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Canonical and non-canonical aryl hydrocarbon receptor signaling pathways

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Abstract

Decades of research on the Aryl hydrocarbon Receptor (AhR) has unveiled its involvement in the toxicity of halogenated and polycyclic aromatic hydrocarbons, and a myriad of normal physiological processes. The molecular dissection of AhR biology has centered on a canonical signaling pathway in an effort to mechanistically reconcile the diverse pathophysiological effects of exposure to environmental pollutants. As a consequence, we now know that canonical signaling can explain many but not all of the AhR-mediated effects. Here we describe recent findings that point to non-canonical signaling pathways, and focus on a novel AhR interaction with the Krüppel-like Factor 6 protein responsible for previously un-recognized epigenetic changes in the chromatin affecting gene expression.

1. Introduction

Since its discovery in the 1980s, the aryl hydrocarbon receptor (AhR) has been a major focus in toxicology due to the fact that it mediates the effects of the halogenated aromatic hydrocarbon environmental pollutants [1]. AhR studies have mainly focused on understanding molecular basis of toxicity induced by the prototypical AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). As a result, the AhR has been implicated in regulatory processes affecting the immune system, liver homeostasis, cardiac development, wound healing, cell proliferation and apoptosis, tumor promotion and metabolic diseases [2–7]. Studies into the underlying molecular mechanisms have centered on a now well-defined canonical signaling pathway. This commentary seeks to compare and contrast canonical signaling with a recently identified non-canonical pathway that promises to enrich our understanding of AhR biology in response to toxic insults as well as normal physiological processes.

2. The aryl hydrocarbon receptor

The murine AhR was first purified in 1991 and subsequently cloned in 1992 [8,9]. This work revealed that the AhR is a member of the basic helix-loop-helix (bHLH) PAS family of transcription factors defined by the Period (PER), AhR nuclear translocator (Arnt), and

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single-minded (SIM) proteins [10]. AhR expression is essentially ubiquitous in mammals consistent with a broad-spectrum homeostatic role, however expression levels varying widely across tissues with the liver, thymus, lung, kidney, spleen, and placenta exhibiting greatest expression [11]. Additionally, AhR expression is developmentally regulated [12], and more recent evidence indicates a role for the AhR in developmental process affecting hematopoiesis, immune system biology, neural differentiation, and liver architecture [13–17]. Structurally, the N-terminal half of the mammalian AhR is well-conserved, suggestive of functional importance in fundamental physiological activities. In contrast, the receptor's C-terminal region exhibits species differences reflected as polymorphisms and variations in protein length which largely account for the size differences observed between species [18]. The functional impact of the polymorphisms remains largely undefined. A basic sequence and juxtaposed helix-loop-helix region in the (bHLH) domain located near the N-terminus is responsible for DNA binding and Arnt protein dimerization, respectively [16]. The PAS domain, specifically the PAS-B region overlaps with the AhR's ligand binding domain, and confers protein–protein interactions with Hsp90 and the Retinoblastoma tumor suppressor protein [19,20]. The C-terminal half of the AhR protein encompasses a glutamine-rich transactivation domain, and harbors the binding sites for several cofactors including p300, SMRT, SRC1, and RIP140 [21–24]. It is noteworthy that the C-terminal region is also responsible for the interaction with the Krüppel-like factor 6 (KLF6) protein [25,26], the focus of the discussion on non-canonical signaling below.

3. Canonical AhR signaling

Canonical AhR signaling has received attention for decades, and is described in detail in several excellent reviews [5,27,28]. In order to provide context in this commentary, a few of the salient findings are presented here. Canonical signaling begins in the cytoplasm where unliganded AhR is bound by a chaperone complex. This chaperone complex includes heat shock protein 90 (Hsp90) [29], immunophilin-like protein XAP2 (also known as ARA9 or AIP) [30–32], and p23 [33]. Transformation of the AhR by ligand binding is thought to involve a conformational change that reveals the nuclear localization sequence to mediate nuclear translocation of the receptor–chaperone complex via β-importins [34]. The conformational change in the PAS-A domain following ligand binding also facilitates subsequent dissociation of the chaperonins and heterodimerization with Arnt protein through the HLH domain inside the nucleus [35]. The AhR–Arnt heterodimer binds DNA at xenobiotic response elements (XRE) defined by the 5'-GCGTG-3' core consensus motif [36] (Fig. 1, canonical signaling pathway). It should be noted however, that while the core sequence is essential for AhR–Arnt complex binding, evidence exists implicating a role for nucleotides flanking the core motif in DNA binding and function [37,38]. Studies revealed 1) that the AhR's DNA binding affinity was influenced by the nucleotide sequence flanking the core motif, 2) that DNA binding affinity and transcriptional responsiveness were not strictly correlated, and 3) that flanking residues could affect AhR-DNA binding in an agonist-specific manner. However, the veracity of the last observation is challenged by more recent findings [39].

The AhR–Arnt–XRE interactions regulate the expression of a plethora of genes including those encoding both phase I and phase II xenobiotic metabolism enzymes (e.g., the *Cyp1a1*,

Cyp1a2, *Cyp1b1*, and the *Gst-ya* genes) associated with adaptive or toxic responses to exogenous agonists [40–42]. While much is now know about the molecular events that lead to AhR activation and DNA binding, the mechanistic basis for AhR-mediated dioxin toxicity is not yet fully understood. Nevertheless, the widely held consensus is that most, if not all of the toxic manifestations caused by TCDD are AhR mediated [43], and several of the better characterized toxicities are clearly mediated by the canonical AhR–Arnt signaling complex [44].

4. Non-canonical signaling

To better understand TCDD toxicity, several groups sought to identify AhR target genes through DNA microarray studies using cell culture and whole animal models [45–48]. A general observation stemming from these studies is that despite computational analyses covering 3 Kb [47] or 6 Kb [48] of the sequence flanking the transcription start sites of responsive genes, many did not contain a readily identifiable XRE. One possible explanation is that expression of genes lacking a XRE reflects indirect AhR-mediated signaling, and indeed, such changes in expression may be attributed to latent secondary effects. However, it is formally possible that the AhR alters transcription directly through a site(s) distinct from the consensus XRE. For instance, the ligand-activated AhR–Arnt dimer can interact directly with unliganded estrogen receptor to promote formation of a transcriptionally active complex binding to estrogen response elements [49]. Correspondingly, the AhR can form a quaternary complex with p300, pRb and E2F to suppress S phase gene expression [50,51], or with an unknown protein(s) upstream of the *CYP1A2* gene [52]. Evidence is also accumulating for direct AhR-DNA binding in conjunction with the RelB protein to a distinct response element located in the interleukin-8 gene regulatory region [53].

5. KLF6 is a novel AhR DNA-binding partner

The plasminogen activator inhibitor-1 (PAI-1) gene represents an example where direct TCDD responsiveness is attributed to a regulatory region devoid of a canonical XRE [54]. Huang and Elferink [55] recently characterized a novel non-consensus XRE (NC-XRE) consisting of a 5’-GGGA-3’ tetranucleotide motif within the PAI-1 promoter that supports direct DNA binding and function by the AhR independently of the Arnt protein. The lack of sequence homology between the XRE and NC-XRE (Fig. 2) presupposes recruitment of a unique AhR DNA binding complex. Our studies confirmed that AhR-DNA binding to the NC-XRE is absolutely dependent on Krüppel-like Factor 6 (KLF6), a novel AhR partner protein [25]. KLF6 (also known as Zf9 or CPBP) is a ubiquitously expressed Cys_2-His_2 transcription factor belonging to a growing family (17 currently) of Krüppel-like zinc finger transcription factors that regulate processes including cell proliferation, signal transduction, differentiation, and development [56,57]. KLFs are evolutionarily highly conserved across vertebrate species, sharing an 81 amino acid C-terminal zinc finger DNA-binding domain that can interact with “GC-box” or “CACCC-box” DNA motifs in responsive promoters [58,59]. Therefore, it was intriguing to discover that the 5’ basic region in KLF6 juxtaposing the first zinc finger conferred binding to the NC-XRE [25]. Likewise, targeted deletion of the AhR’s basic region necessary for XRE binding had no noticeable impact on DNA

binding to the NC-XRE, suggesting that AhR binding to the NC-XRE is fundamentally different from binding to the XRE.

Mutations in KLF6 and loss-of-heterozygosity have been associated with several human malignancies including prostate, colorectal and liver cancer suggesting that KLF6 is a tumor suppressor [60], functioning by regulating expression of the $p21^{Cip1}$ cyclin-dependent kinase inhibitor [61]. Jackson et al. [62] recently showed that transient expression of the G_1 phase cyclin-dependent kinase inhibitor $p21^{Cip1}$ during liver regeneration is dependent on NC-XRE-mediated AhR-KLF6 regulatory control [62]. This study also demonstrated that sustained $p21^{Cip1}$ induction by TCDD is responsible for the previously observed inhibition of normal liver regeneration [63]. Moreover, these data confirm that the AhR-KLF6 complex is responsible for both normal cell cycle control processes, and the deleterious effects of TCDD resulting in disrupted liver regeneration.

6. Carbamoyl phosphate synthase 1 (CPS1) is a novel AhR-KLF6 cofactor

Using the NC-XRE as a DNA affinity reagent to purify the TCDD-inducible AhR-KLF6 complex, mass spectrometry peptide sequencing identified CPS1 as a component of the complex [64]. CPS1 is an ≈160 kDa multidomain protein that catalyzes the irreversible reaction: $2ATP + HCO_3^- + NH_3 \rightarrow 2ADP + P_i + \text{carbamoyl-phosphate}$[65]. While CPS1 is customarily regarded as a mitochondrial enzyme involved in the detoxification of ammonia through the urea cycle [66], the characterization of CPS1 as a component of the NC-XRE-bound AhR-KLF6 complex revealed a novel role for this protein. Specifically, carbamoyl phosphate, the product of CPS1 catalysis, was shown to carbamylate lysine-rich histones to produce homocitrulline through a non-enzymatic mechanism [67]. The proposed mechanism involves nucleophilic attack of lysine residues by the highly electrophilic acylphosphate group on carbamoyl phosphate. A similar process was previously reported for 1,3-bisphosphoglycerate forming a specific, functionally important post-translational modification on glyceral-dehyde-3-phosphate dehydrogenase [68]. Joshi et al. [64] recently demonstrated that a functionally important lysine residue in histone H1 (K34) is reversibly homocitrullinated (H1K34hcit) following AhR-KLF6 recruitment to NC-XRE sites in the genome. Formation of H1K34hcit is dependent on CPS1 expression. It is noteworthy that acetylation of a K34 on the histone H1.4 variant (H1.4K34Ac) is associated with increased H1 mobility and transcriptional activation [69]. In comparison to core histones that remain in place for several hours, H1 proteins are substantially more mobile with a mean residence time at any one binding site estimated to be ≈3 min [70]. It is tempting to speculate that the H1K34hcit post-translational modification may reduce the mean residence time, resulting in a more open chromatin conformation conducive to increased transcription. It is also possible that the H1K34hcit may represent a distinctive modification in the “histone code” read by regulatory proteins to promote transcription [71].

Peptidylarginine deiminase (PADI) enzymes convert arginine and methylarginine residues to citrulline via a hydrolytic process called citrullination or deimination [72]. Recruitment of PADI2 to the genome was shown to citrullinate arginine 26 on histone H3 culminating in local chromatin decondensation and transcriptional activation [73]. Hence the discovery that the PADI2 gene is a transcriptional target for the AhR-KLF6-CPS1 complex implies that

CPS1 and PADI2 function in concert to modify histones H1 (H1K24hcit) and H3 (H3R26cit), respectively, to alter the epigenome in response to AhR activation [64].

7. Conclusions

The discovery of non-canonical AhR signaling pathways represents new opportunities to reconcile TCDD toxicity at a molecular level. The recent identification of the AhR-KLF6 complex dramatically increases the repertoire of AhR target genes, and ongoing studies will ascertain how they contribute to toxic and normal physiological processes. The complexity in understanding AhR biology is highlighted by findings showing that carcinogenic receptor agonists induce the canonical AhR signaling pathway to promote tumor formation, yet AhR-regulated expression of $p21^{Cip1}$ through the non-canonical pathway is consistent with tumor suppression. Hence, future studies will need to reconcile the contribution of both signaling pathways in order to reach a comprehensive understanding. Since both the AhR and KLF6 are ubiquitously expressed, it is reasonable to assume that this complex is functional in many different tissues, however the role for CPS1 is envisioned to be somewhat restricted given that its expression is predominantly hepatic. Hence, involvement of homocitrullination in epigenetic programming may be largely specific to liver functions. Nevertheless, this does not preclude the AhR-KLF6 complex from recruiting other co-factors to regulate tissue-specific gene expression—be they hitherto known, or ones yet to be discovered. What is evident from the recent studies on non-canonical signaling is that the AhR still holds secrets that need to be revealed before a complete understanding of receptor biology is realized.

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Abbreviations

AhR	aryl hydrocarbon receptor
XRE	xenobiotic response element
hcit	homocitrulline
NC-XRE	non-consensus xenobiotic response element
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
KLF6	Krüppel-like factor 6
CPS1	carbamoyl phosphate synthase 1

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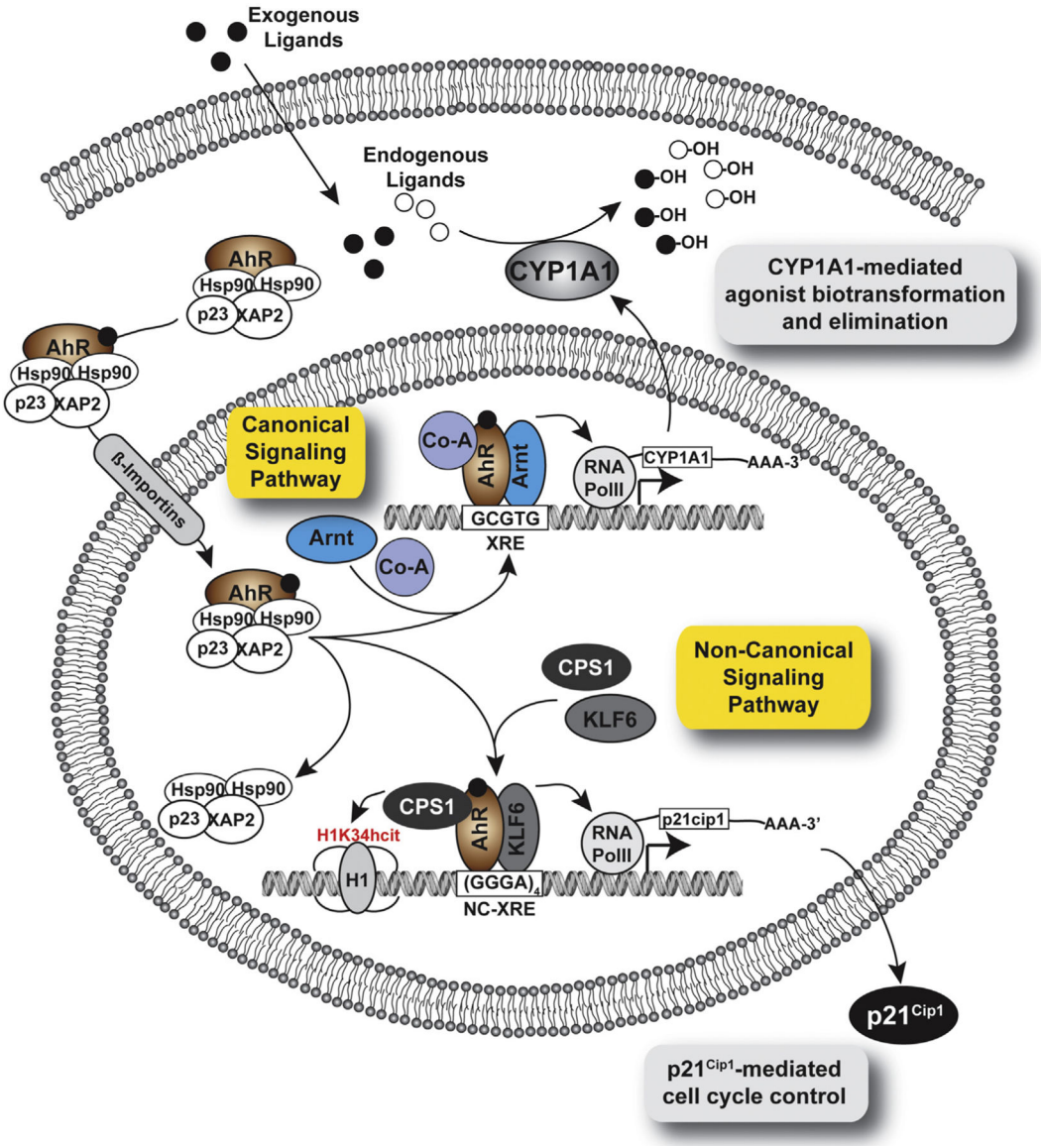

Fig. 1.

The diagram depicts the canonical and non-canonical signaling pathways. The unliganded cytosolic AhR in complex with the chaperones (Hsp90, XAP2, p23) is common to each pathway. Upon ligand binding—by either exogenous or endogenous agonists—the complex translocates into the nucleus, dissociates from the chaperones and forms a DNA-bound complex with the Arnt protein at XRE sites defined by the 5' -GCGTG-3' core recognition motif (canonical signaling), or the KLF6 protein at NC-XRE sites defined by the 5'-$(GGGA)_4$-3' tetranucleotide repeat motif (non-canonical signaling), respectively. The AhR–Arnt complex is known to recruit several co-activators (Co-A) in a context dependent manner, and induces CYP1A1 expression resulting in the biotransformation and depletion of AhR agonists. The AhR-KLF6 complex has been shown to recruit CPS1 as a cofactor responsible for histone H1 homocitrullination on lysine 34 (H1K34hcit). The non-canonical signaling pathway is active in cell cycle control through regulated expression of $p21^{Cip1}$.

Fig. 2.

The CYP1A1 XRE and PAI-1 NC-XRE sequences are presented with the core nucleotides necessary for protein-DNA binding depicted in bold italic type.