Aryl-Hydrocarbon Receptor Activation Regulates Constitutive Androstane Receptor Levels in Murine and Human Liver

Rushang D. Patel, Brett D. Hollingshead, Curtis J. Omiecinski, and Gary H. Perdew

The aryl-hydrocarbon receptor (AhR) is a basic helix-loop-helix/Per-Arnt-Sim transcription factor that can be activated by exogenous as well as endogenous ligands. AhR is traditionally associated with xenobiotic metabolism. In an attempt to identify novel target genes, C57BL/6J mice were treated with β-naphthoflavone (BNF), a known AhR ligand, and genome-wide expression analysis studies were performed using high-density microarrays. Constitutive androstane receptor (CAR) was found to be one of the differentially regulated genes. Real-time quantitative polymerase chain reaction (qPCR) verified the increase in CAR messenger RNA (mRNA) level. BNF treatment did not increase CAR mRNA in AhR-null mice. Time-course studies in mice revealed that the regulation of CAR mRNA mimicked that of *Cyp1A1*, a known AhR target gene. To demonstrate that the increase in CAR mRNA translates to an increase in functional CAR protein, mice were sequentially treated with BNF (6 hours) followed by the selective CAR agonist, TCPOBOP (3 hours). qPCR revealed an increase in the mRNA level of *Cyp2b10*, previously known to be regulated by CAR. This also suggests that CAR protein is present in limiting amounts with respect to its transactivation ability. Finally, CAR was also up-regulated in primary human hepatocytes in response to AhR activation by 2,3,7,8-tetrachlorodibenzo-p-dioxin and benzo[a]pyrene. *Conclusion:* This study identifies a mode of up-regulating CAR and potentially expands the role of AhR in drug metabolism. This study also demonstrates *in vivo* up-regulation of CAR through chemical exposure. (HEPATOLOGY 2007;46:209-218.)

The aryl-hydrocarbon receptor (AhR) is a ligand-dependent basic helix-loop-helix/Per-Arnt-Sim domain transcription factor. It resides in the cytoplasm in a complex with two molecules of heat shock protein 90, HBV X-associated protein 2, and p23. On ligand activation, AhR translocates to the nucleus and heterodimerizes with AhR nuclear translocator. The AhR–AhR nuclear translocator heterodimer binds to a consensus sequence (TNGCGG) known as the dioxin responsive element (DRE) and drives the transcription of its target genes (reviewed by Hankinson[1] and Petrulis and Perdew[2]). Most of the well-characterized AhR target genes belong to phase I and II enzyme families involved in xenobiotic metabolism, principally *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, glutathione-*S*-transferase Ya, NAD(P)H: quinone oxido-reductase 1, and UDP-glucuronosyl transferase 1.[3]

Although several genes have been characterized as regulated by AhR in a DRE-dependent manner, alternative modes of receptor function and novel target genes must be identified to adequately explain the wide spectrum of pathophysiological effects associated with AhR. An emerging aspect of transcription factor biology is the ability of various factors to interact with members of different signaling pathways. Recent reports focusing on receptor crosstalk have highlighted the ability of AhR to influence the activity of other proteins involved in gene regulation, including nuclear factor κB,[4] estrogen receptor,[5-7] and TGF-β1.[8]

Abbreviations: AhR, aryl-hydrocarbon receptor; BaP, benzo[a]pyrene; BNF, β-naphthoflavone; CAR, constitutive androstane receptor; DRE, dioxin response element; KO, knockout; mRNA, messenger RNA; PXR, pregnane X receptor; qPCR, quantitative polymerase chain reaction; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCPOBOP, 1,4-bis[2-(3,5,-dichloropyridyloxy)]benzene.

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The constitutive androstane receptor (CAR), also known as Nr1i3, is a member of the nuclear receptor family. It is found in the cytoplasm in a complex with heat shock protein 90 and CAR cytoplasmic retention protein.[9] A unique feature of CAR is that it can be activated by two distinct mechanisms. Ligands such as 1,4-bis[2-(3,5,-dichloropyridyloxy)]benzene (TCPOBOP) can directly bind to CAR and activate the receptor.[10] Alternately, CAR activity can be induced indirectly by a phenobarbital-responsive protein phosphatase-2A–dependent signaling cascade.[11] Activated CAR undergoes nuclear translocation, heterodimerizes with 9-*cis* retinoic acid receptor to bind its response element, and drives the transcription of its target genes. Using cell culture models, it has been demonstrated that activation of the glucocorticoid receptor can up-regulate transcriptional activity at the CAR promoter[12] and that IL-1β–mediated nuclear factor κB activation inhibits this up-regulation by interfering with chromatin remodeling.[13]

In the current study, we have identified CAR to be an AhR target gene. Previously, it has been shown that both AhR and CAR play a significant role in response to exogenous stimuli as well as pathophysiological events in the liver. Both receptors induce numerous xenobiotic metabolism enzymes. AhR is also known to affect vascular development, as demonstrated by persistent ductus venosus and microvasculature abnormalities in the livers of AhR-null mice.[14] Recently, it has been demonstrated that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure severely impairs the regenerative ability of partially excised mouse livers,[15] an effect most likely mediated through AhR. On the other hand, TCDD is also known to be a potent tumor promoter in mouse liver.[16] CAR is similarly involved in a number of physiological processes in the liver, including bilirubin metabolism and hepatocyte proliferation. As shown in this study, AhR activation increases CAR messenger RNA (mRNA) in the liver as well as extrahepatic tissues and follows a temporal pattern similar to *Cyp1A1*, a known AhR target gene. This increase in CAR mRNA correlates with an increase in the transcriptional activity of CAR. Because a broad range of compounds can activate AhR, an AhR-mediated increase in CAR activity could potentially lead to unexpected effects on drug metabolism. Considering the importance of AhR and CAR in liver biology, knowledge of their interaction will be useful in interpreting the observations made in relation to these two receptors.

Materials and Methods

Mice and Treatment. Adult (10 ± 2 weeks) male wild-type C57BL/6J mice were purchased from the Jackson Laboratory. AhR knockout (AhR-KO) mice in a C7BL/6J background were a kind gift from Dr. Bradfield (McArdle Laboratory for Cancer Research, University of Wisconsin–Madison Medical School). All mice were maintained in a temperature- and light-controlled facility and had free access to water and diet. All experiments were performed in compliance with the standards for animal use and care set by the Pennsylvania State University's animal research program. Mice were injected intraperitoneally with β-naphthoflavone (BNF) dissolved in corn oil or with corn oil alone (control). The volume of injection was adjusted in proportion to body weight. Mice were sacrificed via CO_2 inhalation, and liver tissue samples were collected. Tissues were frozen immediately in liquid nitrogen and stored at −80°C.

Microarray Experiments. Liver samples were homogenized in TRI-Reagent (Sigma-Aldrich Co.) with Ultra Turrax T25 basic disperser from IKA Works, Inc. (Wilmington, NC). RNA was isolated from tissues using TRI Reagent and was further purified with RNeasy kits (Qiagen Inc.) according to the manufacturer's protocol with minor modifications. The quality of RNA was analyzed on 1% agarose-formaldehyde gels and with an Agilent 2100 bioanalyzer and RNA-6000 Nano Chip kit (Agilent Technologies, Inc.). A GeneChip One-Cycle Target Labeling and Control Reagent package (Affymetrix, CA) was used to label 8.0 μg total RNA for each microarray. The GeneChip Hybridization, Wash, and Stain kit (Affymetrix, CA) was used for processing the microarrays. Liver gene expression profiles were compared between BNF (10 mg/kg) treated versus vehicle-control mice using GeneChip Mouse Genome 430 2.0 arrays. The arrays were scanned with a GeneChip Scanner 3000 at the microarray core facility of the Huck Institutes of Life Sciences, the Pennsylvania State University.

Microarray Data Analysis. Background adjustment, normalization, and summarization were performed on the raw data files (.CAB files) using RMAexpress (0.3 Release). Summarized data was further analyzed with Significance Analysis of Microarrays (SAM, version 2.23A). The data was input in the log scale (base 2), and the default settings were accepted as the choice of analysis parameters (*i.e.*, T-statistic, 100 permutations, 10 neighbors for K-nearest neighbors imputer). A δ of 0.35 and 2-fold change was used as the threshold for significant differential expression. Ninety-seven probe sets corresponding to 80 distinct genes/ESTs were found to be significantly changed with a false discovery rate of 5.7% using the settings detailed above.

Postprocessing of the significantly altered genes was performed using the DAVID Bioinformatic Resources 2006 online tool. Genes were clustered with the Gene

Table 1. Sequence Information for Primers Used in qPCR

	Gene	Primer Set
1	CAR	FP: 5′-GGAGCGGCTGTGGAAATATTGCAT-3′
		RP: 5′-TCCATCTTGTAGCAAAGAGGCCCA-3′
2	*Cyp1a1*	FP: 5′-CTCTTCCCTGGATGCCTTCAA-3′
		RP: 5′-GGATGTGGCCCTTCTCAAATG-3′
3	PXR	FP: 5′-TTCATGTGGAGCCAAAGAAACGGC-3′
		RP: 5′-TCCTGGAATGTGGGAACCTTTCCT-3′
4	*Cyp2b10*	FP: 5′-TTCTGCGCATGGAGAAGGAGAAGT-3′
		RP: 5′-TGAGCATGAGCAGGAAGCCATAGT-3′
5	GAPDH	FP: 5′-CATGGCCTTCCGTGTTCCTA-3′
		RP: 5′-GCGGCACGTCAGATCCA-3′
6	CAR (human)	FP: 5′-AGTGCTTAGATGCTGGCATGAGGA-3′
		RP: 5′-TGCTCCTTACTCAGTTGCACAGGT-3′
7	Rpl13a (human)	FP: 5′-CCTGGAGGAGAAGAGGAAAGAGA-3′
		RP: 5′-GAGGACCTCTGTGTATTTGTCAA-3′

Abbreviations: FP, forward primer; RP, reverse primer.

Functional Classification Tool using the lowest classification stringency settings. To reduce redundancy, the list of terms associated with the clustered genes was restricted to biological processes and/or molecular function (according to Gene Ontology classification) with a P value of less than 0.05, as computed by DAVID. A subset of genes was selected to confirm differential expression via real-time qPCR.

Real-Time qPCR. Total RNA isolated from mice livers, as mentioned above, was reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer's protocol. Complementary DNA made from 25 ng of RNA was used for each qPCR reaction. qPCR was performed on a DNA Engine Opticon system using a DyNAmo SYBR Green qPCR Kit purchased from New England Biolabs, Inc. Sequence information for the qPCR primers is provided in Table 1.

Primary Human Hepatocyte Culture. Primary human hepatocytes were obtained from the Liver Tissue Procurement and Distribution System (LTPDS) at the University of Pittsburgh, through NIH Contract #NO1-DK-9-2310, and generously provided by Dr. Stephan C. Strom. Donor organs not designated for transplantation were used to isolate hepatocytes according to a three-step collagenase perfusion protocol.[17] Preparations enriched for hepatocytes were received plated in collagen-coated T25 flasks. Upon arrival, the media was changed to William's Media E supplemented with 1% penicillin/streptomycin, 10 mM HEPES, 20 μM glutamine, 25 nM dexamethasone, 10 nM insulin, 30 mM linoleic acid, 1 mg/mL bovine serum albumin, 5 ng/mL selenious acid, and 5 μg/mL transferrin, as described previously.[18] All cells were maintained at 37°C under 5% CO_2. All culturing materials were purchased from Invitrogen (Carlsbad, CA) unless noted otherwise.

Results

AhR Activation Alters Expression of Various Genes in Mouse Liver. In an effort to identify novel AhR target genes, we performed genome-wide expression profiling studies using high-density microarrays (Affymetrix). Ten-week-old male wild-type C57BL/6J mice were treated with 10 mg/kg BNF in corn oil via intraperitoneal injection for 5 hours, after which RNA was isolated from their livers. The RNA was processed and hybridized to GeneChip Mouse Genome 430 2.0 arrays. After preprocessing and analyzing the data as described in Materials and Methods, significantly altered genes were classified on the basis of gene ontology (biological process and/or molecular function) to identify patterns of biological importance. The Gene Functional Classification module, implemented in the online version of DAVID, clusters genes on the basis of the similarity of different ontology terms associated with the genes. The list of terms for each cluster comprised overlapping and redundant entries and thus had to be manually limited to include those with high statistical significance of association and to prevent repetition of parent/child terms. The results are presented in Table 2. Table 2 also includes whether a DRE is present in the putative regulatory region (−5,000 to +1,000 bases relative to transcription start site) of the respective genes. To validate the microarray results, qPCR was performed on 14 genes that included four previously known AhR targets, and the results were similar to the changes observed on the microarrays. The induction/repression observed by qPCR is given as a fold change in parentheses. The alteration of genes belonging to diverse functional categories further supports the role of AhR in a variety of cellular processes.

AhR Activation Increases CAR mRNA in Mouse Liver. CAR was one of the genes observed to be upregulated in the livers of BNF-treated mice compared with corn oil alone (control). The results obtained from the microarray experiments were verified with real-time qPCR (Fig. 1). qPCR data revealed a 2.1-fold increase in CAR mRNA on BNF treatment, which correlated well with microarray results. The currently accepted model of AhR-dependent transcription is based on the AhR–AhR nuclear translocator heterodimer binding to a consensus DRE in the regulatory region of a target gene. However, sequence analysis of the putative mouse CAR promoter (−5,000 to +1,000 bases relative to transcription start site) did not reveal any sequences matching the DRE (TNGCGTG). This observation generated further interest in examining the role of AhR in regulating CAR expression and whether the observed increase in CAR mRNA had a functional significance.

Table 2. BNF-Mediated Differentially Regulated Genes, Sorted by Biological Process/Molecular Function

	Affy ID	Fold Change	Gene Name	Biological Process/Molecular Function Terms ($P < 0.05$)	Presence of DRE I	qPCR Confirmation
Genes induced by BNF treatment						
Gene group 1 (enrichment score: 2.24)						
1	1425477_x_at, 1451721_a_at, 1450648_s_at	2.8	Histocompatibility 2, class II antigen A, β1	Antigen presentation, exogenous antigen via MHC class II		
2	1435290_x_at, 1452431_s_at, 1438858_x_at	2.6	Histocompatibility 2, class II antigen A, α	Positive regulation of T cell differentiation		
3	1425519_a_at	2.4	CD74 antigen (invariant polypeptide of MHC, class II antigen-associated)	MHC class II receptor activity	+	
4	1417025_at	2.3	Histocompatibility 2, class II antigen E, β	Antigen processing, exogenous antigen via MHC class II	+	
5	1422527_at	2.2	Histocompatibility 2, class II, locus DMa	Immune response	+	
Gene group 2 (enrichment score: 1.98)						
1	1449009_at	5.7	T cell–specific GTPase	Hydrolase activity, acting on acid anhydrides		
2	1420549_at	3.9	Guanylate nucleotide binding protein 1	Nucleoside-triphosphatase activity	+	
3	1419518_at	2.7	Tubulin, α8	Purine nucleotide binding		
4	1425351_at, 1426875_s_at, 1451680_at	2.6	Neoplastic progression 3	GTPase activity		
5	1417141_at, 1458589_at	2.5	IFN-γ–induced GTPase			
6	1419748_at, 1438431_at	2.4	ATP-binding cassette, subfamily D, member 2			
7	1418392_a_at	2.3	Guanylate nucleotide binding protein 4		+	
8	1423597_at	2.2	ATPase, aminophospholipid transporter, class I, type 8a, member 1		+	
9	1425156_at	2.2	RIKEN cDNA 9830147j24 gene			
10	1417101_at	2.1	Heat shock protein 2		+	
11	1443870_at	2.0	ATP-binding cassette, subfamily C (CFTR/MRP), member 4			
Gene group 3 (enrichment score: 1.95)						
1	1422217_a_at	266.6	Cytochrome p450, family 1, subfamily A, polypeptide 1	Monooxygenase activity	+	+(3069)
2	1423627_at	3.4	NAD(P)H dehydrogenase, quinone 1	Generation of precursor metabolites and energy	+	+(5.59)
3	1422904_at, 1422905_s_at, 1435459_at, 1453435_a_at	2.7	Flavin-containing monooxygenase 2	Electron transport		+(2.96)
4	1450715_at	2.6	Cytochrome p450, family 1, subfamily A, polypeptide 2	Oxygen and reactive oxygen species metabolism	+	+(6.18)
5	1454930_at	2.4	Leucine-rich repeat containing 35	FAD binding		
6	1449525_at	2.3	Flavin-containing monooxygenase 3	NADP binding	+	
7	1447411_at	2.3	UDP-glucose dehydrogenase	Heme binding	+	
8	1449565_at	2.2	Cytochrome p450, family 2, subfamily G, polypeptide 1			
9	1416612_at	2.0	Cytochrome p450, family 1, subfamily B, polypeptide 1		+	+(2.05)
Gene group 4 (enrichment score: 0.94)						
1	1419647_a_at	3.1	Immediate early response 3	Establishment of localization	+	
2	1419748_at, 1438431_at	2.4	ATP-binding cassette, subfamily D, member 2	Transport		
3	1421346_a_at	2.1	Solute carrier family 6 (neurotransmitter transporter, taurine), member 6		+	

Table 2. *Continued*

	Affy ID	Fold Change	Gene Name	Biological Process/Molecular Function Terms ($P < 0.05$)	Presence of DRE I	qPCR Confirmation
Gene group 5 (enrichment score: 0.65)						
1	1419647_a_at	3.1	Immediate early response 3	Transmembrane receptor activity	+	
2	1417625_s_at	2.9	Chemokine orphan receptor 1	MHC class II receptor activity	+	
3	1425477_x_at, 1451721_a_at, 1450648_s_at	2.8	Histocompatibility 2, class II antigen A, β1	Cell surface receptor linked signal transduction		
4	1448147_at	2.7	Tumor necrosis factor receptor superfamily, member 19	Defense response	+	+(3.49)
5	1417025_at	2.3	Histocompatibility 2, class II antigen E, β	G protein–coupled receptor activity	+	
6	1446850_at	2.1	Phosphatidic acid phosphatase type 2b		+	
7	1417894_at	2.1	G protein–coupled receptor 97			
Gene group 6 (enrichment score: 0.13)						
1	1421818_at, 1450381_a_at	3.3	B cell leukemia/lymphoma 6	Ligand-dependent nuclear receptor activity		+(3.40)
2	1451814_a_at	3.3	HIV-1 tat interactive protein 2, homolog (human)	Regulation of transcription, DNA-dependent	+	
3	1455267_at	3.2	Estrogen-related receptor gamma	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	+	
4	1416543_at, 1457117_at	2.3	Nuclear factor, erythroid derived 2, like 2	Regulation of cellular metabolism		
5	1425392_a_at	2.3	Nuclear receptor subfamily 1, group I, member 3			+(2.10)
6	1429177_x_at	2.1	Sry-box containing gene 17		+	
Gene group 7 (enrichment score: 0.11)						
1	1418191_at	3.8	Ubiquitin-specific peptidase 18	Ubiquitin-dependent protein catabolism	+	+(4.54)
2	1436532_at	3.6	Doublecortin and CaM kinase-like 3	Purine nucleotide binding	+	
3	1451453_at	3.1	Death-associated kinase 2	Cellular protein metabolism	+	+(3.99)
4	1422962_a_at	2.9	Proteosome (prosome, macropain) subunit, beta type 8 (large multifunctional peptidase 7)	Cellular macromolecule metabolism	+	
5	1419518_at	2.7	Tubulin, α8	Endopeptidase activity	+	
6	1417801_a_at	2.7	Protein tyrosine phosphatase, receptor-type, f interacting protein, binding protein 2	Lipid transport	+	
7	1428484_at	2.5	Oxysterol binding protein-like 3		+	
8	1450696_at	2.2	Proteosome (prosome, macropain) subunit, beta type 9 (large multifunctional peptidase 2)		+	
9	1417101_at	2.1	Heat shock protein 2		+	
10	1424518_at	2.0	RIKEN cDNA 2310016f22 gene		+	
11	1424518_at	2.0	cDNA sequence bc020489		+	
Genes that could not be classified						
1	1427912_at	5.6	Carbonyl reductase 3		+	
2	1452913_at	3.7	Purkinje cell protein 4-like 1			
3	1451486_at	3.7	RIKEN cDNA 1200006f02 gene		+	
4	1456319_at	3.5	Est x83313			
5	1424167_a_at, 1430780_a_at	3.1	Phosphomannomutase 1			
6	1428670_at	2.7	RIKEN cDNA 2610305j24 gene			
7	1443138_at	2.6	Sulfotransferase family 5a, member 1		+	
8	1423410_at	2.5	Meiosis-expressed gene 1		+	
9	1424296_at, 1455959_s_at	2.5	Glutamate-cysteine ligase, catalytic subunit			
10	1428120_at	2.4	F-box and wd-40 domain protein 9		+	
11	1426936_at	2.4	Hypothetical loc433593			
12	1433685_a_at, 1455654_at	2.3	RIKEN cDNA 6430706d22 gene			
13	1417185_at	2.3	Lymphocyte antigen 6 complex, locus A		+	
14	1438855_x_at	2.3	Tumor necrosis factor, alpha-induced protein 2		+	

Table 2. *Continued*

	Affy ID	Fold Change	Gene Name	Biological Process/Molecular Function Terms ($P < 0.05$)	Presence of DRE I	qPCR Confirmation
15	1450699_at	2.3	Selenium binding protein 1		+	
16	1424594_at	2.3	Lectin, galactose binding, soluble 7		+	
17	1431240_at	2.3	C-type lectin domain family 2, member h		+	
18	1433710_at	2.2	Hypothetical protein mgc36552		+	
19	1431806_at	2.2	RIKEN cDNA 4931408d14 gene			
20	1436576_at	2.2	RIKEN cDNA a630077b13 gene		+	
21	1418727_at	2.2	Nucleoporin 155		+	
22	1454890_at	2.2	Angiomotin		+	+(2.59)
23	1418346_at	2.2	Insulin-like 6		+	+(12.3)
24	1419149_at	2.0	Serine (or cysteine) peptidase inhibitor, clade e, member 1		+	
25	1426215_at	2.0	DOPA decarboxylase			
26	1431648_at	2.0	RIKEN cDNA 4930528f23 gene			
27	1438294_at	2.0	Ataxin 1		+	
Genes repressed by BNF treatment						
1	1444296_a_at, 1444297_at, 1448092_x_at	3.5	Serine (or cysteine) peptidase inhibitor, clade a, member 4, pseudogene 1			+(3.23)
2	1433898_at	2.3	Solute carrier family 25, member 30		+	
3	1448724_at	2.3	Cytokine inducible sh2-containing protein		+	+(2.89)
4	1437073_x_at	2.2	Expressed sequence av025504			
5	1418288_at	2.1	Lipin 1		+	
6	1452426_x_at	2.1	Hypothetical protein			
7	1436186_at	2.0	e2f transcription factor 8			

Abbreviations: ATP, adenosine triphosphate; BNF, β-naphthoflavone; cDNA, complementary DNA; DRE, dioxin response element; MHC, major histocompatibility complex; qPCR, quantitative polymerase chain reaction.

Presence of AhR is Essential for BNF-Mediated Increase in CAR mRNA. BNF is an established AhR ligand and to the best of our knowledge, there are no reports confirming the ability of BNF to directly activate any transcription factor other than AhR. However, the possibility of a BNF-mediated effect that is independent of AhR has to be considered. BNF exposure is known to cause oxidative stress, which can possibly activate the nuclear factor erythroid 2 related factor 2—a transcription factor known to regulate genes involved with protection against cellular stress, such as *Nqo1*.[19] Additionally, *Nqo1* is up-regulated directly by AhR. Because the AhR-KO mice are devoid of AhR transcriptional activity, any increase in *Nqo1* mRNA in AhR-KO mice can be attributed to oxidant stress.

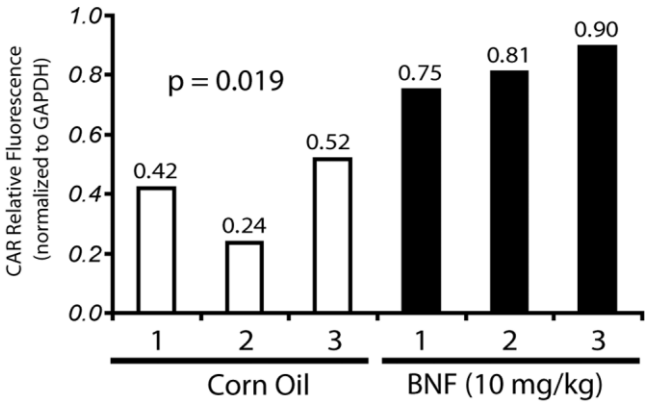

Fig. 1. CAR mRNA levels increase in response to the AhR-ligand BNF. Six adult C57BL/6J mice were injected, either with 10 mg/kg BNF or vehicle control for 5 hours. mRNA isolated from liver samples was reverse-transcribed and quantified via qPCR using CAR-specific primers. The data represent the relative fluorescence units for each sample obtained after normalization to GAPDH. $P < 0.02$ (t test).

To exclude the contribution of such an effect in the observed up-regulation of CAR mRNA, age-matched AhR-KO[20] and wild-type mice were injected with 50 mg/kg BNF or vehicle alone for 5 hours. Compared with the previous experiment, mice were treated with a higher dose of BNF to definitively exclude such an effect. Liver CAR mRNA levels did not demonstrate a significant difference between BNF and vehicle-treated AhR-KO mice, as determined via qPCR (Fig 2B). Also, *Nqo1* mRNA levels were the same between control and BNF-treated AhR-KO mice (data not shown). This suggests that with the experimental parameters used in this study, the observed increase in CAR mRNA is not a result of BNF-generated oxidant stress. Wild-type mice demonstrated an increase in CAR mRNA on BNF treatment similar to that in the microarray experiments (Fig. 2B). *Cyp1A1* mRNA levels were determined as a control (Fig. 2A) for AhR activity. This finding, along with the absence of a consensus DRE in the CAR promoter, makes it necessary

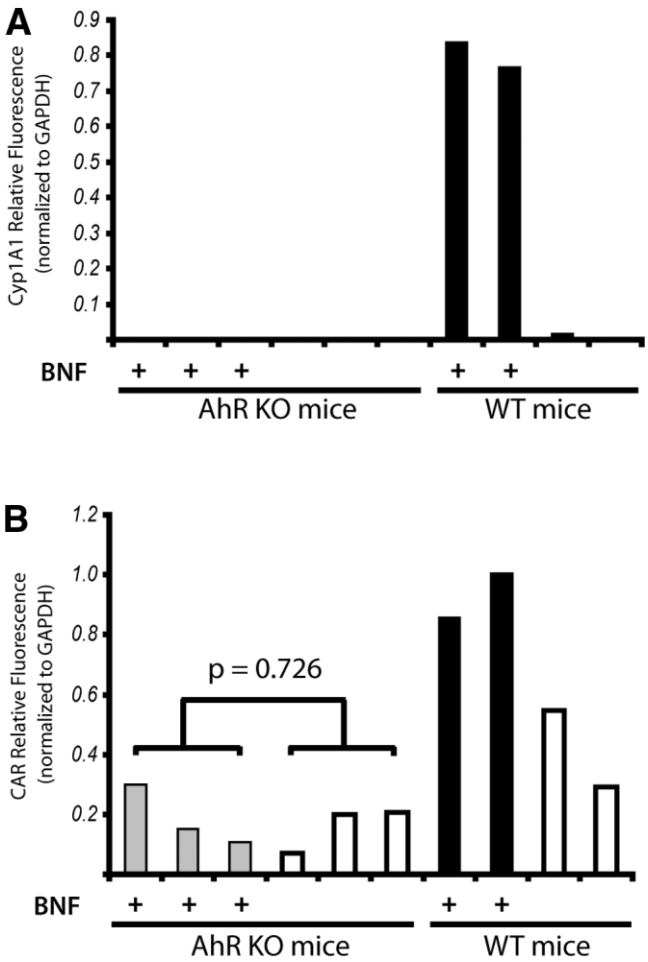

Fig. 2. CAR upregulation is AhR-dependent. AhR knockout (AhR-KO) mice, or wild-type mice, were injected with 50 mg/kg BNF or vehicle alone. Liver mRNA, collected after 5 hours, was reverse-transcribed and quantified via qPCR using (A) *Cyp1A1*- and (B) CAR-specific primers. Normalized relative fluorescence values obtained with *Cyp1A1* (or CAR) primers are presented for individual mice. Statistical analysis was performed using a *t* test.

to examine the mode by which AhR influences CAR mRNA levels. Although it is possible to distinguish a secondary effect from direct transcription with the use of a protein synthesis inhibitor like cycloheximide, the inability to significantly induce transcription of CAR in established cell lines has precluded such experiments.

Temporal and Spatial Patterns of CAR Up-regulation Mimics Cyp1A1. Because CAR plays a significant role in regulating many drug metabolism enzymes, most of which are distinct from the known AhR target genes, an increase in CAR levels in response to AhR activation can significantly expand the role of AhR in controlling xenobiotic metabolism. Time course experiments were performed to determine whether AhR-mediated increase in CAR mRNA was sustained over a longer period. Adult mice were injected with 50 mg/kg BNF or vehicle alone and sacrificed at 6, 12, and 24 hours. Maximal induction (3-fold) of CAR was observed 6 hours after BNF exposure and was sustained until 24 hours (Fig 3A). Induction of *Cyp1A1*, a known AhR target gene, demonstrated a similar temporal pattern, although the level of induction was greater than that of CAR (data not shown). The decline in CAR and *Cyp1A1* mRNA at the later time points is most likely due to a decrease in BNF levels, and subsequent loss of AhR activity, as a result of metabolism.

Increase in CAR levels in extrahepatic tissues could significantly complement the hepatic clearance of xenobiotics. The ability of AhR to up-regulate CAR mRNA in kidney and small intestine, both of which play a significant role in drug metabolism, was determined via qPCR. A statistically significant increase in CAR mRNA levels was observed in the kidney after 6 hours of 50 mg/kg BNF treatment (Fig. 3B). CAR up-regulation (2.2-fold) was also noted in the small intestine (terminal ileum); however, increased intersample variability prevented statistical verification of the results (data not shown).

Increase in CAR mRNA Results in Increased CAR Transcriptional Activity. We wanted to confirm that an increase in CAR mRNA would lead to a corresponding increase in the functional capacity of CAR, as determined by changes in CAR-mediated transcription of its target genes. *Cyp2b10* mRNA levels were chosen as a marker of CAR's transcriptional activity. Previously, it has been reported that activation of CAR by TCPOBOP, a mouse CAR–specific ligand, leads to an increase in hepatic *Cyp2b10* levels in mice.[21] Adult mice were treated with either BNF or vehicle control to up-regulate CAR levels. Six hours later, the mice were treated with TCPOBOP to activate CAR protein or with vehicle control. Three hours after TCPOBOP treatment, liver *Cyp2b10* mRNA levels were measured by qPCR. Mice treated with BNF/TCPOBOP demonstrated a 2.4-fold increase in hepatic

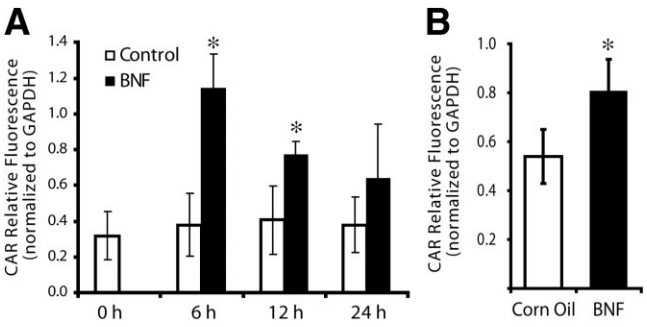

Fig. 3. Temporal and spatial patterns of CAR expression. (A) CAR mRNA levels were quantified via qPCR on liver samples from C57BL/6J mice treated with 50 mg/kg BNF or vehicle for the indicated times. Data are presented as the mean (n = 4) and SD. $*P < 0.05$ (*t* test). (B) qPCR quantification of CAR mRNA levels in kidneys obtained from mice treated for 6 hours in the above time course experiment. $*P < 0.05$ (*t* test).

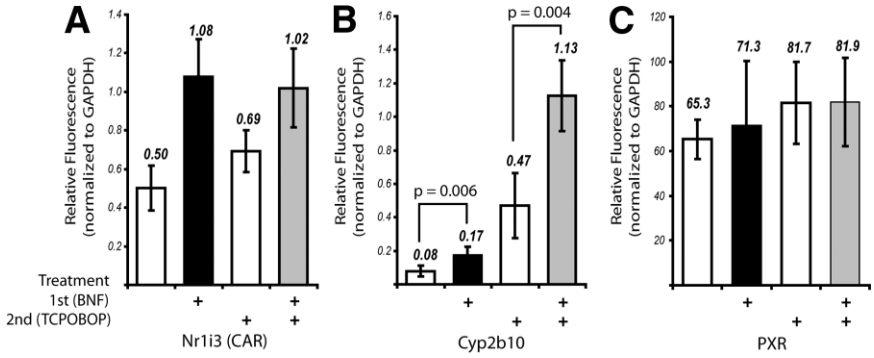

Fig. 4. AhR-dependent CAR up-regulation leads to increased CAR-mediated transcriptional activity. Adult C57BL/6J mice were treated with an AhR ligand (50 mg/kg BNF for 6 hours), to up-regulate CAR levels, or vehicle control. Mice were subsequently treated with a CAR ligand (3 mg/kg TCPOBOP for 3 hours), or vehicle control, to activate CAR protein. (A) CAR, (B) *Cyp2b10*, and (C) PXR mRNA levels were quantified in liver samples by qPCR. A plus symbol (+) indicates BNF/TCPOBOP treatment; absence of a plus symbol indicates vehicle treatment. Data are presented as the mean (n = 4) and SD for each group. Statistical analysis was performed with a *t* test.

Cyp2b10 mRNA compared with vehicle/TCPOBOP (Fig 4B). These results demonstrate that the observed increase in CAR mRNA translates to a similar increase in CAR activity. Interestingly, even in the absence of subsequent TCPOBOP treatment, an increase (2-fold) in *Cyp2b10* mRNA was observed in BNF-treated mice compared with vehicle (Fig 4B). However, the absolute levels of *Cyp2b10* mRNA were significantly lower without TCPOBOP exposure. Based on these results, it is reasonable to assume that the AhR-dependent increase in CAR mRNA leads to a functionally significant change in CAR activity. Although a Western blot would serve to directly confirm an increase in CAR protein, the lack of a quality commercially available antibody to murine-CAR has prevented the demonstration of such an increase in protein level.

Pregnane X receptor (PXR), another transcription factor involved in regulating xenobiotic metabolism enzymes, is also capable of inducing *Cyp2b10* mRNA.[5] Alterations in PXR levels were monitored via qPCR to determine if it contributed to the observed increase in *Cyp2b10*. PXR mRNA levels were found to be similar across all treatments (Fig 4C), confirming that the increase in *Cyp2b10* mRNA was most likely due to the AhR-mediated increase in CAR levels.

CAR Induction in Primary Human Hepatocytes With Different AhR Ligands. CAR mRNA levels were determined after treating primary human hepatocytes with TCDD and benzo[a]pyrene (BaP). Treatment with 10 nM TCDD for 24 hours and 1 μM BaP for 12 hours resulted in a statistically significant increase in CAR mRNA compared with control (Fig 5). Increase in CAR mRNA with BaP treatment for 24 hours was less than that observed at 12 hours, possibly due to faster metabolism of BaP compared to TCDD. Although there is only a modest increase in CAR mRNA levels, it should be noted that this increase is in the presence of 25 nM dexamethasone in the media. As discussed previously, dexamethasone is known to induce CAR level.[12] Increase in CAR mRNA was further confirmed in primary human hepatocytes obtained from a second individual (Fig. 5).

Discussion

Transcription factors play a significant role in coordinating the responses of a cell to various external stimuli. Traditionally, these factors are thought to function by binding to defined consensus DNA sequences and driving the transcription of a certain array of target genes. How-

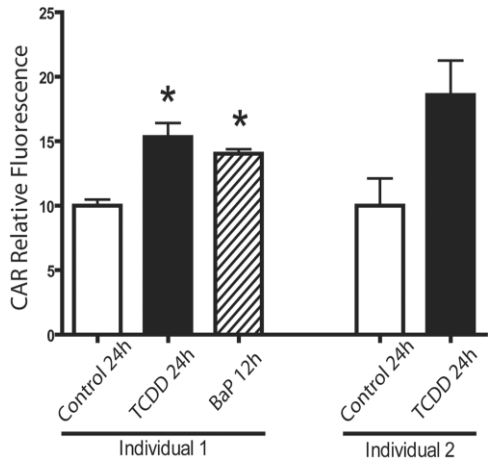

Fig. 5. CAR induction in response to AhR ligands in primary human hepatocyte culture. Primary human hepatocytes were cultured in 6-well plates and treated with 10 nM TCDD or 1 μM BaP for the indicated time periods. RNA was isolated and analyzed via qPCR. Bars represent the mean and SD of triplicate treatments for the first individual. The same experiment was repeated in duplicate for the second individual. $*P < 0.05$ (*t* test).

ever, several receptors have also been found to function in an alternate manner by influencing the functional capacity of other receptors. This significantly expands the range of effects attributed to the individual receptors. At the same time it also makes it challenging to interpret the results of genome-wide studies. As observed in the results of microarray experiments performed as a part of this study, AhR activation leads to alteration in the expression of a variety of genes. The number of genes whose expression was repressed by AhR activation is small compared with the number of up-regulated genes (7 downregulated genes versus 73 upregulated genes). A probable explanation for this discrepancy is the short time period of AhR activation used here. Although 5 hours is adequate to up-regulate the expression of direct as well as, in some cases, indirect target genes, it might not allow adequate time to notice a decline in the levels of down-regulated genes.

AhR has been demonstrated to extensively participate in crosstalk with several other receptors. It can directly interact with nuclear factor κB,[4] the retinoblastoma protein,[22] Sp1 transcription factor,[23] and the estrogen receptor. Interaction of AhR with each of these proteins has been shown to modulate signal transduction by the proteins. In the case of AhR–estrogen receptor, crosstalk has been postulated to arise from multiple mechanisms, including binding to inhibitory DREs; estrogen receptor ligand depletion due to increased metabolism by AhR-induced enzymes; AhR-mediated, proteasome-dependent estrogen receptor degradation; and inaccessibility to their respective response element because of protein–protein interaction.[5-7] In the present study, the ability of AhR to influence CAR-mediated signal transduction exemplifies regulation of receptor quantities as an additional mechanism of AhR crosstalk.

Like AhR, CAR has been implicated in regulating the expression of several xenobiotic metabolism enzymes. CAR and PXR play a significant role in controlling the levels of the *Cyp2B* and *Cyp3A* family of enzymes. These enzymes are believed to influence the metabolism of several currently available drugs.[24] An alteration in the functional capacity of CAR (or PXR) would affect the levels of these enzymes and potentially generate unanticipated changes in the pharmacokinetic properties of their target drugs. The observations recorded in this study demonstrate that *in vivo* activation of AhR alters *Cyp2b10* levels by mediating an increase in CAR, without affecting the expression of PXR. This can have significant practical implications, because AhR can be activated in response to a wide variety of compounds that are ubiquitous and are often encountered by humans. Common examples of AhR ligands include BaP (a constituent of cigarette smoke), TCDD (generated by combustion of organic matter), flavones (present in common food items), indole 3-carbinol (found in cruciferous vegetables), tryptophan metabolites, and drugs such as omeprazole (reviewed by Denison and Nagy[25]). As shown in Fig. 5, AhR-mediated CAR induction occurs in human cells as well and therefore is not limited to the murine model. Furthermore, a variety of AhR ligands—as exemplified by the use of TCDD and BaP—can lead to increased CAR levels. Thus, varying degrees of exposure to AhR ligands can result in significant interindividual differences in CAR activity and consequent disparity in xenobiotic metabolism potential.

The physiological role of CAR extends beyond that of regulating xenobiotic metabolism. Because of its ability to control the expression of genes involved in hepatic uptake and clearance of bilirubin, CAR has been implicated in stress response during periods of hyperbilirubinemia.[26] Also, bilirubin and biliverdin can function as AhR ligands.[27,28] It is possible that AhR-dependent increase in CAR levels is one of the mechanisms by which bilirubin indirectly increases CAR activity to protect the body from the adverse effects of elevated bilirubin levels. Recent reports have also associated CAR activity with thyroid hormone homeostasis during states of restricted calorie intake[16] as well as with hepatocyte proliferation[29] and tumor formation.[30] An AhR-mediated increase in CAR activity may potentially alter the outcome of these states as well.

The activity of many transcription factors is regulated by their tissue-specific expression. Constitutive expression of CAR is principally observed in the liver and small intestine, and most studies related to CAR have explored its functions primarily in a hepatic context. In contrast, AhR is expressed in a wide variety of tissues and can potentially induce CAR levels in these tissues, as demonstrated by the increase in renal CAR expression reported here. Even in hepatic tissue, where maximal constitutive CAR levels are observed, it is possible to increase CAR activity by increasing its levels. Figure 3 clearly demonstrates that an approximately 2-fold increase in CAR resulted in a proportionate increase in its transcriptional activity (as demonstrated by a 2-fold increase in *Cyp2b10* mRNA). Collectively, these results suggest that in extrahepatic tissues, where the constitutive level of CAR is extremely low or absent, AhR-dependent induction of CAR expression can significantly enhance its functions and might reveal additional roles for this receptor. Additional experiments, such as profiling genome-wide expression changes in extrahepatic tissues as well as effects of long-term exposure to AhR ligands in wild-type and CAR KO mice, can provide further information.

In conclusion, these results demonstrate the ability of activated AhR to increase CAR activity, which can alter drug metabolism. Inhalation of cigarette smoke has been known to markedly increase *Cyp1A1* levels in murine livers, thus indicating AhR activation.[31] Similar circumstances can lead to unexpected changes in CAR-dependent drug metabolism. Thus, it may be important to be aware of the effects of AhR activation while determining the dosage of certain drugs in different patient populations. Additionally, the direct transcription models of receptor function cannot explain all the observations from large-scale gene expression studies. There is clearly a need to explore different mechanisms by which transcription factors can influence gene expression. Our results demonstrate a link between AhR and CAR, and although further studies are required to determine the exact molecular mechanism, the information presented here will be helpful in interpreting experimental results related to AhR and CAR, particularly in the liver.

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