

AH RECEPTOR SIGNALING PATHWAYS

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ABSTRACT

The aryl hydrocarbon (Ah) receptor has occupied the attention of toxicologists for over two decades. Interest arose from the early observation that this soluble protein played key roles in the adaptive metabolic response to polycyclic aromatic hydrocarbons and in the toxic mechanism of halogenated dioxins and dibenzofurans. More recent investigations have provided a fairly clear picture of the primary adaptive signaling pathway, from agonist binding to the transcriptional activation of genes involved in the metabolism of xenobiotics. Structure-activity studies have provided an understanding of the pharmacology of this receptor; recombinant DNA approaches have identified the enhancer sequences through which this factor regulates gene expression; and functional analysis of cloned cDNAs has allowed the characterization of the major signaling components in this pathway. Our objective is to review the Ah receptor's role in regulation of xenobiotic metabolism and use this model as a framework for understanding the less well-characterized mechanism of dioxin toxicity. In addition, it is hoped that this information can serve as a model for future efforts to understand an emerging superfamily of related signaling pathways that control biological responses to an array of environmental stimuli.

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EARLY OBSERVATIONS

Individuals are constantly exposed to a variety of xenobiotics, among which are secondary plant metabolites, mycotoxins, venoms, pharmaceuticals, and the byproducts of industrialization. For several decades scientists have been aware that adaptive mechanisms exist to minimize toxicity from these ubiquitous environmental poisons. A metabolic response to polycyclic aromatic compounds (PAHs) was first described in the late 1950s. In these early rodent experiments, the administration of benzanthracene, benzo[a]pyrene or 3-methylcholanthrene led to the induction of a number of liver microsomal enzyme activities collectively referred to as arylhydrocarbon hydroxylase (AHH) (Conney et al 1956). This induced metabolism met the criterion of an adaptive response in that the upregulated enzymes were able to oxidize the same PAH-inducing agents upon short-term re-exposure. Similar adaptive responses were also observed for other classes of structurally unrelated xenobiotics such as phenobarbital and various pesticides. In these cases as well, initial exposures led to increased expression of microsomal and soluble enzymes with metabolic activity toward the inducing agent and the effect of reducing the pharmacological response of subsequent exposure (Snyder & Remmer 1979).

The Ah Receptor and Ahr Locus

Classical murine genetics provided initial insights into the regulation of AHH activity. First, it was observed that the inducibility of AHH activity varied

significantly among inbred mouse strains, with C57 strains being highly responsive to PAHs, whereas the DBA and AKR strains were described as nonresponsive (Nebert & Gelboin 1969). Crosses and back-crosses of these strains indicated that multiple alleles at a single locus controlled inducibility of AHH. This locus initially became known as *Ah*, for aryl hydrocarbon responsiveness (Green 1973, Thomas & Hutton 1973). Although the terms responsive and nonresponsive are still widely used, their application should be limited to induction by PAHs, because halogenated aromatic hydrocarbons (HAHs) such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) soon were found to be orders of magnitude more potent than PAHs and were capable of eliciting AHH induction in nonresponsive strains (Poland et al 1974). These results demonstrated that nonresponsive strains were actually less responsive, requiring 10- to 100-fold higher doses of TCDD to attain the same level of enzyme induction as responsive strains.

Data from a number of laboratories led to the suggestion that a receptor existed for this large class of chemicals and that C57 mice harbored a receptor with a greater affinity for ligand than DBA mice (Poland & Glover 1975). The existence of this *Ah* receptor (AHR) was confirmed using radiolabeled dioxin congeners to demonstrate the existence of low-capacity, high-affinity binding sites in target tissues (Poland et al 1976). As predicted, the binding affinity for TCDD differed between mouse strains, with receptors from the responsive and nonresponsive strains displaying equilibrium dissociation constants of 6 and 37 pM, respectively (Okey et al 1989, Poland et al 1994). The segregation of these alleles, as well as structure-activity studies performed with various dioxin congeners, confirmed the existence of the AHR and its role in regulating the induction of AHH. The idea that the *Ah* locus encoded the AHR resulted in the recent renaming of this locus to *Ahr* by the Mouse Genome Nomenclature Committee (Eppig 1993).

The AHR is highly polymorphic, particularly when compared with other nuclear receptors. This polymorphism extends beyond the classical responsive and nonresponsive phenotypes described above to include significant differences in receptor primary structure. For example, marked differences in AHR molecular weight have been revealed with the use of [¹²⁵I]-photoaffinity ligands and antibodies (Poland et al 1986, Poland & Glover 1987). Three different *Ahr* alleles, denoted with a "b" superscript from the prototype C57BL strain, have been identified that encode high-affinity receptors in responsive strains. The allele found in C57 strains, *Ahr*^{b-1}, encodes a 95-kDa receptor with high affinity for agonists, whereas a 104-kDa high-affinity allele, *Ahr*^{b-2}, is found in most other commonly used laboratory strains such as C3H/He and BALB/c (Poland et al 1987). Several wild-mouse strains, including *Mus spretus*, *caroli*,

and *molossinus*, harbor a third high-affinity allele, *Ahr*^{b-3}, encoding a 105-kDa receptor protein (Poland & Glover 1990). At present, only a single allele has been identified that encodes the low-affinity receptor in nonresponsive strains (*Ahr*^d) (Swanson & Bradfield 1993). This allele is denoted with a “d” superscript, from the prototype DBA strain, and encodes a receptor protein of 104 kDa (Poland & Glover 1990). The structural and functional variability of the AHR is also significant across species. Photoaffinity labeling of hepatic cytosol indicates that the AHR can vary in molecular weight by almost 30 kilodaltons, e.g. C57 mouse, 95; chicken, 101; guinea pig, 103; rabbit, 104; rat, 106; human, 106; monkey, 113; and hamster, 124 (Poland & Glover 1987). Recent cloning studies have demonstrated that this difference in molecular weight is primarily due to differences in the position of the AHR’s translational termination codon, rather than differential splicing or posttranslational modification (Dolwick et al 1993a, Schmidt et al 1993, Carver et al 1994a, Poland et al 1994).

Regulation of Xenobiotic Metabolism

The receptor-mediated upregulation of xenobiotic metabolizing enzymes remains the most clearly understood aspect of AHR biology. Much of our understanding of this pathway comes from analysis of the regulatory regions controlling expression of the genes encoding the cytochrome P4501A1, 1A2, and 1B1 monooxygenases that contribute to AHH activity (Jones et al 1986, Quattrochi et al 1994; W Greenlee, personal communication). Analysis of the 5' regulatory regions of the Cyp1A1 gene revealed the existence of a number of dioxin-responsive enhancer elements (DREs) that were required for induction and were bound by the AHR in response to ligand (Denison et al 1988a,b; Fujisawa-Sehara et al 1988). DNA footprinting of the P4501A1 promoter showed that additional regions were also necessary for transcriptional activation in this system: a TATA box, a G-box element, and two binding sites for the transcription factor NF1 (Jones & Whitlock 1990). These observations led to a model where receptor interactions with multiple upstream DREs facilitate the disruption of local chromatin structure, allowing downstream promoter elements to bind their respective factors and initiate transcription (Wu & Whitlock 1992, Okino & Whitlock 1995). Although considerable evidence indicates a role for chromatin disruption in AHR signaling, the potential for direct contacts between factors bound to upstream DREs and those bound to proximal elements through DNA looping has yet to be determined. The regulation of the Cyp1A1 gene is a useful paradigm. For example, deletion analysis of the human CYP1A2 promoter has demonstrated that two DRE regions are required for TCDD inducibility of this gene as well (Quattrochi et al 1994). Cytochrome P4501B1 is the most recently discovered TCDD-inducible P450 gene, and its

promoter also appears to be regulated by a similar mechanism (Sutter et al 1994; W Greenlee, personal communication). In addition to the cytochrome P450 enzymes, other genes regulated by this mechanism are the glutathione S-transferase Ya subunit, NAD(P)H:quinone reductase 1 and 2, and class 3 aldehyde dehydrogenase (Paulson et al 1990, Favreau & Pickett 1991, Asman et al 1993, Jaiswal 1994).

AHR SIGNALING PATHWAYS

The Adaptive Response Pathway

Although these designations may be overly simplistic, it has been convenient for our laboratory to classify AHR-mediated responses as either adaptive or toxic. We define the adaptive response as being limited to the ligand-induced, DRE-driven, upregulation of genes encoding xenobiotic-metabolizing enzymes. This definition is based on the teleological argument that all genes currently known to be transcriptionally upregulated through a functional DRE encode enzymes with important roles in the metabolism of foreign chemicals. In fact, most of these enzymes appear to have metabolic activity toward PAHs. This observation supports the idea that the AHR system has evolved, at least in part, to minimize an organism's body burden of compounds with extended polycyclic aromatic structures. The reasons that such a metabolic response has arisen specifically for PAHs are still largely speculative.

The selective pressures for maintenance of this detoxification system include the possibility that polycyclic aromatic compounds with extended planarity have deleterious effects for many important cellular processes. Most notable are their capacity to act as nonspecific inhibitors of enzymatic reactions and their ability to intercalate into nuclear and mitochondrial DNA. A complication of this detoxification system is the potential for PAHs to be metabolized to electrophilic intermediates that can alkylate cellular macromolecules, leading to altered cellular function and genotoxicity. This aspect is somewhat problematic given the observation that many AHR-induced enzymes can actually contribute to the generation of these electrophiles.

The Toxic Response Pathway

Our designation of the toxic response pathway is based on the observation that many receptor-mediated effects have a negative impact on the exposed individual and are inconsistent with an adaptive response. It is also a useful classification for receptor-mediated responses that make little sense teleologically or have an unknown mechanism. Many of the agonists of the AHR are familiar environmental toxicants (Skene et al 1989). PAHs are generated from

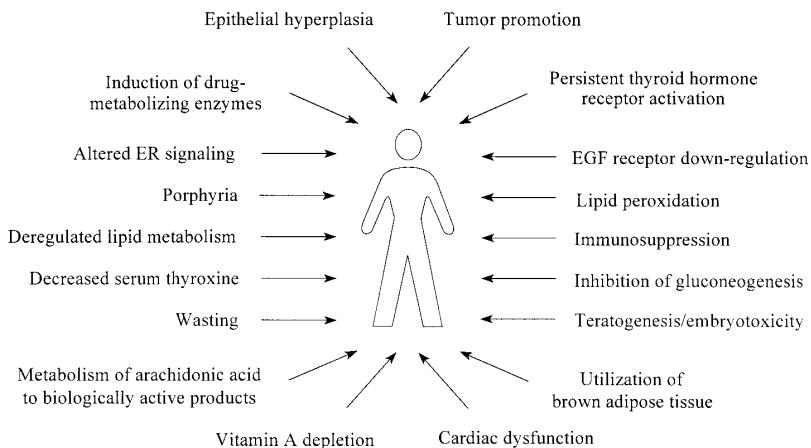


Figure 1 Biological responses to TCDD. A wide variety of cellular processes have been shown to be affected by TCDD.

the incomplete pyrolysis of various carbon sources and are typically found in diesel exhaust, cigarette smoke, and charbroiled foods. HAHs such as TCDD are trace contaminants in industrial processes that involve chlorination in the presence of phenolic substrates. Although both classes of compounds have carcinogenic potential, in recent years HAHs have received greater attention due to their environmental persistence and remarkably acute toxicity.

By the late 1970s, it became clear that in addition to its role in regulating AHH activity, the AHR was also the primary mediator of the toxicity of halogenated dioxins and dibenzofurans. Subsequently, attempts were made to identify the most sensitive endpoints of dioxin exposure and develop estimates of safe environmental levels for these compounds. The proof that a given biological endpoint is mediated by the AHR is twofold. First, the structure-activity studies should indicate that the rank-order potency of a congener to generate the endpoint of interest correlates with agonist-binding affinity for the AHR. Second, the responsiveness of the biological endpoint to agonist should segregate with the *Ahr^b* and *Ahr^d* loci in mice. A surprising number of deleterious biological responses have been shown to result from TCDD exposure, and many have met the above criteria (Figure 1) (Poland & Knutson 1982, Pohjanvirta & Tuomisto 1994 and references therein).

The fact that the same experimental proof has been used to demonstrate the role of the AHR in both adaptive and toxic responses may give the impression that the two mechanisms are the same. This has not been demonstrated and

seems unlikely. The best argument that the adaptive and toxic pathways differ mechanistically comes from the observation that for adaptive responses such as the induction of AHH activity, HAHs and PAHs have parallel dose-response curves (Poland & Knutson 1982). Yet, only potent Ah receptor agonists such as the halogenated dioxins are able to elicit the classic toxic responses described below. This observation suggests the existence of distinct mechanisms for the adaptive and toxic response pathways.

Because of its potency and environmental persistence, TCDD has become the prototype agonist for the study of AHR biology. As a result, the terms TCDD toxicity and AHR biology are often used interchangeably. In most cases, this is a fair simplification, because most, if not all, of the toxicity of TCDD is the result of interactions with the AHR. TCDD toxicity observed in animal models includes tumor promotion, embryotoxicity/teratogenesis, epithelial hyperplasia and metaplasia, lymphoid involution, porphyria, and a severe wasting syndrome followed ultimately by death (Poland & Knutson 1982). In most cases, the proof described above has confirmed the role of the AHR in these events. The ultimate cause of TCDD-induced lethality is unknown, and although wasting seems to be an obvious candidate, it does not appear to be a direct cause of death. When TCDD-treated rats are fed parenterally, such that normal body weight is maintained, they die at approximately the same time as TCDD-treated controls fed ad libitum (Poland & Knutson 1982). Significant species differences are seen in the spectrum of toxicity observed and in the dose of TCDD required to elicit a particular response. For example, the LD₅₀ for acute TCDD exposure varies from 1 $\mu\text{g}/\text{kg}$ in the guinea pig to 20 to 40 $\mu\text{g}/\text{kg}$ in the rat, 70 $\mu\text{g}/\text{kg}$ in the monkey, 114 $\mu\text{g}/\text{kg}$ in the mouse and rabbit, and 5000 $\mu\text{g}/\text{kg}$ in the hamster (Poland & Knutson 1982).

In addition to acute toxicity, TCDD has been shown to act as a potent tumor promoter in the two-stage model of liver carcinogenesis (Pitot et al 1980). TCDD may also act as a complete carcinogen because rodents demonstrate an increased incidence of specific tumors in chronic toxicity studies, without prior exposure to an experimental initiator. Rats maintained for two years on dietary TCDD showed an increased incidence of squamous cell carcinomas of the lung, hard palate/nasal turbinates, and tongue (Kociba et al 1978, Goodman & Sauer 1992). Unlike many potent carcinogens, TCDD appears to be nongenotoxic. It does not covalently bind DNA, RNA, or protein and is not mutagenic in the Ames assay (Poland & Glover 1979, Geiger & Neal 1981). Proposed mechanisms for the carcinogenic effects of TCDD include increased cytochrome P450-mediated metabolic activation of other carcinogens or endogenous compounds such as estrogen, DNA single-strand breaks resulting

from lipid peroxidation, and alterations in cell proliferation through transcriptional regulation of cytokines and growth factors (Huff et al 1994).

The Endogenous Pathway

It is possible that the AHR plays a biological role that is not yet understood. For years, many researchers have entertained the hypothesis that there is an endogenous ligand of the AHR and that toxicity is the result of inappropriate activation of this endogenous pathway. Along similar lines, the AHR may have ligand-independent or constitutive functions in biological processes distinct from the more thoroughly characterized ligand-activated pathways. As is discussed in the section on *Ahr* null mouse models, it appears that the AHR is required for normal liver development and possibly for immune system function. Such evidence could support a role for the AHR in an unknown endogenous pathway or could be indicative of the importance of the adaptive pathway in defending certain cell types from environmental toxicants.

AHR-ARNT INTERACTIONS

An Overview of the bHLH Protein Class

The basic-helix-loop-helix (bHLH) motif has been described in a wide variety of transcription factors such as the mammalian proteins Myc, Max, MyoD, and E2A, and the *Drosophila* proteins Achaete-scute and Daughterless (Murre et al 1989a, Kadesch 1993) that function as sequence-specific transcriptional regulators. This motif has been demonstrated to harbor subdomains that play roles in both DNA binding (basic region) and protein dimerization (HLH) (Murre et al 1989a,b, Davis et al 1990). A feature of many bHLH proteins is the presence of a secondary dimerization surface adjacent to the HLH domain. One well-characterized example of such a secondary dimerization domain is the leucine zipper, and bHLH proteins containing this motif are called bHLH-ZIP proteins (Kadesch 1993).

The myogenic determination protein MyoD and its relatives Myogenin, Myf-5, and MRF4 are among the most widely studied members of the bHLH proteins and illustrate many of the general features of these proteins (Olson 1990, Weintraub et al 1991). The myogenic bHLH proteins were identified based on their ability to activate muscle-specific genes and induce muscle cell differentiation in nonmyogenic cells. These factors autoregulate their own expression and cross-regulate the expression of the other family members. Studies on the regulation of skeletal muscle development provide evidence for distinct roles for each of the myogenic factors in both determination and differentiation of muscle cell phenotype (Braun et al 1992, Rudnicki et al 1992, Cheng et al 1993). All four myogenic factors form heterodimers with the E12 and E47

proteins, which are alternately spliced products of the E2A gene, to generate functional DNA-binding complexes (Murre et al 1989a, Sun & Baltimore 1991, Weintraub et al 1991). Regulation of this system is maintained under different physiologic conditions not only by the complement of dimeric partners that are expressed, but also by restricting the heterodimeric pairs that may form. Key regulators of partner availability are two dominant-negative inhibitory proteins, Id1 and Id2 (Benezra et al 1990, Sun & Baltimore 1991). These proteins have been shown to interact with E12 and E47, as well as with MyoD, forming nonfunctional complexes devoid of DNA-binding ability.

The AHR and ARNT Are bHLH-PAS Proteins

Recently a number of observations have changed our understanding of the AHR. Prior to the cloning of the AHR cDNA, the only nuclear receptors known to exist in mammals were members of the steroid/thyroxin receptor superfamily (Mangelsdorf et al 1995). This led to the assumption that the AHR would ultimately be found to contain zinc-finger DNA-binding domains and have a modular structure similar to that of steroid receptors. Although the steroid receptor homology was ultimately disproven, the presumed relationship led to the finding that the AHR was bound to a dimer of the 90-kDa heat shock protein (Hsp90) (Denis et al 1988, Perdew 1988). This association was correlated with a cytosolic location of the AHR and a receptor state that binds ligand but not DNA. In a manner similar to steroid receptors, this Hsp90 interaction was shown to be destabilized by ligand binding, a process referred to as transformation (Wilhelmsson et al 1990). Correlates of the weakened Hsp90 interaction are the appearance of the AHR in the nuclear fraction of cell homogenates and a higher affinity for DRE sequences in vitro (Pongratz et al 1992).

A finding with great impact on how we think of AHR signaling was the determination that the AHR bound to DREs as part of a heterodimeric complex (Elferink et al 1990, Reyes et al 1992, Dolwick et al 1993b). Genetics experiments in somatic cells indicated that induction of AHR activity required the product of a second locus, which encodes a protein product referred to as the Ah receptor nuclear translocator or ARNT (Hoffman et al 1991). This nomenclature is based on initial reports indicating that ARNT is required for high-affinity association of the AHR with the nuclear fraction of cells upon TCDD binding. Subsequent experiments have suggested that ARNT is not required for nuclear translocation per se, but is required to generate an AHR-ARNT complex with a greater affinity for nuclear extracts upon cell disruption. Two observations support this argument. First, immunocytochemistry was performed to visualize the location of the AHR and ARNT proteins in Hepa cells in the absence and presence of ligand (Pollenz et al 1994). The unliganded AHR is found almost exclusively in the cytoplasm of the cell and treatment with ligand causes

a time-dependent movement of the AHR into the nucleus. The ARNT protein, on the other hand, is found to be exclusively nuclear without respect to ligand. Thus ligand may serve to initiate translocation of the AHR to the nucleus where dimerization of these two partners can occur. Second, in ARNT-deficient cells, the AHR can still translocate to the nucleus *in vivo*, a process therefore independent of ARNT.

Cloning of the AHR and ARNT allowed amino acid sequence alignments, which revealed that these two proteins are similar in primary amino acid sequence to the *Drosophila* proteins Sim and Per (Citri et al 1987, Crews et al 1988, Benezra et al 1990, Hoffman et al 1991, Sun & Baltimore 1991, Burbach et al 1992, Ema et al 1992, Huang et al 1993). The homologous domain present in all four proteins has been termed the PAS domain, for Per-ARNT-Sim. In addition to the PAS motif, the AHR, ARNT, and Sim also have adjacent bHLH domains. Sim is a bHLH-PAS protein involved in the specification of cell fate during midline cell differentiation in *Drosophila* (Nambu et al 1990, 1991). Per, known to be involved in the maintenance of circadian rhythms, is the most unusual member of this family in that it does not contain a bHLH region and may function as a dominant-negative inhibitor in a manner similar to the Id proteins of the MyoD system (Benezra et al 1990). Very recently, a number of new members of the bHLH-PAS superfamily have emerged from cloning studies. The hypoxia-inducible factor 1 α (HIF-1 α), a regulator of cellular responses to hypoxic stress, was purified and cloned from hepatoma cells, and two additional bHLH-PAS members are the products of the *similar* and *tracheless* genes of *Drosophila* (Wang et al 1995, Isaac & Andrew 1996, Nambu et al 1996, Wilk et al 1996). Together these cloning and sequencing studies suggest that a superfamily of PAS proteins exists in a wide variety of cell types and organisms.

The homology between bHLH-PAS superfamily members is also seen at the level of gene structure. Cloning of the structural gene of the mammalian AHR (*Ahr* gene) demonstrated the presence of 11 exons over a region of greater than 35 kb of DNA (Schmidt et al 1993). The AHR is most homologous to *Drosophila* Sim in nucleotide sequence, and analysis of the structural genes of these two proteins has revealed striking conservation of intron-exon pattern despite the evolutionary divergence of their sources (Crews et al 1988, Nambu et al 1990). Within the bHLH-PAS domain, six of eight intron-exon splice junctions are conserved. These results suggest that the *Ahr* and *sim* may have arisen from a common primordial gene. Recent unpublished work from our laboratory has shown that the genomic structure of the murine HIF-1 α PAS domain is highly conserved with the *Ahr* and *sim* genes as well (G Luo & C Bradfield, unpublished data).

As mentioned above, four different *Ahr* alleles can be distinguished among different inbred and wild-mouse strains (Poland et al 1987, 1994, Poland & Glover 1990, Swanson & Bradfield 1993). The cloning of the cDNAs encoding each of these alleles has allowed molecular dissection of observed biochemical differences in ligand-binding affinity between the high- and low-affinity alleles (Ema et al 1994, Poland et al 1994). In vitro translation of the receptors encoded by the *Ahr*^{b-1}, *Ahr*^{b-2}, and *Ahr*^d cDNAs allowed measurement of saturable binding by the radioligand 2-[¹²⁵I]iodo-7,8-dibromodibenzo-*p*-dioxin. Equilibrium dissociation constants for the AHR^{b-1} and AHR^{b-2} receptor proteins were 6 to 10 pM, whereas that for the AHR^d receptor was 37 pM (Poland et al 1994). The *Ahr*^d allele is most similar in sequence to the *Ahr*^{b-2} allele, with only three amino acid differences. Individual mutation of each of these amino acids in the *Ahr*^{b-2} allele demonstrated that the reduced AHR^d ligand affinity was the result of an Ala₃₇₅ → Val₃₇₅ polymorphism. As is discussed below, this amino acid change is localized to a region of the AHR that has been demonstrated to contain the ligand-binding domain.

Given the differences in sensitivity to TCDD toxicity across species, it has been of interest to compare the primary structures of their AHRs. Cloning of the human and rat AHR cDNAs demonstrated strong N-terminal sequence conservation with the mouse protein (Dolwick et al 1993a, Carver et al 1994a). The N-terminus of the human AHR shows 100% amino acid identity with the mouse AHR in the basic region, 97% in the HLH, and 87% in the PAS domain. The rat AHR identity with the mouse is 100% in the bHLH and 96% in the PAS domain, and with the human AHR is 98% identical in the bHLH domain and 86% in the PAS domain. The C-termini of these three proteins are more divergent, however, with 60% identity between human and mouse, 61% between human and rat, and 79% between rat and mouse. The position of the termination codons varies between these three proteins and, with similar data from other mouse alleles, appears to provide a general explanation for the high degree of molecular weight polymorphism observed (Dolwick et al 1993a, Schmidt et al 1993, Carver et al 1994a, Poland et al 1994). A comparison of the amino acid sequence of the human AHR and murine receptors shows that the Ala₃₇₅ → Val₃₇₅ polymorphism responsible for the reduced ligand-binding affinity of the AHR^d receptor is present in the human AHR as well (Dolwick et al 1993a, Ema et al 1994, Poland et al 1994). While further investigation of the significance of this amino acid change on the human AHR is needed, the agonist-binding affinity of humans may be more like that of the nonresponsive than the responsive mouse strains. Such an observation could have a significant impact on the risk assessment of these toxicants.

The primary sequence of the ARNT protein appears to be more highly conserved between species than the AHR. Cloning of a fragment of rat ARNT corresponding to nucleotides 286-1392 of human ARNT shows 98% overall amino acid identity between the two proteins (Carver et al 1994a). A cDNA-encoding mouse ARNT has been isolated from Hepa-1 cells and is highly homologous to human ARNT, with 92% amino acid identity between the two proteins overall (Li et al 1994). Although the C-terminal halves of human and mouse ARNT display somewhat greater diversity than the N-terminal halves, this difference is considerably less pronounced than that seen in the hypervariable C-terminal regions of different species of AHR.

Tissue and Cellular Localization

Northern blot analysis of RNA from eight different human tissues showed that the human AHR mRNA was present in all tissues examined (Dolwick et al 1993a). The highest expression levels were found in placenta and lung, and the lowest levels were found in kidney, brain, and skeletal muscle. Preliminary Northern blot analysis of several human tissues showed that ARNT is expressed in the liver, placenta, and chorion (Brooks et al 1989). Ribonuclease protection assays of numerous rat tissues showed that the rat AHR was highest in lung, thymus, liver, and kidney and lowest in heart and spleen (Carver et al 1994a). In contrast to the human placenta, that of rat did not exhibit a high amount of AHR mRNA. This result may reflect a true species difference in AHR expression or may result from different gestational ages of the two tissue samples. The rat ARNT message was also ubiquitously expressed and by ribonuclease protection was found to be highest in placenta, lung, and thymus and lowest in spleen, brain, and heart (Carver et al 1994a). In general, the AHR and ARNT proteins appear to be coexpressed; however, pronounced differences in relative expression levels exist between the two dimeric partners in some tissues. The non-stoichiometric distribution of these two proteins may have significance for the AHR and ARNT signaling; tissues in which one protein is present in excess over the other may indicate the existence of additional dimerization partners and signaling pathways. Additionally, low levels of ARNT could decrease the sensitivity of a particular tissue to agonist despite high AHR levels.

Developmental expression of the AHR and ARNT has been demonstrated by *in situ* and immunohistochemical methods in C57BL/6 mouse embryos from gestational day (gd) 10-16, although mRNA and protein were not examined in all tissues (Abbott et al 1995, Abbott & Probst 1995). The AHR and ARNT were found to be present at low levels in many embryonic tissues, with markedly higher levels in certain areas. Both the AHR and ARNT are already expressed at gd 10-11, with the highest levels in heart and neuroepithelium and

neuroepithelial/neural crest-derived tissues such as visceral arches and otic and optic placodes. The AHR is also expressed in facial membranous bone and Meckel's cartilage anlagen. At gd 12–13, brain and heart levels of the AHR and ARNT have decreased, and the highest levels are now found in the liver. Areas of bone formation retain high levels of the AHR, which also appears in the epithelium of gut, lung, and kidney, and along the medial edge of the palatal shelves. ARNT is also expressed at significant levels in the tongue. At gd 14–16, both the AHR and ARNT remain strongly expressed in the liver and are also high in adrenal gland and developing bone. The AHR is also expressed in the epidermis. The authors summarize that the relative levels of the two dimeric partners are coordinate overall, with several exceptions, and display the greatest expression in tissues undergoing rapid proliferation and differentiation.

Dimerization and Domain Mapping

Initial observations that the AHR bound to DNA as a heteromeric complex were provided by UV cross-linking experiments with a synthetic DRE. A bromodeoxyuridine-substituted DRE oligonucleotide could be covalently cross-linked to a TCDD-inducible complex in rat liver and visualized by gel-shift assays. SDS-PAGE analysis of this protein complex demonstrated two unique proteins of 110 and 100 kDa; only the 100-kDa protein was able to bind the AHR photoaffinity ligand (Elferink et al 1990, Gasiewicz et al 1991). Gel-shift and immunoprecipitation experiments were used to demonstrate that both the AHR and ARNT proteins were required to form a DRE-binding complex and that the formation of this complex was greatly enhanced by the addition of ligand (Whitelaw et al 1993). Cell culture systems showed that the AHR and ARNT could transactivate a reporter gene under the control of a DRE enhancer in vivo (Matsushita et al 1993, Li et al 1994, Mason et al 1994).

Several groups have analyzed AHR deletion mutants in vitro and in vivo to demonstrate the existence of discrete functional domains (Figure 2). CNBr cleavage products photoaffinity-labeled with 2-azido-3-[¹²⁵I]iodo-7,8-dibromodibenzo-*p*-dioxin showed that ligand bound to a fragment containing amino acids 232–334 within the PAS domain (Burbach et al 1992). Photoaffinity labeling of AHR deletion mutants confirmed that the ligand-binding region lies between residues 166 and 425 (Dolwick et al 1993b). It was demonstrated that both the bHLH and PAS domains are required for DNA-binding and thus presumably for dimerization with ARNT (Dolwick et al 1993b). Immunoprecipitation studies have allowed the dissociation of dimerization and DNA binding and showed that the basic region of ARNT is not required for dimerization, but both helix regions, and either the N-terminal or C-terminal half of the PAS domain, are essential (Reisz-Porszasz et al 1994, Dolwick et al 1996). Another group has recently confirmed these results employing chimeras

of the glucocorticoid receptor DNA-binding domain with the AHR or ARNT (Whitelaw et al 1994).

Deletion constructs and DNA-binding chimeras have been used to map the transcriptional activation domains (TADs) of the AHR, ARNT, Sim, and Per. The AHR and ARNT proteins have a single TAD in their C-terminus, comprising amino acids 521–640 in the AHR and amino acids 582–774 in ARNT (Jain et al 1994, Li et al 1994, Whitelaw et al 1994, Ma et al 1995). Both TADs contain glutamine and hydrophobic residues, whereas the AHR also has acidic amino acids, and ARNT is rich in serine, proline, and threonine residues. Despite their different compositions, in our Gal4 fusion system, the TADs of the AHR and ARNT appear to be equally potent (Jain et al 1994). Full-length AHR-ARNT activation of a DRE-driven reporter plasmid, however, may be

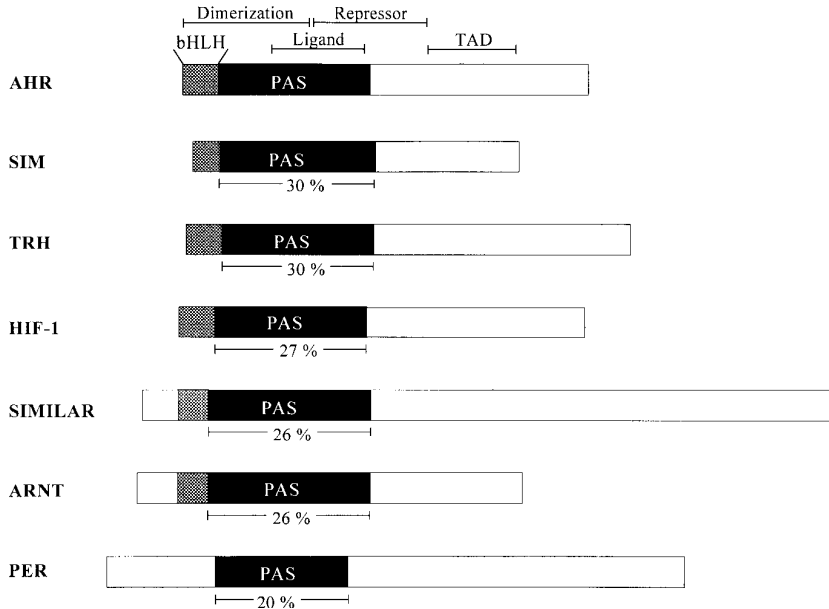


Figure 2 Schematic representation of the bHLH-PAS family proteins. The stippled areas represent the bHLH region and the black areas the PAS domain. For the AHR, the region marked Dimerization indicates bHLH and PAS sequences required for AHR-ARNT dimerization and therefore also for DNA binding. The region marked Repressor indicates the area of Hsp90 interaction, the region marked Ligand indicates the ligand-binding domain as mapped by photoaffinity labeling of deletion constructs, and the region marked TAD indicates the transactivation domain. For the other bHLH-PAS family members, percent amino acid identity to the AHR within the PAS region is indicated beneath each protein. Per does not contain a bHLH domain.

more dependent on the TAD of ARNT (Whitelaw et al 1994). In vivo, the TADs of the AHR and ARNT may synergize, because removal of the Q-rich region of ARNT did not impair AHR-ARNT dimerization but did diminish transactivation of a DRE-driven CAT reporter gene in ARNT-defective Hepa cells (Li et al 1994). Experiments have shown a strong transcriptional activation domain in the C-terminus of the Sim protein as well, whereas the Per protein was devoid of transcriptional activity (Franks & Crews 1994, Jain et al 1994, Whitelaw et al 1994).

The PAS domain of the AHR also harbors the contact region for association with Hsp90. Results from numerous laboratories have demonstrated that the AHR forms a stable complex with Hsp90 and that the region of interaction corresponds to AHR residues 340 to 422 (Denis et al 1988, Perdew 1988, Whitelaw et al 1994). Interestingly, this region colocalizes with a domain our laboratory had previously identified as imposing repression on AHR signaling (Dolwick et al 1993b). It has been hypothesized that Hsp90 functions to keep the AHR in a conformation capable of high-affinity ligand binding and represses the intrinsic DNA-binding affinity of the AHR (Pongratz et al 1992, Whitelaw et al 1994). Our laboratory and others have used a yeast expression system to study AHR-Hsp90 interactions and the role of Hsp90 in AHR signaling (Carver et al 1994b, Whitelaw et al 1995). A LexA-AHR chimera and reporter plasmid expressed in yeast were shown to be a useful model for AHR signaling in mammalian cells. This system was introduced into a yeast strain in which the level of Hsp90 could be regulated. At wild-type Hsp90 levels, normal AHR signaling was seen; however, when Hsp90 levels were decreased to 5% of wild-type, ligand-induced AHR signaling was blocked. From these results it can be concluded that Hsp90 is an essential component of the AHR-signaling pathway, and loss of Hsp90 most likely results in an improperly folded or destabilized receptor protein. More recently it has been shown that an antibody to Hsp90 can precipitate a complex of Hsp90 and the Sim protein (McGuire et al 1995). Hsp90 may thus act as a regulator of Sim activity as well.

A Model for AHR-ARNT Signaling

The work of many laboratories has combined to produce a model for AHR-ARNT signaling (Figure 3). This model holds that the unliganded AHR exists in the cytosol complexed with a dimer of Hsp90, which maintains the AHR in a ligand-binding conformation and prevents nuclear translocation and/or dimerization with ARNT. The hydrophobic AHR ligands enter the cell by diffusion and are bound by the Hsp90-associated AHR. Ligand binding causes a conformational change resulting in a receptor species with an increased affinity for DNA and a much slower rate of ligand dissociation (Bradfield et al 1988). This event is associated with nuclear translocation and an exchange of Hsp90 for

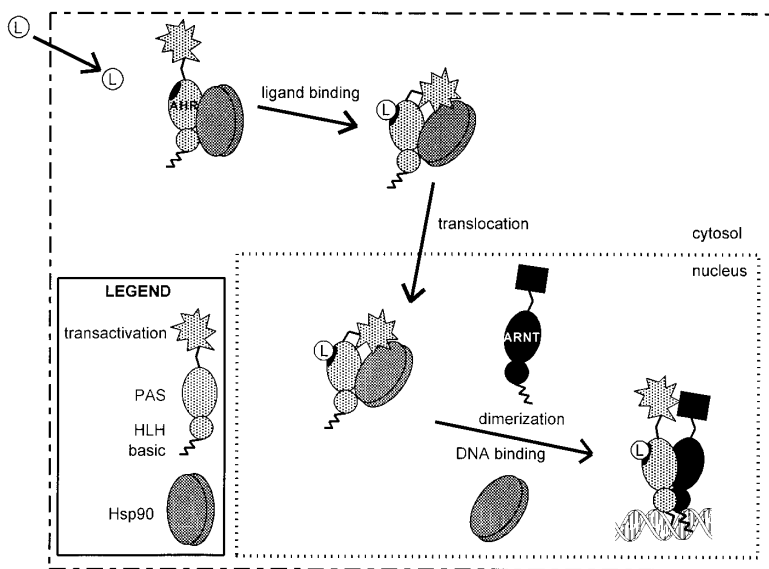


Figure 3 Model of AHR-ARNT signaling. Ligand binding by the AHR results in nuclear translocation and release of Hsp90, followed by dimerization with ARNT, and DNA binding/transactivation. The outer box represents a cell, and the inner box the cell nucleus. The AHR is indicated by the shaded partner, ARNT by the solid black partner, and Hsp90 by the shaded circle. The small circle labeled L represents the AHR ligand.

ARNT. It has been shown that in a purified system the AHR-Hsp90 complex is not dissociated by the addition of ligand (McGuire et al 1994). Addition of a cellular fraction from Hepa cells, but not ARNT-deficient Hepa mutants, can promote Hsp90 dissociation, suggesting that ARNT protein plays an active role in this process (McGuire et al 1994). Ultimately, recognition of DRE enhancer sequences by the AHR-ARNT complex results in the transactivation of target genes.

Sequence-Specific DNA Binding

Site-directed and deletion mutagenesis combined with domain swapping experiments with several bHLH proteins have defined the basic region as the DNA-binding subdomain (Davis et al 1990). This subdomain lies immediately N-terminal to the HLH domain and is generally 12 to 15 amino acids in length with two distinct clusters of basic residues (Figure 4). One of these clusters, a highly conserved ERXR sequence (where E is glutamic acid, R is arginine, and X is any amino acid), is located at a precise distance from helix 1 and is found in the basic region of all the bHLH proteins that have been shown to bind

	BASIC REGIONS	5' HALF-SITES
CAG Binding		
MyoD	ADRRKAATMRERRRLE	CAG
E12	KERRVANNAREERLRV	CAG
E47	RERRMANNAREVRV	CAG
AP4	RIRREIANSNERRRM	CAG
Tal1	VVRRIFTNSRERWRQ	CAG
Consensus	--RR---n-rER-RR	
CAC Binding		
c-Myc	NVKRRRTHNVLERQRR	CAC
Max	ADKRAHHNALERRRR	CAC
USF	EKRRAQHNEVERRRR	CAC
TFE3	RQKKDNHNLIERRRR	CAC
TFEB	RQKKDNHNLIERRRR	CAC
ARNT	RLARENHSEIERRRR	CAC
Consensus	---r--Hn--ERRR	
TPyGC Binding		
AHR	AEGIKSNPSKRHRDR	TC/TGC
Consensus?	----KS----R-R--	
GTPuC Binding		
SIM	MKEKSKNAARTRE	GTA/GC
Consensus?	----KS--AAR-RR-	
TAC Binding		
HIF-1	RRKEKSRDAARSRRS	TAC
SIMILAR	KRKEKSRDAARCRRS	TAC?
TRACHEALESS	LRKEKSRDAARSRRG	TAC?
Consensus	-RKEKSRDAAR-RR-	

Figure 4 The bHLH-PAS proteins have nonconserved basic regions. The basic regions of bHLH proteins have previously been grouped into class A with a consensus sequence ERXR and class B with a consensus sequence ERXRR, which recognize CAG and CAC half-sites, respectively. The residue conferring site specificity is the presence of an R or a nonconserved amino acid in the fifth position. Although the ARNT protein belongs to class B group of proteins, as befits its CAC half-site, the other bHLH-PAS proteins bear little homology to other bHLH proteins in this region. We have thus grouped these proteins according to the non-E-box half-sites they recognize.

to an E-box core sequence (CANNTG) (Murre et al 1989a, Dang et al 1992). Studies have begun to examine which residues in the basic region are important for the sequence-specific DNA binding of individual bHLH proteins. MyoD binds with greatest affinity to the DNA element CAGCTG located upstream of muscle-specific genes but is unable to bind to the c-Myc binding site CACGTG. Likewise, c-Myc is unable to bind to the MyoD recognition element. X-ray crystallography studies have demonstrated that the conserved ER residues recognize the outer CA or TG nucleotides of the E-box motif (Ellenberger et al 1994). Experiments directed toward understanding the determinants for the central two E-box nucleotides have shown that the known bHLH proteins can be divided into two groups: class B proteins that have an R residue directly following the ERXR sequence (ERXRR) and recognize a CACGTG consensus element, and class A proteins that have a nonconserved residue in this position (ERRX) and recognize a CAGCTG element (Figure 4) (Dang et al 1992).

In contrast to the classical bHLH transcription factors, which bind the symmetric E-box sequence, the AHR and ARNT bind an asymmetric recognition site. Because the core DRE consensus TNGCGTG contains one E-box half-site and one nonconsensus half-site, it was of interest to identify where each heterodimeric partner bound to the DRE. ARNT belongs to the class B group of bHLH proteins, with an ERRRR sequence in the basic region (Figure 4) (Hoffman et al 1991). Thus it could be predicted that ARNT would bind to the 3' GTG half-site of the DRE. Because the AHR does not contain the conserved ERXR sequence, it was predicted that the AHR would contact DNA at the 5' half of the DRE that does not conform to the E-box element. UV-cross-linking of TCDD-treated Hepa cytosolic proteins bound to variously substituted DREs allowed the use of AHR- and ARNT-specific antibodies to distinguish the ARNT protein bound to the GTG half-site and the AHR bound to the TNGC site (Bacsi et al 1995). Work from our laboratory has supported these results using an oligonucleotide selection and amplification strategy that allows the purified AHR and ARNT to select their preferred DNA-binding sequences from a pool of random oligonucleotides (Swanson et al 1995).

Analysis of the basic regions of the AHR and Sim and their asymmetric DNA recognition sequences, has resulted in their placement in a new class of bHLH proteins, designated class C (Swanson et al 1995). A comparison of basic region sequences and DNA recognition sites of previously identified bHLH-PAS proteins and several novel bHLH-PAS superfamily members has shown that assignment of these proteins to basic region homology classes is more complicated than for the other bHLH families (Figure 4). The majority of bHLH-PAS proteins, with the exception of ARNT, recognize unique sequences and show little basic region homology to the class A and B proteins. As shown

in Figure 4, we have chosen to group the bHLH-PAS proteins by the DNA E-box half-sites they recognize.

Consideration of the ARNT GTG half-site suggests that an ARNT homodimer might recognize the palindromic E-box sequence CACGTG. The oligonucleotide selection and amplification strategy used by our laboratory demonstrated that the ARNT protein, in the absence of the AHR, selected a consensus-binding site conforming perfectly to this E-box sequence (Swanson et al 1995). Additionally, an ARNT homodimer recognized a CACGTG E-box in gel-shift assays and transactivated an E-box-driven reporter gene in tissue culture cells (Antonsson et al 1995, Sogawa et al 1995).

INTER- AND INTRAMOLECULAR INTERACTIONS

ARNT Dimerizes With HIF-1 α In Vivo

A characteristic of many dimeric transcription factors is their capacity to participate in multiple complexes, both as homodimers and heterodimers. It appears that this may also be a feature of the bHLH-PAS superfamily of proteins, particularly the ARNT protein (Figure 5). ARNT has recently been shown to

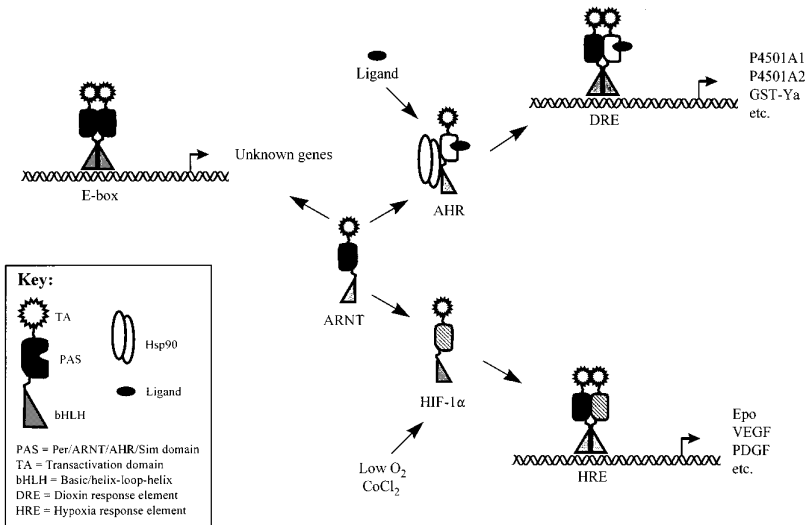


Figure 5 ARNT-mediated signaling pathways. ARNT appears to be a general dimerization partner that can homodimerize or heterodimerize with other bHLH-PAS proteins. The relative proportion of ARNT participating in these different complexes may determine the overall response of a cell to various stimuli.

interact in vivo with a recently identified bHLH-PAS protein known as HIF-1 α to form the HIF-1 transcription factor complex (Wang et al 1995). HIF-1 was initially characterized as a DNA-binding activity induced in cultured human hepatocytes, or in rodent liver, under hypoxic conditions (Semenza et al 1991, Semenza & Wang 1992). This complex appears responsible for mediating the cellular response to hypoxia by transcriptional regulation of genes such as erythropoietin and other mediators of cellular oxygenation through binding to the enhancer element TACGTG (Wang & Semenza 1993, Semenza 1994). The two proteins involved in this DNA-binding complex (designated HIF-1 α and HIF-1 β) have now been identified as HIF-1 α and ARNT (Wang & Semenza 1995), providing strong support for the hypothesis that ARNT is a general dimerization partner for multiple bHLH-PAS proteins where the identity of the partner determines the functional specificity of the complex.

Identification of the HIF-1 α protein as a bHLH-PAS superfamily member and ARNT as its dimeric partner suggests that interactions might occur between the TCDD and hypoxia signaling pathways. Indeed, recent evidence from our laboratory indicates that such cross talk does occur, perhaps through the ARNT subunit or a common accessory factor (W Chan & C Bradfield, unpublished data). Gel-shift and coimmunoprecipitation experiments demonstrate that the AHR and HIF-1 α compete for ARNT in vitro, with approximately equal dimerization efficiencies. Transfection of a P4501A1-driven luciferase reporter construct into the human hepatoma cell line Hep3B revealed that the HIF-1 α inducer cobalt chloride can suppress TCDD-induced expression of luciferase activity. Additionally, cobalt chloride is able to down-regulate the endogenous response to TCDD in Hep3B cells because both cytochrome P4501A1 message and protein levels are suppressed.

Multiple Interactions of bHLH-PAS Proteins In Vitro

Analysis of Baculovirus-expressed ARNT has revealed that ARNT is able to homodimerize in vitro, as well as heterodimerize with Sim and Per (Sogawa et al 1995, Swanson et al 1995). Sim regulates several *Drosophila* genes through interaction with the CNS midline enhancer (CME) core sequence GTACGTG (Wharton et al 1994). This element bears some similarity to the DRE core sequence TNGCGTG, and by comparison with the DRE binding specificities of the AHR and ARNT, Sim is thought to bind the nonconsensus half-site GTAC, whereas a *Drosophila* ARNT-like protein binds the GTG half-site (Wharton et al 1994, Swanson et al 1995). This theory has been supported by the observation that a heterodimeric complex can be formed in vitro by Sim and the human ARNT protein. This complex recognizes the oligonucleotide core sequence GTGCGTG, which differs by one nucleotide from the consensus CME (Swanson et al 1995).

Although the *in vivo* significance of these multiple interactions is unknown, the potential physiologic importance of interactions between members of the bHLH-PAS transcription factor superfamily has been demonstrated recently by the identification of a human Sim homologue that may play a role in the cause of Down's syndrome (trisomy 21) (Chen et al 1995, Dahmane et al 1995). This human Sim protein is found in embryonic CNS and facial tissues, where it may interact with ARNT or an ARNT-like protein to regulate important developmental processes. The role of *Drosophila* Sim as a regulator of midline cell neurogenesis suggests that trisomy for the human Sim gene may be involved in the phenotype of this human disease.

The idea that bHLH-PAS proteins may participate not only in PAS-PAS interactions, but also with unrelated protein sequences, has been documented by AHR-Hsp90 interactions and more recently for the Per protein. The PAS domain of Per was shown to be involved in the formation of Per-Per homodimers and Per-Sim heterodimers (Huang et al 1993). Although these two complexes are not known to have physiological roles, their formation serves as an important model and suggests that the PAS domain is an independent dimerization surface that may cooperate with adjacent bHLH domains. This role as a secondary dimerization motif is similar to that observed for the leucine zipper found in the Myc and Max bHLH-ZIP proteins (Landschulz et al 1988, Turner & Tjian 1989). Additionally, protein interactions within the PAS domain may play a role in the regulation of pairing with other partners. In this regard, it has been shown that the PAS domain of Per can be sequestered through intramolecular interactions with regions within its own C-termini (Huang et al 1995). This idea is supported by the observation that C-terminal truncations of Per, but not full length constructs, are capable of homodimeric interactions. The PAS domain of Per has also been shown to interact in a two-hybrid system with Tim, the product of a recently identified *Drosophila* circadian rhythm gene, *timeless*. Tim is not a PAS protein and contains no other known protein dimerization motif (Gekakis et al 1995). Finally, the PAS domain may also serve as a mechanism to restrict interactions between subclasses of bHLH proteins. Although this idea is difficult to prove, interaction screens with bHLH proteins have identified members of the same subclass, suggesting restrictions exist between bHLH subclass interactions (Staudinger et al 1993).

CELLULAR MECHANISMS OF TCDD TOXICITY

TCDD Teratogenesis Results from Altered Epithelial Cell Differentiation

TCDD was shown to be a classical teratogen by the fact that it induced cleft palate in mice at doses not systemically toxic to the dam or fetus (Courtney &

Moore 1971). Mice appear to be exquisitely sensitive to induction of TCDD terata, whereas most other species display this effect only at overtly toxic doses. All species, however, display a spectrum of embryotoxicity including increased resorptions and fetal mortality and decreased fetal body weight (Couture et al 1990). The physical basis for TCDD-induced cleft palate has been studied in some detail and supports the hypothesis that TCDD acts to alter the proliferation and differentiation of epithelial tissues. Formation of the mouse secondary palate begins with the growth of the palatal shelves, which consist of mesenchyme covered by a two-cell layer of medial edge epithelium (MEE) (Fitchett & Hay 1989). Contact and fusion of the shelves occurs on embryonic day 14 to 15 and is preceded by the death and sloughing of the outer layer of MEE. Once contact occurs, the remaining basal cell MEE layer undergoes a mesenchymal transformation facilitating complete fusion (Fitchett & Hay 1989). In mice treated with TCDD, growth of the palatal shelves occurs normally, but they do not fuse, and intervening medial epithelial cells persist and adopt a stratified squamous epithelial appearance (Pratt et al 1984, Abbott & Birnbaum 1989). Induction of cleft palate has been shown to be most sensitive to TCDD given on embryonic day 10, and TCDD appears to act at this time to alter the differentiation state of the MEE, interfering with their later competency to undergo mesenchymal transformation (Pratt et al 1984, Abbott & Birnbaum 1989).

Recently, gene targeting has been used to generate a mouse line null for the TGF- β 3 protein (Kaartinen et al 1995, Proetzel et al 1995). These mice display clefting of the palate resulting from a failure of the opposing palatal shelves to adhere and fuse and/or the midline epithelial seam to disappear. This mechanism of cleft palate is remarkably similar to that seen with TCDD treatment and is very different from the mechanism of other teratogens such as glucocorticoids (Pratt 1983). This similarity suggests that the AHR may be involved, directly or indirectly, in the regulation of TGF- β 3 in the developing palate. TGF- β proteins have a broad range of effects on cellular proliferation and differentiation. Alternately, TGF- β 3 may be positively regulated by ARNT alone or by a heterodimer of ARNT and an uncharacterized PAS protein. If such a situation exists, TCDD exposure would increase the formation of AHR-ARNT dimers, decreasing the amount of ARNT available for other interactions and resulting in decreased TGF- β 3 expression. Interestingly, the expression of various isoforms of TGF- β in the palate of TCDD-treated mice has been previously investigated (Abbott & Birnbaum 1990). TCDD, when administered on embryonic day 10 to 12, decreased the expression of the TGF- β 1 isoform in palatal epithelium and mesenchyme at embryonic day 14 to 16. The TGF- β 3 isoform has not been examined, but future experiments may show it to be a

gene whose altered expression contributes to at least one aspect of the TCDD toxic syndrome.

TCDD Immunosuppression May Result from Altered Lymphocyte Differentiation

Thymic involution and immunosuppression are the most consistently observed toxic effects of TCDD across species, occurring at doses well below those which cause systemic toxicity. Thymic atrophy has been shown to be dependent on the presence of the AHR (Poland & Glover 1980). The thymus appears to be exquisitely sensitive to TCDD because involution can be seen in mice after a single dose of 4 $\mu\text{g}/\text{kg}$ TCDD, and suppression of cell-mediated immunity (CMI) is apparent using isolated lymphocytes at 4 ng/kg (Clark et al 1981). The generation of bone marrow chimeras between B6 and DBA mice demonstrates that the immune response of the grafted lymphocytes to TCDD is dependent on the genotype of the host, suggesting that the suppression of CMI in the responsive mice is dependent on a nonlymphoid tissue (Nagarkatti et al 1984). It was suggested that this tissue may be the thymic epithelium (TE), because TCDD treatment of TE cells caused altered maturation and a decreased response to mitogens such as concanavalin A or phytohemagglutinin in cocultured thymocytes (Greenlee et al 1985). The ability of several different congeners to induce this effect was shown to correlate with their affinity for the AHR (Greenlee et al 1985).

TCDD effects on humoral immunity appear to involve the suppression of B lymphocyte responses. TCDD treatment of B6 and DBA mice decreased the antibody response to sheep red blood cells and this immunosuppression segregated with the B6 genotype in B6D2F₁ \times DBA back-crosses (Vecchi et al 1983). In mice congenic at the *Ahr* locus, TCDD and related congeners were shown to decrease antibody production in response to both T lymphocyte-dependent and T lymphocyte-independent antigens, in accordance with structure-activity relationships for AHR binding (Tucker et al 1986, Davis & Safe 1988, Kerkvliet et al 1990). Decreased antibody production resulted from a decreased number of antibody-producing cells, suggesting that TCDD inhibits B lymphocyte differentiation into plasma cells (Tucker et al 1986). Examination of the mechanism of TCDD effects on the B cell have shown that while TCDD is required early in B cell activation, it has no effect on initial proliferation but rather exerts its inhibition later at the differentiation stage (Luster et al 1988). TCDD may thus affect the B lymphocyte at a very early point in the commitment to differentiation. As is discussed below, the perturbation of cellular differentiation may play a key role in the mechanism of TCDD toxicity.

Alterations in humoral immunity appear to be one of the rare cases where some of the effects of HAHs may be independent of the AHR. For example,

although subchronic treatment of B6C3F mice with TCDD suppressed the antibody response in correspondence with known structure-activity relationships, the 2,7-dichlorodibenzodioxin congener, which does not appear to bind the AHR, caused a similar immune suppression (Holsapple et al 1986b). This effect was further supported in spleen cells from *Ahr* congenic mice where in vitro exposure to 2,7-dichlorodibenzodioxin showed an equivalent effect in both strains, suggesting this compound may be acting through a mechanism independent of the AHR (Holsapple et al 1986a). Additionally, when lymphocytes from B6 and DBA mice were treated in vitro with polychlorinated dibenzofuran congeners having a range of potency varying 15,000-fold in vivo, all compounds were approximately equipotent in suppressing the antibody response in both strains of mice (Davis & Safe 1991). Although often contradictory, the weight of evidence suggests that TCDD and related HAHs may exert an inhibitory effect on the maturation or differentiation of B lymphocytes. It is still unclear, however, if the AHR is required for all or part of this effect.

MOLECULAR MECHANISMS OF TCDD TOXICITY

Possible Mechanisms for TCDD Toxicity

Despite our knowledge of AHR-regulated induction of xenobiotic metabolizing enzymes, the mechanisms by which the AHR mediates the broad spectrum of TCDD toxicity remain unknown. However, emerging knowledge of the diversity of the PAS gene superfamily along with parallels from other transcription factor families allows us to begin to speculate on possible mechanisms for these effects (Figure 6). The most evident possibility is that TCDD toxicity may be the result of persistent transcriptional activation of genes regulated by the AHR-ARNT dimeric complex. Such a mechanism would be most similar to the induction of xenobiotic-metabolizing enzymes. As yet, none of the genes known to be regulated by the AHR and ARNT proteins can explain all the diverse toxic effects seen, although additional regulated genes with distinct activities will undoubtedly be identified. Initial support for this mechanism has been provided by subtractive hybridization of a TCDD-treated human keratinocyte cell line (Sutter et al 1991). In these experiments, two RNAs were isolated, encoding interleukin-1 β and plasminogen activator inhibitor-2, that were increased by TCDD treatment. TCDD toxicity might also result from hypothetical, low-affinity AHR-ARNT binding sites in the promoters of certain genes involved in toxicity. These elements may have a degenerate sequence that precludes interactions at levels of activated receptor generated by the majority of PAH-type agonists. Activation of the AHR by the potent and persistent ligand TCDD, however, could achieve a rate of receptor occupancy that allows

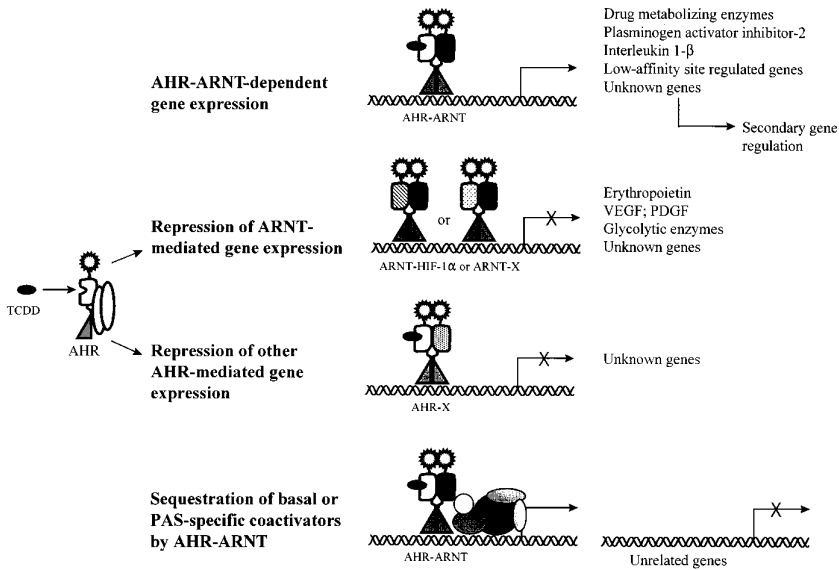


Figure 6 Possible models for the mechanism of TCDD toxicity, which probably results from alterations in gene expression induced by AHR-ARNT activity. This may be either a direct effect of the activation of AHR-ARNT-regulated genes or an indirect effect resulting from a decrease in the availability of either the AHR or ARNT to participate in different transactivation complexes.

activation from these low-affinity sites, resulting in expression of a normally restricted battery of genes. Yet another possibility is that the AHR and ARNT may recognize DRE sequences involved in transcriptional repression rather than activation, as has been shown for the glucocorticoid receptor (Sakai et al 1988). TCDD toxicity might result from altered repression of specific genes by the activated AHR-ARNT complex. Characteristics of TCDD toxicity predict that future implicated gene products, whether positively or negatively regulated by high- or low-affinity sites, will be involved in the control of cellular proliferation and differentiation.

Alternately, the mechanism of TCDD toxicity may be unrelated to direct transcriptional regulation by the AHR-ARNT complex. In fact, there is no data to date proving that ARNT is required for any of the toxic effects of dioxins. Rather, the production of this complex in response to TCDD may have repercussions for other bHLH-PAS protein pairs. Decreased concentrations of free ARNT owing to recruitment by the liganded AHR may shift the balance of this general dimeric partner away from HIF-1α or other partners, or from the formation of ARNT homodimers. In this model, TCDD toxicity could result

from decreased activity of other ARNT-dependent transcription pathways. A related explanation is that the AHR may have other dimeric partners with which it regulates additional physiologic processes. Toxicity in this case could result from decreased activity of the limiting AHR with its other partners.

Emerging information about the constraints on protein dimerization has shown that these interactions need not be limited to domains of like type. For example, the zinc-finger domain of the glucocorticoid receptor has been shown to form a complex with an unrelated region of the basic/leucine-zipper (bZIP) protein Fos, preventing it from interacting with its bZIP partner Jun (Kerppola et al 1993). These considerations must be included in the types of responses that TCDD may perturb; either the AHR or ARNT may have dimeric partners outside the bHLH-PAS protein family. As mentioned above, such a phenomenon has already been shown for AHR-Hsp90 and Per-Tim interactions (Gekakis et al 1995).

Another possible mechanism for toxicity is that TCDD activation of the AHR may result in such dramatic and sustained activation of cytochrome P4501A1 and related genes that squelching of unrelated transcriptional processes occurs through saturation of coactivators. For example, overexpression of one steroid hormone receptor can diminish the activity of another, presumably through sequestration of common accessory factors or coactivator proteins (Meyer et al 1989). A similar mechanism may apply to the bHLH-PAS proteins and play a role in TCDD toxicity.

The Limited-Restricted Pleiotropic Response Model

An early model to explain TCDD toxicity proposed a limited and restricted pleiotropic response (Poland & Knutson 1982). In this model, most tissues challenged with TCDD respond with a limited response consisting primarily of the induction of xenobiotic-metabolizing enzymes (adaptive response pathway). Under certain conditions, particular tissues might be permissive for the expression of a set of genes that is normally restricted, or unexpressed. The activation of these additional genes presumably results in the characteristic toxic response to TCDD, and variations in the identity of these genes between different cell types may account for the species and tissue differences seen (toxic response pathway). This earlier model fits well with the models proposed above and recent evidence demonstrating tissue-specific differences in TCDD induction of certain genes. In human keratinocytes, for example, the plasminogen activator inhibitor-2 and TGF- α genes are induced in response to TCDD treatment; however, in TCDD-treated rat hepatocytes neither gene is induced (Choi et al 1991, Sutter et al 1991, Vanden Heuvel et al 1994). In contrast, the P4501A1 gene is strongly induced by TCDD in both cell types (Sutter et al 1991, Vanden Heuvel et al 1994). Variably expressed or inducible

cell-type specific and species-specific coactivator proteins may be the molecular mechanism behind the restricted response that results in TCDD toxicity. Those species and/or cell types showing this broad spectrum response may express accessory factors that allow the activated AHR-ARNT complex to transcribe a set of genes resulting in the TCDD toxic phenotype. This type of regulation has recently been described for the POU homeodomain transcription factor Oct-1, with the identification of a B cell-specific coactivator called alternately Bob1 or OBF-1 (Gstaiger et al 1995, Strubin et al 1995). This protein interacts with Oct-1, allowing this ubiquitous transcription factor to regulate a set of B lymphocyte-restricted genes.

Ahr KNOCKOUT MICE

Despite our increased understanding of the AHR signaling pathway, fundamental questions remain concerning the endogenous function of the AHR and its role in the toxicity of TCDD. The use of gene targeting technology to inactivate murine genes *in vivo* (knockout mice) has been a powerful technique to elucidate protein function, confirming predicted actions in some cases while uncovering unexpected roles in others. The *Ahr* gene is an ideal candidate for targeted inactivation; *Ahr* null mice might demonstrate an unknown AHR function (endogenous pathway) and provide a valuable model system for investigation of TCDD toxicity. *Ahr* null mice have been generated independently by two groups, yielding very different phenotypes (Fernandez-Salguero et al 1995, Schmidt et al 1996). These phenotypes, as well as possible reasons for the differences between them and their implications for AHR function, are discussed below.

Our laboratory has used gene targeting to delete exon 2, which encodes the bHLH DNA-binding and dimerization domain, generating an *Ahr* null mouse line (Schmidt et al 1996). RT-PCR analysis detects the presence of a full-length alternately spliced *Ahr* transcript, lacking exon 2, produced from the targeted allele. This splicing event generates a frame-shift, and detailed Western blot, as well as functional assays, detect no AHR protein in this model system. We believe, therefore, that our *Ahr*^{-/-} mice represent a true knockout and that the phenotype we observe results from the loss of AHR activity. Our *Ahr*^{-/-} animals are viable and fertile; however, they exhibit a spectrum of hepatic defects suggesting that the AHR may play a previously unrecognized role in liver growth and development. *Ahr*^{-/-} mice appear normal at birth but display slowed growth for the first few weeks of life. At 1 week of age, these animals show a dramatic yet transient liver phenotype including decreased liver weight, fatty metamorphosis, and increased residual extramedullary hematopoiesis. Detailed analysis of the decrease in liver weight has shown that this aspect of the *Ahr*^{-/-}

phenotype is present at all ages examined so far, from birth through 6 weeks. The fatty change of the liver, however, develops after birth and resolves entirely by 3 weeks of age. The residual extramedullary hematopoiesis resolves by this age as well. Older *Ahr*^{-/-} mice (beyond 3 weeks of age) begin to develop mild portal hypercellularity with thickening and fibrosis, and approximately 50% of animals have enlarged spleens by 6 weeks. Although the underlying basis for this phenotype is unknown and will be the subject of much future study, we believe it may represent a hepatic developmental delay. The phenotype we have described may indicate a role for the AHR in liver growth and maturation to a functionally metabolic organ. In addition to providing additional functional roles for the AHR, these mice will serve as valuable tools to delineate receptor-mediated from nonreceptor-mediated effects of various AHR agonists.

Ahr^{-/-} mice that display a quite different phenotype from that seen in our mice were first generated by Fernandez-Salguero et al (1995). This group targeted their inactivating mutation to the first exon of the *Ahr*, deleting the initiation methionine and a portion of the basic region. For convenience in contrasting the two *Ahr* null mouse lines, we have designated these mice alleles by the exon that has been deleted, i.e. the Fernandez-Salguero et al mice as *Ahr*^{Δ1/Δ1} and our mice as *Ahr*^{Δ2/Δ2}. Demonstration of a true null allele by analysis of AHR protein or message was not included in the *Ahr*^{Δ1/Δ1} report. However, these mice do not induce P4501A1 in response to TCDD. The mice display a 50% neonatal mortality rate, with inflammation of several major organ systems. Surviving *Ahr*^{Δ1/Δ1} mice have decreased liver weights and portal fibrosis similar to that seen in the *Ahr*^{Δ2/Δ2} mice; however, both phenotypes appear to be more severe in the *Ahr*^{Δ1/Δ1} mice. Additionally, the *Ahr*^{Δ1/Δ1} animals have a severely depressed immune system, with an 80% decrease in total splenic lymphoid cells at 2 weeks of age that gradually resolves over time. Despite experiments designed to specifically address these differences, we see no evidence of neonatal lethality or immune cell depletion in our *Ahr*^{Δ2/Δ2} mice. The reasons underlying the phenotypic differences between the two *Ahr* null mouse lines remain unclear.

In our attempts to explain these differences, we have considered possibilities such as genetic background effects, partial inactivation of the allele, and differences in environmental factors. Because both *Ahr*^{-/-} mouse lines were generated on 129 × C57BL/6 backgrounds, differences in genetic background are an unlikely cause. The possibility that the phenotypic differences result from a partial knockout in one of the mouse lines deserves consideration. We have demonstrated that the formation of any functional protein from our targeted *Ahr* allele is unlikely because we detect no AHR protein in *Ahr*^{Δ2/Δ2} mice by Western blot using three domain-specific antibodies. Additionally, we

have shown that our *Ahr*^{Δ2/Δ2} mice are not inducible for cytochrome P4501A1 activity by TCDD. Analysis of AHR message or protein was not provided in the characterization of the *Ahr*^{Δ1/Δ1} mice; however, the lack of P4501A1 induction in response to TCDD in these animals suggests that this mouse line represents a functional inactivation of the *Ahr* gene as well. A third possibility is that the differences may result from environmental factors. Two experiments argue against such differences. We have attempted to provide our *Ahr*^{Δ2/Δ2} mice with an environment free of PAH compounds through the use of highly purified food and bedding material. These environmental modifications had no effect on the described pathology. Additionally, we have established *Ahr*^{Δ2/Δ2} colonies at Northwestern University and the University of Chicago with no differences in *Ahr*^{Δ2/Δ2} phenotype. Clarification of the differences between these two *Ahr* null mouse lines may require intercrossing and direct comparison of the *Ahr*^{Δ1/Δ1} and *Ahr*^{Δ2/Δ2} animals.

CONCLUSION

The AHR lies at the heart of two important toxicological problems: the regulation of xenobiotic metabolism by PAHs and the receptor-mediated toxicity of halogenated dioxins. Recent years have seen a remarkable clarification in our understanding of AHR biology. Despite these advances, our satisfaction is tempered by the realization that many of the most interesting questions remain unanswered. Why does the AHR signaling pathway exist? What are the selective pressures that have led to the conservation of this pathway in organisms from diverse environments? How does this receptor mediate toxic responses such as immune suppression, wasting, epithelial hyperplasia, cancer, terata, and death? How closely does the mechanism of xenobiotic metabolism induction mirror the pathway to toxic endpoints? An unexpected result of this research has been the realization that the AHR represents a novel mechanism for transduction of environmental signals to the nucleus. The structural similarities of the AHR with the ARNT, HIF-1 α , and Per proteins suggest that this model system may yield information important to our understanding of organismal responses to environmental cues that influence biological rhythms and regulate metabolism under conditions of low oxygen.

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