Chapter 16

The Aryl Hydrocarbon Receptor in Immunity: Tools and Potential

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Abstract

The signaling pathway of the evolutionary old transcription factor AhR is inducible by a number of small molecular weight chemicals, including toxicants such as polycyclic aromatic hydrocarbons, bacterial toxic pigments, and physiological compounds such as tryptophan derivatives or dietary indoles. AhR activation is of immunological importance, but at the same time mediates toxicity of environmental pollutants, such as immunosuppression by dioxins. Measuring AhR activity and identification of ligands is thus of great interest for a variety of research fields. In this chapter, I briefly introduce the AhR signaling pathway, its role in immunology, and the tools and assays needed to analyze AhR signaling. Both are also needed when therapeutic applications are envisioned.

Key words Aryl hydrocarbon receptor, PAS-bHLH proteins, T cells, Innate lymphoid cells, AhR ligand binding and activation assays, TCDD, Immunotoxicity, Dioxin

1 Introduction

Interaction with the environment and building meaningful physiological responses is pivotal for organisms. In fact, much in the science of biology is about the study of cellular differentiation and interaction. Signaling and induction of gene expression are at the core of differentiation and response to external triggers. Signaling is mediated via signaling molecules and their receptors. Major signaling pathways are known. Surface receptor (e.g. cytokine receptors, G proteins) mediated transmission eventually amplify a signal via a cascade of downstream events (such as MAP kinase signaling). In contrast, nuclear receptors (e.g. steroid receptor or thyroid receptors) are transcription factors themselves. The aryl hydrocarbon receptor (AhR) is a nuclear receptor which can sense and respond to certain chemicals. AhR has been studied for a long time by toxicologists because it binds to and mediates toxicity to the environmental pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other, often anthropogenic, polycyclic aromatic

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hydrocarbons (PAH). However, it has always been considered unlikely that AhR has evolved to recognize a modern environmental pollutant. In recent years, the physiological functions of AhR signaling have been studied increasingly and revealed numerous pivotal functions of AhR for cell differentiation, proliferation, and function of immune cells [1–3]. AhR might also be a pattern recognition factor for bacterial pathogens [4]. AhR research is highly interdisciplinary. This chapter provides a discussion on the role for AhR in immunity and major tools in measuring and assessing AhR activation. Both are needed when therapeutic applications are envisioned.

2 The Family of PAS-bHLH Proteins

The AhR belongs to the family of Per-ARNT-Sim-basic-helix-loop-helix (PAS-bHLH) proteins [5, 6], one of the three main families of bHLH proteins. PAS-bHLH proteins are transcriptional regulators controlling essential gene expression in adaptive responses. The acronym PAS indicates a domain, which was first identified in the drosophila proteins PER and SIM, and in ARNT. The PAS domain is common for proteins which can sense environmental clues, such as oxygen in the case of the PAS-bHLH member HIF-1α, or polycyclic aromatic hydrocarbons in the case of the AhR [7]. Also bacterial PAS-containing proteins are known, e.g. redox sensor DOS from *Escherichia coli* [8]. Some plants use PAS domain containing proteins for photoreception [9]. The PAS domain is approximately 300 amino acids long and some PAS-bHLH proteins have two degenerate repeats, PAS-A and PAS-B. Characteristically, PAS-bHLH proteins form functional homo- or heterodimers via their PAS domains. Recently, the murine PAS-A domain has been crystallized [10], and the authors could detail the heterodimerization of the AhR with its partner molecule AhR-nuclear translocator (ARNT) via PAS. Also, the PAS domain includes the ligand binding domain (LBD). The LBD appears spatially conserved. AhR binds its ligand in the PAS-B domain [11, 12]. PAS-bHLH proteins are evolutionary old, members exist in both vertebrates and invertebrates, e.g. in the nematode *Caenorhabditis elegans*. A crystal structure of the full AhR has not been reported, although it would be of enormous interest. Figure 1 shows the basic sections of AhR protein. Table 1 lists members of PAS-bHLH proteins.

3 Biochemistry of AhR Signaling

The biochemistry of so-called canonical signaling via AhR is well-known (reviewed by [13]). AhR resides in the cytoplasm in a multi-protein complex, which consists of two heatshock protein

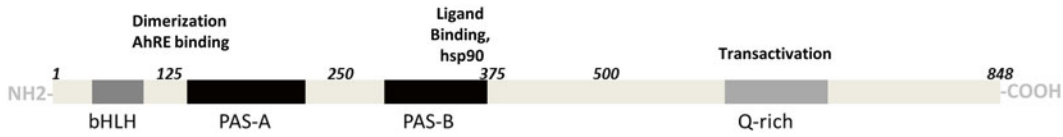

Fig. 1 Graphic scheme of domains in AhR. In humans, AhR is 848 amino acids long. A basic amino acid stretch is placed near the N-terminus, followed by a domain with two alpha-helices connected by a loop. This region binds to DNA and is important for nuclear translocation as well. At around amino acid 120 the first PAS domain begins. PAS domains are about 100 amino acids long. Dimerization with ARNT, ligand binding (at PAS-B), and also attachment with chaperoning proteins such as AIP occur in this region. Finally, toward the C-terminus a transactivation domain is found, necessary for the transcription factor activity of AhR

Table 1
Some members of PAS-bHLH protein family

"Sensor" Class I (class α)	Major function	Mouse model(s) available[a]	"Partner" Class II (class β)	Mouse model(s) available[a]
AhR	Chemical sensing/ immunity, metabolism	Yes	ARNT (=HIF1β)	Yes
AhRR	Suppression of AhR	Yes	ARNT2	Yes
HIF1α	Hypoxia sensing	Yes	BMAL1 (ARNTL1; MOP3)	Yes
HIF2α		Yes	BMAL2 (ANRTL2)	
HIF3α (IPAS)		–		
Sim1	Embryogenesis			
Sim2		Yes		
Clock	Circadian rhythm			
Per (lacks bHLH)		Yes		
NPAS2				
NPAS1	Neurogenesis			
NPAS3	Suppression of HIF			
NPAS4	Memory	Yes		

[a]To date

(hsp)-90 molecules, co-chaperon p23, and the immunophilin-related AhR-interacting protein (AIP, formerly known as XAP2 or ARA9). Hsp90 molecules prevent proteolytic degradation, while AIP prevents binding of the molecule importin [14], and thus premature nuclear import. The AhR complex disintegrates once a ligand binds into the AhR ligand binding pocket, and at the same time a nuclear translocation site (NLS) is exposed, and AhR is imported into the nucleus [15, 16]. Ligands of AhR can be either present in the cells, or must cross the cell membrane (and therefore are likely to be very lipophilic). There is no known AhR ligand transporter system, but import apparently requires dephosphorylation in

the NLS region [16]. Due to the omnipresence of ligands, a constitutive activity of AhR must be assumed. Within the nucleus, AhR dimerizes with ARNT molecules and binds to a short DNA sequence with the substitution intolerant core sequence (5′-GCGTG-3′). This sequence is called "dioxin-responsive element" (DRE), "xenobiotic response element" (XRE), or AhR-responsive element (AhRE). To initiate transcription, co-activators (such as SP1, NCOA1, p300) are recruited and chromatin structure changes. Eventually specific and general transcription factors enable that RNA polymerase II starts transcribing the gene [15, 17–20]. AhR-induced gene transcription is highly cell- and tissue-specific [21–23]. Many genes contain putative AhREs, but only few are actually targeted by AhR. The specificity is controlled by factors such as DNA accessibility, AhRE sequence and placement within promoter and other promoter elements, or availability of cofactors. Nonetheless, much of this is not understood and requires further research.

In addition to the canonical gene induction by AhR:ARNT, interactions of AhR with proteins from other signaling pathways have been described in recent years, most notably in cells of the immune system. AhR can associate with a number of proteins, including retinoblastoma protein, NFκB, STATs, c-maf, or β-catenin [24–31]. Apparently, this ability to tie into other signaling pathways allows for great flexibility and builds a network of cellular responses in cell proliferation, development, or inflammation. The interactions are highly cell- and situation-specific. Finally, purely cytoplasmic events can be initiated by dissociation of the AhR complex. A rapid increase in Ca^{2+} concentration or phosphorylation of the EGF receptor by c-src has been described in this context [32, 33].

4 Immunosuppression by TCDD and PAHs

PAHs are persistent in the environment and continue to be a regulatory challenge and ecological concern [34]. Measures have been taken in the last decades to remove PAHs from the environment but much remains to be done. New sources of pollution, such as electronic waste reclamation in developing countries have emerged, which lead to intolerably high intakes of toxic equivalents for workers and people living in the respective areas [35]. These new pollution scenarios must be dealt with by national governments in accordance with international conventions.[1] Toxicological assessment looks at various endpoints. One of them is immunotoxicity. Immunosuppression is caused by very low doses of TCDD in laboratory animals [36–38]. TCDD has a broad range of effects,

[1] http://chm.pops.int/Convention/ConventionText/tabid/2232/Default.aspx (accessed June 8, 2015).

causing atrophy of secondary lymphoid organs (thymus, lymph node, spleen) and functional impairment of many immune cells [39–42]. As a result, TCDD is immunosuppressive on the systemic level. Epidemiological evidence of TCDD immunotoxicty in humans was gathered after major poisoning incidents, such as the release of TCDD into the environment in 1976 after an explosion in a plant in Seveso, Italy. However, results were sometimes conflicting, and correlation with exposure is not always available. In general though, the data support the view that the human immune system is a target of dioxin-like substances [43]. Similarly, in vitro studies with human cells have shown that immune functions are adversely affected by dioxins, albeit a robust human biomarker for "immune competence impairment" is still lacking. This remains a major challenge for immunotoxicologists.

5 AhR and Immunity

Studies of AhR-deficient mice have highlighted its role for differentiation and function of immune cells. AhR is expressed highly in several hematopoietic cells from both the innate and adaptive immune system. Microarrays and studies using cell sorting combined with real-time PCR and Western blotting have identified Lin-Sca$^+$ and Sca$^-$ progenitor cells in bone marrow (BM), double-negative (CD4$^-$CD8$^-$) DN4 cells in thymus, CD4$^+$Th17 cells, innate lymphoid cells type 3 (ILC3), BM-derived dendritic cells (DC), γδ T cells, and Langerhans cells (LC) as subpopulations with high AhR levels [44–51]. Also keratinocytes, mast cells, and immune cells of the skin express AhR [52]. Dendritic cells (DC) and γδ T cells have simultaneously a constitutively high expression of AhR repressor (AhRR). The significance of this is not known.

5.1 T Cells

T cells are part of the adaptive immune system. Naïve T cells differentiate upon recognition of their cognate antigen and co-stimulatory signals provided by antigen-presenting cells (APC). Cytotoxic CD8+ T cells are capable of killing infected cells or cancer cells. T helper (Th) cells, on the other hand, orchestrate specific and innate immune responses by secretion of cytokines; for instance they help B cells to differentiate and undergo immunoglobulin class switching, and provide pro-inflammatory or immunosuppressive cytokines for other immune cells. Differentiation from naïve CD4+ T cells into T helper (Th) 1, Th2, or Th17 cells is driven by combinations of cytokines in the micromilieu, which are also provided by APC. AhR expression is not equal among T-cell subsets, or indeed other immune cells. It remains debated whether high AhR expression levels are indicative of physiological importance. However, AhR expression levels can be inducible in T cells and other cells [53–55].

One T-cell subset, Th17 cells, expresses higher constitutive amounts than other T helper cells [47]. Under in vitro Th17 differentiating culture conditions, AhR ligands promote the generation of Th17 [56]. It was further shown that AhR is needed for the expansion of this subset and secretion of IL-22 by Th17 cells [47, 57]. As IL-22 is very important for fighting bacterial infections, lack of AhR can lead to high susceptibility to certain infection [51]. However, Th17 cells and their cytokines are also known for their contribution to tissue destruction in autoimmunity. Unexpectedly, in experimentally induced autoimmunity models exposure to persistent and easily degradable AhR ligands (TCDD, ITE, or FICZ) ameliorated the disease, rather than exacerbating it [57–60]. Disease amelioration was explained by a shift toward generation of regulatory T-cell subsets (Treg). Likely, this is not a direct AhR effect. First, because evidence for induction of FoxP3 by AhR is inconclusive, and second, because mice with a constitutively active AhR in T cells have no increase in Treg [57, 60, 61]. It could be a question of a tolerogenic cytokine micromilieu generated by AhR effects on DC [62, 63]. Clearly, the in vivo situation is more complex, i.e. AhR ligands may induce Th17 directly, but Treg indirectly via DC, and this is balanced out by other parameters such as onset, route of exposure, immune status, and so on.

Regulatory T cells (Treg) secrete IL-10, an immunosuppressive cytokine, which also helps prevent tissue damage. Inducible Treg, natural Treg, and regulatory Tr1 cells were shown to be increased in mice injected with AhR ligands [29, 47, 57, 58, 64]. AhR cooperates with c-Maf in Tr1 cells to induce IL-10 transcription [29, 65]. Kynurenines, tryptophan metabolites, and high-affinity endogenous AhR ligands have been reported to promote Treg formation [57, 63, 66]. Thus, AhR is involved in the balance between Treg and Th17. However, there are still many unknowns, and more research is necessary if this is to be pharmacologically exploited. In particular, understanding ligand-specific and cell-specific interference with immune responses will be pivotal for any therapeutic approach [67].

5.2 Innate Lymphoid Cells

DC are professional antigen-presenting cells, which can sense pathogenic challenges via their Toll-like receptors. DC secrete cytokines upon antigen uptake and thereby generate either a tolerogenic or inflammatory micromilieu, adapted to the type of pathogen and the immunological situation. Their activities thus range from ensuring immune tolerance against dietary antigens to the initiation of a potent immune response upon entering bacteria into skin wounds. Of note, DC express high levels of AhR. In LC, AhR is needed for maturation and function of the cells [49]. Also, expression of the immunosuppressive enzyme indolamine-2,3-dioxygenase (IDO) by DC needs the presence of AhR, and is driven by kynurenine, a ligand of AhR which is produced by IDO;

AhR is in this case a negative regulator of immunogenicity [49, 63]. Triggering of immunosuppression via production of kynurenine as AhR ligands is even used by glioblastoma cancer cells to evade immunity [68]. Consequently, some researchers have looked at the potential of AhR ligands to manipulate immune responses. For instance, one such compound, VAF347, can suppress allergies or suppress graft rejection in a mouse model [62].

AhR signaling has recently been shown to be important for the differentiation and function of other cells of the innate immune system as well. AhR and AhR signaling is necessary for γδ T cells, innate lymphoid cells of the gut and NK cells. Its presence is required for proliferation and expansion in the respective tissues, and for secretion of IL17 and IL22. AhR-deficient mice thus lack important immune cells in their gut and skin, with potentially devastating consequences during bacterial infection and inflammation [45, 51, 53, 59, 69].

5.3 Epithelial Cells

The epithelia of the gut, skin, lung, and genitals present barriers to the environment. They are the first line of defense against unwanted chemicals, but also must allow some passage of chemicals, e.g., from the diet. Maybe not surprisingly, AhR expression is high in most cells of the epithelia, at least as far as analyzed. Immune cells and epithelial cells of the skin, gut, and lung have high AhR levels (reviewed in Esser and Rannug, 2015). For many of these cells, AhR was shown to be important for specific cell responses, such as the ultraviolet (UV) B stress response in keratinocytes of the skin [32, 70], or proliferation of ILC3 in the gut [51]. Intriguingly, ILC3 proliferation is also impaired when the AhR ligands are removed from the diet, highlighting that the AhR signaling evolved as a sensor for environmental triggers.

5.4 Therapeutic Potential

Immunotoxicology and immunopharmacology are two sides of one coin. Both analyze and describe how chemicals change immune responses. The immune system is very complex and thus the search for drugs which can be used in specific situations reflects this complexity. AhR activity is modulated by the type and affinity of ligands, as well as the target cell type. Persistent or short-term activation of the AhR by ligands can lead to changes in immunity, as known from the effects of TCDD and other polycyclic aromatic hydrocarbons, and from studies using FICZ or other endogenous ligands. As described above 4, immunosuppression is a hallmark of TCDD exposure. TCDD affects numerous immune cells, and similar effects of any AhR ligands ought to be considered carefully. A number of chemicals have been proposed as potential drugs, but so far no clinical trials have been reported. Of particular interest may be drugs, which are already marketed for certain diseases (and thus have undergone phase I and II trials), and have later been identified as AhR activators and thus maybe eligible for new fields of

application. Tranilast is one such example. Originally used as a drug for allergic diseases, it is now suggested as a breast cancer drug [71]. StemRegenin was found to promote proliferation of HSC [72], and kynurenine inhibitors as cancer drugs [68]. UVB irradiation-induced skin damage is dampened by the chemical BDDI [73], and coal tar, a mixture containing many AhR ligands, is a long-standing effective treatment for psoriasis, an inflammatory skin disease. With an ever-increasing knowledge of AhR ligands and their biochemistry and pharmacokinetics, the stage is set, however, for AhR-signaling related drugs. Conceivably, the situation in vivo integrates AhR activation in a more complex fashion than deduced from in vitro data, and in vitro data must be viewed with caution [60].

6 Major Tools

Research on AhR function and detection of novel ligands requires a range of tools. In the following text, I briefly describe such tools and how they can be used in AhR research.

6.1 Anti-AhR Antibodies

More than 200 monoclonal antibodies (mAb) against AhR are currently commercially available. In contrast, only a handful of polyclonal anti-AhR antisera are sold. The majority of anti-AhR mAbs are raised against short peptides from the N-Terminus of AhR. Fewer mAbs exist, which were developed using peptides from the C-terminus of AhR or against the phosphorylated form of AhR (e.g. against pSer-36). Phosphorylation of AhR contributes to nuclear import and DNA binding [16, 74]. Mouse and human AhR have about 80 % homology. Because of this high cross-species homology of AhR, anti-AhR antibodies are often cross-reactive and will detect AhR from human and several "laboratory" animals/rodents.

Before choosing and buying an antibody for AhR detection, it is useful to consider the way the antibody has been quality tested. Because most cells contain ARNT, another member of the PAS-bHLH family with sequence similarity to AhR, cross-reactivity is a risk. Quality tests thus should go beyond showing the size of the "anti-AhR" antibody on a Western blot. Rigorous negative controls must be done as well. The gold standard is testing the antibody or antiserum in question on AhR-negative cells or tissues. Thus Western blots should be done using cell lysates from AhR-negative/low tissues, lysates from cells from AhR knock-out mice, or siRNA knock-down cells. Similarly, for immunohistochemistry, a control with AhR-negative cells or tissues should be provided by the company and done as a routine control when using the antibody.

6.2 AhR Ligand Binding and Activation: Assays and Cell Lines

Identification of AhR ligands and their affinities/capacities to induce AhR-dependent transcription is an important tool in the search for therapeutic ligands, or evaluation of environmental exposure [16, 75]. Many endogenous and endogenous potential AhR ligands have been identified [11]. Ligand binding is the first and decisive step in AhR activation. The affinities between AhR and its ligands are relevant for the outcome of activation of AhR. Toxicological research has used this fact for developing the Toxic Equivalent Factor (TEF) concept, where 2,3,7,8-TCDD, the substance with the highest affinity to AhR, is assigned the value "1," and other substances get factors in relation to their affinity [76, 77]. This then allows describing the toxicity of a chemical mixture, albeit the metabolic stability of a substance and AhR expression levels also influences the toxicity in biological scenarios [78, 79].

Both direct affinity of an AhR ligand to AhR, and the ability to mediate transcription of AhR-dependent genes—most often *cyp1a1* is the gene of choice—can be measured. The latter has to be interpreted with greater caution, excluding indirect gene induction mechanism and the involvement of other transcription factors, such as retinoid X receptor, NF-κB, and others [80–83]. Identification of a true AhR ligand or inhibitor is not trivial [84]. Ligand binding, capacity to trigger nuclear translocation, AhRE binding, and eventually gene transcription are steps in the signaling pathway. Ideally, all of these are measured. Several methods exist, which are described briefly below. An example can be found here [85], where a new class of AhR ligands was recently identified with immune-modulating potential.

6.3 Measuring Gene Induction

For measuring activation of AhR transcriptional activity (rather than direct ligand binding), gene induction is the method of choice. The cytochrome P4501A1 (*cyp1a1*) gene has several DREs in its promoter [21], and is often used for assessing the AhR-activating ability of a ligand. Measurements are done by RT-PCR. Liver has high AhR expression levels, making liver-derived cells such as HepG2 (human), H4IIE (rat), or Hepa1c1 (mouse) suitable and common tools. For control purposes, one can also assay cell lines which have lost or deleted AhR or ARNT. A set of murine liver hepatoma cells (Hepa1c1c7 (wild-type), Hepa1c12 (AhR-deficient), Hepa1c4 (ARNT deficient)) has been published by Oliver Hankinson many years ago [86]. In addition, a transient transfection with respective siRNAs is a fast and easy alternative to prove the involvement of AhR and/or ARNT in the regulation of a certain gene of interest.

6.3.1 CYP1 Activity Assays

Rather than CYP450 induction, enzyme activity of CYP1 isoenzymes is often measured. In the so-called ethoxyresorufin-O-deethylase (EROD) assay, 7-ethoxyresorufin is preferentially converted into

resorufin by CYP1A1 won from cell lysates treated with putative AhR ligands. For a more specific measurement of CYP1A2 activity, the methoxyresorufin-*O*-deethylase (MROD) assay, with 7-methoxyresorufin serving as substrate, is often used. The EROD/MROD product—if present—can easily be measured fluorometrically, and the 50 % effective concentration (EC_{50}) values quantified.

6.3.2 Luciferase Reporter Assay

Reporter gene assays are often used in lieu of affinity as well. AhR-expressing cells are stably or transiently transfected with a plasmid reporter vector containing *Photinus* luciferase under the control of a DRE-sequences containing promoter (e.g. derived from rat *cyp1a1*). These cells can then be treated with putative AhR ligands, and AhR activation measured as luminescence. In transient transfection experiments, parallel transfection with a plasmid coding for a different luciferase (e.g. from *Renilla* under a strong constitutive promoter) should be used. The so-called CALUX assay consists of stable transfectants of the plasmid pGudLuc1.1 into rat hepatoma cell line H4IIE [87–90]. In Fig. 2a, the scheme of such a reporter plasmid is shown. The pGudLuc plasmid contains four functional DREs from the murine *cyp1a1* gene that confer TCDD responsiveness upon a MMTV promoter and adjacent luciferase gene. A human HepG2 cell line with a luciferase reporter plasmid was described as well [89]. A dose–response curve can then be derived which gives EC_{50} values (Fig. 2b). These assays are interesting in particular because they can simultaneously assess and quantify the presence of agonizing and antagonizing AhR ligands in samples, including mixtures. They are very useful for screening and monitoring, especially in environmental studies. In a note of caution, inhibition of luciferase activity by the test substance must be excluded.

6.3.3 Nuclear Translocation

To study translocation of AhR into the nucleus, another important parameter of AhR activation upon ligand binding, an expression vector plasmid has been developed. It contains AhR fused to a fluorescent EGFP gene in the plasmid pEGFP-C1 [32]. Cells which are transfected with this plasmid can be treated with the AhR ligand in question and the translocation of the AhR-EGFP fusion protein into the nucleus can then be followed in a fluorescent microscope. Nuclear translocation can also be assessed in Western blots by comparing band intensity of nuclear versus cytosolic fractions upon ligand treatment of the cells. Finally, immunohistochemistry with a sensitive antibody can reveal nuclear translocation.

6.3.4 Competition Assay

The above methods measure indirectly, whether AhR activity leads to transcription. Varying background levels of the natural high-affinity ligand 6-formylindolo[3,2-*b*]carbazole (FICZ) in cell culture media leads to a particular problem for assays of AhR activation in cultured cells [91, 92]. FICZ binds to the AhR with very

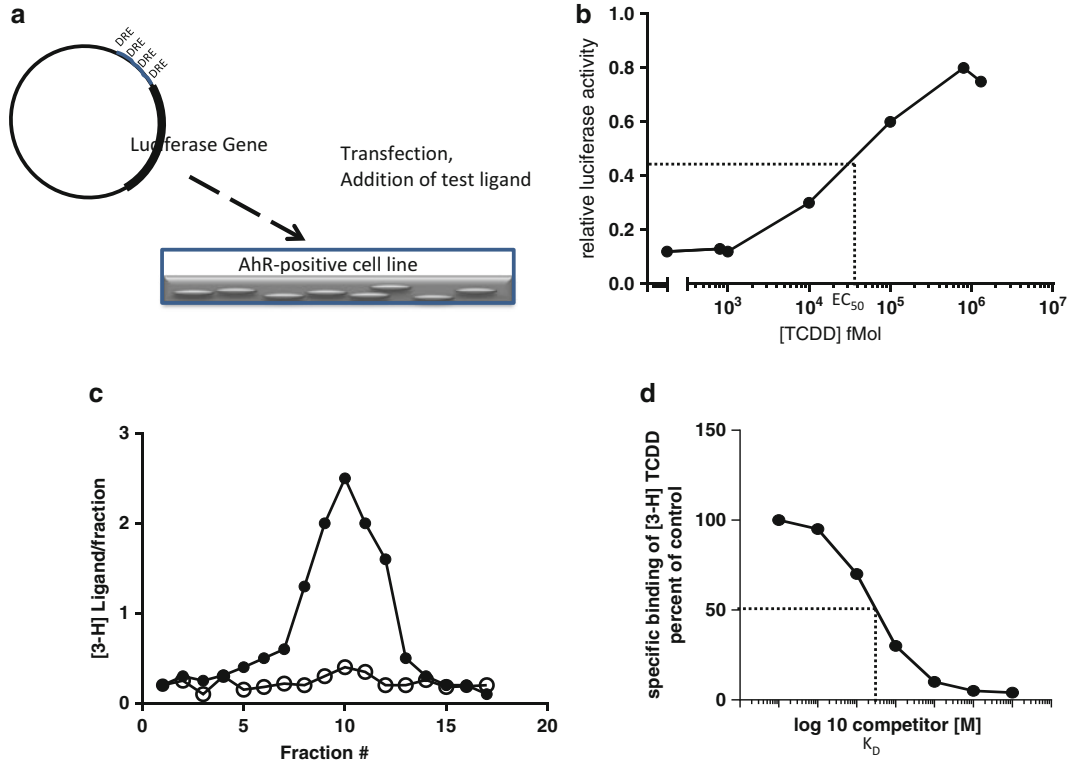

Fig. 2 Measuring AhR activity and AhR ligand affinity. A number of methods to screen for AhR activity and determine ligand binding affinity exist, as described in the text. (**a**) The luciferase reporter assay. A plasmid with the gene for luciferase under the control of a DRE-containing promoter is transfected transiently or stably into an AhR-proficient cell line. The ligand or environmental sample to be tested is added to the transfected cells. Finally, luciferase enzyme activity is measured as luminescence after addition of luciferin as the substrate. (**b**) The intensity of luminescence is correlated to ligand concentration, and can be expressed as EC_{50}. Note that the assay does not allow comparing absolute ligand affinities across labs, unless conditions were exactly the same. (**c**) Proving ligand binding—rather than induction of AhR-dependent transcription—requires biochemical competition assay. Radioactively labeled ligand, e.g. TCDD, is incubated with AhR-containing cell lysates and the amount of bound activity measured across fractions collected from a sucrose gradient. Cold TCDD is added in excess to show that binding has indeed occurred. (**d**) A graph derived from a competition assay, where binding is expressed in percentages across the molar input of ligand. The absolute affinity K_D in molar concentration can then be read off the graph, and compared between various ligands

high affinity ($<10^{-12}$ M), efficiently induces *cyp1a1* transcription and is quickly degraded by CYP1A1 enzyme activity [93]. Chemicals which inhibit CYP1A1 could thus appear as AhR activators in all the assays above [94]. Measuring direct binding of a putative ligand in competitive binding assays is therefore the only way to prove that a given chemical is an AhR ligand [95, 96]. Serial dilutions of the competitors to be tested are added to AhR-containing liver cytosol and incubated with radioactively labeled TCDD ([^{3}H]-TCDD). To control for nonspecific binding, samples are treated with radioactive TCDD together with an excess

amount of "cold" TCDD or TDCF. Unbound radioactive TCDD is removed by dextran-charcoal and the remaining solution separated on a linear sucrose gradient. Fractions are collected and the radioactivity in the fractions is determined by liquid scintillation counting. Finally, the specific binding to the AhR is calculated by subtracting the amount of radioactivity in the fractions containing radioactive TCDD together with the excess "cold" TCDD from the activity in the fractions containing radioactive TCDD [75]. Binding affinity can then be determined by plotting the specific binding relative to the concentrations of the competitor (Fig. 2c, d). Several different protocols exist and it is common that [^{125}I]2-iodo-7,8-dibromodibenzo-p-dioxin, DBDD, is used instead of [^{3}H]-TCDD. Alternatively, the hydroxyapatite (HAP) assay can be done, which was first described in 1982 [97], and has been modified since [98]. In this assay, bound radioactivity is retrieved from the mixture with hydroxyapatite. Aliquots of cytosol are incubated with [^{3}H]TCDD and different concentrations of the ligand to be tested. Thereafter, hydroxyapatite suspension is added to the different reaction mixtures and incubated. The suspension is pelleted, washed, and measured in a scintillation counter. Displacement of [^{3}H]-TCDD by the test ligand is then calculated, and corrected for nonspecific binding [99].

6.3.5 Chromatin Immunoprecipitation Assay

This assay directly identifies the binding of a transcription factor to a promoter such as AhR:ARNT to the AhRE element. Briefly, cells (approximately 1×10^6 are needed per sample) are incubated with ligand, then fixed with, e.g. 1 % formaldehyde, to crosslink DNA–protein complexes. Cells are lysed and DNA is sheared to fragments of approximately 300–500 bp size. Cell debris is cleared away, and the DNA–protein complexes are precipitated by a specific antibody against AhR. The antibody can be coupled to magnetic beads, sepharose or similar to enable and enhance precipitation or isolation from the mixture. Finally, the DNA–protein complex is "decrosslinked," protein-digested with proteinase K, and the remaining DNA fragments amplified by RT-PCR [100, 101]. PCR fragments can be electrophoresed and further identified by sequencing, cloning or on a microarray (ChIP-on-chip). The quality of the assay depends of course on the quality of the antibody (see above). ChIP assay kits are commercially available by now, and ChIP technology is quickly evolving, in particular with a view to reduce the number of cells needed, and solve specificity issues.

6.3.6 EMSA Electrophoretic Mobility Shift Assay

Finally, a somewhat older method to assess the capacity of a ligand to induce AhR-DNA binding must be included. Again, cytosolic extracts are incubated with the ligand in question. The cytosolic extracts are then incubated with radioactive [γ-32P]-labeled DNA, i.e. the AhRE consensus sequence. The products are separated and visualized on a polyacrylamide gel. Specificity of the binding of

Table 2
TCDD-resistant and susceptible rodent strains

	High susceptibility	Low susceptibility
Rats (*Rattus norvegicus*)	Long-Evans (*Turku*/AB) (inbred) LnC Sprague–Dawley (outbred)	Han/Wistar (*Kuopio*, closed colony) LnA
Mice (*Mus musculus*, *Mus spretus*)	AhRb-1 C57BL/6 AhRb-2 CH3/HeJ A/J BALB/c AhRb-3 *Mus spretus*	AhRd 129 DBA/2 NZB AKR SJL/J

AhR:ligand can be confirmed by using unlabeled AhREs as competitors. Addition of anti-AhR or anti-ARNT antibodies to induce a supershift on the gel (i.e. a slower electrophoretic movement due to the larger complex) is a further proof of the specificity of the AhR:ligand:DNA complex [98, 99].

6.4 Rats and Mice

Mice and rats with AhR alleles leading to different dioxin sensitivity are known [102, 103] (Table 2). High-susceptibility rat strains are Long-Evans (Turku/AB), Sprague–Dawley, and LnC (bred from L-ExH/W). Low-susceptibility strains are Han/Wistar and LnA [103, 104]. In rats, the difference in susceptibility is caused by a deletion in the transactivation domain, resulting in the loss of transcriptional induction of genes important for TCDD toxicity [104]. High-affinity alleles ($AhR^{b\text{-}1}$, $AhR^{b\text{-}2}$, and $AhR^{b\text{-}3}$) and a low-affinity allele (AhR^{d}) exist in mice. The d allele (e.g. found in DBA/2) differs from the b-1 allele found in C57BL by 10 nucleotides, five of which represent amino acid changes. Affinity of these AhR proteins to TCDD differs approximately by a factor of 100. Congenic C57BL/6 strains have been bred, i.e. strains which differ only at the AhR locus. Several strains of AhR gene-deleted mice have been developed independently in the 1990s. In addition, ARNT-deficient, AhR repressor-deficient, AhR repressor-EGFP reporter strains, humanized transgenics, constitutively active AhR, nuclear translocation signal (NLS) hypomorphs, floxed AhR or ARNT mice and others were developed, offering a comprehensive "zoo" of mice related to AhR signaling. Many of these lines are commercially available. For a discussion of the phenotypes see [102]. In addition, conditional mouse strains, where the AhR is deleted only in certain cell types are increasingly used in research. These mice are generated by breeding $AhR^{flox/flox}$ mice (which were generated by Christopher

Bradfield, University of Wisconsin) with an appropriate Cre-expressing mouse strain. For instance, this has been done for keratinocytes, liver cells, gut epithelial cells, recombination activation gene (RAG)-positive cells, and more [45, 51, 105, 106].

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