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Topic 1.13

Transcriptional roles of AhR in expression of biological effects induced by endocrine disruptors*

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Abstract: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), one of most toxic man-made chemicals, binds arylhydrocarbon receptor (AhR or dioxin receptor), whose endogenous ligand remains unknown, with an extremely high affinity and expresses pleiotropic biological effects.

From analysis of the primary structures, AhR belongs to a distinct group of a supergene family from that of nuclear receptors. PCB, 3-methylcholanthrene, and benzo(a)pyrene are also ligands to the AhR, and these polycyclic aromatic chemicals are considered to display pleiotropic biological effects such as induction of a variety of drug-metabolizing enzymes, teratogenesis, tumor promotion, immunodeficiency due to thymic involution, and liver damage. Generation of the AhR-deficient mice by gene knock-out technology revealed that these biological effects described are mediated by AhR, because AhR(¯/_) mice lost susceptibility to these effects by TCDD and benzo(a)pyrene. It has recently been revealed that AhR is also involved in reproduction of female mice. Although the detailed mechanisms of involvement of AhR in exerting these effects are not always clarified, AhR is generally considered to function as a transcription factor, which activates the expression of genes by binding directly the XRE sequence in their promoter in a heterodimer form with Arnt. From analysis of DNA transfection and GST pull-down assays, it is revealed that AhR and Arnt interact with various coactivators such as RIP140, SRC-1/NcoA, and CBP/p300 to transmit their transactivation activity to general transcription factors (GTFs). AhR has also been shown to interact with various regulatory factors including Rb, NF-κB, and SP1, resulting in mutual inhibition or synergistic enhancement of their activities depending on the mode of localization of their cognate binding sequences in the target genes. Agonistic and antagonistic properties of various ligands to AhR also are discussed.

INTRODUCTION

When taken up into the animal bodies, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and its related compounds induce pleiotropic biochemical and toxicological effects, such as induction of drug-metabolizing enzymes, teratogenesis, immunosuppression due to thymic involution, tumor promotion, and

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liver damage. AhR (arylhydrocarbon receptor) is a receptor type transcription factor and has been believed to mediate these effects as a cellular factor. Under normal conditions, it exists in the cytoplasm in association with a Hsp90 (heat shock protein 90 kD) complex [1]. Upon binding TCDD with an extremely high affinity, the liganded AhR translocates to nuclei where it switches it partner molecule from Hsp90 complex to Arnt (AhR nuclear translocator). Thus, the formed AhR/Arnt heterodimer binds to the XRE (xenobiotic responsive element) sequence in the promoter region of the target genes to activate their expression [1–4]. Extensive studies of AhR have revealed that most, if not all, of the toxic effects of TCDD are mediated by AhR. Here, we briefly summarize recent studies on AhR. Because of limited space, we apologize that this review is not comprehensive.

MOLECULAR ANATOMY OF AhR AND MECHANISM OF TRANSACTIVATION

AhR and its partner molecule, Arnt, belong to a superfamily of structurally related transcription factors characterized by structural motifs designated bHLH and PAS [1,3] (Fig. 1). In the N-terminal region, these proteins contain a basic helix-loop-helix (bHLH) motif, which is shared by another family of transcription factors such as Myc and MyoD, and is involved in DNA binding and hetero- or homodimerization. Adjacent to the C-terminus of the bHLH domain, the sequence consisting of about 250 amino acids named as PAS continues. The PAS domain was originally identified as a conserved sequence between *Drosophila* PER, human ARNT, and *Drosophila* SIM, and includes two imperfect repeats of 50 amino acids, PAS-A and -B, the function of which is considered to be an interactive surface for hetero-

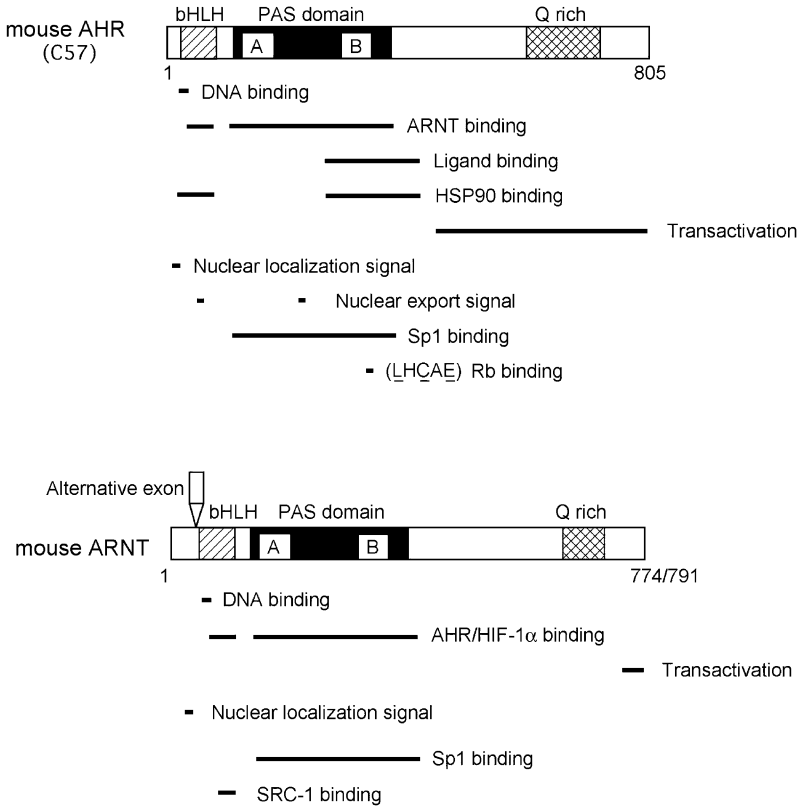

Fig. 1 Map of the functional domains of AhR bHLH: basic helix-loop-helix, PAS: Per/Arnt/Sim homology, Q rich: glutamine rich.

or homodimer formation, with Hsp90 complex and with ligands. The PAS domain is distributed in a wide variety of regulatory proteins involved in circadian rhythm (Per, Clock, BMAL1), hypoxic response (Hif-1α, Hif-2α/HLF), neurogenesis (Sim), and transcription coactivation (SRC-1, TIF2) in the animal kingdom and is also found in bacterial proteins that function as a light and oxygen sensor [4,5].

The ligand-binding domain of AhR is located in the sequence of 230-431 a. a. containing the PAS-B region, and this region overlaps the binding site for Hsp90, which makes it structurally competent to bind a ligand [6]. In addition, Hsp90 bound to the PAS-B domain is also known to interact with the bHLH region to mask the nuclear localization signal (NLS) therein, resulting in the cytoplasmic localization of AhR [7,8]. Under normal conditions, AhR exists in cytoplasm in a complex with Hsp90 containing XAP2 (hepatitis B virus X-associated protein) and P23. Upon binding with a ligand, AhR most probably changes its conformation to expose the NLS and translocates to the nucleus where it switches its partner molecule from Hsp90 to Arnt. As a result, the AhR/Arnt heterodimer recognizes and binds the XRE sequence in the promoter of the target genes to enhance their expression. A recent report has suggested that the Rb pocket sequence, LXCXE in the C-terminal region of PAS-B binds to Rb, thereby affecting the cell cycle in hepatoma cell lines [9]. The transactivation activity is distributed broadly in the C-terminal half of the AhR molecule, which has been reported to interact with multiple coactivators including Myb-binding 1a [10], BRG-1, RIP140 (receptor-interacting protein), and SRC-1/NcoA [11], while that of Arnt is localized at the very C-terminal 43-amino acid sequence [12] which interacts with CBP/p300 as coactivator [13,14]. Arnt contains a constitutive NLS in the N-terminal region of the bHLH domain [15]. As soon as it is synthesized in the cytoplasm, Arnt is transported into the nucleus. In addition, AhR contains a nuclear export signal (NES) in its second helix of the bHLH domain that mediates the nuclear export of the AhR protein, followed by proteasome degradation [16].

It has been reported that liganded AhR was rapidly degraded via the ubiquitin/proteasome pathway after transactivation of the target genes [17,18]. Nuclear localization and phosphorylation of AhR were necessary for the degradation. Interestingly, an inhibitor of protein synthesis, cycloheximide, inhibited the ligand-dependent degradation of AhR [18], although its precise mechanism remains unknown.

Phosphorylation of the AhR/Arnt heterodimer is reported to be also important for transactivation, because the binding activity of the AhR/Arnt heterodimer to the XRE sequence is abolished by the phosphatase treatment [19].

AhR HOMOLOGS

cDNAs for AhR were cloned from various species of animals ranging from mammals to flies and nematodes [3] (Fig. 2). cDNAs for two AhR homologs from *Fundulus* (FhAhR1 and FhAhR2) [20] have been cloned and sequenced, whereas only one AhR cDNA has been identified in mammalian species despite extensive efforts. These two homologs of fish AhR mRNAs are derived from their independent genes. Rainbow trout also expresses at least two isoforms of AhR (rtAhR2α and rtAhR2β). Interestingly, the expressions of rtAhR2α and rtAhR2β mRNAs were remarkably enhanced by TCDD, indicating that expression of AhR may be positively autoregulated in fish [21]. Concerning invertebrate species, structurally related AhR and Arnt homologs have been found in both *Drosophila* and *Caenorhabditis elegans* [22,23]. An AhR homolog in *Drosophila* is designated spineless. Deletion of the spineless gene caused morphological transformation in distal legs and antennae of adult flies. Spineless can form a heterodimer with Tango (an Arnt homolog of *Drosophila*), which recognizes the XRE sequence. AHR-1 is a *C. elegans* homolog of AhR and forms a heterodimer with an Arnt homolog of *C. elegans*, AHA-1, which binds the XRE. However, it remains unknown whether or not these invertebrate AhR homologs can mediate the toxicological effects of xenobiotics including dioxin, because nematode AHR-1 appears to exhibit no ability of binding to β-naphthoflavone like the mammalian AhR [23].

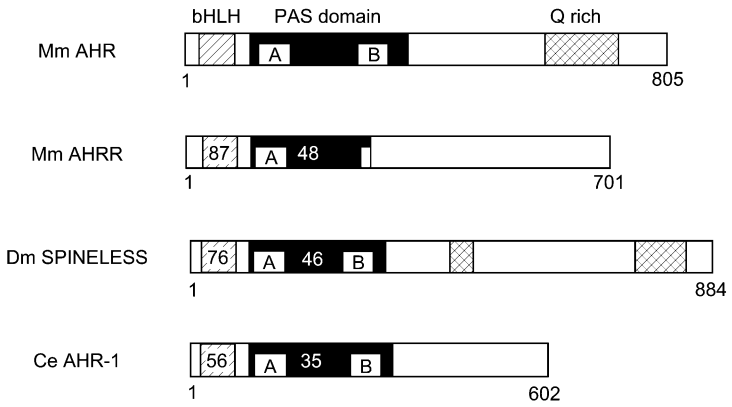

Fig. 2 Amino acid sequence comparison of mouse AhR (Mm AhR), mouse AhRR (Mm AhRR), fruit fly spineless (Dm spineless) and nematode AHR-1 (Ce AHR-1). Figures indicate the percent amino acid identity with mouse AhR.

TARGET GENES OF AhR

A number of phase I (CYP1A1, 1A2, 1B1, and 2A8) and phase II (NADPH-quinone xidoreductase, GST-Ya, UDP-glucronosyltransferase) drug-metabolizing enzymes (DMEs) are known to be induced by AhR ligands. In addition to these enzymes, AhR ligands upregulate expression of the genes involved in cell proliferation (TGF-β, IL-1β, and PAI-2), cell cycle regulation (p27 and jun-B), apoptosis (Bax), ADH3, and DNA polκ [1,24,25]. One or several copies of the XRE sequences are found in their regulatory region, indicating that ligand-activated AhR upregulates the expression of these genes by directly binding to the XRE sequences (i.e., primary effects). On the other hand, indirect mechanisms (i.e., secondary effects) for the induction of multidrug resistance gene mdr1 by 3-MC (3-methylchoranthrene) were also reported [26]. In HepG2 cells, the DNA microarray analysis revealed that 310 genes were either up- or down-regulated by the TCDD treatment [27]. Of the 310 genes whose expressions were affected by TCDD, 108 genes still were observed to be affected even by the simultaneous treatment with cycloheximide, while the altered expression of the remaining 202 genes by the TCDD treatment became unchanged by the treatment with the protein synthesis inhibitor. These results suggested that the expression of the former 108 genes was directly regulated by TCDD without protein synthesis, while regulation of the remaining 202 genes by TCDD required protein synthesis.

Safe et al. reported that the enhanced expression of cathepsin D and c-fos with estrogen treatment was inhibited in the presence of TCDD through the XRE sequence [28]. Although precise mechanisms of this inhibition still remain controversial, there is some possibility that AhR may play an inhibitory role in the transcription by binding to a specific sequence of XRE, designated as inhibitory XRE.

MODULATION OF AhR FUNCTION

As described, AhR exists in the cytoplasm in the complex of Hsp90, p23, and XAP2 in the absence of ligands. It is also reported that the pp60 src protein is also found in this AhR-chaperone complex [29]. As soon as AhR binds a ligand, src kinase is released from the AhR-Hsp90 complex and activates Ras-dependent signaling cascades [30]. At the initiation step of transcription, the AhR/Arnt heterodimer and Sp1 synergistically bind their respective cognate DNA sequences by interacting with each other [13]. In the promoter of the target genes of AhR, there usually exist one or several copies of the GC-box sequence together with the XRE. Synergistic transactivation by SP1 and AhR/Arnt heterodimer is considered to be a common mechanism for inducible expression of the drug-metabolizing enzymes by xenobiotics. Recent studies have demonstrated that Rb and NF-κB also interact with AhR. Liganded

AhR stabilizes the complex of Rb and E2F by binding to Rb, thereby enhancing the repression of the transcriptional activity of E2F and, hence, cell cycle is arrested [31]. It is also suggested that Rb functions as a coactivator of AhR by binding with AhR [32]. The ligand-activated AhR and the RelA subunit of NF-κB interacted with each other to interfere mutually in binding to their respective recognition sequence in the separate genes [33]. On the other hand, TCDD and TPA synergistically enhanced the expression of the AhRR gene driven by the XRE and NF-κB binding sequences in the promoter, probably through the interaction between AhR and NF-κB [34].

AhRR has a bHLH-PAS sequence with a high similarity to that of AhR and inhibits the transcription activity of AhR by competing with AhR for binding to Arnt and then to the XRE sequence [35] (Figs. 2 and 3). Since AhRR expression is induced by TCDD via AhR/Arnt heterodimer, AhR and AhRR form a regulatory feedback loop in the xenobiotic signal transduction.

A hypoxia signal also inhibits AhR-dependent transactivation [36]. In hypoxia, Hif-1α is stabilized and translocates to the nucleus where it heterodimerizes with Arnt in competition with AhR. Thus, xenobiotic and hypoxia signal transduction pathways interfere with each other by competing for a common partner molecule Arnt.

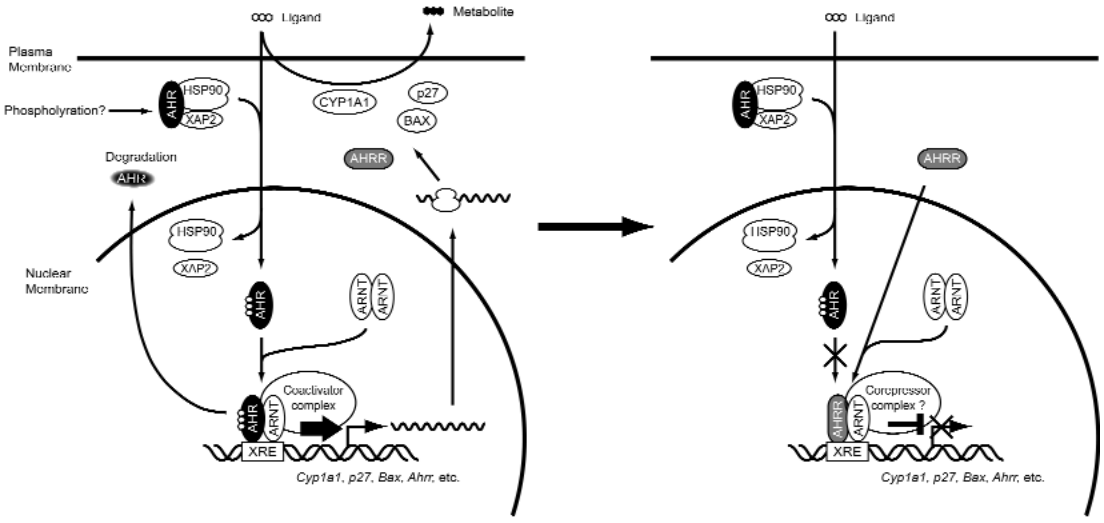

Fig. 3 AhR signaling pathway and negative feedback regulation of AhR function by AhRR.

POLYMORPHISM OF AhR

It is well known that there is a marked strain and species difference in sensitivity to TCDD [1,3]. LD_{50} values of TCDD varies over a 5000-fold range among different species. For example, LD_{50} values vary from 1 μg/kg for the guinea pig, the most sensitive animal to >5000 μg/kg for the hamster, the most resistant. In mice, difference in the responsiveness to TCDD among strains depends on the AhR alleles. cDNA cloning of a responder (C57BL) and nonresponder (DBA) mice revealed that Ala-to-Val substitution at codon 375 and the C-terminal extension due to a mutation in the termination codon in DBA mice reduced the binding affinity of AhR toward TCDD by about 10-fold [37]. Polymorphism in the AhR locus is also found in the rat. TCDD-sensitive Long–Evans (L-E) (LD_{50}, 10–20 μg/kg) and -insensitive Han/Wistar (H/W) (LD_{50}, >9600 μg/kg) rats show about 1000-fold difference in sensitivity. cDNA cloning indicated that L-E AhR cDNA sequence is the same as that of Sprague–Dawley rat previously determined [38]. In contrast, H/W AhR cDNA was revealed to carry a point mutation in the first base of intron 10, resulting in a frame shift with a different sequence of the C-terminal transactivation domain. In human, one genetic variation has been reported at codon 554, resulting in amino acid change

from Arg to Lys [39,40]. However, this mutation does not seem to be related to any altered phenotype of TCDD toxicity in human.

AhR GENE-ENGINEERED MICE

In general, there are two ways to investigate the function of AhR—loss-of-function and gain-of-function studies. As loss-of-function study, three groups reported the generation of AhR-knock-out mice by using the gene targeting technology [41–43]. AhR-null mice were born in normal Mendelian genetics by cross-mating of the heterozygous AhR($^{+}$/_) mice, but their growth was retarded for the first three weeks of life, but returned to normal thereafter. AhR-null mice were revealed to be defective in hepatic development, immune system development, and retinoic acid metabolism. It is reported that female AhR-null mice had difficulty in maintaining conception, lactation, and rearing pups to weaning [44]. Recently, AhR deficiency has been revealed to cause abnormal hepatic vascular structures [45]. Concerning xenobiotic metabolism, the lack of AhR abolished the inducible expression of CYP1A1, 1A2, and 1B1 in mice in response to polycyclic aromatic hydrocarbons, and lost the susceptibility to chemical carcinogenesis by benzo(a)pyrene and teratogenesis such as cleft palate and hydronephrosis caused by TCDD [43,46]. Although the genes responsible for TCDD-induced teratogenesis remain to be identified, the loss of the inducible expression of CYP1A1, 1A2, and 1B1 which metabolically activate various procarcinogens to the ultimate carcinogens, is considered to be a cause of resistance to chemical carcinogenesis. In addition, AhR together with Arnt has been shown to upregulate the expression of DNA polymerase κ, which duplicates DNA in an error-prone manner [47]. This is a good reason why AhR is also involved in the promotion of chemical carcinogens.

As gain-of-function study, analysis of transgenic mice with the expression of a constitutively active AhR has very recently been reported [48]. The constitutively active form of AhR was constructed by deleting a region containing the PAS-B domain and expressed under the control of a modified SV40 promoter and mouse IgH intron enhancer. In the transgenic mice, AhR was rather ubiquitously expressed and CYP1A1 was constitutively expressed in various tissues such as liver, lung, muscle, heart, and others. The constitutively active AhR expressed in the transgenic mice reduced the life span of the mice and induced tumors in glandular part of the stomach, indicating the oncogenic potential of the AhR and implicating the receptor in regulation of cell proliferation.

AhR LIGAND

TCDD is the most potent ligand of AhR with an extremely low K_d value. Other types of halogenated aromatic hydrocarbons such as PCB and polycyclic aromatic hydrocarbons such as benzo(a)pyrene and 3-MC also function as ligands to AhR. Some flavonoids function as an antagonist at low concentrations (~0.5 μM), while they are agonistic for AhR at higher concentrations (>10 μM) [49,50]. It has been reported that 7-ketocholesterol functions as AhR antagonist and actually antagonizes the TCDD effects in vivo [51]. This antagonistic effect of 7-ketocholesterol has been suggested to have physiological significance, when its physiological plasma concentration was taken into account. Resveratrol, a plant steroid, was also reported to be an antagonist for AhR [52]. It has been an interesting and long-lasting problem to identify a true and intrinsic ligand of AhR. Recently, a number of endogenous AhR ligands have been reported to have a high affinity toward AhR. Most of these chemicals are tryptophan derivatives. UV-irradiated or ozone-treated tryptophan products function as AhR agonist [53]. Some of the purified products were reported to function as potent ligand of AhR. Indirubin and indigo isolated from urine were reported to function as even more potent ligands of AhR than TCDD in yeast assay system [54]. It would be difficult to identify a true and intrinsic ligand of AhR, if any, until the intrinsic role of AhR other than induction of drug-metabolizing enzymes is clarified.

CONCLUSION

In summary, AhR is originally identified as a mediator of dioxin toxicities. However, recent studies have suggested that AhR plays a regulatory role in homeostasis and development of animals, and its functional roles in these processes remain to be clarified in future studies.

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