

The ability of TCDD and other AhR ligands (i.e., PAHs) to produce both rapid (within minutes) and sustained calcium influx into exposed cells (Karras et al., 1996; Puga et al., 1997), coupled with an ability of TCDD to produce biological effects in AhR knockout mice and alter gene expression in AhR (−/−) cells, suggests that these ligands can also produce rapid and significant effects in an AhR-independent manner (Tijet et al., 2005). Interestingly, although recent evidence has indicated that TCDD can increase intracellular calcium levels through its ability to open plasma membrane calcium channels and by releasing intracellular calcium stores through an action on ryanodine receptors (Biswas et al., 2008; Kim et al., 2009), the exact mechanism by which TCDD and perhaps other AhR ligands accomplish this remains an open question. However, ligand-dependent increases in intracellular calcium can produce diverse intracellular signaling events that can contribute to AhR-dependent signaling. Calcium can stimulate protein kinase C (PKC) activity and calcium-dependent stimulation of cAMP production from adenylate cyclase. Not only can increased intracellular PKC activity synergistically enhance the transcriptional activity of ligand-activated AhR (Chen and Tukey, 1996; Long et al., 1998), but cAMP can also reportedly directly activate the AhR by an as yet undefined mechanism (Oesch-Bartlomowicz et al., 2005). Additionally, PKC and cAMP result in activation of protein kinase A (PKA) that can
produce its own diverse spectrum of intracellular responses that could contribute to the overall effects of AhR ligand exposure. In fact, recent studies have reported a role for PKA in the inflammatory response to TCDD and other AhR agonists and involvement of the AhR in stimulating the cAMP and PKA pathway (Li and Matsumura, 2008; Matsumura, 2009). The ability of PKA to stimulate activity of the epidermal growth factor receptor (EGFR) and its downstream signaling events, coupled with the ability of PKA and the AhR pathways to stimulate expression of known EGFR ligands would contribute to EGFR-dependent activation of the MAPK pathway and MAPK-dependent changes in gene expression (Haarmann-Stemmann et al., 2009; Marlowe and Puga, 2010; Puga et al., 2009). Expression of prostaglandin-endoperoxide synthase 2/cyclooxygenase 2 (PTGS2/Cox-2) can be stimulated both directly by the classical AhR signaling pathway and indirectly through activation of the EGFR-mediated pathway (Kraemer et al., 1996; Matsumura, 2009). AhR-dependent induction of Cox-2 expression was shown to be mediated by nonclassical and nongenomic mechanisms in transgenic mice that contained a mutant AhR that was unable to translocate into the nucleus (Li et al., 2010a). Although the mechanisms responsible for most of the effects of AhR ligands on these cell signaling pathways remain to be elucidated, these results highlight additional levels of complexity and diversity in the response of cells to AhR ligands and are exciting areas of future research.

LIGAND-DEPENDENT MECHANISMS AND EFFECTS OF PERSISTENT AhR ACTIVATION

The above mechanisms highlight some of the pathways that can be affected by the AhR and how AhR ligands can contribute to the observed diversity and complexity in AhR response; however, not all ligands produce the same spectrum of biochemical and toxicological responses between species. Although the best-characterized high-affinity ligands for the AhR include numerous HAHs, PAHs, and PAH-like chemicals, a relatively large number of natural, endogenous, and synthetic AhR agonists have also been identified in recent years whose structure and physicochemical characteristics are dramatically different from the prototypical HAH and PAH ligands (Denison and Nagy, 2003; Denison et al., 1998a; Nguyen and Bradfield, 2008), suggesting that the AhR has an extremely promiscuous ligand-binding pocket. Interestingly, these studies have not only revealed significant differences in the specificity of AhR ligand binding but also ligand- and species-specific differences in AhR-dependent gene expression (Aarts et al., 1996; Denison and Wilkinson, 1985; Denison et al., 1986, 1998a).

HAHs, such as TCDD and related halogenated DLCs, represent the best-characterized and highest affinity ligands for the AhR (with binding affinities in the pM to nM range), and although these compounds can produce the full spectrum of AhR-dependent toxic and biological effects, significant species differences in these responses exist. In contrast, there are numerous PAHs and PAH-like chemicals that are also relatively high-affinity AhR ligands (with binding affinities in the low nM to μM range), and although they can produce a similar spectrum of biological effects as the HAHs (i.e., alteration in gene expression and others), these chemicals rarely produce the spectrum of what we define as classical TCDD-like AhR-dependent toxic effects (i.e., wasting, teratogenicity, dermal toxicity, lethality, thymic involution; Table 1). One major difference between these classes of chemicals is that HAHs are poorly metabolized, whereas PAHs and most other structurally diverse AhR ligands are readily degraded by metabolism. The primary mechanism of toxicity of PAHs involves their metabolic activation by CYPs into reactive metabolites (Conney, 1982; Nebert and Jensen, 1979). Although it is well established that TCDD and related HAHs induce persistent activation of AhR-dependent gene expression, PAHs, PAH-like chemicals, and other structurally diverse ligands typically induce AhR-dependent gene expression only transiently. These results, combined with the observation that metabolically labile AhR ligands can also affect the nonclassical AhR signaling pathways described above and the observation that inhibition of their metabolism can significantly increase the magnitude and longevity of their induction response (Chiaro et al., 2007; Wei et al., 2000; Luecke et al., 2010), strongly suggest that the ability of a ligand to produce AhR-dependent toxicity is predominantly driven by its resistance to metabolism and ability to persistently activate AhR-dependent signaling in the responsive cells.

How does the metabolic stability of an AhR ligand contribute to persistent activation of the AhR signaling pathway? The essentially irreversible binding of TCDD to the AhR (Bradfield et al., 1988; Henry and Gasiewicz, 1993), combined with the requirement for ligand:AhR:ARNT:DRE complex formation in AhR-dependent gene expression initially, suggested that AhR bound by persistent ligands might have longer nuclear residence times and thus more persistent gene expression. However, the similar extremely slow rate of dissociation of β-naphthoflavone (BNF), a nontoxic PAH-like AhR ligand, suggests that other AhR ligands exhibit similar characteristics of persistent binding (Bohonowycz and Denison, 2007). Additionally, several studies have demonstrated the existence of multiple mechanisms that can contribute to downregulation of AhR nuclear signaling events, including nuclear export of the AhR and its subsequent proteolytic degradation (Pollenz, 2002) (with release of the ligand into the cytosol) and AhR-dependent induction of expression of the AhR repressor (AhRR) protein (Baba et al., 2001). AhRR is a ligand-independent AhR-like protein that constitutively translocates into the nucleus and dimerizes with ARNT and through both competition for ARNT and nonproductive binding of AhRR:ARNT complexes to DREs, AhRR can reduce ligand:AhR:ARNT complex formation and prevent...
binding by functional ligand:AhR:ARNT complexes that could activate gene transcription (Baba et al., 2001; Hahn et al., 2009). Together these activities can reduce overall AhR signaling by decreasing nuclear AhR concentrations, decreasing the concentration of ARNT available for AhR dimerization and reducing available DRE-binding sites as a result of their occupancy by AhRR:ARNT (Fig. 1). However, although these mechanisms can reduce AhR signaling, the demonstration of persistent gene expression indicates that they do not block it completely. Accordingly, this suggests that persistent AhR signaling primarily results from the continued expression and synthesis of nascent AhR complexes. In this scenario, AhR-dependent induction of CYP1A1 and other metabolic enzymes contribute to the degradation of the nonpersistent AhR ligands, leading to a time-dependent reduction in their intracellular concentration and ability of these ligands to bind to and activate newly synthesized AhR complexes. In contrast, metabolically stable AhR ligands are not readily degraded, and intracellular concentrations can be maintained at a relatively stable level that allows for continual activation and nuclear translocation of newly synthesized AhR complexes and essentially result what appears to be an irreversible activation of AhR-dependent gene expression. Thus, the time course of induction by a given AhR ligand is a combination of its relative affinity for the AhR and its intracellular concentration, with metabolically stable ligands (such as TCDD) producing a greater spectrum of time-dependent toxic and biological effects. Although the metabolic persistence of AhR agonists appears to be the major characteristic of a ligand that leads to AhR-dependent toxic effects, other factors and pathways will certainly be identified that contribute to these effects.

THE AhR LBD AND LIGAND SPECIFICITY OF BINDING

A molecular understanding of AhR ligand binding, ligand specificity, and the mechanisms of AhR activation by diverse ligands requires detailed structural information about the AhR PAS B LBD. Because of an inability to obtain sufficient amounts of purified AhR protein, X-ray or nuclear magnetic resonance (NMR)—determined structures of the liganded or unliganded AhR are lacking, and this has represented a major limitation to furthering our understanding of the mechanisms of AhR action. However, the availability of crystal and NMR structures of homologous proteins of the PAS superfamily allowed development of theoretical models for the LBD of the mouse AhR by applying homology modeling techniques (Pandini et al., 2007). The most recent homology model of the mouse AhR LBD (Fig. 3; Pandini et al., 2009) was built using the NMR-determined structures of the PAS B domains of HIF-2α (Erbel et al., 2003) and ARNT (Card et al., 2005) proteins as templates, given their higher degree of sequence identity and similarity with the AhR PAS B domain and their functional relationship to the AhR (Kewley et al., 2004). Comparison of the LBD models developed for six mammalian AhRs, which exhibit high affinity for TCDD, also revealed that the physicochemical characteristics and residues of the modeled internal cavity spaces were well conserved in each, despite some significant differences in the residues within each LBD (Pandini et al., 2009). Site-directed mutagenesis, functional analysis, and ligand-docking studies were used to validate the improved AhR LBD model and to identify the residues needed for optimal TCDD binding (Fig. 3) (Bisson et al., 2009; Goryo et al. 2007; Murray et al., 2010a; Pandini et al., 2007, 2009). These analyses as well as future improvements in the AhR LBD model will provide avenues in which to examine mechanisms contributing to the observed structural diversity of AhR ligands, species differences in ligand-binding specificity, and the mechanisms of ligand-dependent AhR signaling.

The AhR can bind and be activated or inhibited by a remarkably wide variety of structurally dissimilar compounds (Denison and Nagy, 2003; Denison et al., 1998a; Nguyen and Bradfield, 2008), and significant species-specific differences in ligand-binding specificity and ligand-specific AhR functionality have been reported (Aarts et al., 1996; Denison and Wilkinson, 1985; Denison et al., 1986, 1998a). A selection of structurally diverse AhR ligands described in the following sections are presented in Figure 4. Given the restrictions of the AhR LBD model, it remains an open question as to how the AhR LBD can accommodate such a diversity of ligand structures and how binding of these ligands results in differential AhR activity. The extreme structural diversity of AhR ligands is similar to the ligand promiscuity reported for some members of the nuclear hormone receptor superfamily, such as that of the pregnane X receptor (Noy, 2007), and by analogy suggests that AhR ligand-binding promiscuity may results from differential binding of ligands within the AhR ligand-binding pocket. This hypothesis is supported by structure-activity relationship analysis of a large group of structurally diverse AhR agonists that suggested the presence of at least two distinct binding patterns, one that is similar to TCDD and one that is similar to coplanar polychlorinated biphenyls (PCBs) (Petkov et al., 2010). More recently, a novel ligand-selective AhR antagonist (CH223191) was identified that preferentially inhibits the activity of TCDD and related HAHs to bind to and activate the AhR and AhR-dependent gene expression but has little inhibitory effect on the AhR agonist activity of BNF, PAHs, flavonoids, and indirubin (Zhao et al., 2010). These results suggest differences in binding by these groups of AhR agonists. Although it is clear from previous 3H-TCDD competitive binding studies in many laboratories that HAH and non-HAH AhR ligands can directly compete with each other for binding to the AhR and thus must have identical or overlapping binding sites and/or common binding residues within the ligand-binding pocket, CH223191 must interact with the AhR in such a way as to preferentially inhibit the binding of HAHs, but not non-HAH ligands. Similar
The preferential antagonism of HAHs by these inhibitors is consistent with the hypothesis that significant differences exist in the binding of the HAHs and non-HAH AhR agonists within the ligand-binding pocket. Differential binding of structurally diverse ligands within the AhR LBD is supported by site-directed mutagenesis/ligand-binding analysis studies (Backlund and Ingelman-Sundberg, 2004; Goryo et al., 2007; Whelan et al., 2010) and the structures of selected ligands are shown in Figure 4. Mutation of a conserved tyrosine residue into phenylalanine (Y320F) in the human AhR LBD (comparable to residue 316 in the mouse AhR) reportedly resulted in the selective loss of binding and/or activation by several low-affinity non-HAH AhR ligands (i.e., 2-mercapto-5-methoxybenzimidazole, primaquine, and omeprazole), but not by the high-affinity ligand TCDD (Backlund and Ingelman-Sundberg, 2004). In contrast, insertion of a single mutation at a closely associated position into the mouse AhR LBD (F318L) reportedly produced a receptor that could be activated by the ligand 3MC, but not by BNF or TCDD (Goryo et al., 2007). In the homology model of the mouse AhR LBD (Pandini et al., 2007, 2009), these mutations reside in the same small alpha helix (Ea) present at the top of the ligand-binding cavity (Fig. 3). Interestingly, although the loss of the aromatic group in the F318L mutation eliminates binding by TCDD/BNF (Goryo et al., 2007), the Y316F mutation, which retains an aromatic ring in this position, eliminates binding by low-affinity non-HAH ligands, but not TCDD (Backlund and Ingelman-Sundberg, 2004). These results, combined with previous mutagenesis results (Pandini et al., 2007, 2009), indicate that aromatic residues in these positions are critical for AhR ligand binding/functional activity and that changes in the specific residues within this helix differentially affect the binding of different ligands. Another key residue, histidine 285, is contained within a central strand of the beta sheet (Aβ) of the LBD with its side chain pointing into the center of the binding cavity (Fig. 3). Previous studies (Pandini et al., 2009) suggest that it plays a role in stabilizing TCDD binding through aromatic interactions because its mutation to alanine eliminated ligand binding and ligand-dependent activation and mutation to phenylalanine only reduced the potency/affinity of TCDD. Interestingly, the results of Whelan et al. (2010) indicate that the presence of a tyrosine residue in this position allows binding and activation of the AhR by the novel agonist YH439, but not TCDD, consistent with distinct differences in the specific binding of these two agonists within the ligand-binding pocket. Taken together, these studies support the hypothesis that the observed structural promiscuity of AhR ligands is derived, at least in part, from differential binding by ligands or classes of ligands within the AhR ligand-binding pocket. This differential binding could lead to ligand-dependent differences in the overall structure of the activated AhR that may contribute to differences in its overall functionality (i.e., differences in coactivator recruitment and transcriptional activity), similar to that reported for some steroid hormone receptors (Kazmin et al., 2006; Ozers et al., 2005).

FIG. 3. Homology model of the mouse AhR LBD and residues critical for TCDD ligand binding.

SPECIES-SPECIFIC DIFFERENCES IN AhR LIGAND BINDING AND FUNCTION

Mutagenesis and functional analysis based on the current mouse AhR LBD model has provided a framework for studies into the mechanisms of ligand binding and activation of the AhR from other species; however, similar approaches will be necessary to understand the mechanisms responsible for species variation in ligand binding and ligand-dependent responsiveness. Although it is apparent that some of the species diversity in response to AhR ligands can be attributed to differences in species-specific biochemical and physiological characteristics (particularly as they relate to differences in ligand pharmacokinetics, pharmacodynamics, and metabolism), the binding specificity and rank-order potency of many ligands are similar for AhRs among species and tissues, but
they are not identical (Aarts et al., 1996; Denison and Wilkinson, 1985; Denison et al., 1986; Head et al., 2008). For example, although TCDD-inducible, AhR-dependent gene expression and TCDD binding to the mouse AhR have been shown to be antagonized by several di-ortho PCBs, these compounds only partially antagonized the rat AhR and did not exhibited antagonistic effect on human or guinea pig AhRs (Aarts et al., 1996). Similarly, species-selective AhR antagonism by the purine derivative StemRegenin 1 (SR1) (Fig. 4) was recently reported, with complete inhibition of TCDD-inducible AhR-dependent transcription by SR1 observed in human cells, weak inhibition in mouse cells, and no effect on rat AhR signal transduction (Boitano et al., 2010). Interestingly, although a series of benzimidazole drugs (omeprazole, thiabenzazole, and lansoprazole) can stimulate AhR-dependent gene expression in human hepatoma (HepG2) cells, they were unable to activate the AhR in mouse hepatoma (Hepa1c1c7) cells (Kikuchi et al., 1996). Species-specific differences in ligand binding have also been observed with a series of PAHs (Denison et al., 1986) and single hydroxylated benzo(a)pyrene molecules (Denison and Wilkinson, 1985). Other examples include the ability of phenobarbital and gamma-amino butyric acid to bind to and activate the rainbow trout AhR (Sadar et al., 1996a,b), while not affecting mammalian AhRs, and studies

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<tr>
<th>HAH AhR Agonists</th>
<th>PAH and PAH-like AhR Agonists</th>
<th>AhR Agonists</th>
<th>AhR Antagonists</th>
<th>Selective AhR Modulators (SAhRMs)</th>
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<tr>
<td><img src="image1" alt="2,3,7,8-Tetrachlorodibenzo-p-dioxin" /></td>
<td><img src="image2" alt="3,4,3',4',5-Pentachlorobiphenyl" /></td>
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<td><img src="image7" alt="Benzo(a)pyrene" /></td>
<td><img src="image8" alt="Indirubin" /></td>
<td><img src="image9" alt="CH223191" /></td>
<td><img src="image10" alt="SGA560" /></td>
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<td><img src="image11" alt="3,3'-Diindolylmethane" /></td>
<td><img src="image12" alt="6-Methyl-1,3,5-Trichlorodibenzofuran" /></td>
<td><img src="image13" alt="YH439" /></td>
<td><img src="image14" alt="GNF351" /></td>
<td><img src="image15" alt="3',4'-Dimethoxy-α-Naphthoflavone" /></td>
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**FIG. 4.** Representative structures of functional classes of AhR agonists, antagonists, and SAhRMs.
identifying mono-ortho PCBs that can activate the human AhR, but were ineffective in activating rainbow trout and zebrafish AhRs (Abnet et al., 1999).

Although the majority of AhR LBD modeling has been done using the mouse AhR sequence, LBD models for rat, human, and zebrafish AhRs have also been reported and comparison of the primary sequence and homology models of the LBD from several species reveals significant variation that could contribute to species differences in ligand binding and response (Bisson et al., 2009; Pandini et al., 2009). Mutagenesis studies have already begun to identify several amino acids that contribute to species-specific differences in ligand binding. Although 3′-methoxy-4′-nitroflavone is a antagonist/partial agonist of TCDD-induced AhR DRE binding and reporter gene induction in rat cells, it is a full agonist in guinea pig cells, and this species variation appears to result from a single amino acid difference in the LBD of these two species (R355 in the mAhR and I360 in the guinea pig AhR [Henry and Gasiewicz, 2008]). Similarly, Hahn and coworkers (Karchner et al., 2006) demonstrated that the 250-fold lower TCDD sensitivity of the tern AhR, compared with the chicken AhR, was due to a two-amino acid difference in the tern LBD (Val-325 and Ala-381) that reduced the affinity of ligand binding. Similar differences have been observed in a broad range of other avian species (Head et al., 2008). The binding affinity of the human AhR and DBA mouse AhR (the AhR^b allele) is 5-10-fold lower than that of the high-affinity C57 mouse AhR (i.e., the AhR^a allele) and results from a valine residue at the same position in the middle of the binding pocket of the human and DBA mouse AhR LBDs that is an alanine in the high-affinity mouse AhR^b allele (position 381 in the human AhR and 375 in the mouse AhR) (Pandini et al., 2007; Poland et al., 1994). The role of this residue was confirmed by a significant decrease in the binding affinity of TCDD to a C57 mouse AhR containing an alanine to valine mutation at position 375 (Pandini et al., 2007; Poland et al., 1994). More recently, Perdew and coworkers (DiNatale et al., 2010; Flaveny and Perdew, 2009; Schroeder et al., 2010) demonstrated that the human AhR exhibited a significantly higher ligand-binding affinity and responsiveness to a variety of natural and putative endogenous ligands (i.e., indirubin, quercetin, kynurenic acid and 3-indoxyl sulfate) compared with that of the mouse AhR, although the responsible amino acids that contribute to this species specificity remain to be determined. Species differences in amino acids within the AhR itself can contribute to variations in ligand-binding specificity, ligand-dependent gene expression, and thus the spectrum of biological and toxic responses. As described above, microarray analysis revealed dramatic differences in the number and spectrum of gene products induced and repressed by TCDD treatment in mouse hepatocytes expressing either human or mouse AhRs, with only a moderate number of genes regulated by both receptors (Flaveny et al., 2010), and these results are not only consistent with differences in how each AhR modulates gene expression but they demonstrate that results from studies carried out in one species may not accurately predict the response in another. Thus, differences in the AhRs, their associated regulatory factors, and signaling mechanisms are major contributors to the dramatic species variation observed in the biochemical and toxic effects of TCDD and other AhR ligands.

The importance of factors other than variations in the AhR itself in modulating overall AhR responsiveness is clearly evidenced from studies of the biological and toxic effects of TCDD and other DLCs in humans. Available epidemiological evidence and results from in vitro analyses suggest that humans are significantly less sensitive than most mammalian species with regards to TCDD-dependent toxicity, and although this reduction in sensitivity is due in part to the lower binding affinity reported for the human AhR, other modulatory factors appear to play a key role (reviewed in Connor and Aylward, 2006; Okey, 2007; Sweeney and Mocarelli, 2000). For example, examination of human lung samples from smokers revealed dramatic differences (up to 200-fold) in CYP1A1-dependent activity between donor samples, with CYP1A1 undetectable in some samples (Anttila et al., 2000, 2001). Considering the results of TCDD ligand-binding analysis of human placental samples which demonstrated that the relative affinity of the human AhR for TCDD can differ by more than 10-fold (Harper et al., 2002; Nebert et al., 2004), this variation in relative response may not be surprising. Interestingly, however, sequence analysis revealed no identified polymorphism in the AhR, ARNT, CYP1A1, and/or CYP1A1 promoter region that could account for the observed differences in the TCDD-binding affinities in any of these studies. Similarly, studies of TCDD-exposed humans from an industrial accident at Seveso, Italy, did not reveal an obvious relationship between serum concentrations of TCDD levels and the development of chloracne (Mocarelli et al., 1991), suggesting involvement of additional regulatory mechanisms that contribute to this AhR-dependent toxic effect. The lower overall sensitivity/response of humans to the adverse effects of TCDD and other AhR ligands is due in part to the lower ligand-binding affinity of the human AhR; however, although other factors and mechanisms must clearly contribute to this diversity in response, they have yet to be identified.

**LIGAND-SELECTIVE MODULATION OF AhR FUNCTIONALITY**

The structural diversity and differential binding of AhR ligands and the diversity in mechanisms of action described above suggest the existence of ligands that act as selective modulators of the AhR, similar to that reported for nuclear steroid hormone receptors. The functional activity of nuclear hormone receptors can be altered in a ligand-selective manner, and these functional changes appear to be directly related to ligand-specific changes in the overall structure of the receptor...
that directly affects its specific interaction with nuclear factors (i.e., coactivators) that can interact with it, thus altering the overall biological response of the receptor (De Bosscher, 2010; Norris et al., 2009). Similarly, given the established ligand promiscuity of the AhR and differences in binding of ligands within the AhR LBD, one can envision ligand-selective alterations in its structure that differentially modulate AhR function (i.e., the existence of selective AhR modulators [SAhRMs]). In fact, several SAhRMs have been identified based on their ability to selectively produce some AhR-dependent responses and not others (Fig. 4). The compounds 3,3'-diindolylmethane (DIM) and 6-methyl-1,3,8-trichlorodibenzo-furan (MCDF) are classic SAhRMs in that they produce AhR-dependent antiestrogenic activity in vitro and in vivo with minimal induction of classical AhR-responsive genes (i.e., CYP1A1), whereas TCDD and other full AhR agonists produce both responses (Chen et al., 1998; McDougal et al., 1997). Murray et al. (2010a,b, 2011) described novel SAhRMs, namely WAY-169916, SGA360, and 3',4'-dime-thoxy-α-naphthoflavone (DMF), that repressed expression of a variety of acute phase inflammatory genes (namely several of those for serum amyloid A [SAA1, 2 and 3]) in an AhR-dependent manner, yet produce little or no AhR:ARNT:DRE binding or DRE-dependent transcriptional activation. TCDD was also shown to repress SAA gene expression in an AhR-dependent manner, and subsequent studies using targeted AhR mutagenesis revealed that the repressive effect of TCDD was a direct effect on SAA gene expression and required AhR nuclear translocation and dimerization with ARNT (Patel et al., 2009). Interestingly, repression by TCDD did not depend on AhR DNA-binding activity, as repression of SAA gene expression was still observed when the AhR contained a mutation that eliminated its ability to bind to DRE-containing DNA (Patel et al., 2009). The ability of several SAhRMs (WAY-169916, SGA 360 and DMF) to antagonize classical AhR- and DRE-dependent gene expression by TCDD and to repress acute phase inflammatory gene expression in a non-classical AhR-dependent and DRE-independent manner identify them as a unique class of AhR ligands (Murray et al., 2010a,b, 2011). Although the exact mechanism(s) by which SAhRMs produce their novel nonclassical repressive effects is unclear, ChIP analysis showed that AhR activation could alter the presence of the RelA, the (p65) subunit of NF-κB, and CAATT-enhancer–binding protein at the SAA gene promoters, but no AhR DNA binding was observed. The ability of ligand-activated AhR to also repress induction of various acute phase genes (including cAMP-receptor protein, lipopolysaccharide-binding protein, haptoglobin, α2-macro-globin, and α-1-glycoprotein-1) suggests that AhR represses these genes though a common but unidentified nonclassical (DRE independent) mechanism of action (Patel et al., 2009). Interestingly, identification of GNF351 as a pure antagonist of the AhR (i.e., one that inhibits both the action of AhR agonists and SAhRMs; Smith et al., 2011) suggests that there must exist some subtle differences in the binding of a given ligand within the AhR-binding pocket that result in it being an agonist, antagonist, or SAhRM. Whether other previously identified AhR antagonists can also produce complete inhibition of AhR action remains to be determined. Taken together, the above results suggest that differential ligand binding can result in the AhR assuming unique ligand-dependent protein conformations that allows it to interact with distinct subsets of nuclear factors to induce and/or repress expression of different genes in both DRE-dependent and -independent manners. Consistent with this hypothesis are the results of limited proteolysis studies that demonstrate a difference in the structure of the AhR when it is bound by an agonist or antagonist (Henry and Gasiewicz, 2003). Additionally, treatment of cells in culture with geldanamycin (an inhibitor that disrupts hsp90-AhR interactions) can also produce an artificially transformed (i.e., activated) AhR that is structurally distinct from the ligand activated AhR (Lees and Whitelaw, 1999).

If the AhR can assume ligand-specific conformations, then differential recruitment of coactivator proteins and/or other coregulatory factors would be predicted. Although the AhR antagonist (partial agonist) DIM has been shown to be less efficient at recruiting RNA polymerase II to the CYP1A1 gene promoter as compared with BNF, no difference in recruitment was observed between DIM and TCDD. This may reflect a true ligand-specific difference between BNF and TCDD or may result from cell line– or other tissue-specific differences (Hestermann and Brown, 2003). More recently, ligand-specific differences in coactivator recruitment were observed for AhRs bound by different HAHs (i.e., polychlorinated dioxins, furans, or biphenyls) (Zhang et al., 2008). This result was surprising and raised the possibility that even within a structurally related group of AhR ligands (agonists) minor physiochemical/structural differences could produce a unique AhR conformation/functionality, recruit different coactivators, and result in differential gene transcription. Interestingly, the kinetics of AhR recruitment to DREs may be ligand specific, with BNF and DIM causing oscillatory recruitment of the AhR to the CYP1A1 promoter and enhancer regions, but TCDD does not (Hestermann and Brown, 2003; Matthews et al., 2005). However, although oscillatory recruitment of the AhR to the CYP1A1 promoter in human breast carcinoma (T47D) cells can be detected with the AhR ligands TCDD and 3MC if the time course is extended to 4.5 h, these effects become species- or cell line–specific as they were not observed in mouse hepatoma cells (Pansoy et al., 2010). Although current evidence is limited, it does suggest the existence of some ligand-dependent differences in AhR structure/function that can contribute to its diversity in response. Assuming that different ligands can induce a unique AhR conformation(s), then an additional possibility to consider is that ligand-dependent changes in AhR structure could lead to alterations in its nucleotide specificity of DNA binding.
Classically, the ligand:AhR:ARNT complex has been shown to bind to the DRE, which contains the extended core conserved sequence 5′-T/GNGCGTGA/C-3′ (Denison et al., 1998b; Swanson et al., 1995; Yao and Denison, 1992). This core sequence has been shown to be necessary, but not sufficient, for TCDD-induced AhR:ARNT complex binding and transcriptional activation, and nucleotides adjacent to the core sequence are also required (Lusska et al., 1993; Yao and Denison, 1992). Although early site-directed DRE mutagenesis failed to demonstrate any ligand-specific differences in AhR DRE-dependent DNA binding (Bank et al., 1992), several studies have suggested that this can occur. Gouéard et al. (2004) reported that a nonclassical DRE (4/5 consensus fit with the invariant DRE core sequence of 5′-GCGTGA-3′) present in the upstream region of the human paraoxonase 1 (Pon-1) gene was sufficient to allow the ligands 3MC and quercetin, but not TCDD, to stimulate AhR-dependent gene expression. Similarly, Matikainen et al. (2001) reported that two degenerate DREs present in the upstream regulatory region of the Bax gene were able to confer AhR-dependent responsiveness to a PAH metabolite (7,12-dimethylbenz[a]anthracene-3,4-dihydrodiol), but not to TCDD. However, in this study, insertion of a single-nucleotide mutation that restored the consensus DRE sequence reportedly restored TCDD responsiveness. Additionally, novel AhR-responsive DNA-binding sites that show little sequence similarities to the classical DRE sequence have been identified in the upstream region of the human ATP-binding cassette subfamily G member 2 gene and in the second intron of the mouse phospholipase A2α gene that reportedly confer ligand inducibility upon these genes (Kinehara et al., 2009; Tompkins et al., 2010). Application of various chromatin immunoprecipitation approaches allowed isolation of AhR-bound DNA fragments from intact cells exposed to TCDD and while these analyses resulted in enrichment of DRE-containing DNA fragments, numerous AhR-bound DNA fragments were isolated that lacked identifiable DRE sequences (De Abrew et al., 2010; Kinehara et al., 2008). Although these studies suggested the presence of alternative AhR DNA-binding sites in these latter DNA fragments, the specific binding sites and their functional activity were not determined. Although differences in the DNA-binding specificity of the AhR have been suggested from these studies, in vitro approaches to unambiguously identify AhR DNA-binding sites by PCR-based DNA-binding site selection analysis (Swanson et al., 1995) failed to identify any AhR DNA-binding site lacking a recognizable DRE consensus sequence. Additionally, although it is well established that ligand-dependent transcription factors, such as the steroid hormone receptors, can bind to imperfect or degenerate consensus sequences, we are not aware of any ligand-activated transcription factor that can bind to and/or stimulate gene expression from a unique DNA recognition site driven solely by the specific ligand to which it is bound. Although we are unable to explain the above results, based on existing information about nuclear receptors, ligand-dependent variation in receptor response more likely resides in the specific proteins that can differentially interact with the AhR when it is bound by various ligands rather than through an alteration of its DNA-binding specificity. However, detailed biochemical and functional characterization of these reported ligand-selective AhR DNA-binding sites and the ability of different ligands to dramatically alter the functional activities of the AhR is an exciting area of future research that may expand our understanding of the diversity in AhR response.

**CONCLUSIONS**

Our current understanding of the details of the molecular mechanisms of action of the AhR have been principally derived from research into the ability of TCDD and other chemicals to stimulate AhR-dependent gene expression (primarily that of CYP1A1) and the extension of this model to studies of other AhR ligand–dependent toxic and biological effects. The AhR not only plays a key regulatory role in a wide variety of endogenous physiological functions and processes, adaptive responses, and toxic effects but also AhR responses and responsiveness occur in cell-, tissue-, species-, and ligand-specific manner and can be complicated by ligand promiscuity, structural diversity, and functional activities of the AhR. It is clear that although the AhR and its classical mechanism of action is required for the majority of the toxic responses resulting from exposure to TCDD and related DLCs, nonclassical mechanisms certainly contribute to many of these responses. This review has highlighted the mechanistic complexity by which AhR ligands and components of the AhR signaling pathway can produce their diverse spectrum of toxic and biological effects. We have primarily emphasized those specific mechanisms of cross talk between the AhR and cellular signaling pathways where there is both clear evidence for direct interactions between the ligand, AhR, and/or ARNT with key components of these pathways and where these interactions result in some change in their functional or modulatory activity. However, additional AhR-dependent regulatory interactions have been observed and others are sure to be identified in the near future. Although not discussed in this overview, the reciprocal cross talk occurs between cell signaling and AhR pathways that can modulate both the level and functional activity of the AhR and alter the specific cellular responses and responsiveness to AhR ligands. The involvement of both classical and nonclassical mechanisms as contributors to the diversity in responses that are observed following ligand-dependent activation of the AhR signaling pathway, while well documented, requires additional research to further define the responsible mechanisms in greater detail.
Substantial research progress has been made over the past 35 years in our understanding of the molecular mechanisms of action of AhR ligands and the biochemical and toxic effects resulting from AhR activation. However, there still remain many unresolved questions that are critical for our understanding of the diversity in AhR responses and the role the AhR plays in both normal endogenous functions and in toxicity. Although it is clear that the AhR can bind and be activated by a structurally diverse array of chemicals, the extent and significance of this ligand promiscuity from both an adaptive and endogenous perspective remain to be established. Although a single high-affinity endogenous AhR ligand has not been identified, numerous endogenous agonists have been described and their structural diversity is consistent with the idea that multiple endogenous AhR ligands exist in different tissues at different times. The identification of the spectrum of endogenous AhR ligands and their physiological roles are important future research areas that will not only provide insights into both the endogenous functional activities of the AhR but also into its mechanisms of toxicity. Although knowledge of the structural diversity of AhR ligands is important, there is a critical need for accurate crystal/NMR protein structure information of the AhR LBD to help resolve issues of ligand-binding specificity and promiscuity and species differences in ligand binding. Additionally, the availability of structural information of the AhR LBD and other functional domains would facilitate studies into the basic mechanisms of ligand-dependent AhR activation and allow detailed analysis of ligand-dependent differences in AhR conformation, differential interactions with coactivators, corepressors, chromatin, and other protein factors and their role in the diverse biological and toxic effects produced by AhR ligands.

Beyond aspects of the AhR itself, an improved understanding of the diverse biochemical and molecular mechanisms of toxicity of TCDD and related DLCs is critically needed to allow adequate evaluation of human sensitivity to those compounds and to improve human risk assessment. Although a few detailed mechanisms of AhR-dependent toxic action have been elucidated, the demonstration that the major toxic effects of TCDD requires alterations in gene expression suggest that identification of such target genes can provide insights into these effects. High-throughput transcriptomic and genomic approaches should allow identification of the genes that are modulated by TCDD and responsible for or contribute to toxicity. However, the time delay in manifestation of TCDD-dependent toxicity coupled with the large number of induced and repressed primary and secondary TCDD-responsive genes has complicated this approach and slowed progress in this area. Such approaches do provide an avenue in which to identify all the gene products whose concentration is altered in response to TCDD (by classical and nonclassical mechanisms), and future studies will allow investigators to build upon these extensive analyses to increase our understanding of the responsible mechanisms. Such studies can also further define the role that the nonclassical genomic and nongenomic AhR-dependent mechanisms play in modulating the effects of endogenous and exogenous ligands. Comparative transcriptomic analysis of TCDD- and AhR-dependent gene expression in tissues of various species is necessary to facilitate the identification of both common and species-specific gene products important in the biological and toxic effects of AhR ligands. Additionally, the ability of these microarray studies to identify genes regulated by the AhR in the absence of exogenous ligand can provide novel insights into the endogenous physiological functions of the AhR. The application of genomic, transcriptomic, proteomic, and metabolomic approaches, coupled with recent developments in bioinformatics, is certain to facilitate the future identification and characterization of gene products involved in AhR-dependent toxic and biological effects. These approaches will also provide important avenues for comparative studies that could help answer what is perhaps the most well-known open question in AhR biology and that is determining what is responsible for the dramatic species differences in the sensitivity to toxicity of TCDD and related DLCs. For example, it remains to be determined what accounts for the dramatic 5000-fold greater resistance of hamsters to the lethal effects of TCDD as compared with guinea pigs. Numerous other species differences in ligand-dependent AhR responsiveness and endpoint responses have been reported, and as described above, current evidence suggests that humans appear to be less sensitive to TCDD than most species and that there are significant differences between humans in their responsiveness to these chemicals. Does the striking difference in responsiveness results from differences in the structure and function of the AhR and/or its associated factors or are these differences determined by variations in classical and nonclassical AhR-dependent mechanisms? Although the use of transgenic mice containing human AhRs have already provided some novel mechanistic insights into the specific differences in AhR action and gene regulation or mouse and human AhRs, future studies with these novel humanized mouse models may identify human-specific responses and mechanisms. Additionally, one can envision that development of a series of transgenic mice containing AhRs from other species (i.e., guinea pig or hamster) may provide useful novel model systems to further analyze species diversity in response to TCDD and other AhR ligands and to identify key mechanisms of AhR-dependent toxicity.

Taken together, current ongoing and future studies are certain to provide insights into many of the open questions that remain. An appreciation of the complexity of the mechanisms by which the AhR and AhR-dependent signal transduction pathway can produce its diverse biological and toxicological effects indifferent species is essential for our understanding of the mechanisms by which the AhR can regulate the diversity of response of cells to both endogenous ligands and xenobiotics. With this information, we will be able to appreciate more fully the role of the AhR in normal physiological processes and toxicological outcomes and to apply this knowledge to the ultimate goal of human health risk assessment.
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**REFERENCES**


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