Modulation of aryl hydrocarbon receptor (AHR)-dependent signaling by peroxisome proliferator-activated receptor β/δ (PPARβ/δ)

M. G. Borland, et al

Supplementary materials and methods

Chemicals and Materials

GW0742 was provided by Drs. Andrew Billin and Timothy Willson. B[*a*]P, DMBA, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and 5-aza-2'-deoxycytidine (5-Aza-dC), and the MISSION™ shRNA plasmid construct for Non-target (SHC002) and human PPARβ/δ (TRCN0000010647) were purchased from Sigma-Aldrich (St. Louis, MO). 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) was purchased from AccuStandard (New Haven, CT), and β-naphthoflavone (β-NF) was purchased from IndoFine (Hillsborough, NJ). Indolo[3,2-*b*]carbazole (ICZ) and 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) were provided by Dr. Jan Bergman and Dr. Steven Safe, respectively. 2-Azido-3-^{125}I-iodo-7,8-dibromodibenzo-*p*-dioxin (^{125}I-N_3Br_2DpD) and 2-^{125}I-iodo-7,8-dibromo-*p*-dioxin (^{125}I-Br_2DpD) were synthesized as previously described [1,2]. Lipofectamine® and Plus Reagent® were purchased from Invitrogen (Carlsbad, CA). Protein A/G sepharose resin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Wizard® Genomic DNA Purification kit was purchased from Promega (Madison, WI), and puromycin was purchased from EMD Biosciences (Gibbstown, NJ). All primers for quantitative real-time PCR (qPCR) and bisulfite sequencing were purchased from Integrated DNA Technologies (Coralville, IA) and are listed in Supplemental Table 1.

shRNA lentivirus stable HaCaT cells

Briefly, the virus packaging plasmids and non-target or shPPARβ/δ plasmids were co-transfected into HEK293T cells to produce retrovirus using the Lipofectamine® transfection reagent and manufacturer's recommended protocol. Forty-eight hours after transfection, the cellular supernatant containing the retrovirus was filtered and used to spinoculate HaCaT cells [3]. Two days post-infection, HaCaT cells were selected with DMEM supplemented with 1 µg/mL puromycin and cultured below 50% confluency for 4 days to select for stable plasmid integration. Cells were maintained in selection media throughout all experiments. Western blotting to examine expression of PPARβ/δ, AHR, ARNT, HSP90, XAP2, and ACTIN was performed as described above. To characterize the responsiveness of non-target and shPPARβ/δ HaCaT cells to AHR and PPARβ/δ ligands, HaCaT cells were treated with vehicle, 0.2 µM GW0742, or 1 µM B[*a*]P for 1-24 h. Total RNA was then prepared and qPCR for *PPARβ/δ*, *ANGPTL4*, *CYP1A1*, and *CYP1B1* mRNA expression was examined as described above.

In vivo liver analysis and isolation and treatment of primary mouse hepatocytes

Wild-type or *Pparβ/δ*-null mice were gavaged with corn oil (control) or β-NF (50 mg/kg) and euthanized 8 h post-treatment. Livers were removed and snap frozen until further analysis. Primary hepatocytes from wild-type and *Pparβ/δ*-null mice were isolated as previously described [4], and cultured in HepatoZYME SFM media (Invitrogen, Carlsbad, CA) supplemented with 2.5% dimethylsulfoxide (DMSO), 10 nM dexamethasone, 1% glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin.

Epigenetic studies

To examine whether methylation altered transcriptional regulation mediated by the AHR, the methylation inhibitor 5-Aza-dC was supplemented into the culture media of primary keratinocytes from wild-type, *Pparβ/δ*-null mice and/or HaCaT stable cell lines. Cells were cultured with the inhibitor for 72 h, and then treated with either vehicle or 1 μM B[*a*]P and fresh 5-Aza-dC for 8 h.

For bisulfite sequencing, DNA was isolated from primary keratinocytes from wild-type or *Pparβ/δ*-null mice and bisulfite conversion was completed using the EZ DNA Methylation-Direct kit (Zymo Research, Orange, CA) and the manufacturer's recommended protocol. A total of 2 x 10^5 cells per genotype were utilized for bisulfite conversion. The promoter of the *Cyp1a1* gene was analyzed by PCR amplification using primers designed for cytosine-phosphate-guanine (CpG) islands in the *Cyp1a1* promoter using MethylPrimer Expression (Applied Biosystems, Foster City, CA). Primers for amplification were 5'-GTTTTGGTTATAGAGTAGATATTAATG-3' (forward) and 5'-TTAAACTAACTCTATACCCCACAAATT-3' (reverse). The bisulfite converted promoter of *Cyp1a1* was amplified using the following protocol: 95°C for 1 min, 60°C for 1 min, and 72°C for 2.5 min, repeated for 35 cycles. The appropriately amplified amplicon was cloned in a sequencing vector using the TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA) and the manufacturer's recommended protocol. Plasmids containing the amplicon were sequenced at the Penn State University Nucleic Acid Facility, and five sequencing plasmid amplicons were analyzed from wild-type and *Pparβ/δ*-null keratinocyte bisulfite-converted DNA using the CyMATE program [5] to identify the patterns of DNA methylation in the two genotypes.

Supplementary Information

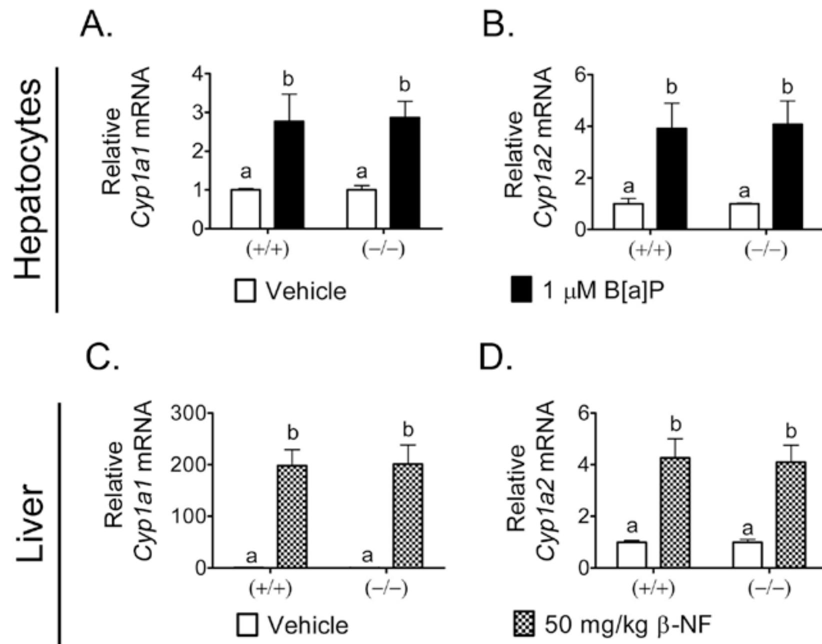

Fig. S1. Activation of the AHR is not influenced by PPARβ/δ in hepatocytes or liver. Primary hepatocytes from wild-type (+/+) or *Pparβ/δ*-null (–/–) mice were treated with or without B[*a*]P and used to quantify mRNA encoding the AHR target genes (**A**) *Cyp1a1* or (**B**) *Cyp1a2* by qPCR. mRNA was isolated from liver of (+/+) or (–/–) mice treated with or without β-naphthaflavone (β-NF) and used to quantify mRNA encoding the AHR target genes (**C**) *Cyp1a1* or (**D**) *Cyp1a2* by qPCR. Values with different letters are significantly different than control, $P \leq 0.05$.

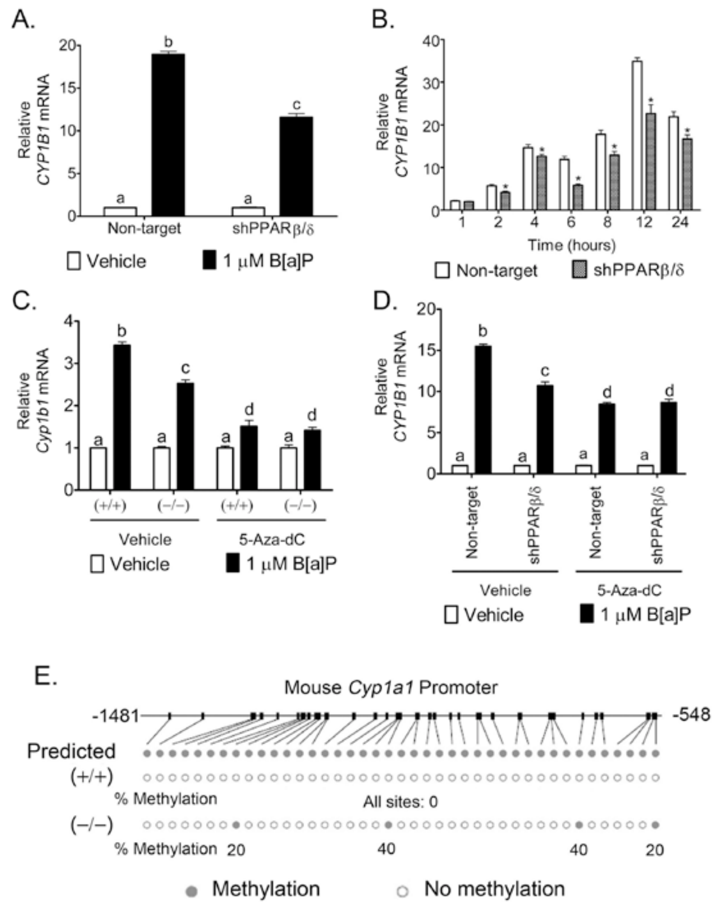

Fig. S2. Modulation of AHR-dependent signaling by PPARβ/δ is conserved in mouse and human keratinocytes and could be due in part to altered methylation of the *Cyp1a1* promoter. qPCR was performed using total RNA isolated from the HaCaT shRNA stable cell lines to quantify the mRNA expression of (**A**) *CYP1B1* in response to B[*a]*P. qPCR was performed using total RNA isolated from the HaCaT shRNA stable cell lines to quantify temporal mRNA expression of (**B**) *CYP1B1* in response to B[*a*]P. Wild-type (+/+) and *Pparβ/δ*-null (–/–) primary keratinocytes as well as HaCaT shRNA cell lines were treated with 5 μM 5-Aza-dC for 72 h prior to an 8 h 1 μM B[*a*]P treatment. qPCR was performed using total RNA to quantify the expression of *Cyp1b1*/*CYP1B1* mRNA in (**C**) primary keratinocytes or (**D**) HaCaT shRNA cell lines. Values were normalized to

the respective genotype or stable cell line vehicle control and represents the mean ± S.E.M. of N = 4 biological replicates. Values with different letters are significantly different than control (*P* ≤ 0.05). *significantly different than control (*P* ≤ 0.05). (**E**) Bisulfite converted DNA from (+/+) and (–/–) primary keratinocytes was used to amplify a putative methylation region within the *Cyp1a1* promoter. Five plasmids containing the promoter region were sequenced for each genotype to map and examine the methylation patterns. Percent (%) methylation indicates the incidence of methylation at a particular cytosine residue.

References

1. Bradfield, C.A., Kende, A.S. and Poland, A. (1988) Kinetic and equilibrium studies of Ah receptor-ligand binding: use of [125I]2-iodo-7,8-dibromodibenzo-p-dioxin. *Mol Pharmacol*, **34**, 229-37.
2. Poland, A., Glover, E., Ebetino, H. and Kende, A. (1986) Photoaffinity labelling of the Ah receptor. *Food and Chemical Toxicology*, **24**, 781-787.
3. Pear, W.S., Miller, J.P., Xu, L., Pui, J.C., Soffer, B., Quackenbush, R.C., Pendergast, A.M., Bronson, R., Aster, J.C., Scott, M.L. and Baltimore, D. (1998) Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood*, **92**, 3780-92.
4. Stoehr, S.A. and Isom, H.C. (2003) Gap junction-mediated intercellular communication in a long-term primary mouse hepatocyte culture system. *Hepatology*, **38**, 1125-35.
5. Hetzl, J., Foerster, A.M., Raidl, G. and Mittelsten Scheid, O. (2007) CyMATE: a new tool for methylation analysis of plant genomic DNA after bisulphite sequencing. *Plant J*, **51**, 526-36.

Supplementary Table 1. Primers used for qPCR

Species	Gene	Genbank Accession	Forward Primer (5'-3')	Reverse Primer (5'-3')
Mouse	*Atf3*	NM_007498	TCAAGGAAGAGCTGAGATTCGCCA	GTTTCGACACTTGCGAGCAGCAAT
	Cox2	NM_011198	TTGCTGTACAAGCAGTGGCAAAGG	TGCAGCCATTTCCTTCTCTCCTGT
	Cyp1a1	NM_009992	AGGTTAACCATGACCGGGAACTGT	TTCGCTTGCCCAAACCAAAGAGAG
	Cyp1a2	NM_009993	ACATTCCCAAGGAGCGCTGTATCT	GTCGATGGCCGAGTTGTTATTGGT
	Cyp1b1	NM_009994	GCTAGCCAGCAGTGTGATGATATT	GGTTAGCCTTGAAATTGCACTGAT
	Gapdh	NM_008084	AAATGGTGAAGGTCGGTGTGAACG	TGGCAACAATCTCCACTTTGCCAC
	Gsta1	NM_008181	ACTAGACCGTGAACCACAGTTGCT	ACCATGGGCACTTGGTCAAACATC
	Hox1	NM_010442	CCTCACTGGCAGGAAATCATC	CCTCGTGGAGACGCTTTACATA
	Nqo1	NM_008706	AGATGGCATCCAGTCCTCCAT	TTAGTCCCTCGGCCATTGTT
	Nrf2	NM_010902	AGCACTCGCTGGAGTCTTCCATTT	TGTGCTTTAGGGCCGTTCTGTTTG
	p53	NM_001127233	AAAGGATGCCCATGCTACAGAGGA	GCAGTTTGGGCTTTCCTCCTTGAT
	Ugt1a2	NM_013701	AAGGCTTTCTGACCAACTGGA	GGCAAATGTACTTCAGGACCAGAT
Human	*ANGPTL4*	NM_139314	TCACAGCCTGCAGACACAACTCAA	CCAAACTGGCTTTGCAGATGCTGA
	CYP1A1	NM_000499	AGTGGCAGATCAACCATGACCAGA	CCGCTTGCCCATGCCAAAGATAAT
	CYP1B1	NM_000104	CATGCGCTTCTCCAGCTTTGT	GGCCACTTCACTGGGTCATGA
	GAPDH	NM_002046	TGCACCACCACCTGCTTAGC	GGCATGGACTGTGGTCATGAG

Supplementary Table 2. Comparison of Complete Carcinogen Bioassay and Two-Stage Skin Cancer Bioassay

Complete Carcinogen Bioassay				**Two-Stage Carcinogen Bioassay**			
Chemical carcinogen[a]	AHR-dependent bio-activation of carcinogen?	Chemical carcinogen application	Tumor promoter application[b]	Chemical carcinogen[a]	AHR-dependent bio-activation of carcinogen?	Chemical carcinogen application	Tumor promoter application[d]
B[a]P	Yes	Weekly (34 wks)	None	B[a]P	Yes	Once[c]	Weekly
DMBA	Yes	Weekly (27 wks)	None	DMBA	Yes	Once[c]	Weekly
MNNG	No	Weekly (25 wks)	None	–	–	–	–

[a]B[a]P and DMBA cause DNA damage after bio-activation to reactive intermediates that is mediated primarily by the AHR in the Complete Carcinogen Bioassay and the Two-Stage Carcinogen Bioassay. B[a]P and DMBA are two of the most common chemical carcinogens used for both the Complete Carcinogen Bioassay and the Two-Stage Skin Cancer Bioassay. By contrast, MNNG does not require bio-activation by the AHR and served as a control for these studies. [b]The only chemical applied weekly is the chemical carcinogen, no tumor promoter is applied in the Complete Carcinogen Bioassay. The tumor promoting activity/activities in the Complete Carcinogen Bioassay is not completely understood, but could be due to both the chemical carcinogen and/or signaling in the keratinocyte. [c]The chemical carcinogen is applied once at the start of the experiment. One week after topical application of the chemical carcinogen, the tumor promoter is applied 1-3X per week. [d]There are a number of different tumor promoters used for the Two-Stage Carcinogen Bioassay with phorbol ester being the most common. The tumor promoter is applied weekly in this bioassay as compared to the complete carcinogenesis bioassay.