Regulation of Constitutive and Inducible AHR Signaling: Complex Interactions Involving the AHR Repressor[1]

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[1] Supported by P01-ES11624 (D.H.S.), ArtBeCAUSE (D.H.S.), R01ES006272 (M.E.H.), P42ES007381 (M.E.H. and D.H.S.)

Running Title: The AHR/AHRR Feedback Loop

Number of Figures: 4

Number of Tables: 0

Keywords: aryl hydrocarbon receptor; aryl hydrocarbon receptor repressor; PAS proteins; Cancer; Toxicology

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Abstract

The AHR is well known for regulating responses to an array of environmental chemicals. A growing body of evidence supports the hypothesis that the AHR also plays perhaps an even more important role in modulating critical aspects of cell function including cell growth, death, and migration. As these and other important AHR activities continue to be elucidated, it becomes apparent that attention now must be directed towards the mechanisms through which the AHR itself is regulated. Here, we review what is known of and what biological outcomes have been attributed to the AHR repressor (AHRR), an evolutionarily conserved bHLH-PAS protein that inhibits both xenobiotic-induced and constitutively active AHR transcriptional activity in multiple species. We discuss the structure and evolution of the AHRR and the dominant paradigm of a xenobiotic-inducible negative feedback loop comprised of AHR-mediated transcriptional up-regulation of *AHRR* and the subsequent AHRR-mediated suppression of AHR activity. We highlight the role of the AHRR in limiting AHR activity in the absence of xenobiotic AHR ligands and the important contribution of constitutively repressive AHRR to cancer biology. In this context, we also suggest a new hypothesis proposing that, under some circumstances, constitutively active AHR may repress *AHRR* transcription, resulting in unbridled AHR activity. We also review the predominant hypotheses on the molecular mechanisms through which AHRR inhibits AHR as well as novel mechanisms through which the AHRR may exert AHR-independent effects. Collectively, this discussion emphasizes the importance of this understudied bHLH-PAS protein in tissue development, normal cell biology, xenobiotic responsiveness, and AHR-regulated malignancy.

1. Introduction

Historically, the AHR has been studied for its transcriptional regulation of genes encoding cytochrome P450 enzymes that metabolize environmental AHR ligands, sometimes producing mutagenic and toxic intermediates. Given this focus, it is not surprising that the outcomes of AHR activation most frequently studied have been malignant transformation and cell toxicity. However, the highly conserved structure of the AHR and its expression in embryonic as well as adult organs as disparate as the liver, lung, lymphatics, and brain hint at the possibility that the AHR plays other, potentially more important physiologic roles in the absence of xenobiotic ligands. Indeed, recent studies demonstrated a myriad of biologic activities ascribed to AHR activated in the absence of exogenous ligands, ranging from control of mammary tumorigenesis [1-6] to regulation of autoimmunity [7-9]. That other members of the PAS family are associated with critical biologic functions such as regulation of neurologic development and the circadian cycle, responses to hypoxia, and angiogenesis [10, 11] is consistent with the hypothesis that PAS proteins in general evolved to regulate important biologic processes. In this context, several studies summarized here and in accompanying articles in this issue of *Biochemical Pharmacology* now demonstrate a wide range of AHR-regulated cellular activities. What is not completely understood is how these AHR-mediated activities are controlled. Nevertheless, negative regulation by transcriptional repressors is an important feature of other PAS proteins involved in critical signaling functions; examples include the negative regulation of HIF by iPAS [12] and of Sim1 and NXF by Sim2 [13, 14]. Similarly, a compendium of transcriptional co-activators and co-repressors that physically associate with and regulate AHR transactivation is being compiled (reviewed in [15-17]).

Herein we extend these discussions to consider an important but poorly understood regulator of the AHR, the AHR repressor (AHRR). Many studies now show that the AHRR plays a critical role in modulating "normal" as well as xenobiotic-induced AHR transcriptional activity. To highlight the significance of both temporal (developmental) and spatial (cell and tissue-specific) regulation of AHR signaling by the AHRR, we begin with a brief summary of some of the important, physiologically relevant activities of the AHR and note how AHRR might be involved in their regulation.

2. Controlling the AHR, an important physiological process

A large body of work demonstrates that the AHR contributes to regulation of cell growth [1, 2, 18-34]. For example, there is an association between increased AHR expression or activity and fibroblast, lymphocyte, hepatocyte, and mammary tumor growth [1, 2, 18-22]. AHR-deficient hepatoma cells grow more slowly than wildtype cells, and transfection of *AHR* cDNA into the mutant cells increases their growth rate to that of wildtype cells [25]. Progression of murine or human hepatoma cells from G_1 to S is prolonged by transfection of *AHR* antisense cDNA or inhibitory siRNA [25, 26]. *AHR siRNA*-mediated growth inhibition is accompanied by down-regulation of cyclin D1, cyclin E, cdk2, and cdk4 [26]. Expression in HeLa cells of *BRCA1*, an important regulator of cell growth and DNA integrity [35], requires binding of the constitutively active AHR [36] to the *BRCA1* promoter [37]. Possible pathways for AHR agonist-induced growth regulation include altered TGF-β signaling [38], induction of $p27^{Kip1}$ [33, 39], AHR-Rb interaction [34, 40], and AHR displacement of p300 on the E2F promoter [39, 41]. In estrogen receptor-α (ERα) positive mammary tumors, AHR activation with exogenous ligands induces AHR-ERα interaction [42, 43], ERα degradation [44, 45], suppression of ERα transcription [46], and inhibition of estrogen-driven *c-fos* transcription [47]. Since ERα is critical

to the growth of mammary tumors, particularly early in the transformation process, these AHR-ER signaling pathway interactions suggest an obvious mechanism for AHR-mediated growth inhibition, at least in some tumor subsets. Interestingly, the ability of AHR activation to decrease estrogen-induced hsp27 [48] and BRCA1 [49] transcription suggest mechanisms through which the AHR may actually increase growth and/or survival in ER^+ cells. Studies evaluating AHRR activity during the cell cycle might reveal that the equilibrium between AHR and AHRR activity, rather than just the level of AHR expression, is the critical measure of AHR contribution to cell growth.

Regulation of apoptosis represents a second critical area in which the AHR appears to play an important role [41, 50-52, 53, 54] and for which AHRR regulation of the AHR has not yet been evaluated. The AHR provides a survival advantage to murine hepatoma cells in which the intrinsic apoptosis pathway is initiated by UV irradiation [50]. In contrast, the AHR is required for optimal TNF-α-plus-cytoxan-induced apoptosis through a lysosomal-dependent process [53] and for Fas-mediated apoptosis [54]. Furthermore, constitutively active or PAH-induced AHR induces apoptosis in murine and human oocytes through AHRE binding and transactivation of *Bax* [51, 52]. This latter observation points to an extremely critical AHR function, regulation of fetal or neonatal oocyte atresia and thereby female fertility. Recent evidence linking AHRR to reproductive disorders (see below) and the control of apoptosis [55] suggest a possible role for AHRR in regulating mammalian reproduction.

Similarly, there now exists evidence for a role for the AHR in migration of normal and malignantly transformed cells (reviewed in [4]). For example, there is an acute increase in human mammary tumor cell invasiveness within 24 hours of B[*a*]P treatment and AHR inhibitors block that increase [56]. Furthermore, immortalized mammary myofibroblasts from $AHR^{-/-}$ mice

exhibit a decrease in migration *in vitro* and *in vivo*, lamellipodia formation, and VEGF receptor expression *in vitro*, all of which have been implicated in cell motility and/or invasiveness [57]. At least part of this influence on migration or invasion may be mediated by AHR transcriptional control of *Slug* [6, 58, 59], a master regulator of epithelial to mesenchymal transition, or *CK2α*, a serine/threonine kinase that regulates Snail, a second critical regulator of cell invasion. Both *Slug* and *CK2α* promoters contain multiple AHREs. The ability of the AHRR to control these or other AHR-mediated activities in tumor models has only begun to be studied [55, 60], although early results indicate that the AHRR can down-regulate AHR activity and Slug protein expression in murine mammary tumor cells [6].

Another important physiological role of AHR is in vascular development. In $AHR^{-/-}$ or AHR-hypomorphic mice, the ductus venosus fails to close after birth, resulting in persistent shunting of portal blood flow [61]. Additional studies show that this effect on vascular development involves AHR expressed in vascular endothelial cells [62]. The vascular defects in AHR-hypomorphic mice can be rescued by treatment with AHR agonists [63], implying that endogenous activation of the AHR is involved in vascular development. Whether AHRR may have a role in regulating the AHR during vascular development has not been investigated.

It is increasingly appreciated that the AHR plays an important role during immune responses. For example, activation of human B cells with stimuli that mimic signals delivered to B cells either during an innate or adaptive immune response, i.e, CpGs or CD40 ligand respectively, up-regulates AHR expression and results in constitutive AHR activation [18]. Constitutively active AHR in these activated B cells likely regulates cell growth, as demonstrated by the modest but statistically significant decrease in B cell growth following AHR down-regulation with siRNA (Fig. 1). These results notwithstanding, the ability of the AHRR to

repress AHR activity in the immune system has not been examined. For example, the ability of constitutively active AHR to up-regulate AHRR expression in a feedback loop and the ability of AHRR, presumably expressed in these cells, to limit AHR-regulated cell growth is unknown. A similar situation exists in what is a rapidly growing area of study, defining the role of the AHR in development of regulatory (Treg) and IL-17-secreting (Th17) T cells. These T cell subsets play critical roles in autoimmunity, graft rejection, and tumor immunity. While several very recent studies implicate the AHR in development and/or function of these important T cell subsets [7-9, 64, 65], none have evaluated AHRR control of AHR activity in the immune system during autoimmune or tumor-specific responses, despite the argument that the balance between AHR and AHRR activity is a major determinant in whether or not pathologic Th17-mediated autoimmune responses are enabled or protective anti-(self) tumor responses are manifest.

3. The AHR/AHRR feedback loop

Before considering the contribution of the AHRR to the control of physiological AHR functions and its hypothesized role in disease, we will review the current understanding of AHRR structure, evolution, expression in normal tissues, and possible roles in embryonic development. Additional background on AHRR can be found in other recent reviews [66, 67].

3.1. AHRR structure and evolution

The AHRR was first identified by Fujii-Kuriyama and colleagues [68] as an AHR-related cDNA cloned from a mouse intestine cDNA library. When expressed by transient transfection in mammalian cells, the murine AHRR inhibited AHR-dependent transactivation of a luciferase reporter gene, earning it the designation “AHR repressor” [68]. Similar *in vitro* repressor activity has been demonstrated for AHRRs from other vertebrate species [69-71], supporting the idea that

negative regulation of AHR signaling is an important and evolutionarily conserved function of this protein.

The AHRR shares high amino acid identity with AHR in the N-terminal third of the protein (~275 aa) containing the basis helix-lop-helix (bHLH) and Per-ARNT-Sim "A" (PAS-A) domains, but the two proteins are highly divergent thereafter. Not surprisingly, considering the demonstrated roles of the AHR bHLH and PAS-A domains in DNA binding (basic regions) and dimerization to ARNT (HLH and PAS-A domains), the AHRR is capable of interacting with ARNT and binding to AHR response elements (AHREs). Consistent with this, it was initially hypothesized that the mechanism of AHRR-mediated AHR repression involves competition between AHR and AHRR for binding to ARNT and competition of AHR-ARNT and AHRR-ARNT dimers for binding to AHREs [68]. As described in later sections, the mechanisms through which AHRR inhibits AHR activity now appear to be more complicated [72]. Consistent with the lack of a recognizable PAS-B domain, which in the AHR functions as the ligand-binding domain, AHRR does not bind TCDD [69] and appears to act in a ligand-independent manner [68].

AHRR orthologs have been identified and characterized in several mammalian species [68, 71, 73, 74], in an amphibian [75], and in bony fishes [69, 70, 76]. Zebrafish is notable for possessing two *AHRR* paralogs, which, based on phylogenetic analysis and conserved synteny, appear to be co-orthologs of the mammalian *AHRR* [76]. An *AHRR* gene has not been identified in any earlier diverging vertebrates or invertebrates, including an invertebrate chordate (*Ciona intestinalis* [77]) or a deuterostome (sea urchin *Strongylocentrotus purpuratus* [78]. Thus, the *AHRR* is considered a vertebrate-specific member of the *AHR* subfamily within the PAS gene family [69, 79]. Consistent with the close phylogenetic relationship of *AHRR* to *AHR*, the

structures of the *AHRR* and *AHR* genes are highly similar [70, 80]. The *AHRR* clearly arose from a duplication of an ancestral *AHR* gene; a better understanding of when this occurred in vertebrate or chordate evolution awaits the characterization of genomes from cartilaginous and jawless fishes.

3.2. AHRR tissue expression, regulation, and role in development

As shown earlier for the AHR (reviewed in [81]), *AHRR* mRNA is expressed constitutively in a variety of adult tissues, although expression of AHRR appears to be more tissue-specific than that of AHR. Although results vary by species and study, the adult tissues that consistently express the highest amounts of *AHRR* mRNA are testis, lung, spleen, heart, and kidney [71, 73, 82-84]. In the case of testis, the level of mRNA expression is three times as high as the level expressed in the next highest tissues (lung and ovary) and orders of magnitude higher than in liver [83]. Notably, in some tissues that express high AHR levels, e.g., liver, basal *AHRR* expression is low and *AHRR* mRNA only becomes easily measurable after induction with 3-MC, B[*a*]P, TCDD or other strong AHR ligands [68, 73, 84, 85]. Interestingly, adult *AhRR* levels may be imprinted during embryogenesis as exposure of murine embryos to TCDD elevates *AHRR* mRNA levels in the embryo and in the resulting adult, presumably through epigenetic signaling [86].

An important consideration in interpreting results concerning tissue-specific expression of *AHRR* mRNA, as with any data on mRNA expression, is that relative mRNA expression may not exactly correlate with relative protein levels, and expression of AHRR protein across tissues has not yet been assessed in a systematic way. Nevertheless, the data on *AHRR* mRNA expression in adult and embryonic tissues (below) is likely to provide useful information on potential cell- and tissue-specific roles of the AHRR protein.

Given that activated AHR regulates *AHRR* transcription [80, 83, 87] it is not surprising that four putative AHREs are located in the murine 5' proximal *AHRR* promoter located on chromosome 13 (Fig. 2) and that at least three of these AHREs are required for AHR-dependent transactivation [68, 80]. A highly homologous, putative regulatory region is present at the 5' end of intron 1 of the human *AHRR* gene located on chromosome 5 [88, 89] and functional AHREs also occur in the promoter of fish AHRRs [69]. Overlapping GC sequences enable binding of SP1 and SP3 transcription factors to the murine AHRR regulatory region [80]. Importantly, these GC boxes are required for optimal constitutive as well as AHR ligand-induced *AHRR* transcription in murine or human models [68, 89]. Furthermore, *AHRR* transcription is induced with 12-*O*-tetradecanoylphorbol-13-acetate, an NF-κB activator [68]. An NF-κB-binding site is located immediately downstream of AHRE #4, overlapping with a GC box (Fig. 2). These observations suggest that these highly promiscuous transcription factors regulate *AHRR* transcription and, by inference, constitutive AHR activity in the absence of AHR ligands.

AHRR is expressed in fish and amphibian embryos [70, 75, 76], and in mammalian fetuses, where expression appears to be low relative to that in adult tissues [83, 90, 91]. The embryo-fetal expression suggests a possible developmental role for the AHRR. Nevertheless, $AHRR^{-/-}$ mice are fertile and the offspring appear to develop normally [92], suggesting that AHRR may not be required for embryonic development in mice. In zebrafish, however, knock-down of one of the two *AHRR* paralogs (*AHRRa*) using morpholino-modified antisense oligonucleotides causes developmental abnormalities like those seen in TCDD-exposed embryos [93]. The authors suggest that AHRR may serve to suppress constitutive AHR signaling during development. This intriguing possibility, as well as an explanation for the differential appearance

of phenotypes resulting from AHRR knock-out or knock-down in mouse *versus* fish embryos, will require further investigation and clarification.

In contrast to the still uncertain role of AHRR in regulating constitutive AHR signaling during development, there is good evidence for a role for the AHRR in regulating the response to AHR agonists in embryos and adults. For example, AHRR-null mice exhibit greater 3-MC-mediated induction of *CYP1A* mRNA in skin, stomach, and spleen than wildtype mice, although such an enhanced inducibility is not seen in other tissues [92]. Similarly, zebrafish embryos, in which expression of *AHRRa* has been reduced with morpholino oligonucleotides, show an increase in TCDD-induced developmental abnormalities. Embryos in which both *AHRRa* and *AHRRb* have been knocked down display enhanced induction of *CYP1A* by TCDD at 72 hours post fertilization [93]. The role of the AHRR in regulating responsiveness to AHR agonists *in utero* is further suggested by the ability of these compounds to induce high levels of *AHRR* mRNA in fish or murine embryos, the latter occurring via placental transfer [70, 76, 86, 91].

As a consequence of emerging studies implicating an inducible AHR/AHRR feedback loop in embryos as well as in adult tissues, there has been great interest in evaluating a possible role for the AHRR in governing species-, population- or strain-specific differences in sensitivity to dioxins and related compounds. For example, elevated AHRR expression has been considered as a potential mechanism for the PCB resistance that has evolved in fish inhabiting highly contaminated environments. However, studies in three different resistant populations and two different species have consistently shown no elevation in constitutive expression of *AHRR* transcripts in the resistant fish [69, 76]. In contrast, in all three studies, the *AHRR* resembled *CYP1A* in being refractory to induction by exposure to AHR agonists in the laboratory. The possible role of AHRR in the well-known insensitivity of frogs to dioxins also has been

hypothesized; however, the developmental pattern of *AHRR* expression in *Xenopus laevis* is not consistent with such a role [75]. AHRR also has been evaluated as a contributing factor in the AHR-dependent dioxin resistance of the Han/Wistar (Kuopio) rat strain. However, the basal expression and inducibility of *AHRR* mRNA does not differ between dioxin-sensitive and dioxin-resistant strains [73].

3.3. The central AhR/AHRR feedback paradigm-Is it always valid?

The context in which the *AHRR* was first and most frequently described involves its induction by exogenous AHR ligands such as TCDD [69], 3-methylcholanthrene (3-MC)[80], or benzo[*a*]pyrene (B[*a*]P)[84]. These studies generally point to a straightforward feedback loop wherein AHR-induced AHRR suppresses AHR activity. Accordingly, *AHRR* mRNA levels generally increase after AHR activation with TCDD or PAH, and *CYP1A1* induction with xenobiotics appears to be repressed in primary cells and some cell lines with high basal *AHRR* levels [82, 94]. For example, HeLa cells express very high basal *AHRR* mRNA levels and are relatively resistant to *CYP1A1* induction with TCDD or 3-MC [82]. Reduction of AHRR expression using siRNA restores *CYP1A1* inducibility, demonstrating the ability of high basal AHRR levels to constitutively repress AHR activity [89]. Some, but not all (see below) studies evaluating AHR activated in the absence of xenobiotics ("constitutively active AHR"), suggest that this model applies to "physiologically" activated AHR as well. For example, mammalian lung constitutively expresses relatively high levels of both AHR and AHRR, a result consistent with regulation of basal *AHRR* levels by endogenously activated AHR [83]. Furthermore, *AHRR* mRNA is decreased in brain and heart from naïve $AHR^{-/-}$ mice [84] and basal *CYP1A1* levels are low in testis, where basal *AHRR* expression is relatively high [73, 83]. These results suggest that

basal *AHRR* levels are regulated, at least sometimes, by constitutive AHR activity, which in turn is limited by the AHRR.

However, as with many aspects of AHR-target gene interactions, the outcome of AHR-AHRR interactions is likely to be more complex than suggested by a simple AHR-induced, AHRR-mediated feedback model. That is, the ability of the AHR to transactivate the *AHRR* gene, and the ability of AHRR to repress AHR activity, at least as defined by *CYP1* induction, is likely to be tissue-, cell-, and context-specific. A hint of this complexity is provided by the observation that B[*a*]P induces significant *CYP1A1* transcription in the murine kidney (reaching approximately half of the *CYP1A1* induced in the liver), but does not induce detectable levels of *AHRR* mRNA [84], a result demonstrating that ligand-activated AHR may not be able to transactivate *AHRR* in all tissues. Similarly, some cell lines derived from testis, lung, kidney, or bladder express low or moderate basal *AHRR* levels that are not increased following AHR ligand exposure [82].

The feedback component of the model also may be tissue specific. The testis notwithstanding, the overall tissue expression of the AHRR and the ability of AHR ligands to induce *CYP1A1* mRNA are not always inversely correlated [84]. Thus, while basal *AHRR* levels in the murine heart are approximately 5 fold higher than in the liver, *CYP1A1* inducibility is comparable in both organs [84]. Similarly, TCDD-induced *AHRR* and *CYP1A1* transcription do not inversely correlate in rat kidney, spleen, heart [73] or hypothalamus [85]. These data suggest that AHRR levels alone do not necessarily dictate the magnitude of AHR responses and that AHRR activity may be modulated in a tissue-specific fashion. An important caveat here is that all of these studies measured AHRR transcripts, rather than protein. Therefore, the relationship

between AHRR protein levels and AHR ligand-mediated *CYP1A1* inducibility still requires elucidation.

4. AHRR polymorphisms and disorders of human reproduction

One of the most intriguing *and controversial* hypothesis to arise concerning the physiological activity of AHRR concerns its possible role in human reproduction. Because the constitutively active and xenobiotic activated AHR has long been linked to reproductive physiology [51, 95-98], an involvement of AHRR in reproduction has been viewed as plausible, although a review of the literature demonstrates some significant inconsistencies. Studies to date have focused on the Pro185Ala single-nucleotide polymorphism first reported by Watanabe et al. [87]. In this initial study, no link was found between *AHRR* genotype and the occurrence of uterine endometriosis in a Japanese population. However, two subsequent studies did find such an association. Japanese women with at least one copy of the Ala185 allele were reported to be at increased risk for both the occurrence and increased severity of endometriosis [99]. Similarly, in a study of Korean women, the frequency of the Ala185 allele was slightly but significantly increased in women with advanced stage endometriosis as compared to women without the disease [100].

The same *AHRR* polymorphism also has been linked to male reproductive abnormalities. Two reports [90, 101] have shown an association of the Pro185 allele and Pro/Pro genotype with the incidence of micropenis, a condition characterized by undermasculinized external genitalia in the absence of other abnormalities. Fujita et al. [90] suggested that the Pro185 allele might be a hypomorphic allele with a weaker inhibitory effect on AHR. The Pro/Pro genotype also has been associated with male infertility (azoospermia or severe oligospermia) in a Japanese population [102]. In contrast, Estonian men with the Ala185 allele and Ala/Ala genotype were at increased

risk for male infertility [103]. The Ala185 allele also contributed to an association between organochlorine exposure and the X:Y ratio of sperm in a group of Swedish fishermen [104].

The frequent association between *AHRR* genotype and increased risk for male and female reproductive abnormalities is striking. However, the lack of consistency in the specific AHRR variant associated with the abnormal condition (Ala or Pro) raises questions about the underlying explanation for these observations. There are no published studies evaluating the functional characteristics of the two variant AHRR proteins. However, recent work suggests that both variants exhibit similar activity as repressors of AHR signaling [105, 106]. If differences in the function or expression of the variant *AHRR* alleles are not identified, the epidemiological results might be explained not by the AHRR itself but by polymorphisms in a nearby gene that is in linkage disequilibrium with *AHRR*. Thus, despite the intriguing results emerging from these epidemiological studies, the link between AHRR and reproductive disorders remains to be elucidated.

5. AHRR and cancer

Another remarkable aspect of AHR biology, and by inference AHRR biology, is the apparent contribution of the AHR to malignancy. The earliest AHR research demonstrated that the AHR is required for optimal *CYP1A1* or *CYP1B1* induction, production of mutagenic metabolites, and tumor initiation [107]. Since then, the role of the AHR in both genetic and epigenetic regulation in a variety of tumors has been well documented. Indeed, it is now generally accepted that both environmental chemical-activated [56] and constitutively active AHR [4] contribute to tumorigenesis and aberrant cell behavior.

Despite these important observations, relatively little is known of AHRR expression and function in tumors. Nevertheless, some recent data are beginning to shed light on what may be a

critical role for the AHRR in cancer. For example, we have noted increased expression of *AHRR* mRNA in non-metastatic murine mammary tumors induced by oral gavage with DMBA, relative to *AHRR* levels in normal mammary glands. In human mammary tumor cell lines, *AHRR* knock-down with siRNA reduces AHR activity (data not shown), confirming the assumption that the AHRR constitutively represses AHR activity in tumors. Interestingly, murine breast tumors induced with DMBA generally express extremely high *AHR* levels [2], although the AHR protein and *AHRR* mRNA levels do not necessarily correlate in any given tumor. Indeed, individual tumors with high AHR levels tend to express low basal *AHRR* levels, suggesting the intriguing possibility that the AHR actually may suppress *AHRR* transcription and thereby maximize AHR activity.

The ability of the AHRR to regulate important tumor cell functions was exemplified by the demonstration that stable *AHRR* transfection decreases E2F, cyclin E1, and PCNA and slows the growth of ERα positive MCF-7 cells [60]. Interestingly, this growth inhibition may be mediated by direct interaction of the AHRR with ERα [108]. (This and other potential AHR-independent AHRR effects are discussed later in this article). AHR and AHRR control of mammary tumor cell growth also extends to ERα negative cells, as we have demonstrated that ectopic *AHRR* expression slows the growth of immortalized MCF-10F mammary epithelial cells expressing constitutively active AHR but no ERα [4].

Regardless of the mechanism through which the AHRR affects mammary tumor cell growth, these studies suggest the intriguing possibility that the *AHRR* is essentially a tumor suppressor gene. Indeed, this thesis has recently been put forth by Zudaire *et al* [55]. These investigators noted that the short arm of human chromosome 5, the region containing the *AHRR* gene (5p15), is frequently deleted in a variety of human cancers including cervical, colorectal,

ovarian, bladder, esophageal and lung cancers. Consequently, this region is thought to encode an important tumor suppressor gene(s). The finding that the AHRR promoter is hypermethylated in a variety of tumor cell lines and primary tumors from multiple organs, leading to decreased levels of *AHRR* mRNA levels as compared with corresponding "normal" tissue, suggests that the AHRR may be one such tumor suppressor [55]. A correlation between *AHRR* promoter hypermethylation and tumor grade in cervical and esophageal malignancies and a modest decrease in *AHRR* mRNA in pre-cancerous colon polyps as compared with a more profound decrease in primary invasive colon carcinomas implies a continuous process of *AHRR* down-regulation and AHR activity up-regulation during malignant transformation. Importantly, it was shown that *AHRR* down-regulation with siRNA increases the growth and invasiveness of lung carcinoma cell lines and enables non-malignant MCF-10A cells to grow in soft agar [55]. These important studies highlight the importance of both the AHR and the AHRR in tumorigenesis and further underscore the relevance of a balance between AHR and AHRR in physiologic processes occurring in the apparent absence of environmental AHR ligands.

These results bring up yet another intriguing possibility. The association between AHRR silencing by hypermethylation *in situ* and increased tumorigenicity suggests that progressing tumors would display enhanced growth resulting from AHRR down-regulation, regardless of the mechanism. We and many others have shown that AHR behavior is tissue and stimulus specific. For example, while TCDD-activated AHR induces *CYP1A1* and *CYP1B1* [20], it represses genes encoding hsp27 [48] and cathepsin D [109] in mammary tumor cell lines. Furthermore, in cells in which TCDD induces both *CYP1A1* and *CYP1B1*, constitutively active AHR preferentially up-regulates *CYP1B1*, but does little or nothing to transactivate *CYP1A1* [3] and actually represses *c-myc* [20]. These results provide precedents for AHR-mediated repression of some

target genes. Therefore, it seems plausible that, under some circumstances where AHR activity enhances cell growth or survival, the AHR will be found to suppress *AHRR* transcription, thereby relieving the AHR of a negative feedback loop. Indeed, recent results showing increased *AHRR* expression in spleen and thymus of $AHR^{-/-}$ mice [84] are consistent with this hypothesis.

6. Mechanisms of AHRR action

An understanding of the molecular mechanisms by which the AHRR inhibits AHR signaling is important for elucidating the regulatory interactions of these two proteins in the context of cell growth. Similarly, understanding the specificity of the AHRR, i.e. whether it also controls other signaling pathways, also is essential. Both of these aspects of AHRR function, its mechanism of repression and specificity with respect to AHR, are often assumed to be well understood. However, this is not yet the case; key questions about AHRR function remain to be resolved, as described below.

6.1 Molecular mechanisms of repression

There is no doubt that the AHRR is a transcriptional repressor of AHR. This has been demonstrated repeatedly with reporter constructs in transfected cells [68-70, 72, 110] and with endogenous target genes such as *CYP1A* and c-*myc* [20, 55, 68, 89]. Two additional features of AHRR's mechanism that are not disputed include its ligand-independence and constitutive nuclear localization. For example, the association of AHRR with ARNT in co-immunoprecipitation experiments did not require, and was not enhanced by addition of an AHR ligand [68]. Karchner *et al.* [69] directly evaluated the ability of mammalian and fish AHRRs to bind [^{3}H]TCDD, and found no evidence of specific binding like that seen for AHR. Both of these results are consistent with the lack of conservation between AHRR and AHR in the PAS-B domain, which in the AHR is part of the ligand-binding pocket [68]. Several studies have shown

that AHRR-GFP fusion proteins, and presumably native AHRRs, are localized primarily to the nucleus of transfected cells [68, 72, 111]. Kanno *et al.* [111] showed that AHRR contains both nuclear localization and nuclear export sequences, and that AHRR undergoes nucleocytoplasmic shuttling, with equilibrium favoring nuclear localization. They also suggested a role for ARNT in AHRR nuclear transport. Together, these studies reveal that the AHRR is a ligand-independent, nuclear repressor of (AHR) transcription.

The exact molecular mechanism by which the AHRR represses transcription is more uncertain. The initial report identifying the AHRR as a repressor of AHR transactivation proposed two mechanisms of repression: competition with AHR for binding to ARNT and competition between AHR-ARNT and AHRR-ARNT complexes for binding to DNA [68]. The authors presented data and described additional, unpublished results in support of these proposed mechanisms. For example, Mimura *et al.* [68] used co-immunoprecipitation studies to demonstrate that the AHRR associates with ARNT, a requirement for both putatitive mechanisms. In addition, these authors showed that AHRR-ARNT complexes bind to AHR response elements (AHREs; also called DREs or XREs), making it plausible that competition between AHR-ARNT and AHRR-ARNT complexes is involved in the mechanism of repression.

While the hypothesized mechanisms of AHRR-mediated AHR suppression are appealing in their simplicity, closer examination reveals that the mechanism of repression is likely to be more complex. Recently, we performed experiments to directly test both hypothesized mechanisms of repression [72, 112]. In these studies, ARNT overexpression had no effect on AHRR-mediated repression of AHR, demonstrating that competition for ARNT is not the primary mechanism of repression. In addition, through the use of an AHRR point mutant (AHRR-Y9F) in which nuclear localization was not affected but the ability to bind AHREs was

abolished, we showed that AHRE binding was not required for repression, although a slight decrease in repressive potency of this mutant suggested that AHRE binding may contribute to AHR repression. The results obtained with this point mutant were similar to unpublished results reported earlier involving use of an AHRR lacking the basic region [68], although in that case the ability of the mutant to still localize to the nucleus was not confirmed. In a key experiment, we showed that DNA-binding mutant AHRR-Y9F, even in the presence of excess ARNT, was still a potent repressor of AHR [72]. Thus, when both hypothesized mechanisms were precluded through experimental manipulations, the ability to repress was maintained, indicating that there may be an additional mechanism of repression. These results [72] suggest that this additional mechanism might involve transrepression (repression through protein-protein interactions, but independent of direct DNA binding by the repressor). A repression mechanism that is independent of DNA binding is reminiscent of recent findings showing that the AHR can act as a coactivator to activate transcription without binding to DNA [17, 113]. The transrepression hypothesis, although consistent with the published results [72], remains to be verified through identification of the specific protein-protein interactions that are involved. Nevertheless, there appear to be at least two mechanisms by which AHRR can repress AHR-dependent transcription (summarized in Fig. 3).

Two groups have attempted to localize the regions of the AHRR protein that are important for repression. We constructed two deletion mutants of a zebrafish AHRR, AHRR1Δ270-550 (truncated after the portion of the AHRR PAS domain that is conserved in AHRs and AHRRs) and AHRR1Δ189-550 (lacking most of the PAS domain but retaining the PAS-A repeat) [72]. In transient transfection assays, AHRR1Δ270-550 was as effective as the full-length AHRR at repressing AHR transactivation of an AHRE-luciferase reporter gene.

AHRR1Δ189-550 was still fully active as a repressor, but required higher amounts of transfected DNA [72]. We concluded that the C-terminal half of AHRR is not required for repressor activity.

In what at first appears to be contradictory results, Oshima *et al.* [110] found that the C-terminal segment of AHRR (aa 555-701 of mouse AHRR) contains a repressor domain. This result was obtained with an engineered system involving chimeric proteins containing the DNA binding domain of the yeast Gal4 protein fused to various parts of the AHRR, with repressor activity measured as the ability to inhibit expression of a constitutively active luciferase reporter gene driven by the TK promoter and Gal4 binding sites. Thus, the assay involves binding of the chimeric proteins to Gal4 binding sites on the reporter vector, and therefore measures repression that is dependent on DNA binding of the AHRR protein (albeit to a heterologous sequence). In support of the repressive role of the AHRR C-terminal portion, Oshima *et al.* identified ANKRA2 as a possible co-repressor that interacts with aa 342-701 of AHRR to recruit histone de-acetylases HDAC4 and HDAC5.

Although the experiments of Evans *et al.* [72, 112] and Oshima *et al.* [110] identified different ends of the protein as being involved in repression and thus at first seem contradictory, the results may in fact not be in conflict. In the latter study, the role of ANKRA2 in repression of *CYP1A* expression was assessed by blocking ANKRA2 expression with siRNA in mouse embryo fibroblast cells. Only a small effect on basal *CYP1A* expression (2-fold) was seen, and there was no effect of ANKRA2 knock-down on 3-MC-induced CYP1A expression. In contrast, blocking AHRR expression with siRNA affected both basal expression of *CYP1A* (5-fold increase) and 3-MC-induced expression (2-fold increase). The authors concluded that AHRR can act by an ANKRA2-independent mechanism of repression. The results of Evans *et al*. [72, 112] would suggest that the ANKRA2-independent mechanism involves the N-terminal, bHLH-PAS-

domain-containing part of the protein. This conclusion is consistent with the evolutionary conservation of AHRR function in vertebrates, despite the low degree of sequence conservation in the C-terminal half of the protein [70].

6.2 AHRR specificity

The initially proposed mechanisms responsible for AHRR-mediated AHR suppression involving competition for ARNT and AHREs would predict that AHRR is a specific repressor of AHR, with possible effects (although likely reduced) on other ARNT-dependent bHLH-PAS proteins such as HIF or SIM. In contrast, a transrepression mechanism independent of AHRE binding allows for the potential of AHRR to have broader specificity; just how broad depends on the exact mechanism by which the transrepression occurs. Thus, the question of AHRR specificity is intimately tied to its mechanism of action. Indeed, the very name "AHR repressor" creates a mindset that may prevent us from considering other possible targets of this protein.

Hints of a broader range of AHRR targets were contained in the original report on the existence of an AHR repressor [68]. In that study, AHRR repressed the ability of a Gal4-ARNT chimeric protein to transactivate a reporter construct driven by Gal4 binding sites. In addition, these authors reported (but did not show) that AHRR "…moderately inhibited transactivation by the HIF-1α/Arnt heterodimer" [68]. More recently, Karchner *et al.* [106] showed that AHRR repressed HIF-1α activation of an HRE-luciferase reporter construct. Consistent with this result and the known role of HIF proteins in angiogenesis, Zudaire *et al.* [55] showed that AHRR expression in tumor cells was inversely correlated with their angiogenic potential.

One important question is whether AHRR can repress proteins other than those in the bHLH-PAS family. Karchner *et al.* [106] showed that the AHRR did not affect the ability of the

nuclear receptors ER or PXR (pregnane-X-receptor) to activate transcription through their respective enhancer elements. The results with ER are in contrast to another recent report showing that AHRR can repress ERα-mediated transactivation of reporter genes and endogenous target genes, and that the AHRR interacts directly with ER at multiple sites on the ER protein [108]. This difference remains to be resolved. It seems clear, however, that AHRR is capable of repressing more than just the AHR, and that its targets may not be limited to bHLH-PAS proteins. Identification of all such targets of AHRR repression will help elucidate the role of this protein in potentially regulating many biologic functions.

7. Conclusions and future directions

This article and others presented in this *Biochemical Pharmacology* issue underscore the importance of the AHR as an inducible signal-transducing transcription factor in many biologic contexts. While a nearly exponential increase in the number of studies on the AHR has occurred over the last few years, progress on the analysis of its repressor has been considerably more modest. (At the time of this writing, a search of PubMed using the words "aryl" or "aromatic" + "hydrocarbon" + "receptor" yielded 5,085 hits while a search using "aryl" or "aromatic" + "hydrocarbon" + "receptor" + "repressor" yielded 137 hits). If it is conceded that the AHR is an important intracellular signal transducer under "normal" physiological conditions, after exposure to xenobiotic agonists, or during tumor progression, then it should be concluded that whatever regulates the AHR is of equal importance. The analysis of AHR co-activators and co-repressors [15-17] is one important step in that direction. Elucidation of AHRR expression and function is another.

Studies summarized here suggest that, like the AHR and at least in part because of the AHR, an evolutionarily conserved AHRR plays important physiological roles during

embryogenesis. A generally increased level of *AHRR* expression in adult as compared with embryonic tissues [83], and an asymmetric tissue-specific expression of *AHRR* mRNA (e.g., 3 times to greater than 30 times higher *AHRR* levels in testis than any other organ)[83], suggest an important and tissue-specific contribution to adult organ function as well. Additional studies suggest important roles for the AHRR in disease including but not limited to cancer.

The classical analysis of AHR function by stimulation with well-characterized xenobiotics has led to a central paradigm in which xenobiotic-mediated AHR activation results in AHR-mediated transcriptional up-regulation of *AHRR*, the protein product of which feeds back to limit AHR activity. Some studies, for example with brain and heart from $AHR^{-/-}$ mice, which exhibit decreased *AHRR* expression, suggest a similar feedback loop when the AHR is activated *in situ* either through an endogenous ligand or through some as yet undefined ligand-independent mechanism. However, alternative consequences must be considered. For example, an increase in *AHRR* transcript levels in spleens and thymi of $AHR^{-/-}$ mice [84] and the inverse relationship between AHR expression (high) and *AHRR* transcripts (low) in primary murine mammary tumors [2](data not shown) suggest the intriguing possibility that the AHR, under some circumstances, may actually repress transcription of its own repressor, thereby maximizing AHR activity. If it is accepted that, at least under some [1, 2, 4-6, 21] though perhaps not all [29] circumstances, increased AHR activity contributes to malignancy, then this alternative pathway of AHR-mediated repression of AHRR expression would be consistent with the view of the AHRR as a tumor suppressor protein, as recently proposed by Zudaire *et al* [55]. Indeed, it would provide a second epigenetic mechanism, in addition to *AHRR* hypermethylation [55], responsible for *AHRR* transcriptional repression.

Finally, plausible mechanisms through which the AHRR inhibits AHR activity, i.e., competitive binding to ARNT, competitive binding of ARNT/AHRR dimers to AHREs [68, 114], and recruitment of co-repressors [110], have been proposed and supported by some experimental data. However, recent studies demonstrating that AHRR mutants incapable of binding AHREs still suppress AHR activity, even in the presence of excess ARNT [72, 106, 112], suggest that, as is often the case, mechanisms are more complex than initially appreciated. Furthermore, studies demonstrating AHRR-mediated inhibition of HIF-1 signaling [68, 106] and physical association of the AHRR with the ER [108] encourage caution in ascribing all AHRR activity to its propensity to block AHR activity. Future studies on this very important transcriptional regulator must consider these putative "off-target" effects.

Clearly, many important questions concerning the function of AHRR remain to be answered. In what cells and developmental stages does AHRR limit the activity of constitutively active or xenobiotic-activated AHR? Which other transcription factors are targets for repression by AHRR? What are the exact molecular mechanisms of repression and do they differ for different transcription factors and target genes? What is the precise role of AHRR and its polymorphic variants in reproductive disease and cancer? Answers to these and many other questions about the AHRR will enrich our understanding of AHR biology and quite possibly the biology of other signaling pathways as well.

8. Acknowledgments

We are grateful to Professors Matsumura and Puga for the opportunity to contribute to this special issue. We thank Drs. Sibel Karchner, Matthew Jenny, and Brad Evans for helpful discussions on AHRR biology.

9. Legends:

Figure 1. Silencing of AHR expression with AHR-specific siRNA slows human B cell growth

(A) Purified human B cells from a minimum of 5 human donors were activated by culturing on CD40 ligand (CD40L)-transfected L cells plus rIL-4 for 1 week. CD40L-activated B cells were transfected with AHR-specific siRNA or, as a negative control, lamin A/C-specific siRNA. Cells were harvested after 24 hours, protein extracted and assayed for AHR levels by immunoblotting. Blots were re-probed with ß-actin-specific antibodies to control for protein loading. Data from a representative experiment (5 total) are shown. (B) B cells activated with CD40 ligand as in (A) were transfected with AHR or Lamin A/C siRNA. Twenty four hours later, cells were restimulated with CD40L and rIL-4, ^{3}H-thymidine added, and ^{3}H-thymidine incorporation assayed 18 hours later. The means of the triplicate counts were averaged for each experiment. Data are presented as mean ± SE from 4 donors, cells from which were transfected in separate experiments. An asterisk (*) indicates a significant decrease in ^{3}H-thymidine uptake of AHR-specific siRNA-transfected cells as compared with lamin-specific siRNA-transfected cells ($p < 0.05$; paired t-test).

Figure 2: AHR, NF-κB, and SP-1/3 binding sites in *AHRR* regulatory regions

The murine *AHRR* proximal promoter on chromosome 13 contains 4 AHREs (closed ovals), 2 overlapping and 1 additional GC boxes (open rectangles), and 1 NF-κB binding site which overlaps with one of the GC boxes (closed diamond). Each of these sites has been functionally implicated in regulating constitutive and AHR ligand-induced *AHRR* transcription.

A highly homologous regulatory sequence is located on the 5' end of the human *AHRR* gene on chromosome 5.

Figure 3: Mechanisms of repression by AHRR

Two hypothesized mechanisms by which AHRR can repress transactivation by AHR. The AHR illustrated in this figure represents AHR that is either constitutively active or has been activated by exogenous ligand (not shown). One mechanism of repression involves competition with AHR for binding to AHR response elements (AHREs) in the promoters of AHR target genes, as originally proposed [68]. The AHRE-bound AHRR may recruit co-repressors such as ANKRA2 and histone deacetylases [110]. This mechanism is likely to require ARNT as part of an AHRR-ARNT complex, but does not involve competition for dimerization with ARNT [72, 106, 112]. A second hypothesized mechanism is independent of competition for ARNT or AHREs, and may involve transrepression [72, 106, 112]. The hypothesized transrepression mechanism is illustrated using an AHRR-ARNT dimer, but whether this mechanism requires ARNT or occurs in an ARNT-independent manner (i.e. by the AHRR alone or as complex with other proteins) is not yet known. The transrepression mechanism could be involved in the AHR-independent effects of AHRR on other transcription factors. For additional details, see text.

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Figure 1
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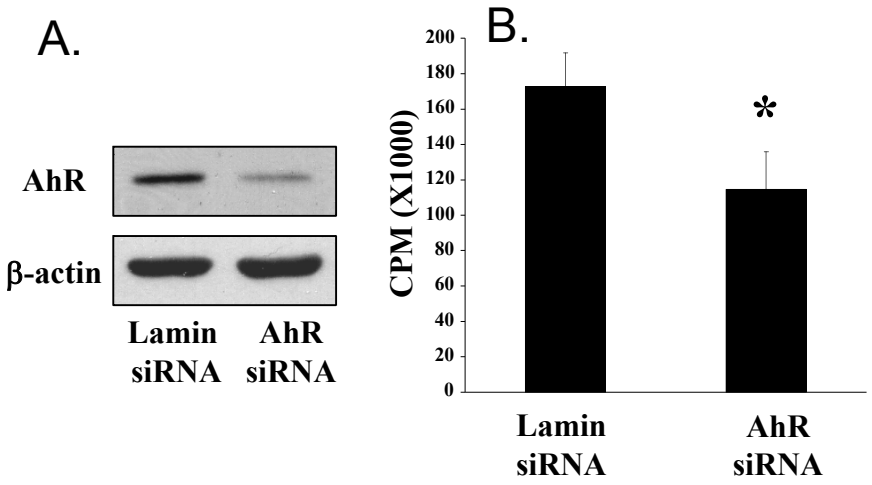

Figure 2
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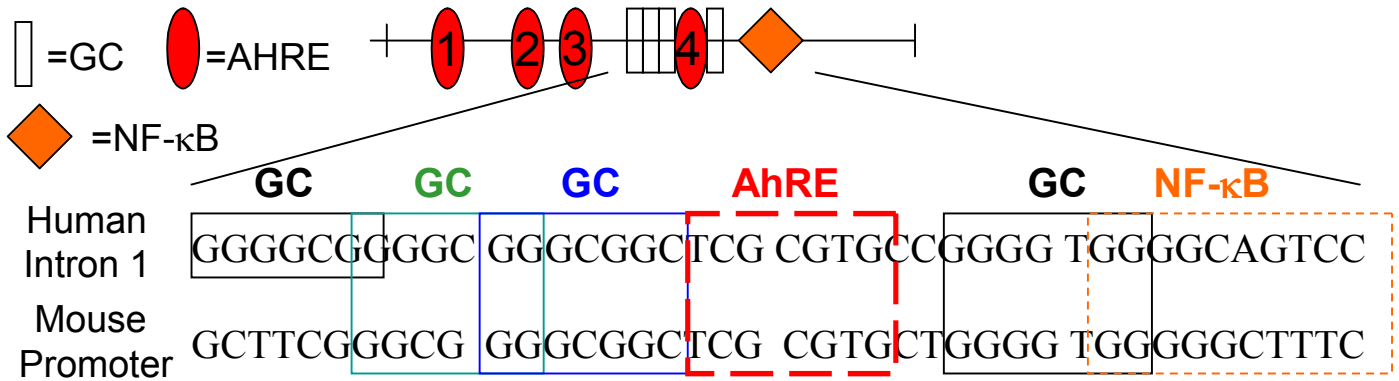

Figure

Figure 3
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