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The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways

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ABSTRACT

Exposure to toxic polycyclic aromatic hydrocarbons raises a number of toxic and carcinogenic responses in experimental animals and humans mediated for the most part by the aryl hydrocarbon – or dioxin – receptor (AHR). The AHR is a ligand-activated transcription factor whose central role in the induction of drug-metabolizing enzymes has long been recognized. For quite some time now, it has become clear that the AHR also functions in pathways outside of its role in detoxification and that perturbation of these pathways by xenobiotic ligands may be an important part of the toxicity of these compounds. AHR activation by some of its ligands participates among others in pathways critical to cell cycle regulation, mitogen-activated protein kinase cascades, immediate-early gene induction, cross-talk within the RB/E2F axis and mobilization of crucial calcium stores. Ultimately, the effect of a particular AHR ligand may depend as much on the adaptive interactions that it established with pathways and proteins expressed in a specific cell or tissue as on the toxic responses that it raises.

1. Introduction

The aryl hydrocarbon (dioxin) receptor (AHR) is a cytosolic ligand-activated transcription factor that mediates many toxic and carcinogenic effects in animals and possibly in humans [1,2]. It is generally accepted that its activation in vertebrates causes the toxic and carcinogenic effects of a wide variety of environmental contaminants such as dioxin (TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin), coplanar polychlorinated biphenyls (PCBs) and polycyclic or halogenated aromatic hydrocarbons (PAHs or HAHs). As a consequence of AHR activation, many detoxification genes are transcriptionally induced, including those coding for the Phase I xenobiotic-metabolizing cytochrome P450 enzymes CYP1A1, CYP1A2, CYP1B1, and CYP2S1, and the phase II enzymes UDP-glucuronosyl transferase UGT1A6, NAD(P)H-dependent quinone oxydoreductase-1 NQO1, the aldehyde dehydrogenase ALDH3A1, and several glutathione-S-transferases. AHR is a member of the bHLH/PAS family of heterodimeric transcriptional regulators (basic-region helix-loop-helix/Period [PER]-Aryl hydrocarbon receptor nuclear translocator [ARNT]-single minded [SIM]) [3,4] involved in regulation of development [5] and in control of circadian rhythm, neurogenesis, metabolism and stress response to hypoxia. Evidence from AHR knockout mice, however, points to functions of the receptor beyond xenobiotic metabolism at several physiologic roles that may contribute to the toxic response. Ablation of the *Ahr* gene in mice leads to cardiovascular disease, hepatic fibrosis, reduced liver size, spleen T-cell deficiency, dermal fibrosis, liver retinoid accumulation and shortening of life span (reviewed

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in [6]), suggesting that it has biological functions other than xenobiotic detoxification that likely contribute to the overall toxic response resulting from its activation.

The AHR is widely expressed in practically all mouse tissues [7], and in humans expression is high in lung, thymus, kidney and liver. In the absence of ligand, the AHR exists as part of a cytosolic protein complex containing two HSP90 chaperone molecules, the HSP90-interacting protein p23 and the immunophilin-like protein XAP2 (also AIP or ARA9) [8–10]. Activation by ligand is followed by translocation of the complex into the nucleus, dissociation from the chaperone proteins and heterodimerization with ARNT. This AHR-ARNT heterodimer interacts with several histone acetyltransferases and chromatin remodeling factors [11–15], and the resulting complex binds to consensus regulatory sequences termed AhREs (aryl hydrocarbon response elements; also XREs or DREs), located in the promoters of target genes, and by mechanisms not yet well characterized, recruits RNA polymerase II to initiate transcription. The activated AHR is quickly exported to the cytosol where it is degraded by the 26S proteasome [16], hence preventing constitutive receptor activity.

Activation of the AHR by high-affinity HAH or PAH ligands results in a wide range of cell cycle perturbations, including G_0/G_1 and G_2/M arrest, diminished capacity for DNA replication, and inhibition of cell proliferation. These alternative functions of the AHR are often accomplished in the absence of an exogenous ligand, but the underlying molecular mechanisms governing these processes remain elusive in part because no definitive endogenous ligands have been identified (reviewed in [17]). At present, all available evidence indicates that the AHR can trigger signal transduction pathways involved in proliferation, differentiation or apoptosis by mechanisms dependent on xenobiotic ligands or on endogenous activities that may be ligand mediated or completely ligand independent. These functions of the AHR coexist with its well-characterized toxicological functions involving the induction of Phase I and Phase II genes for the detoxification of foreign compounds.

In this review, we will address novel experimental evidence relating to these less orthodox AHR functions, focusing on new data appearing since our previous review of this subject [17] dealing with the role of the AHR in the activation of mitogen-activated protein kinases, cell cycle regulation, apoptosis and cell differentiation, with a focus on the cross-talk between AHR signaling pathways and the effectors, regulatory events and cell cycle checkpoints responsible for normal cellular functions. Key steps in the activation of AHR signaling are schematically shown in Fig. 1.

2. Cross-talk between cellular kinases and the Ah receptor

Post-translation modifications such as phosphorylation play a major role in the regulation of gene expression and function in

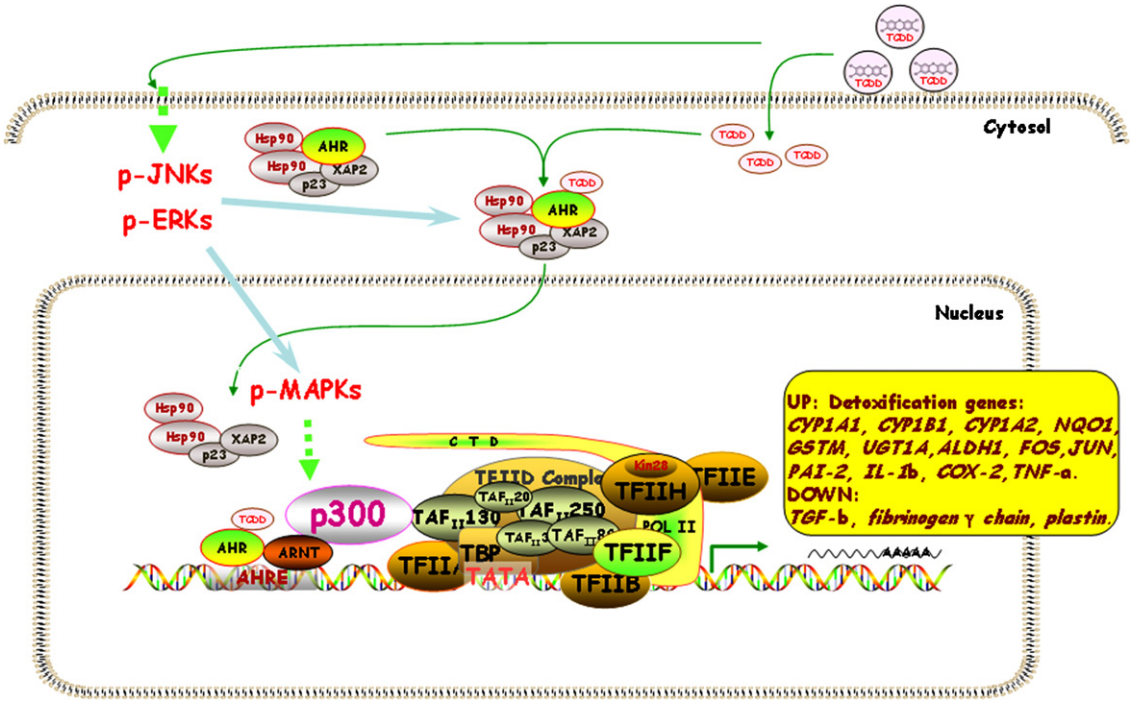

Fig. 1 – *AHR signaling*. Shown are the key events in signaling through the Ah receptor. Entry of ligand (TCDD in the figure) through the cell membrane leads to binding to the receptor followed by translocation of the cytosolic heat-shock chaperone complex to the nucleus. Various MAP kinases are involved in this step. Once in the nucleus, the AHR dissociates from the heat-shock complex, and forms a complex with ARNT that recruits p300 and binds to the cognate sites in DNA. Probably through a DNA-looping step, the complex recruits the basal transcription factors and RNA pol II needed for initiation of transcription. Not shown in the scheme is the obligatory removal of a HDAC1-DNMT1 complex bound in the proximity of the TATA box that blocks RNA pol II recruitment and effectively maintains the gene in a silent state.

eukaryotic cells. These covalent modifications control intracellular distribution, transcriptional activity and stability of growth factors, hormone receptors and transcription factors, including the AHR, and the physiologic activity of a number of genes too large to be discussed within the confines of this chapter (see [18] for a recent review covering this subject). *In silico* analysis reveals a multiplicity of potential phosphorylation sites in the AHR primary structure, but evidence for their actual phosphorylation and for the functional role of such phosphorylated residues in determining receptor activity has been limited. Inhibition of protein kinase C (PKC) blocks ligand-induced DNA-binding of AHR/ARNT heterodimers and leads to the suppression of *Cyp1* gene expression [19–21]. Several studies have also shown that PKC is required for AHR activity in DNA-binding and gene transactivation [19,22–26] and that serine/threonine phosphatase inhibitors such as okadaic acid increase AHR-directed gene expression, suggesting the involvement of serine/threonine protein kinases in the activation of the AHR complex [27,28]. Recent insight into the precise signaling mechanism by which PKC regulates the activities of the AHR complex comes from Ikuta and colleagues, whose results have revealed a set of PKC-dependent phosphorylation events that decrease AHR activity. The AHR protein has both a nuclear localization signal (NLS) and a nuclear export signal (NES), which both play important roles in AHR translocation and intracellular distribution. Ikuta et al. [29] have shown that the NLS, located between amino acid residues 13–39, consists of two separate basic amino acid domains, one comprising residues 13–16 (Arg-Lys-Arg-Arg) and the other containing residues 37–39 (Lys-Arg-His). Ligand-dependent AHR nuclear import is inhibited by phosphorylation of either of two serine residues, Ser-12 or Ser-36, located one amino acid upstream from each of the two basic domains. Replacement of these Ser residues for Ala does not affect receptor translocation, but their replacement with Asp, which mimics the negative charge of phosphorylation, retains the mutant AHR in the cytoplasm. These observations were supported by *in vitro* nuclear transport assays and a luciferase reporter assay in which Ala and Asp replacement mutants had much lower transcriptional activity than the wild-type, suggesting a two-step mechanism in ligand-dependent nuclear translocation of the AHR.

Phosphorylation also plays an essential role in the transformation of the unliganded AHR into a fully functionally active AHR/ARNT heterodimer. Phosphorylation sites in two domains of the carboxyl-terminal half of the AHR have been localized by chemical cleavage patterns. These domains include four phosphotyrosine residues (Tyr-372, Tyr-408, Tyr-462, and Tyr-532) within residues 368–605, and a fifth (Tyr-698) within residues 639–759, that are highly phosphorylated *in vivo* [23]. Additionally, an amino-terminal tyrosine residue, Tyr-9, although not itself phosphorylated, is essential for proper recognition of the AHR for PKC-dependent phosphorylation, for binding of the AHR to its cognate DNA sequence, and for full transcriptional activity [30].

The known association of serine/threonine kinases with cytosolic HSP90 complexes [31] has prompted the search for the role of co-chaperone phosphorylation on receptor function. Ogiso and coworkers have shown that HSP90 phosphorylation modulates the formation of a functional cytosolic AHR multiprotein complex. These authors examined the cytosolic AHR complex in Chinese hamster ovary cells stably transfected with mouse AHR. Using mass spectrometry to determine site-specific phosphorylation, they identified phosphorylation of Ser-225 and Ser-254 of HSP90β and Ser-230 of HSP90α. Replacement of Ser-225 and Ser-254 with alanine increased the binding affinity of HSP90 for the AHR, which exhibited more potent transcriptional activity than when the residues were replaced by glutamic acid, suggesting that phosphorylation of the charged linker region of the HSP90 molecule modulates the formation of a functional cytosolic AHR complex [32].

3. Cross-talk of mitogen-activated protein kinases with the Ah receptor

The three families of mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal/stress-activated protein kinases (JNK/SAPK), and the p38s are important intracellular signal transduction mediators. They control gene expression and various other events in eukaryotic cells through the phosphorylation of transcription factors and the modulation of their function. MAPKs can phosphorylate a large panel of substrates on serine and threonine residues directly or via down-stream MAPK-activated protein kinases. MAPK activities are controlled by the MAPKKK-MAPKK signaling cascades [33] in which the MAPKs are activated by MAPK kinase-dependent phosphorylation, and the MAPK kinases are in turn are activated by MAPKK kinase-mediated phosphorylation. As a general rule, ERK1 and 2 are involved in regulating mitogenic and developmental events and the four p38 kinase isoforms play important roles in the inflammatory response, apoptosis and the cell cycle. The three JNK isoforms play important roles in cellular signaling, the immune system, stress-induced and developmentally programmed apoptosis, carcinogenesis, and in the pathogenesis of diabetes [34].

TCDD activates ERK and JNK [35], but TCDD-stimulated MAPKs do not converge upon the transcriptional activities of ELK-1 or c-JUN, the well-known nuclear targets of the ERKs and the JNKs, respectively, but rather on AHR activity and receptor-dependent gene expression [35]. Thus, MAPK activation may represent an alternative mechanism by which TCDD regulates AHR function, contributing to the diversity of TCDD-dependent toxicity in a cell-lineage and gene-specific manner [36]. Three AHR ligands, TCDD, benzo[*a*]pyrene (BaP) and benzo[*a*]pyrene-diolepoxide (BPDE), activate JNK in mouse hepatoma Hepa-1 cells, human lung carcinoma A549 cells, AHR-negative CV-1 cells and in both AHR-positive and AHR-negative mouse embryonic fibroblasts, suggesting that MAPK activation by TCDD does not actually require the presence of the AHR. However, TCDD-stimulated MAPKs appear critical for the induction of AHR-dependent gene transcription and CYP1A1 expression [35], indicating that AHR ligands elicit AHR-independent non-genomic events that are essential for AHR activation and function.

AHR activation by TCDD or by 3-methylcholanthrene, another often-utilized AHR ligand, has recently been shown to

induce morphological changes that modulate epithelial cell plasticity through a JNK-dependent mechanism [37]. Prolonged treatment with either ligand caused the cells to undergo prominent cytoskeletal changes resulting from increased interaction with extracellular matrix with simultaneous relaxing of cell–cell contacts. This pro-migratory activity of TCDD and 3-methylcholanthrene on epithelial cells correlates with activation of JNK and is reversible using a JNK inhibitor. Furthermore, all of the aforementioned dioxin-mediated effects were mimicked by constitutive expression and activation of the AHR. These novel effects on cell plasticity support a mechanistic role for the AHR in cancer progression as mediated by many of its ligands.

c-JUN is a down-stream JNK target induced by TCDD [38,39], which in turn contributes to the induction of the DNA-binding activity of the AP-1 transcription factor. Work with TCDD-treated rat hepatoma 5L cells expressing AHR has also shown induction of c-Jun mRNA and an increase in AP-1 levels and activity via an unconventional Ah receptor-mediated p38-dependent pathway [40] unrelated to JNK activity. Weiss and colleagues used an ELK-1-responsive reporter gene as a sensitive assay for p38 activation and showed that TCDD-induced p38 phosphorylation. Moreover, ELK-1 activation was undetectable in BP8 cells, an AHR-deficient subclone of the 5L line. None of the kinases known to phosphorylate p38 were activated by the AHR, although phosphorylation did not require transcription activation, suggesting a novel activation mechanism not currently understood. In an extension of this work, Weiss et al. have recently reported the induction of JUN-D in rat liver oval cells [41], which was also reported from our laboratory in mouse hepatoma cells [38,39].

The cross-talk between p38 and AHR signaling pathways and the role of p38 in AHR signaling has been explored in several studies. In human keratinocytes, cell density appears to be critical in determining AHR subcellular distribution, with the AHR maintaining a predominantly cytoplasmic localization under confluent cell culture conditions while becoming nuclear at sparse cell densities. Ikuta et al. found that nuclear accumulation of the activated AHR was associated with p38-dependent phosphorylation of Ser-68 in the NES and that nuclear export was suppressed by substitution of Ser-68 for aspartic acid [42]. These findings strongly suggest the existence of a functional relationship between cell density, phosphorylation, intracellular localization, and AHR activity that likely plays a pivotal role in AHR function.

Activation of p38, and possibly other MAP kinases, by Ah receptor ligands seems to be a cell-specific consequence of ligand exposure. TCDD activates p38 and ERK1/2 in RAW 264.7 murine macrophages by an AHR-independent mechanism [43], and it also activates JNK and ERK, but not p38, in mouse embryonic fibroblasts and African Green Monkey kidney CV-1 cells [35]. In macrophages, TCDD does not cause any apparent changes in JNK activity, although it does induce caspase-3 activity, whereas in fibroblasts and CV-1 cells the JNKs are activated. Equilibrium between the ERK and p38 pathways may be critical to the determination of cell fate following TCDD exposure.

The use of MAP kinase inhibitors has established a potential connection between MAPK and AHR signaling pathways, but these studies must be interpreted with caution in light of the extensive evidence that many MAPK inhibitors, particularly those derived from flavonoids, pyridinyl imidazole compounds, and others, are AHR agonists, antagonists or both [35,36,44–48]. With this caveat in mind, the two p38 MAP kinase inhibitors SB203580 and SB202190 were shown to suppress CYP1A1 induction by TCDD in mouse hepatoma Hepa-1 cells and in human hepatoma HepG2 cells. These inhibitors also suppressed CYP1B1 induction in human breast adenocarcinoma MCF7 cells, although over-expression of a dominant-negative p38 MAP kinase did not suppress induction of a Cyp1a1 reporter gene by TCDD in Hepa-1 cells [49]. Hence, suppression of *Cyp1a1* transcription by these pyridinyl imidazole compounds might be due not to p38 inhibition, but to an alternative effect on AHR function. SB203580 did not inhibit AHR transformation by TCDD *in vitro*, indicating that this compound did not act as a simple AHR antagonist. Instead, it decreased TCDD-induced histone acetylation levels in the TATA box region of the *Cyp1a1* gene promoter, suggesting the possibility that pyridinyl imidazole compounds might suppress the recruitment of co-activators needed for initiation of Cyp1a1 mRNA transcription [49]. A third imidazole-based p38 inhibitor, SB203580, blocked the spontaneous translocation of ectopic AHR into the nucleus of African Green Monkey kidney COS-7 cells and suppressed AHR transcriptional activity [50], suggesting two alternative conclusions that either the compound is an AHR antagonist that blocks its translocation or that p38-mediated phosphorylation is somehow involved in AHR translocation. These results nicely illustrate the difficulties inherent to the interpretation of inhibitor data.

The extracellular signal-regulated kinases comprise two isoforms, a 44 kDa protein called ERK1 and a 42 kDa protein known as ERK2. ERK1 and ERK2 share 83% identity in amino acid sequence and are expressed in virtually all tissues. ERKs are activated by growth factors and mitogens and are involved in the processes of cell growth and differentiation. AHR ligands can activate ERK in many different cell systems. TCDD, B*a*P and BPDE were reported to activate JNK and ERK in mouse hepatoma Hepa-1 cells, human lung carcinoma A549 cells, AHR-negative CV-1 cells and in AHR-negative and AHR-positive mouse embryonic fibroblasts [35]. Because induction occurred equally well in AHR-negative as in AHR-positive cells, it was concluded that this induction was mediated via an AHR-independent pathway. Many recent studies have established a close connection between ERK1/2 function and Ah receptor signaling. Promotion of *N*-nitrosomethylamine-initiated lung adenocarcinomas in mice by TCDD is accompanied by a tumor-suppressive function of K-RAS and a positive role for RAF-1 and ERK1/2 in lung tumorigenesis. TCDD may promote tumors by contributing to the down-regulation of K-RAS and the stimulation of RAF-1 [51]. Characterization of the role of the AHR in ERK1/2 activation was not attempted in these experiments, although the AHR is reasonably expected to mediate the effects of TCDD under these conditions. Studies in primary human macrophages, African green monkey kidney cells, mouse fibroblasts and mouse hepatoma cells have shown that AHR ligands can induce the activation of an ERK-dependent pathway that, in the case of the human macrophages, leads to TNFα induction [52], and in mouse cells culminates in activation of AHR/ARNT

transcriptional activity [35,36]. Along these same lines of evidence, over-expression of constitutively active MEK1, the MAPKK upstream of ERK1/2, reduced total AHR levels and enhanced the TCDD-initiated transactivation potential of the receptor, confirming the direct modulation of the AHR transcriptional response by ERK1/2 activity. Concomitantly, ERK inhibitors delayed TCDD-induced AHR degradation, suggesting that ERK kinase is critically linked to AHR function and expression by facilitating ligand-initiated transcriptional activation while targeting the AHR for degradation. Immunoprecipitation experiments were suggestive of a ligand-independent association of AHR and ERK, suggesting that ERK might be important in the regulation of AHR function perhaps by targeting receptor phosphorylation or blocking ubiquitinylation [53]. Consistent with the above studies, over-expression of a dominant-negative variant of MEK1 or treatment with a MEK1 inhibitor reduced TCDD-dependent transcription of a reporter gene and inhibited the binding of the AHR to its cognate DNA motif in the *Cyp1a1* gene promoter [54].

In summary, a relatively strong body of evidence indicates that there is a two-way cross-talk between MAP kinase pathways and AHR signaling. In general, it seems that AHR ligands activate one or another MAP kinase, possibly depending on the specific ligand and cell or tissue type examined, and that the kinase in turn mediates an ill-defined step in the process of AHR activation, facilitating its binding to DNA and its ability to transactivate target genes. Undoubtedly, as new tools to study the mechanisms of chromatin regulation are developed, the deeper mysteries of this signaling cross-talk will be revealed.

4. Cross-talk of the Ah receptor with cell cycle progression and apoptosis

4.1. *Cell cycle progression*

The cell cycle results from a recurring sequence of molecular events that leads to the duplication of the DNA content of a cell and to the subsequent division of that cell. The cell cycle consists of five distinct phases: G_0, G_1, S, G_2 and M. In G_0, cells are in a state of quiescence in which they have temporarily or reversibly stopped dividing. In response to growth factors and mitogens, cells come out of quiescence into the G_1 phase, during which cyclins and cyclin-dependent kinases (CDKs) become activated to promote DNA replication. Entry into S phase results from the CDK-mediated phosphorylation of the retinoblastoma protein, which when hyperphosphorylated, can no longer repress the activity of E2F, the main transcription factor responsible for the induction of S-phase specific genes. The G_1, S and G_2 phases collectively constitute the interphase, during which cells prepare for mitosis. The M phase is itself composed of two tightly coupled processes, including mitosis, in which chromosomes are distributed to the two daughter cells, and cytokinesis, in which the cytoplasm divides to form two distinct cells. For a more detailed description of the cell cycle, the reader is directed to a number of excellent reviews in the field [55–57].

Several published accounts point to a role for the AHR in cell cycle control, although the precise mechanism remains ill-defined [58–63]. Early studies revealed that an AHR-defective variant of the mouse hepatoma Hepa 1c1c7 cell line exhibited a prolonged doubling time compared with its wild-type counterpart [59]. This effect was attributed to delayed progress through the G_1 phase, suggesting that AHR action facilitates cell cycle progression. Mouse embryo fibroblasts (MEF) from AHR null mice were also found to grow more slowly, but this was attributed to an accumulation of cells in the G_2/M phase due to altered expression of the G_2/M kinases CDC2 and PLK [64]. Using the same AHR-null MEF cells, the AHR was shown to contribute to p300-mediated induction of DNA synthesis during S-phase by the adenovirus E1A protein [65]. Collectively, these observations suggested that in the absence of an exogenous ligand, the AHR promotes progression through the cell cycle. In contrast, evidence spanning more than 20 years has shown that TCDD, the prototypical AHR ligand, can inhibit cell proliferation. Confluent mouse epithelial cell cultures exhibit a diminished capacity for DNA replication in the presence of as little as 10 pM TCDD [66] and TCDD also inhibits DNA synthesis in rat primary hepatocytes [67] and in rat liver following partial hepatectomy [68].

There is extensive evidence in several different cell lines supporting the conclusion that in the presence of exogenous ligands, particularly TCDD, the activated AHR inhibits cell proliferation and induces cell cycle arrest in normally cycling cell populations (reviewed in [17,69]). While the mechanisms of this effect are complicated and multifactorial, analysis of the molecular basis of the inhibition has shown that at least one factor contributing to the inhibition of cell cycle progression by AHR ligands is a direct interaction between the AHR and the RB/E2F axis. As the major target of CDK2 activity, phosphorylation of RB is critical for most cells to enter into S-phase. Using yeast two-hybrid assays and co-immunoprecipitation experiments, AHR was found to form complexes *in vitro* and *in vivo* with the hypophosphorylated RB protein and to block its phosphorylation in G_1 [60,62]. At least two Ah receptor domains were found to be involved in this interaction. One is the cyclin D LXCXE motif, common to many RB-interacting oncoproteins [70]. The other is present within the glutamine-rich region transactivation domain of the AHR (reviewed in [69]).

Direct interaction between the ligand-activated AHR and hypophosphorylated RB constitutes a major G_1 checkpoint in cells exposed to AHR ligands [69]. The synergy between these two proteins reinforces the repression of E2F-dependent gene expression, slowing down the progression of cells from G_1 into S-phase [62]. In addition, AHR functions as a co-repressor of RB in a manner similar to that of BRG-1, mediating repression of RB target genes such as CDK2 and cyclin A to cause cell cycle arrest [71]. Additional data from our laboratory [72] and from Huang and Elferink [73] reinforces this effect of AHR on RB-mediated signaling and suggests that the recruitment of AHR to RB-regulated promoters results in repression of transcription through the formation of specific protein–protein interactions and the exclusion of co-activator proteins from these promoters. Elevated expression of the AHR and hyperphosphorylated RB were also found in DMBA-induced mammary tumors and in rat oval cells treated with TCDD [74], contrasting the idea that AHR signaling induces growth arrest through RB in every context [75]. AHR expression was also found to be

significantly repressed in DEN-induced liver tumors from RB-positive and RB-negative mice [76] and in several primary human acute lymphoblastic leukemias [77], indicating that AHR silencing may be associated with cancer progression. Finally, at least one report suggests that the RB is required for maximal transcriptional activity mediated by the AHR-ARNT complex at the *Cyp1a1* promoter [63], suggesting that RB can function as an AHR coactivator protein under certain contexts.

Two different molecular mechanisms have been proposed to explain the G_1 arrest induced by AHR ligands, with significant supportive evidence for both. One mechanism proposes that AHR activation and interaction with RB blocks its phosphorylation and reinforces repression of S-phase specific gene transcription. Alternatively, AHR activation is proposed to induce CDK inhibitors that arrest the cell cycle in G_1. We used mouse hepatoma Hepa-1c1c7 cells to study the expression of E2F-regulated S-phase specific genes and found that the AHR-RB-interaction caused the transcriptional repression of several of these genes, including Cyclin E, *Cdk2*, DNA polymerase α, and *Dhfr*. Further analyses using chromatin immunoprecipitation assays showed that TCDD recruited the AHR to E2F-regulated promoters from which it displaced the histone acetyl transferase p300 [72]. This result suggests that the interaction between AHR and RB acts as a negative regulator of cell cycle progression by inhibiting E2F-dependent transcriptional activity, preventing the expression of genes required for cell cycle progression through S phase. However, we also found a significant increase in the expression of the CDK inhibitor $p27^{kip1}$, indicating that there is also a positive regulatory mechanism at play involving the up-regulation of cyclin kinase inhibitors. We found similar results in a study of the inhibition of androgen-dependent proliferation in prostate cancer LNCaP cells by TCDD, in which TCDD repressed cyclin D1 and blocked RB phosphorylation, but it also induced expression of $p21^{Waf1/Cip1}$ [78]. Hence, the evidence also supports an alternative mechanism in which AHR/ARNT dimers interact with RB to play a positive role in the induction of genes encoding CDK inhibitory proteins. Huang and Elferink used dominant-negative, DNA-binding-defective AHR and ARNT mutants in rat hepatoma cells and found that TCDD-induced, AHR-mediated G_1 arrest was only partially regulated by direct AHR transcriptional activity, suggesting that both co-activation and co-repression were responsible for the arrest. When they used small interfering RNA to down-regulate ARNT protein expression, they found that TCDD-induced G_1 arrest was dependent on the ARNT protein [73]. Taken together, these results point to the conclusion that the TCDD-activated AHR may inhibit cell cycle progression not only by directly interacting with RB and repressing the expression of genes required for entry into S-phase and cell cycle progression, but also by inducing G_1 phase regulatory proteins such as $p27^{Kip1}$ that directly inhibit CDK2/4 activity, causing RB inactivation.

While these observations provide a plausible mechanistic rationale for the role of the ligand-activated AHR in cell cycle regulation, additional data suggest that other mechanisms may be equally important in the absence of exogenous ligands. Recent work in our laboratory used stably integrated, Tet-OFF-regulated AHR variants in fibroblasts from AHR-null mice to further investigate the AHR role in cell cycle regulation. $Ahr^{+/+}$ fibroblasts proliferated significantly faster than $Ahr^{-/-}$ fibroblasts, and exposure to TCDD or deletion of the ligand-binding domain did not change their proliferation rates, indicating that the AHR function in the cell cycle is ligand-independent. Growth-promoting genes such as cyclins and cyclin-dependent kinases, were significantly down-regulated in $Ahr^{-/-}$ cells, whereas growth-arresting genes such as transforming growth factor-β1 (TGF-β1), extracellular matrix-related genes and cyclin-dependent kinase inhibitors were up-regulated, suggesting that that the Ah receptor may possess intrinsic cellular functions in regulating cell proliferation that are independent of activation by either exogenous or endogenous ligands [79].

Inhibition of cell proliferation does not seem to be a universal effect of Ah receptor activation by its ligands. Weiss et al. have found that TCDD treatment of rat liver oval cells, presumably a hepatic cell lineage of progenitor cells, leads to AHR-dependent induction of the transcription factor JUN-D and the transcriptional up-regulation of cyclin A, triggering a release from contact inhibition [41]. Similar results in the same cells were obtained using benzo[*a*]anthracene, B[*a*]P and benzo[*b*]fluoranthene [74], suggesting that an AHR-dependent pathway of promoting cell proliferation through induction of JUN-D and cyclin A may constitute a novel mechanism in mediating AHR-induced deregulation of cell cycle control.

4.2. *Apoptosis*

Tumor promoters are generally believed to act by affecting the rate of division, terminal differentiation, or death of tumor precursor cells. One widely accepted mechanism of tumor promotion/progression is the inhibition of apoptosis [80]. The capacity of at least one AHR ligand, TCDD, to act as a tumor promoter, particularly in rodent liver, has been attributed to its ability to inhibit the apoptotic elimination of initiated cells bearing genotoxic lesions [81]. However, the precise mechanism(s) of this effect remains elusive, and differs with the organism, tissue, or cell type examined. A number of contradictory reports indicate that AHR activation by TCDD can induce apoptosis in some cases and inhibit it in others (reviewed in [69]). In studies of liver tumor promotion in the two-stage hepatocarcinogenesis model, TCDD was shown to mediate clonal expansion of initiated cells by inhibiting apoptosis and bypassing AHR-dependent cell cycle arrest [82]. In contrast, other reports using thymocytes have shown that TCDD induces apoptosis [83–85]. Several explanations have been advanced to explain these paradoxical differences in cell responses. Nebert et al. have suggested that AHR-mediated oxidative stress generated by the induction of cytochrome P450 enzymes may be a critical upstream event in the apoptosis cascade [86]. In agreement with this hypothesis, apoptosis initiated by BaP in human hepatoma HepG2 cells was linked to the induction of the p38 MAP kinase, to activation of the AHR and to induction of *CYP1A1*, an event that lead to the formation of the ultimate carcinogen BPDE. Confirmatory evidence showed that p38-null mouse embryo fibroblasts were resistant to BPDE-induced apoptosis, indicating that the Ah receptor plays a critical role in BaP-induced apoptosis while p38 links the actions of an electrophilic metabolite like BPDE to the regulation of programmed cell

death [87]. To this day, both induction and inhibition of apoptosis appear to be consequences of TCDD exposure, pointing at the possibility that the ligand-activated AHR modulates apoptotic cell fate in a tissue- or cell lineage-specific manner.

It has been proposed that AHR contributes to mammary tumor cell growth by inhibiting apoptosis while promoting the transition to an invasive, metastatic phenotype [88]. If this were correct, it would follow that loss of AHR would be pro-apoptotic. Indeed, in AHR-null mice, liver pathology is associated with an accelerated rate of apoptosis, possibly related to an abnormal accumulation of hepatic retinoic acid that causes activation of TGFβ, resulting in stimulation of apoptosis [89]. Consistent with the anti-apoptotic role of the AHR, tumor promotion in the liver by TCDD treatment is absent in rat strains lacking a functional AHR, which also show an increased rate of apoptosis, characterized by activation of MDM2 and attenuation of p53 by increased ubiquitinylation [90,91]. Similarly, activation of AHR by TCDD in a number of leukemia and lymphoma cells resulted in loss of apoptosis response, associated with an increase in expression of cyclooxygenase-2 and decrease of apoptosis-related BCL-2 family members BCL-XL and MCL-1. Both a cyclooxygenase-2 inhibitor and an AHR antagonist abolished TCDD-induced apoptosis resistance *in vitro*, strongly suggesting that AHR activation and the ensuing cyclooxygenase-2 over-expression are directly involved in a mechanism of resistance to apoptosis in lymphoma cell lines [92] (see also elsewhere in this volume).

An endogenous role of the AHR in the intrinsic apoptotic process was also uncovered in studies directed at the effect of differing AHR levels on apoptosis susceptibility. Analyses of a number of different endpoints of apoptosis in murine hepatoma Hepa-1c1c7 cells and in its AHR-deficient derivative LA1, revealed that the LA1 cells were more sensitive to intrinsic apoptosis-induced stresses (UV irradiation, hydrogen peroxide, serum starvation) than wild-type cells, suggesting that the endogenous AHR plays a cytoprotective role in the face of stimuli that initiate the intrinsic apoptotic pathway and that it can regulate cell fate directly. Lack of the AHR appeared to lead to an impaired survival response mediated by PI3 kinase-AKT/PKB and EGFR activation [93].

In contrast, other studies have shown that the AHR modulates susceptibility to pro-apoptotic agents and that it regulates critical players in mitochondrial functions related to apoptosis. Treatment with TNFα plus cycloheximide-induced apoptosis in murine hepatoma Hepa-1c1c7 cells, which have high levels of AHR protein, but not in Tao cells, an AHR-deficient variant of the Hepa-1c1c7 line [47]. Extensive characterization of these cell lines led to the conclusion that in the absence of an exogenous ligand, the AHR regulates lysosomal disruption and permeability, essential to trigger the apoptotic process. In good agreement with this conclusion, analysis of TCDD-induced proteome changes in rat 5L cells showed VDAC2, the voltage-dependent anion channel-selective protein-2, to be an AHR target, and to be dependent on the presence of a functional AHR. VDAC2 plays a central role as an inhibitor of the activation of the pro-apoptotic protein BAK and consequently of the regulation of the mitochondrial apoptotic pathway. Thus, these data point to VDAC2 as a possible effector of AHR-mediated apoptosis [94].

Studies in human fetal testis suggest that the developing germ cells may be a target for regulation by AHR ligands and that AHR activation may be one mechanism responsible for the reduction of spermatogenesis in men exposed to environmental toxicants. Expression of the AHR in germ cells was detected in the human testis between 7 and 19 weeks of gestation. Immunohistochemical analyses showed that treatment with AHR ligands led to increased evidence of apoptosis, which was suppressed by AHR antagonists [95]. If confirmed, these results may point at a mechanism to explain a number of male reproductive syndromes.

Many hypothesis have been proposed to explain the role of the AHR in apoptosis [64,89,96,97]. Recent data from our laboratory suggest that at least part of the answer may lie in an interaction between the AHR and E2F1 proteins. The E2F proteins control the transcription of a variety of essential cell cycle control genes, including cell cycle regulators, RB and related pocket proteins, enzymes for nucleotide biosynthesis, and proteins required for DNA replication [98]. In addition, several E2F family members, in particular E2F1, are able to activate apoptosis. Evidence suggests that the ability of E2F1 to act as a tumor suppressor lies in its ability to initiate apoptosis in cells that lose normal cell cycle control [99]. Recent results from our laboratory show that the AHR inhibits this response through transcriptional repression of the pro-apoptotic genes regulated by E2F1 [100]. We found that AHR and E2F1 can physically interact both *in vitro* and *in vivo* in an association that is independent of the interaction between AHR and RB. Inhibition of AHR expression in MEF cells results in elevated oxidative stress levels and E2F1-dependent apoptosis, which is inhibited by transfection of a siRNA for E2F1. AHR activation represses the induction of the pro-apoptotic E2F1 target genes *Tap73* and *Apaf1* that results from expression of a constitutively active CHK2 protein or ectopic over-expression of E2F1 itself, possibly through the formation of AHR-E2F1 complexes on the promoters of these genes. Such findings may shed light on the role of AHR and TCDD in mediating tumor progression, although these mechanisms have yet to be tested *in vivo*. The obvious implication is that the AHR has a pro-proliferative, anti-apoptotic function that is likely to play a major role during tumor progression.

5. Conclusions

It might be premature to speculate on the connections between the different signal transduction pathways that cross-talk with the AHR and their possible role in adult disease, although it is an inescapable conclusion that those connections exist. In this context, it is obvious that AHR ligand-mediated repression of previously active genes that might have little connection with detoxification pathways, and concomitant induction of previously silent genes, are equally likely to derail cellular homeostasis. The exact molecular mechanisms by which AHR ligands exert their effects still remain ambiguous. It is clear, however, that AHR signaling impinges upon numerous molecular pathways both in its physiological state and in its ligand-activated form, a

conclusion that is reinforced by the preponderance of evidence presented in this volume concerning the diversity and breadth of AHR interacting proteins and signaling pathways. The relative importance of these pathways in mediating AHR-dependent toxicities is still being determined. Mapping these pathways in various systems for different ligands, cell types, and exposure conditions will aid in predicting safe exposure levels and identifying susceptible human populations.

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