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AH receptor antagonist inhibits constitutive *CYP1A1* and *CYP1B1* expression in rat BP8 cells

Steven Roblin,[a,b] Allan B. Okey,[b] and Patricia A. Harper[a,b,*]

[a] *Developmental Biology Program, The Hospital for Sick Children, Toronto, Ont., Canada M5G 1X8*
[b] *Department of Pharmacology, University of Toronto, Toronto, Canada*

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Abstract

The BP8 variant of the 5L rat hepatoma cell line is completely devoid of aryl hydrocarbon receptor (AHR) and is a useful model to examine AHR function. Previous studies showed that BP8 cells, when transfected with mouse AHR, exhibit induction of a plasmid-based reporter even in the absence of exogenous ligands. We transfected BP8 cells with full-length human AHR and found that presence of the AHR alone was sufficient to induce substantial CYP1A1 and CYP1B1 mRNA without any exogenous AHR ligand. An AHR antagonist, 3,4-dimethoxyflavone, inhibited CYP1A1 and CYP1B1 expression in a dose-dependent manner. When we transfected BP8 cells with a mutated human AHR that is defective in ligand binding, expression of CYP1A1 and CYP1B1 was diminished but not abolished. Inhibition by the AHR antagonist along with the diminished response to the mutated AHR indicates that BP8 cells contain some agent that acts as an agonist ligand for the AHR.

Keywords: Aryl hydrocarbon receptor; BP8 cells; Endogenous; Ligand; CYP1A1; CYP1B1; P450; TCDD; Dioxin

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates most, if not all, toxic effects of dioxin-like halogenated-aromatic hydrocarbons (HAH) including the prototype agonist, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [1–3]. Many AHR agonists produce a wide range of toxic, developmental, and/or carcinogenic effects. The spectrum of effects varies with the ligand, tissue, animal species, and strain [4,5]. TCDD is classed by IARC as a human carcinogen based on animal studies as well as on data from human industrial exposure and accidental exposure [6–9].

The AHR is a member of the bHLH/PAS protein superfamily and is the critical mediator of a cell signaling system whose activation results in altered regulation of a wide array of target genes [10–13]. The diverse members of the bHLH/PAS protein superfamily share similar functional attributes in that many of them participate in "sensor pathways" which mediate or facilitate intercellular signaling. The AHR shares this function as an environmental sensor, serving as a ligand-activated transcription factor to mediate intracellular changes provoked by external chemical stimuli [13].

The AHR resides in the cytoplasm bound to protein chaperones such as hsp90 and XAP2 [14]. Ligand binding results in transformation of the AHR, release of associated proteins, nuclear translocation, and heterodimerization with another bHLH/PAS protein, ARNT. The ligand·AHR·ARNT complex then associates with specific DNA sequences denoted dioxin response elements (DRE) upstream of target gene promoters and thereby regulates expression of the gene [12,15].

Numerous target genes regulated by the AHR include the cytochromes P450: CYP1A1, CYP1A2, CYP1B1, and CYP2S1 [16,17]. Expression of these enzymes increases bioactivation of pro-toxicants and pro-carcinogens (many of which are AHR agonists) to their ultimate genotoxic state [18,19]. Extensive analysis of these AHR-regulated P450 genes suggests that their dysregulation probably is not responsible for the classical major forms of toxicity from dioxins such as

* Corresponding author. Fax: +1-416-813-7562.
E-mail address: pharper@sickkids.ca (P.A. Harper).

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TCDD. Therefore, continued studies of AHR function are essential to elucidate its role in mediating xenobiotic toxicity and normal cellular processes.

Several AHR-deficient cell lines have been created and used as tools to examine AHR functions in vitro [11,12,20–22]. The BP8 cell line, derived from 5L rat hepatoma cells, was selected by exposing the parental cell line to the genotoxic AHR agonist, benzo[*a*]pyrene [20]. BP8 cells have no detectable expression of AHR at either the mRNA or protein level [23]. We transiently transfected BP8 cells with expression constructs for the human AHR with the goal of testing the ability of polymorphic forms of the human AHR to regulate CYP1 genes. However, we observed upregulation of the classical AHR-regulated genes, CYP1A1 and CYP1B1, in the absence of exogenous ligand. TCDD-independent induction of AHR-responsive genes previously has been described in various reconstituted cell systems which have defect(s) in the AHR pathway [23–25] and the authors postulated that activation of the AHR in the absence of exogenous ligand may be due to an endogenous ligand for the AHR which is a substrate for an AHR-responsive gene. Results of several assays we performed to examine TCDD-independent induction of CYP1A1 and CYP1B1 in AHR-reconstituted BP8 cells suggest that this phenomenon is caused by an unidentified ligand which is present within the BP8 cells.

Materials and methods

Plasmids. A human AHR wildtype plasmid expression construct (phAHRwt) was created as follows: the expression plasmid pRC/CMV2 (Invitrogen) was modified to include an internal ribosomal entry site (IRES) cloned from the *VegF* gene. cDNA from the human AHR (hAHR) was ligated downstream of the IRES. The hAHR 5′ start site was modified using site directed mutagenesis (Stratagene) to create a consensus Kozak sequence (ATGgccAGC). We prepared a mutant (phAHRasp381) that is defective in ligand binding by site directed mutagenesis (Stratagene) using phAHRwt as the template with the following primers: 5′ GGA AGA CCA GAT TAT ATC ATT GAT ACT CAG AGA CCA CTA AC 3′, and the reverse complement 5′ GTT AGT GGT CTC TGA GTA TCA ATG ATA TAA TCT GGT CTT CC 3′. Plasmid DNA for transfections was prepared using an endotoxin-free maxiprep kit (Qiagen).

Cell culture and transfection. Rat hepatoma BP8 cells and human hepatoma HepG2 cells were grown in αMEM (Wisent) supplemented with 10% fetal bovine serum (Hyclone). Cells were grown to near-confluency and passaged using trypsin digestion. Culture medium was replaced every 48 h. For transfection, BP8 cells were plated at a concentration of 1.0×10^5 cells/35 mm tissue culture plate (Nunc) and incubated for 24 h. Cells were transfected with plasmid DNA using Effectene Transfection Reagent (Qiagen). Conditions were optimized for transfection as follows: 0.5 μg DNA, 110 μl EC, 3.5 μl enhancer, 11 μl effectene, and 2.0 ml final transfection volume. TCDD (Chemsyn), 3,4-dimethoxyflavone (DMF, Lancaster) or the vehicle, Me_2SO4 (Sigma), was added to the final transfection medium as appropriate. Cells were incubated for 24 h and then harvested. HepG2 cells were plated at a concentration of 4.0×10^5 cells/35 mm tissue and incubated for 24 h. Media were changed and DMF or DMSO was added as noted in the figure legends. Cells were incubated for 24 h and then harvested for RNA extraction.

RT-PCR. QiaShredder (Qiagen) was used for cell homogenization. Total RNA was prepared using the RNeasy Kit (Qiagen); DNase was added to the RNeasy elution column as recommended by the manufacturer. Reverse transcription (RT) was performed using M-MLV reverse transcriptase (Invitrogen). Purified total RNA (1.5 μg) was used in each reaction. PCR was performed using AmpliTAQ DNA Polymerase (Perkin–Elmer) according to the manufacturer's instructions. RT reaction mixture (2.5 μl) was used for each PCR. Agarose gel electrophoresis was used to separate PCR-amplified products. Ethidium bromide was added to the gel before solidification. PCR products were visualized under a UV transluminator and digitally recorded. Band intensity was quantitated using ImageQuant software (Amersham–Pharmacia). Primer sequences used were: CYP1A1 forward, ACG TTA TGA CCA CGA TGA CC; reverse, AGG CCG GAA CTC GTT TG and CYP1B1 forward, CTT ATT AGA CGC CTT CTG AC; reverse, CTC CAG CCA GTC TTA AGA GA.

Statistics. Three independent experiments, each containing three replicates, were performed for all studies unless otherwise indicated. Intra-experimental replicates were averaged and the inter-experimental means were compared. Local-average background correction was employed during densitometry (ImageQuant). Individual samples were normalized to β-actin. Intra-experimental replicates were averaged and expressed as a percent of one intra-experimental value to allow comparison of independent experiments. Error bars represent standard deviation.

Results and discussion

BP8 cells transfected with full-length human AHR exhibit CYP1A1 and CYP1B1 upregulation in the absence of any exogenous ligand

A primary research goal in our laboratory is to determine how variation in AHR structure affects the receptor's function. An AHR-deficient cell line would be a valuable tool for testing function of genetic variants of the AHR. Unfortunately, there are few AHR-deficient cell lines suitable for this purpose. We endeavored to use the BP8 rat hepatoma cell line, which is devoid of endogenous AHR, to test function of variant human AH receptors. Weiss et al. [23] previously studied the response of an AHR-driven reporter construct in BP8 cells transiently transfected with a mouse AHR expression construct and observed a significant increase in DRE-driven reporter activity, even in the absence of TCDD. This unexpected phenomenon confounded examination of TCDD-dependent activation by the murine AHR in the BP8 cells.

Plasmid reporter gene activation is a highly sensitive assay but does not necessarily represent how native genes in the cells will respond. We postulated that basal levels of native chromosomal AHR target genes would not be significantly elevated after transient transfection of a human AHR into the BP8 cells unless the cells were exposed to an AHR agonist. To test this hypothesis, we expressed a full-length wildtype human AHR construct, phAHRwt, in BP8 cells through non-stable transfection.

Total RNA was harvested after 24 h and RT-PCR measurements were done for the AHR target genes, CYP1A1 and CYP1B1. Transient reconstitution of hAHRwt in BP8 cells resulted in substantial mRNA expression of classical AHR-responsive genes, CYP1A1 (Fig. 1A) and CYP1B1 (Fig. 1A), without exposure to any exogenous AHR ligand. Transfection of vector alone did not result in a significant change in basal mRNA expression for these genes (Figs. 1A and B); therefore, the observed increase in CYP1 expression is truly AHR-dependent and not an artifact from the stress of transfection.

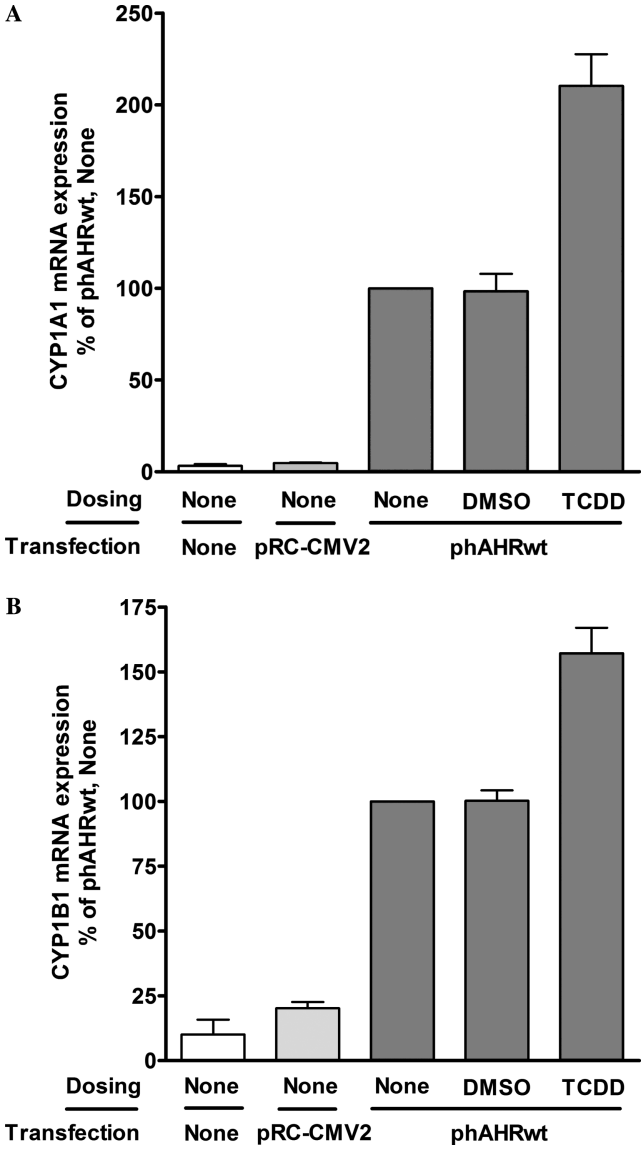

Fig. 1. Effect of AHR transfection on AHR target gene expression in BP8 cells. Empty vector (pRC–CMV2) or human AHR (phAHRwt) was transfected into AHR-deficient BP8 cells for 24 h before cells were harvested for RNA isolation. "None" indicates non-transfected cells used as a negative control. Solvent (0.1% DMSO) or 50 nM TCDD was present in the medium throughout transfection. RT-PCR was performed for AHR target genes. (A) Mean (±SD) expression of *CYP1A1* mRNA expressed as a percent of the response in transfected cells that received no chemical treatment. (B) Mean (±SD) expression of *CYP1B1* mRNA expressed as a percent of the response in transfected cells that received no chemical treatment. TCDD did not cause expression of CYP1A1 or CYP1B1 in BP8 cells unless they had been transfected with a full-length AHR expression construct (data not shown).

When BP8 cells that had been transfected with hAHRwt were exposed to a high concentration of TCDD (50 nM) for 24 h there was a modest increase in expression of both CYP1A1 and CYP1B1 above the basal level achieved from transfection alone (Figs. 1A and B). However, the maximum fold induction over basal expression of these genes was lower than would be expected in usual cell lines at this TCDD concentration. When BP8 cells that had not been transfected with an AHR expression construct were exposed to TCDD, there was no significant elevation in CYP1A1 or CYP1B1 over the levels in untreated cells (data not shown). These results support previous evidence that transfection of BP8 cells with a functional AHR alone results in a substantial increase in expression of AHR target genes without a need for any exogenous ligand.

It has been hypothesized that cells that lack AHR or have low endogenous AHR levels may accumulate an AHR activator which normally is metabolized by AHR target genes such as CYP1A1 [24,25]. This hypothesis is supported by the observation that stable reconstitution of BP8 cells with mouse AHR resulted in decreased basal expression of AHR-responsive reporter genes while restoring the normal response to TCDD treatment [23]. Our observations of increased AHR target gene expression in BP8 cells reconstituted with human AHR further support the existence of an activator which accumulates in BP8 cells and which is eliminated in cells that possess AHR.

CYP1A1 and CYP1B1 expression in cells transfected with hAHRwt is decreased by treatment with the AHR antagonist, DMF

Several investigators hypothesized that the observed AHR target gene activation following transfection with AHR is caused by an unknown intracellular ligand. However, a ligand-binding assay to test whether an endogenous substance could compete with [^{3}H]TCDD for AHR binding in a co-culture of AHR-responsive + AHR-non-responsive BP8 cells was inconclusive [23]. Therefore, we designed an experiment to determine if the expression of AHR target genes in transfected BP8 cells is the result of an endogenous ligand. For one approach, we employed a competitive antagonist: BP8 cells were transfected with phAHRwt and exposed to increasing concentrations of the competitive AHR antagonist, DMF [26], for 24 h. DMF exposure resulted in a dose-dependent reduction in mRNA levels for both CYP1A1 (Fig. 2A) and CYP1B1 (Fig. 2B). A DMF concentration of 10 μM almost completely abolished

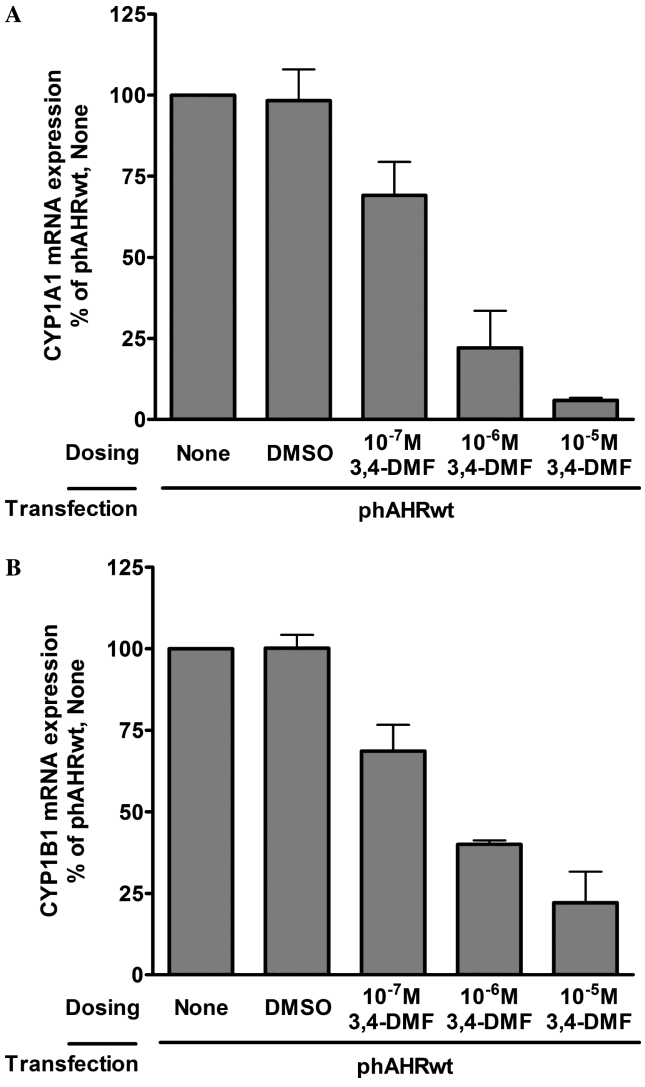

Fig. 2. Decreased expression of CYP1A1 and CYP1B1 mRNA in the presence of an AHR antagonist. BP8 cells were transfected with phAHRwt as described in the legend to Fig. 1. Solvent (0.1% DMSO) or the AHR-antagonist, DMF, was present in the medium throughout the transfection period. RT-PCR was performed for AHR target genes. (A) Mean (±SD) expression of CYP1A1 mRNA expressed as a percent of the response in transfected cells that received no chemical treatment. (B) Mean (±SD) expression of CYP1B1.

CYP1A1 and CYP1B1 mRNA expression. This ability of a well-characterized AHR antagonist to reduce the "basal" levels of CYP1A1 and CYP1B1 supports the hypothesis that the AHR activation in BP8 cells is caused by an endogenous ligand, and that DMF competes with this unknown ligand for the ligand-binding site of the AHR.

Altered expression of CYP1A1 and CYP1B1 in BP8 cells transfected with an AHR mutant that is defective in ligand binding

To further test the hypothesis than an endogenous ligand drives CYP1 expression in BP8 cells, we transfected the cells with a mutant human AHR construct modeled on a mouse construct that was engineered by Ema et al. [27] to be defective in ligand binding. This variant contains a mutation in the ligand-binding domain which converts residue 381 from val381 to asp381. Ligand-binding assays show that this mutant is deficient in its ability to bind [^{3}H]TCDD [27]. phAHRasp381 and phAHRwt were transfected into separate plates of BP8 cells and incubated for 24 h. CYP1A1 mRNA expression was lower in cells expressing the binding-deficient mutant (phAHRval381-asp) than in cells transfected with the wildtype AHR construct (phAHRwt) (Fig. 3) but the cells transfected with the binding mutant still responded with expression of more than half the CYP1A1 mRNA level found with the full-length wildtype transfectant. It is not certain that the phAHRval381-asp mutant has completely lost ability to bind ligand since this originally was assessed by a relatively insensitive assay. If the mutant retains at least a low affinity for agonists, that may be sufficient to stimulate transactivation of CYP1A1, a highly sensitive AHR-mediated response.

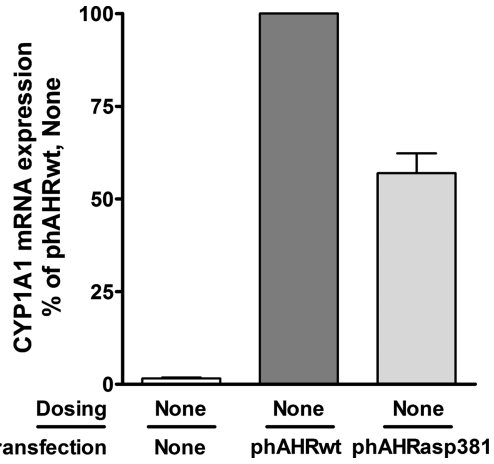

Fig. 3. CYP1A1 expression in BP8 cells transfected with an AHR-mutant construct that is defective in ligand binding. Human AHR (phAHRwt) or a human AHR mutant defective in ligand binding (phAHRasp381) was transfected into AHR BP8 cells for 24 h. "None" indicates non-transfected cells used as a negative control. Mean (±SD) CYP1A1 mRNA level is expressed as a percent of the response in transfected cells that received no chemical treatment.

Effect of an AHR antagonist on constitutive induction of CYP1A1 in HepG2 cells

In order to confirm the presence of a physiologically relevant endogenous ligand it is necessary to eliminate the possibility that the cell is being unintentionally exposed to an exogenous ligand in the growth medium. For example, tryptophan derivatives, known to activate the AHR, have been found in tissue culture media exposed to UV or to visible light

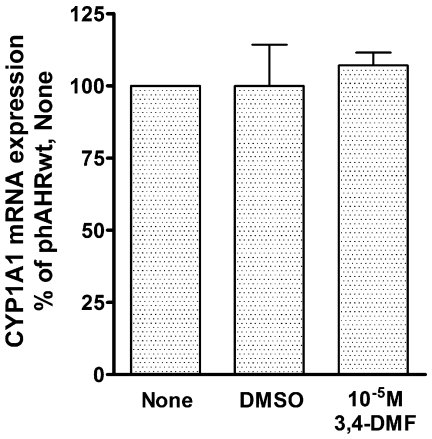

Fig. 4. The effect of an AHR antagonist on constitutive expression of CYP1A1 in HepG2 cells. HepG2 cells were exposed to the solvent (0.1%) DMSO or to 10 μM 3,4-DMF for 24 h before RNA was harvested. "None" indicates untreated cells used as a negative control. Mean (±SD) CYP1A1 mRNA level is expressed as a percent of that in untreated cells.

radiation and also may occur naturally in sera used in culture media [28,29]. It was possible that other uncharacterized ligands were active components of the extracellular environment [30]. To determine if the tissue culture medium contained AHR agonists we tested the response of HepG2 hepatoma cells to the medium. HepG2 cells have substantial levels of their own AHR [31]. They exhibit low CYP1A1 expression in the absence of exogenous agonists but are highly responsive to the addition of TCDD or other known AHR agonists to the medium [31,32]. To determine if a component in the medium was responsible for "basal" CYP1A1 expression we exposed HepG2 cells to 10 μM DMF for 24 h. Total RNA was harvested and RT-PCR quantitation was performed for CYP1A1 (Fig. 4). The results indicate that a high concentration of DMF had no effect of expression of "basal" CYP1A1 mRNA levels in HepG2 cells whereas this DMF concentration was able to nearly abolish the TCDD-independent CYP1A1 and CYP1B1 mRNA expression in reconstituted BP8 cells (Figs. 2A and B). We conclude that the constitutive levels of CYP1A1 in HepG2 cells are likely not regulated by ligand-activated AHR; therefore, basal CYP1A1 expression in HepG2 cells may be regulated in an AHR-independent manner. Furthermore, since no AHR-mediated induction of CYP1A1 was observed in HepG2 cells, we conclude that the culture medium that we used in our experiments with BP8 cells does not contain a cryptic ligand for the AHR. Furthermore, co-culture of BP8 cells and HepG2 cells did not result in increased CYP1A1 mRNA expression in the HepG2 cells, indicating that the postulated endogenous ligand in BP8 cells is not released into the medium (data not shown).

Conclusion

The AHR is a promiscuous receptor that binds a wide range of synthetic and natural ligands [29,33]. Therefore, it is not surprising that the AHR participates in a diverse array of cellular processes following exposure to these ligands [13,29,33,34]. Numerous studies have been performed to catalog AHR ligands [35,36] in order to understand classical roles of the AHR in gene regulation/dysregulation after xenobiotic exposure [37,38].

Many AHR functions are not necessarily linked to exposure to exogenous xenobiotic ligands [39]. In vitro studies show that perturbation of AHR expression, in the absence of exogenous ligands, can alter cell proliferation [40]. Studies of AHR knockout mice [41] provide the most compelling evidence for a role of the AHR independent of chemical exposure. AHR-null mice manifest hepatic and cardiac abnormalities, implicating the AHR as a factor critical to normal development [42–44] but the specific pathways that cause these aberrant phenotypes have not yet been elucidated. However, numerous studies provide indirect evidence for activation of the AHR by endogenous ligands [45–48]. It has been hypothesized that such endogenous ligands may activate the AHR during critical phases of development, and that the absence of a functional AHR pathway disrupts this process. Studies to identify and characterize "natural" and endogenous ligands for the AHR have produced several candidates. These include chemicals derived from plant materials, tryptophan derivatives, metabolic intermediates including tetrapyroles and arachadonic acid metabolites as well as cholesterol derivatives [29,36]. It is unclear however what role, if any, these compounds play in the normal physiological activities of the AHR.

CYP1A2 is constitutively expressed in liver of AHR-null mice [49,50] as is CYP1B1 [49]. CYP1A2 and CYP1B1 also each can be induced in AHR-null mice by compounds such as phenobarbital [51], piperonyl butoxide or acenapthylene [49]. Clearly expression of CYP1A2 and CYP1B1 can be regulated by factors other than the AHR whereas CYP1A1 expression appears to be highly dependent on the AHR [51]. Therefore, CYP1A1 expression in the absence of an exogenous ligand in the BP8 cells is very likely to be dependent on the AHR. The fact that CYP1A1 is expressed in the absence of an exogenous ligand when BP8 cells are transfected with full-length AHR and the fact that this expression is suppressed by the AHR antagonist, DMF, strongly support the existence of an endogenous ligand in these cells.

Our results reinforce a model originally proposed by Hankinson et al. [24] for mutants of the mouse Hepa-1 cell line which postulates that CYP1 enzymes in "normal" cells degrade an intracellular compound. Cells that are deficient in CYP1 enzymes (due to defects in the

enzymes themselves or in factors regulating their expression) accumulate this compound that acts as an endogenous AHR agonist. The identity of compounds that act as endogenous ligands in cells in culture remains to be determined. The substantial AHR-mediated expression of CYP1A1 and CYP1B1 in BP8 cells in the absence of exogenous ligands indicates that these cells are a suitable model in which to attempt to identify endogenous AHR ligands.

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