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

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Whether peroxisome proliferator-activated receptor β/δ (PPARβ/δ) reduces skin tumorigenesis by altering aryl hydrocarbon receptor (AHR)-dependent activities was examined. Polycyclic aromatic hydrocarbons (PAH) increased expression of cytochrome P4501A1 (CYP1A1), CYP1B1 and phase II xenobiotic metabolizing enzymes in wild-type skin and keratinocytes. Surprisingly, this effect was not found in *Pparβ/δ*-null skin and keratinocytes. *Pparβ/δ*-null keratinocytes exhibited decreased AHR occupancy and histone acetylation on the *Cyp1a1* promoter in response to a PAH compared with wild-type keratinocytes. Bisulfite sequencing of the *Cyp1a1* promoter and studies using a DNA methylation inhibitor suggest that PPARβ/δ promotes demethylation of the *Cyp1a1* promoter. Experiments with human HaCaT keratinocytes stably expressing shRNA against PPARβ/δ also support this conclusion. Consistent with the lower AHR-dependent activities in *Pparβ/δ*-null mice compared with wild-type mice, 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced skin tumorigenesis was inhibited in *Pparβ/δ*-null mice compared with wild-type. Results from these studies demonstrate that PPARβ/δ is required to mediate complete carcinogenesis by DMBA. The mechanisms underlying this PPARβ/δ-dependent reduction of AHR signaling by PAH are not due to alterations in the expression of AHR auxiliary proteins, ligand binding or AHR nuclear translocation between genotypes, but are likely influenced by PPARβ/δ-dependent demethylation of AHR target gene promoters including *Cyp1a1* that reduces AHR accessibility as shown by reduced promoter occupancy. This PPARβ/δ/AHR crosstalk is unique to keratinocytes and conserved between mice and humans.

Abbreviations: ^{125}I-Br2DpD, 2-^{125}I-iodo-7,8-dibromo-p-dioxin; ^{125}I-N3Br2 DpD, 2-azido-3-^{125}I-iodo-7,8-dibromodibenzo-*p*-dioxin; 5-Aza-dC, 5-Aza-2′-deoxycytidine; AHR, aryl hydrocarbon receptor; ANGPTL4, angiopoietin-like protein 4; ARNT, aryl hydrocarbon receptor nuclear translocator; ATF3, activating transcription factor 3; B[*a*]P, benzo[*a*]pyrene; ChIP, chromatin immunoprecipitation; COX2, cyclooxygenase-2; CpG, cytosine-phosphate-guanine; CYP, cytochrome P450; CYP1A1, cytochrome P450 1A1; DiB[*a*,*l*]P, dibenzo[*a*,*l*] pyrene; DMBA, 7,12-dimethylbenz[*a*]anthracene; FBS, fetal bovine serum; GSTA1, glutathione S-transferase alpha 1; GW0742, (4-(((2-(3-fluoro-4-(trifluoromethyl)phenyl)-4-methyl-5-thiazolyl)methyl)thio)-2-methylphenoxy acetic acid; HOX1, heme oxygenase 1; LDH, lactate dehydrogenase; mEH, microsomal epoxide hydrolase; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; NQO1, NAD(P)H quinone oxidoreductase 1; NRF2, NF-E2-related factor 2; PAH, polycyclic aromatic hydrocarbon; PeCDF, 2,3,4,7,8-pentachlorodibenzofuran; qPCR, quantitative real-time polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; UGT1A2, UDP-glucuronosyltransferase 1a2; XAP2, hepatitis B virus X-associated protein 2.

Introduction

Pparβ/δ-null mice exhibit an enhanced hyperplastic response in the epidermis following treatment with the tumor promoter 2-*O*-tetradecanoylphorbol-13-acetate compared with wild-type mice (1–3). This effect has been found in three different *Pparβ/δ*-null mouse models by three independent laboratories (1–3). *Pparβ/δ*-null primary keratinocytes, the progenitor cell type of many skin tumors, are also more proliferative than wild-type keratinocytes (4–6). These studies indicate that one function of PPARβ/δ in skin is to inhibit epidermal hyperplasia. This is consistent with the fact that ligand activation of the PPARβ/δ inhibits cell proliferation in both mouse and human skin and keratinocyte models (reviewed in refs. 7 and 8). A number of mechanisms have been elucidated that may mediate the inhibitory effect of PPARβ/δ on keratinocyte proliferation, including ubiquitin-dependent degradation of protein kinase C α (6,9), reduced MAP kinase signaling (10), induction of terminal differentiation markers (11–13), inhibition of cell cycle progression (10,14,15), increased apoptosis (4) and crosstalk with E2F signaling (15).

Given the fact that PPARβ/δ regulates epidermal cell proliferation and differentiation, it is not surprising that *Pparβ/δ*-null mice exhibit enhanced sensitivity and greater tumor multiplicity in a two-stage chemically induced skin cancer model compared with wild-type mice (6,11,16–18). Further, ligand activation of PPARβ/δ inhibits the onset of tumor formation, tumor incidence and tumor multiplicity in wild-type mice in two-stage skin chemical carcinogenesis bioassays. These effects require PPARβ/δ because they are not found in similarly treated *Pparβ/δ*-null mice (11,16,18). Although there is strong evidence that PPARβ/δ inhibits chemically induced skin cancer by inhibiting cell proliferation and inducing terminal differentiation, which would significantly modify the promotion phase of tumorigenesis, it remains unclear whether PPARβ/δ could influence the initiation of DNA damage by chemical carcinogens.

Chemical carcinogens are modified by phase I and II xenobiotic metabolizing enzymes that catalyze detoxification and excretion. Phase I enzymes, including the cytochrome P450s (CYPs), can generate DNA-reactive diol-epoxide intermediates from chemical carcinogens such as the polycyclic aromatic hydrocarbons (PAH) benzo[*a*]pyrene (B[*a*]P) and 7,12-dimethylbenz[*a*]anthracene (DMBA). These bioactivated intermediates can either form DNA adducts that may or may not be repaired, or be further conjugated by phase II enzymes to stable, detoxified derivatives (19). Although the expression of phase I and II enzymes is regulated by a number of transcription factors, expression of several key phase I and II enzymes involved in PAH metabolism are primarily regulated by the aryl hydrocarbon receptor (AHR) (reviewed in ref. 20). Thus, the AHR is considered a key modulator of chemical carcinogenesis that influences the balance between bioactivation and detoxification. The AHR exists in the cytoplasm bound to heat shock protein 90 (HSP90), p23 and hepatitis B virus X-associated protein 2 (XAP2). Similar to other soluble receptors that act dynamically, the AHR translocates to the nucleus after ligand binding, heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) and binds to dioxin response elements often upstream of target genes such as *Cyp1a1*. The activated AHR/ARNT complex facilitates coactivator recruitment, chromatin remodeling and transcription of target genes, which include phase I and II enzymes (reviewed in ref. 20). Transcriptional regulation mediated by the AHR is dynamic because similar to other soluble receptors, the AHR is continually interacting with chromatin in the presence of endogenous and exogenous ligands and the fact that binding sites for the AHR in chromatin are continually regulated in cells by many different transcription factors and mechanisms (21–24).

Whether PPARβ/δ can alter the initiation of DNA damage caused by chemical carcinogens and influence PPARβ/δ-dependent modulation

of chemically induced skin tumorigenesis has not been examined to date. Analysis of *Ahr*-null mice indicates that the AHR is required for the metabolism of PAH to form genotoxic DNA adducts (25–27) and PAH-induced skin tumorigenesis (26,27). However, although *Ahr*-null mice are completely refractory to the carcinogenic effects of some PAH, this effect is not found for all PAH (26,27). It is of interest to note that there is evidence that AHR activity can be inhibited or altered by PPARα (28–31) because there are many studies showing that all three PPARs can interact with other proteins through similar mechanisms (i.e. all three PPARs can directly bind with and interfere with various proteins (8)). Combined, this supports the hypothesis that there could be an interaction between the PPARβ/δ and the AHR. This study examined the hypothesis that PPARβ/δ reduces AHR-dependent activities associated with PAH-induced skin cancer.

Materials and methods

Chemicals

See Supplementary Materials and methods, available at *Carcinogenesis* Online.

Cell culture

The human embryonic kidney cell line HEK293T and HaCaT human keratinocytes, provided by Dr Yanming Wang and Dr Stuart Yuspa, respectively, were cultured in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin. Hepa1c cells were cultured in α-modified minimal essential media supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin. All cells were cultured at 37°C and 5% carbon dioxide.

Isolation and treatment of primary mouse keratinocytes and dermal fibroblasts

Primary keratinocytes and dermal fibroblasts from wild-type and *Pparβ/δ*-null mice were isolated from neonatal skin and cultured as described previously (32). Keratinocytes were cultured in low calcium (0.05 mM) Eagle's minimal essential medium with 8% chelexed FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin. Dermal fibroblasts were cultured in Dulbecco's minimal essential medium supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin. All cells were cultured at 37°C and 5% carbon dioxide. Fibroblasts and keratinocytes for mRNA expression analyses were treated for 8 h with vehicle or the indicated treatment unless otherwise stated.

In vivo *studies*

Wild-type or *Pparβ/δ*-null mice (3) in the resting phase of the hair cycle were shaved and treated with acetone (control) or the indicated PAH. Studies using mice were approved by The Pennsylvania State University Institutional Animal Care and Use Committee. Mice were euthanized and the dorsal skin regions isolated and snap frozen until further analysis.

RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was prepared from samples using RiboZol RNA Extraction Reagent (AMRESCO, Solon, OH) and the manufacturer's recommended protocol. The mRNA encoding AHR target genes, DNA damage markers, PPARβ/δ and a PPARβ/δ target gene were measured by quantitative real-time polymerase chain reaction (qPCR) analysis as described previously (4). The relative mRNA value for each gene was normalized to the relative mRNA value for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). The following genes were examined, with the primers described in Supplementary Table 1, available at *Carcinogenesis* Online: activating transcription factor 3 (*Atf3*), angiopoietin-like protein 4 (*Angptl4*), cyclooxygenase-2 (*Cox2*), cytochrome P450 1A1 (*Cyp1a1*), *Cyp1a2*, *Cyp1b1*, glutathione S-transferase alpha 1 (*Gsta1*), heme oxygenase 1 (*Hox1*), NAD(P)H quinone oxidoreductase 1 (*Nqo1*), NF-E2-related factor 2 (*Nrf2*), p53, *Pparβ/δ* and UDP-glucuronosyltransferase 1a2 (*Ugt1a2*).

Western blot analysis

Protein samples were isolated from mouse skin microsomes, primary keratinocytes or HaCaT shRNA stable cell lines as described previously (4,33). The expression of AHR, ARNT, CYP1A1, CYP1B1, microsomal epoxide hydrolase (mEH), COX2, HSP90, PPARβ/δ, XAP2, β-ACTIN and lactate dehydrogenase (LDH) was examined by western blot analysis as described previously (4). Hybridization signals for specific proteins were normalized to hybridization signal for β-actin or LDH. The following antibodies were used: anti-AHR MAb Rpt1 (Affinity Bioreagents, Golden, CO), anti-ARNT, anti-CYP1A1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-HSP90 (34), anti-XAP2 (Novus Biologicals, Littleton, CO), anti-CYP1B1 (35), anti-human PPARβ/δ (Abcam, Cambridge, MA), anti-LDH and anti-β-actin (Rockland, Gilbertsville, PA).

B[a]P DNA adduct post-labeling

B[*a*]P DNA adduct formation was quantified as described previously (36–38). Briefly, triplicate 100 mm dishes of wild-type or *Pparβ/δ*-null primary keratinocytes were treated for 24 h with 1 μM B[*a*]P, and genomic DNA was isolated. Five microgram of genomic DNA was labeled with γ-^{32}P-ATP and polynucleotide kinase. Two-dimensional thin layer chromatography on PCI-cellulose plates was used to separate and identify γ-^{32}P-labeled B[*a*]P-adducted nucleotides compared with standards. B[*a*]P-DNA adducts were quantified and normalized to the total amount of nucleotides examined, and are presented as adducts per 10^9 nucleotides.

Photoaffinity ligand ^{125}I-N$_3$Br$_2$DpD binding assay

Primary keratinocytes from wild-type and *Pparβ/δ*-null mice were trypsinized, pelleted and homogenized in MENG buffer (25 mM 3-(*N*-morpholino) propane sulfonic acid (MOPS), 2 mM ethylenediaminetetraacetic acid (EDTA), 0.02% NaN_3 and 10% glycerol pH 7.4) containing 20 mM sodium molybdate and protease inhibitor cocktail (Roche, San Francisco, CA). Cytosol was obtained by centrifugation at 100 000*g* for 1 h. All binding experiments were conducted in the dark until ultraviolet-mediated cross-linking of 2-azido-3-125I-iodo-7,8-dibromodibenzo-p-dioxin (^{125}I-N$_3$Br$_2$DpD) was completed as described previously (39). Briefly, 150 μg of cytosolic protein was incubated at room temperature with increasing amounts of ^{125}I-N$_3$Br$_2$DpD. Ligand was allowed to bind protein for 30 min at room temperature and samples photolyzed at 8 cm with 402 nm ultraviolet light. Three percent dextran-coated charcoal was added to the photolyzed samples for 5 min followed by centrifugation to remove free ligand. Labeled samples were resolved using 8% acrylamide-sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane and visualized using autoradiography. Radioactive AHR bands were then excised and counted using a γ-counter to quantify radioligand binding.

Reversible ligand ^{125}I-Br$_2$DpD mediated AHR nuclear translocation

Wild-type and *Pparβ/δ*-null primary keratinocytes were cultured and treated for 1 h with 2-125I-iodo-7,8-dibromo-p-dioxin (^{125}I-Br$_2$DpD), washed twice with Dulbecco's phosphate buffered saline, trypsinized and pelleted. Nuclear translocation was examined as described previously (39). Bovine serum albumin (4.4 S) and catalase (11.3 S) were used as external markers of sedimentation in the sucrose gradients.

Chromatin immunoprecipitation

Wild-type and *Pparβ/δ*-null primary keratinocytes were treated for 3 h with vehicle or 1 μM B[*a*]P. Chromatin was isolated and used for chromatin immunoprecipitation (ChIP) as described previously (40) with specific antibodies for either AHR (Enzo, Farmingdale, NY), anti-acetylated histone H4 (Upstate Biotechnology, Lake Placid, NY) as a positive control, or rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) as a negative control. Immunoprecipitated DNA was used for qPCR analysis to quantify occupancy of the AHR in the proximal promoter of the *Cyp1a1* gene because this is a specific AHR target gene. The primers for *Cyp1a1* were 5′-GTCGTTGCGCTTCTCACGCGA-3′ (forward) and 5′-CACTGAGGGAGGGGTTTGAGG-3′ (reverse). The specific values were normalized to treatment inputs and verified to be greater than rabbit IgG controls. Promoter occupancy was determined based on fold accumulation to normalized vehicle values.

Mouse complete skin carcinogenesis bioassay

Female, wild-type (+/+), *Pparβ/δ*-null (–/–) (3)) and *Ahr*-null (41), mice in the resting phase of the hair cycle (6–8 weeks of age) were shaved and topically treated weekly with 100 μg of DMBA or B[*a*]P or 300 μg of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) dissolved in 200 μl acetone (Supplementary Table 2, available at *Carcinogenesis* Online). The B[*a*]P and DMBA dosing regimen was chosen based on previous bioassays using the C57BL/6 strain (42–44). The studies were carried out for 34 weeks (B[*a*]P), 27 weeks (DMBA) or 25 weeks (MNNG), respectively. The onset of lesion formation, lesion number and lesion size was assessed weekly, and mice were euthanized by overexposure to carbon dioxide at the end of the study.

Statistical analysis

In vitro data were analyzed for statistical significance using Student's *t*-test, one-way or two-way analysis of variance with the Bonferroni's multiple comparison test (Prism 5.0, GraphPad Software Inc., La Jolla, CA) or the Mann-Whitney test (DNA adducts). Tumor data were analyzed for significance using Fisher's exact test (lesion incidence) or Student's *t*-test (lesion/mouse and average lesion size). All results are reported as mean ± SEM.

Results

PPARβ/δ specifically reduces PAH-dependent signaling in the skin and keratinocytes

To determine if PPARβ/δ reduces PAH-dependent signaling in the skin, wild-type and *Pparβ/δ*-null mice were treated topically with DMBA. The expression of both CYP1A1 and CYP1B1 mRNA and protein were increased in DMBA-treated wild-type mice but this effect was not found in similarly treated *Pparβ/δ*-null mice (Figure 1A and B). Expression of mEH, which can also metabolize PAH (45), was not different between genotypes (Figure 1B). Expression of COX2 was increased by treatment with DMBA and this effect was not found in similarly treated *Pparβ/δ*-null mouse skin (Figure 1B). However, basal expression of COX2 was higher in *Pparβ/δ*-null mouse skin compared with wild-type (data not shown). This is consistent with a previous study (17) and could be due to PPARβ/δ-dependent repression of gene expression (46). B[*a*]P and DMBA both increased expression of *Cyp1a1* and *Cyp1b1* mRNA in wild-type keratinocytes and this effect was markedly lower in similarly treated *Pparβ/δ*-null keratinocytes, in particular for *Cyp1a1* mRNA (Figure 1C and D). This suggests that the keratinocyte is at least one of the cell types within the epidermis where PPARβ/δ could reduce AHR-dependent effects induced by PAH.

The specificity of this effect was examined in primary dermal fibroblasts, the liver and primary hepatocytes to determine if this regulation is a global or tissue-specific phenomenon. Dermal fibroblasts were examined because this cell type is directly adjacent to keratinocytes. The liver and primary hepatocyte cultures were examined because they are a primary site of PAH metabolism mediated by AHR. Interestingly, B[*a*]P increased expression of *Cyp1a1* and *Cyp1b1* mRNA in mouse primary dermal fibroblasts (Figure 1E and F) and *Cyp1a1* and *Cyp1a2* mRNA in mouse primary hepatocytes in both genotypes (Supplementary Figure S1A and B, available at *Carcinogenesis* Online). Moreover, the AHR agonist β-naphthoflavone increased expression of *Cyp1a1* and *Cyp1a2* mRNA in mouse liver of both genotypes (Supplementary Figure S1C and D, available at *Carcinogenesis* Online).

The ability of PPARβ/δ to reduce AHR-dependent signaling in response to structurally diverse PAH/AHR agonists was also examined. Treatment with 11 different PAH/AHR agonists caused an increase in expression of *Cyp1a1* and *Cyp1b1* mRNA and this effect was diminished in similarly treated *Pparβ/δ*-null keratinocytes

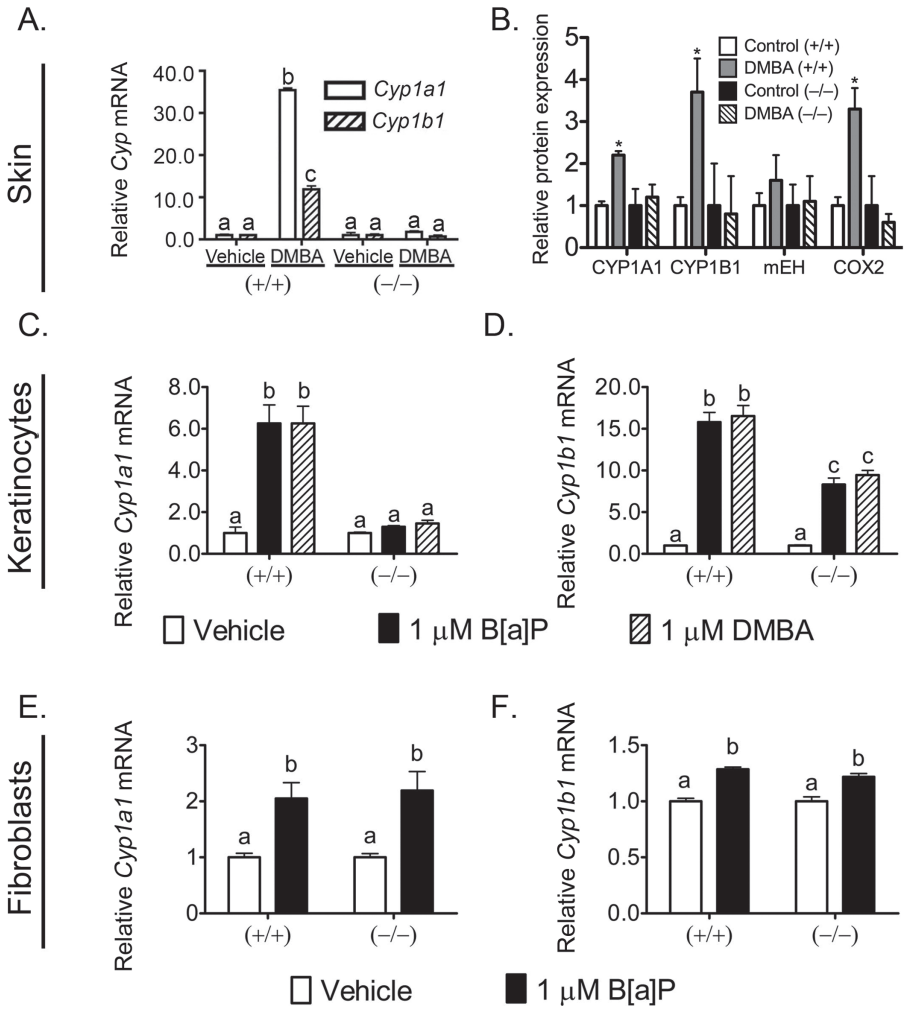

Fig. 1. PPARβ/δ specifically reduces PAH-induced changes in P450 expression in mouse skin and primary keratinocytes. (**A** and **B**) Wild-type (+/+) and *Pparβ/δ*-null (–/–) mice were topically treated with 50 μg DMBA or (**C** and **D**) primary keratinocytes and (**E** and **F**) primary dermal fibroblasts from (+/+) and (–/–) mice were treated 8 h with 1 μM B[*a*]P or DMBA. (A) qPCR of total RNA to quantify the mRNA expression of *Cyp1a1* and *Cyp1b1* in response to DMBA. Values are the average normalized fold change compared with (+/+) vehicle control and represent the mean ± SEM of $N = 5$ biological replicates. (B) Protein expression of CYP1A1, CYP1B1, mEH, COX2 was normalized to LDH and is shown as 'relative protein expression'. (C–F) qPCR of primary keratinocyte or primary dermal fibroblast total RNA to quantify the expression of (C and E) *Cyp1a1* or (D and F) *Cyp1b1*. Values were normalized to the respective genotype vehicle controls and represent the mean ± SEM of $N = 4$ biological replicates. Values with different letters are significantly different than controls ($P \leq 0.05$). *Significantly different than wild-type control ($P \leq 0.05$).

(Figure 2A and B). This effect was even observed with the highly potent AHR full agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. It is worth noting that treatment with the PPARβ/δ agonist GW0742 did not alter the mRNA levels of *Cyp1a1* or *Cyp1b1* in either genotype (Figure 2A and B). Temporally, the absence of PPARβ/δ expression attenuated B[*a*]P-induced expression of *Cyp1a1* and *Cyp1b1* mRNA even over a 24 h period (Figure 2C and D). PAH exposure beyond 24 h resulted in high keratinocyte toxicity and minimal recovery of quality RNA for gene expression analyses (data not shown).

PPARβ/δ reduces phase II enzyme expression, markers of oxidative stress and markers of DNA damage

Activation of the AHR by PAH directly regulates genes encoding phase I and II xenobiotic metabolizing enzymes known as the 'AHR gene battery' (47). Additionally, PAHs can increase oxidative stress and modulate NRF2 activity causing changes in expression of both AHR and/or NRF2 target genes, which include phase II xenobiotic metabolizing enzymes (48). Thus, the ability of PPARβ/δ to alter AHR-dependent expression of genes encoding phase II xenobiotic metabolizing enzymes was examined. DMBA and B[*a*]P increased expression of mRNA encoding the phase II enzymes *Nqo1*, *Ugt1a2* and *Gsta1* in wild-type keratinocytes, an effect not found in *Pparβ/δ*-null keratinocytes (Figure 3A–C). DMBA and B[*a*]P also increased expression of mRNA encoding *Nrf2*, *Cox2* and *Hox1* in wild-type keratinocytes, but these effects were not observed in *Pparβ/δ*-null keratinocytes (Figure 3D–F). It is also known that DNA damage by PAHs enhances p53 signaling, particularly in the skin, and *Atf3* mRNA expression has recently been identified as an indirect marker of DNA damage (49). B[*a*]P had no effect on expression of mRNA encoding *p53* or *Atf3* at 8 h post-PAH treatment but was increased in wild-type keratinocytes 24 h post-PAH treatment (Figure 3G and H). These effects were not observed in *Pparβ/δ*-null keratinocytes (Figure 3G and H).

AHR nuclear function and DNA damage are reduced by PPARβ/δ

To begin to assess the mechanism(s) by which PPARβ/δ reduces AHR signaling, intrinsic functions of AHR, including protein expression, ligand binding, nuclear translocation, promoter occupancy, chromatin remodeling and DNA adduct formation were all examined. Quantitative western blot analysis showed no significant alterations in the expression of AHR, ARNT, HSP90 or XAP2 between wild-type

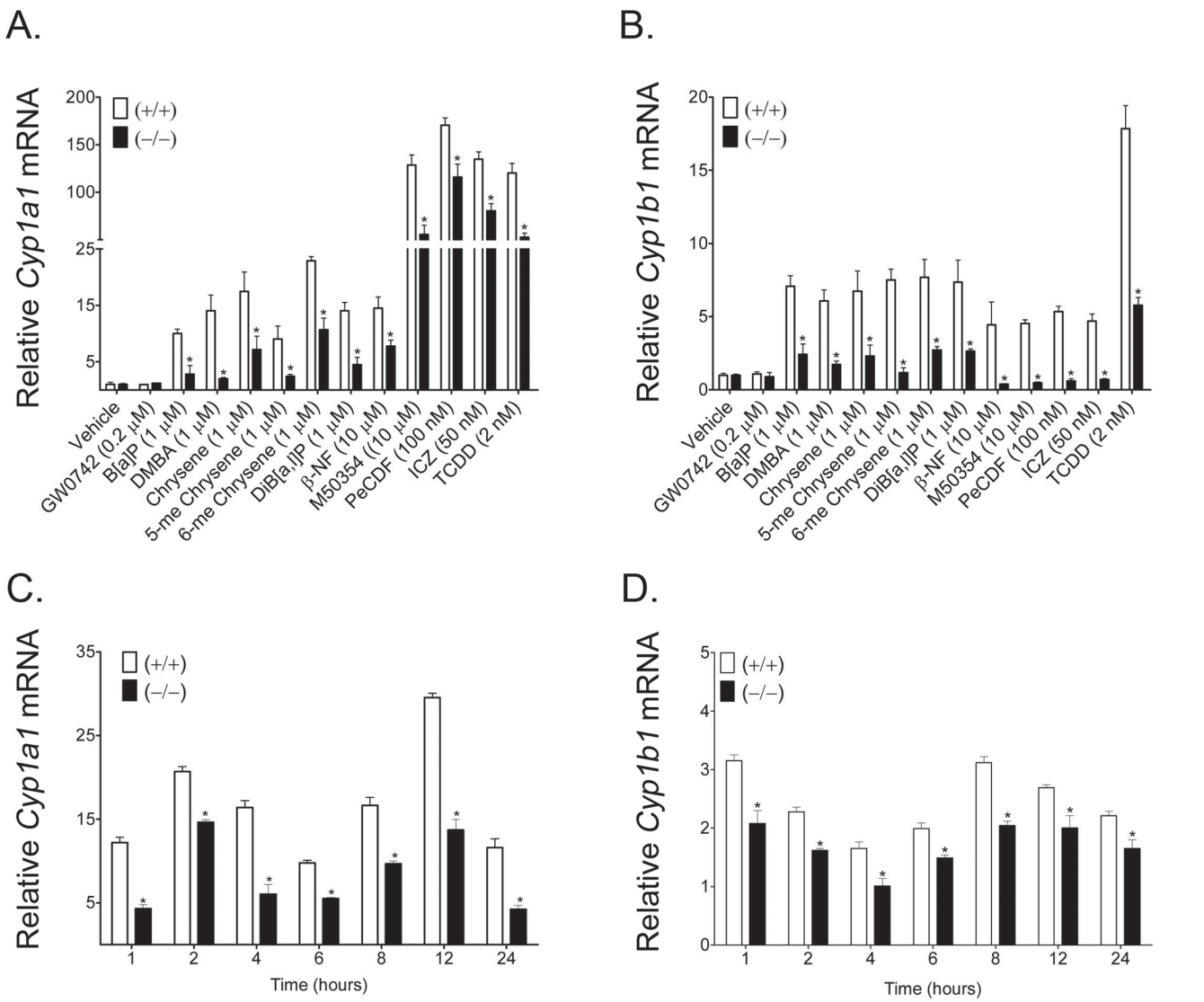

Fig. 2. Modulation of AHR-dependent signaling by PPARβ/δ by multiple PAH. Wild-type (+/+) and *Pparβ/δ*-null (–/–) primary keratinocytes were treated with the indicated compounds (**A** and **B**) or 1 μM B[*a*]P (**C** and **D**) for either 8 (A and B) or 1 to 24 h (C and D). qPCR was performed using total RNA isolated from primary keratinocytes to quantify the mRNA expression of (A and C) *Cyp1a1* or (B and D) *Cyp1b1*. Values were normalized to the respective genotype vehicle controls and represent the mean ± SEM of $N = 3$ biological replicates. *Significantly different than control ($P \leq 0.05$).

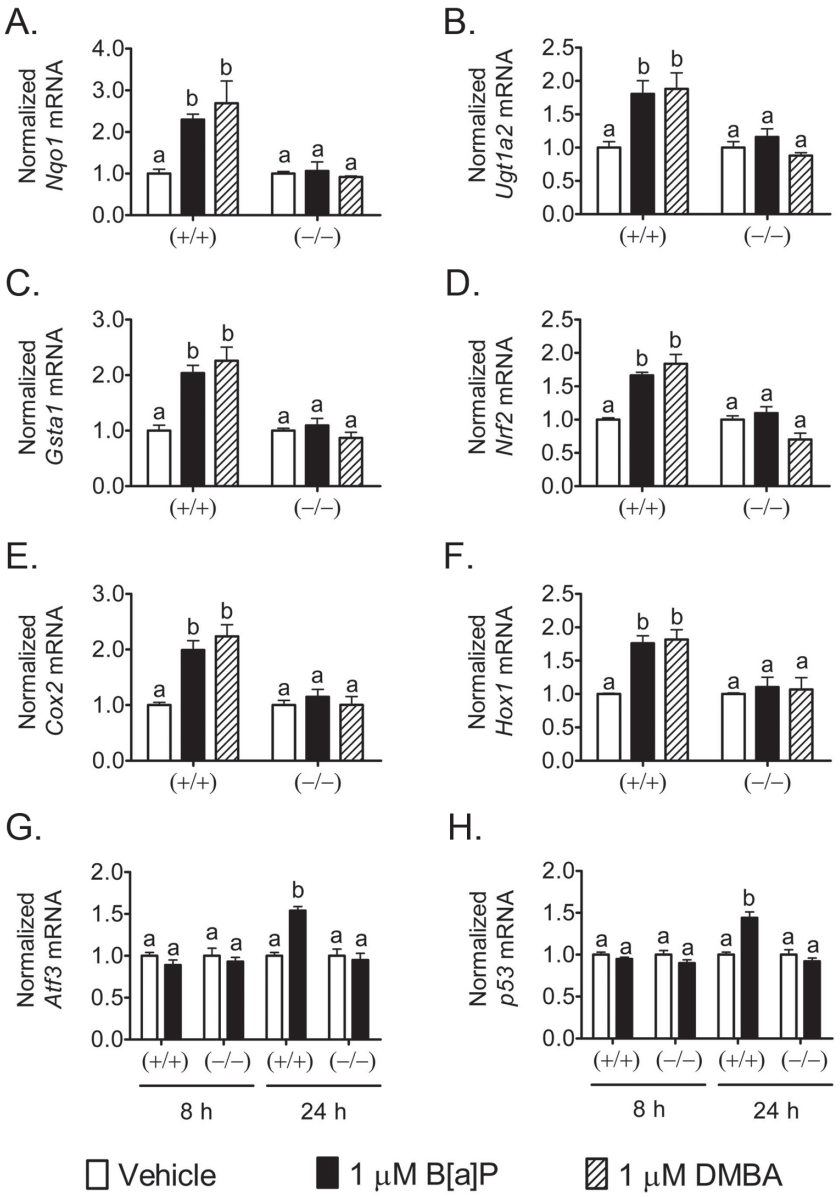

Fig. 3. PPARβ/δ reduces PAH-dependent mRNA expression of phase II enzymes, oxidative stress markers and DNA damage markers. Wild-type (+/+) and *Pparβ/δ*-null (–/–) primary keratinocytes were treated with 1 μM B[*a*]P or DMBA for 8 or 24 h. qPCR was performed using total RNA isolated from primary keratinocytes to quantify the expression of (**A**) *Nqo1*, (**B**) *Ugt1a2*, (**C**) *Gsta1*, (**D**) *Nrf2*, (**E**) *Cox2*, (**F**) *Hox1*, (**G**) *Atf3* or (**H**) *p53*. Values were normalized to the respective genotype vehicle controls and represent the mean ± SEM of $N = 4$ biological replicates. Values with different letters are significantly different than controls ($P \leq 0.05$).

and *Pparβ/δ*-null keratinocytes (Figure 4A). AHR ligand binding was assessed using a radioactive irreversible and reversibly bound dioxin derivatives (50,51). ^{125}I-N_3Br_2DpD is a high-affinity AHR ligand that can be irreversibly cross-linked to AHR by ultraviolet exposure. A dose-dependent increase in ligand binding based on this assay was observed in both genotypes (Figure 4B). AHR ligand binding and function was also assessed with the reversible ^{125}I-Br_2DpD ligand binding assay used in a previous study that showed a difference in sedimentation between the unliganded AHR/HSP90 complex (9S) and ligand-bound AHR/ARNT complex (6S (50)). No differences in the amount of 6S receptor complex present in nuclear extracts were observed between wild-type and *Pparβ/δ*-null samples (Figure 4C).

ChIP was performed to assess whether PPARβ/δ altered the ability of AHR to bind a target gene promoter and cause chromatin remodeling in response to B[*a*]P exposure. Compared with control, B[*a*]P treatment resulted in increased accumulation of acetylated histone H4 and increased occupancy of AHR in chromatin of wild-type keratinocytes containing the *Cyp1a1* promoter, and these effects were not found in similarly treated *Pparβ/δ*-null keratinocytes. In addition, decreased DNA adducts were noted in *Pparβ/δ*-null keratinocytes treated with B[*a*]P compared with wild-type keratinocytes (Figure 4E).

PPARβ/δ reduces AHR signaling in a human keratinocyte cell line

To examine whether PPARβ/δ-dependent modulation of AHR-dependent signaling found in mouse keratinocytes also occurs in human keratinocytes, a stable human HaCaT keratinocyte cell line was generated to knockdown PPARβ/δ expression. A 36% decrease in PPARβ/δ protein expression was observed in the shPPARβ/δ HaCaT cell line compared with control shRNA HaCaT cells (Figure 5A). Knockdown of PPARβ/δ expression significantly reduced ligand-dependent regulation of the PPARβ/δ target gene *ANGPTL4* compared with controls (Figure 5B). Notably, expression of AHR, ARNT, HSP90 and XAP2 was not altered by reduced PPARβ/δ protein expression

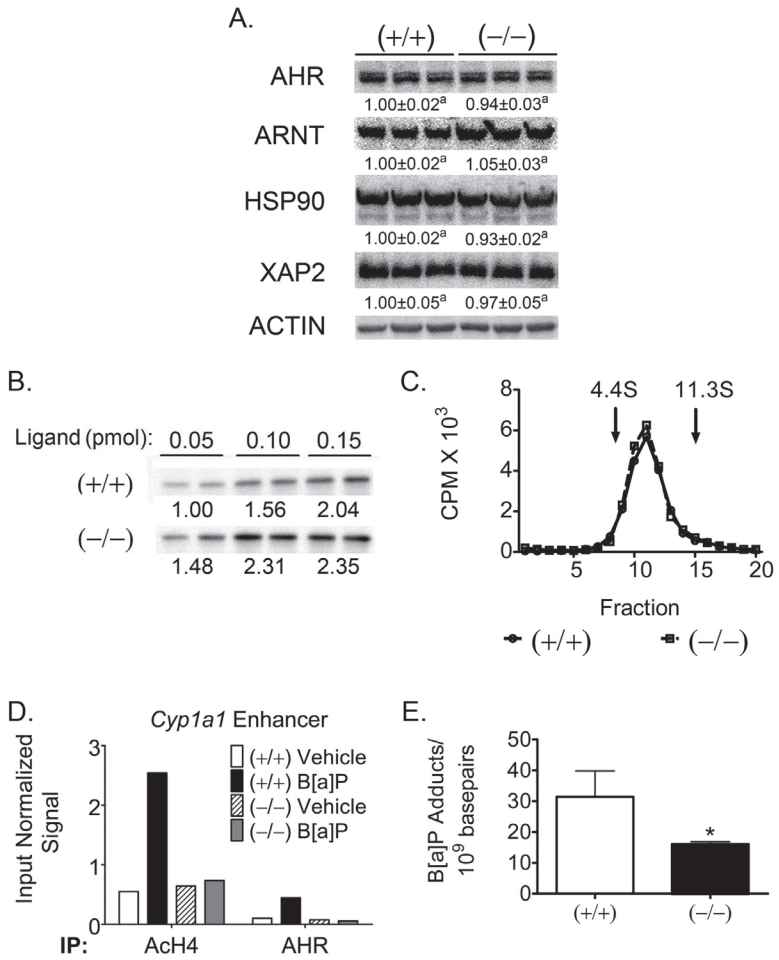

Fig. 4. Functional differences in AHR-dependent signaling are reduced by PPARβ/δ. (**A**) Quantitative protein expression of AHR and AHR accessory proteins in wild-type (+/+) and *Pparβ/δ*-null (−/−) primary keratinocytes normalized to ACTIN expression. Normalized expression values are fold expression relative to (+/+) and represent the mean ± SEM of $N = 3$ biological replicates. Values with different letters are significantly different than controls ($P \leq 0.05$). (**B**) Level of cytosolic AHR binding of the irreversible photoaffinity ligand ^{125}I-N$_3$Br$_2$DpD. Cytosol from (+/+) and (−/−) keratinocytes was incubated with increasing amounts of radioaffinity ligand with $N = 2$ technical replicates. Relative binding was determined by gamma counting of the excised bands from the protein blots and normalized to the (+/+) 0.05 pmol average signal. (**C**) AHR nuclear translocation using reversible ligand ^{125}I-Br$_2$DpD. (+/+) or (−/−) keratinocytes were treated 1 h with ^{125}I-Br$_2$DpD, and nuclear extracts were isolated and subjected to sucrose density gradient ultracentrifugation. Isolated gradient factors were quantified by gamma counting, and the marker proteins bovine serum albumin (4.4S) and catalase (11.3S) were used as external standards to evaluate AHR complex status. (**D**) ChIP to assess promoter occupancy at the *Cyp1a1* enhancer element in response to B[*a*]P. Keratinocytes were treated for 3 h with vehicle or 1 μM B[*a*]P, and ChIP and qPCR quantification was performed. Results are from one biological replicate pooled from keratinocytes isolated from $N = 3$ neonates. Acetylated histone H4 immunoprecipitation was used as a positive marker of transcriptional activation compared with specific AHR occupancy. (**E**) Quantitative DNA adduct formation as determined by γ-^{32}P-post-labeling of B[*a*]P-adducted nucleotides. Keratinocytes were treated 24 h with 1 μM B[*a*]P, and DNA isolation and post-labeling were performed. Values are normalized to nucleotide content and represent the mean ± SEM of quantified number of adducts per 10^9 basepairs from $N = 3$ biological replicates. *Significantly different than control ($P \leq 0.05$).

in HaCaT keratinocytes (Figure 5A). B[*a*]P increased expression of *Cyp1a1* and *Cyp1b1* mRNA in control shRNA HaCaT cells and this effect was reduced in shPPARβ/δ HaCaT cells (Figure 5C and Supplementary Figure S2A, available at *Carcinogenesis* Online). The attenuated expression of *Cyp1a1* and *Cyp1b1* mRNA was observed as early as 2 h post-B[*a*]P treatment in shPPARβ/δ HaCaT cells and continued until 24 h post-B[*a*]P treatment compared with control shRNA HaCaT cells (Figure 5D and Supplementary Figure S2B, available at *Carcinogenesis* Online).

PPARβ/δ-dependent differences in promoter methylation of the AHR target gene, Cyp1a1

Methylation of DNA can cause silencing of gene expression and can modulate initiation and progression of cancers (reviewed in refs 52 and 53). To examine whether DNA methylation may be a mechanism by which PPARβ/δ alters the occupancy of AHR on the *Cyp1a1* promoter, mouse keratinocytes or human HaCaT keratinocytes were treated with a cytosine analog, 5-aza-2′-deoxycytidine (5-aza-dC), which cannot be methylated. This results in ablation of methylation patterns following extended treatment (72 h) (54). B[*a*]P increased expression of *Cyp1a1* and *Cyp1b1* mRNAs in control wild-type keratinocytes and this effect was diminished in *Pparβ/δ*-null keratinocytes (Figure 5E and Supplementary Figure S2C, available at *Carcinogenesis* Online). B[*a*]P increased expression of *Cyp1a1* and *Cyp1b1* mRNAs to similar levels in keratinocytes from both genotypes co-treated with 5-aza-dC in control wild-type and *Pparβ/δ*-null keratinocytes (Figure 5E and Supplementary Figure S2C, available at *Carcinogenesis* Online). Similarly, B[*a*]P increased expression of *CYP1A1* and *CYP1B1* mRNAs in control shRNA HaCaT cells and this effect was diminished in shPPARβ/δ HaCaT cells (Figure 5F and Supplementary Figure S2D, available at *Carcinogenesis* Online). B[*a*]P increased expression of *CYP1A1* and *CYP1B1* mRNAs to similar levels in both control shRNA HaCaT and shPPARβ/δ HaCaT cells co-treated with 5-aza-dC (Figure 5F and Supplementary Figure S2D, available at *Carcinogenesis* Online).

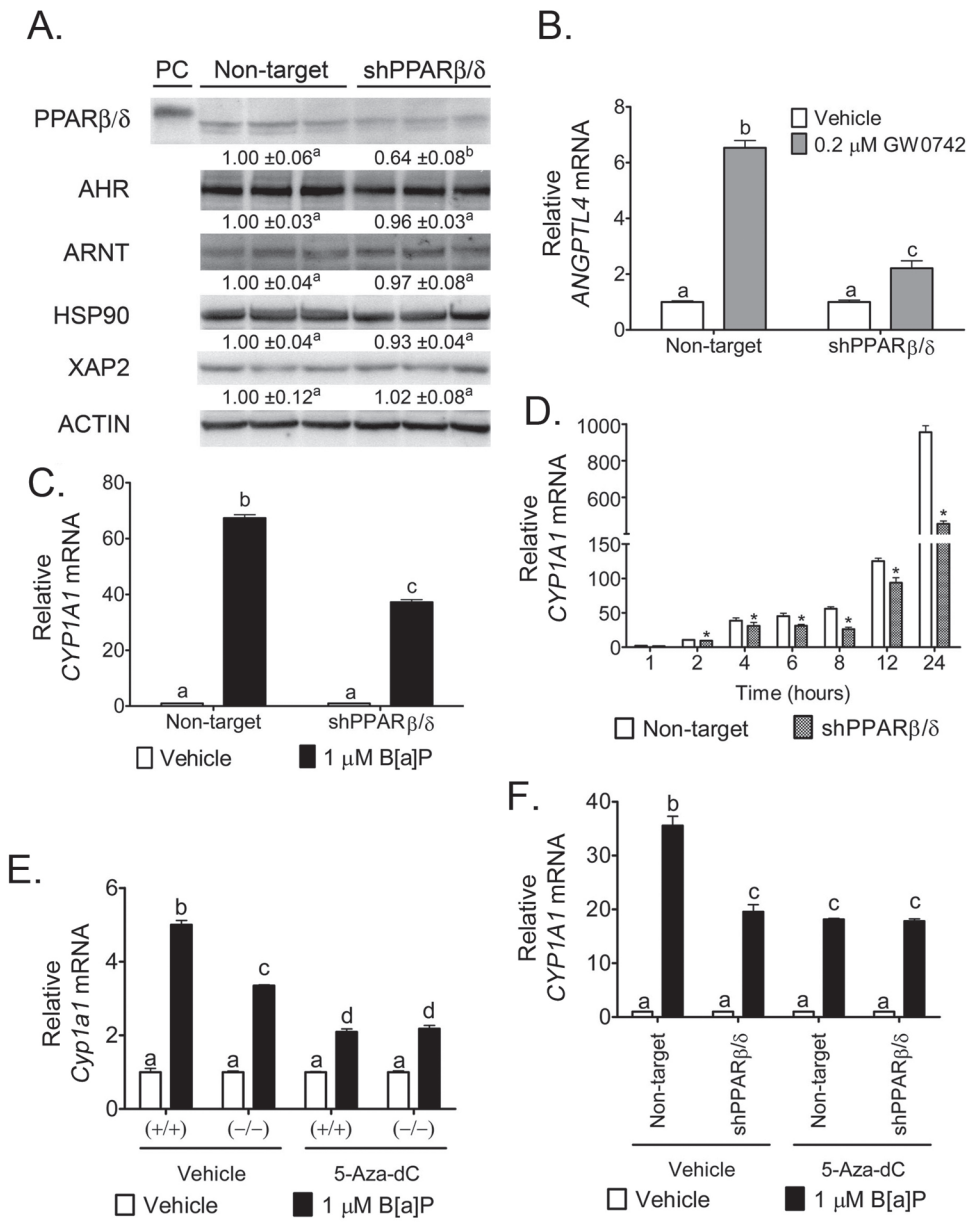

Fig. 5. Modulation of AHR-dependent signaling by PPARβ/δ is conserved in human HaCaT keratinocytes and possibly influenced by epigenetic modification of AHR target genes. (**A**) Quantitative protein expression of PPARβ/δ, AHR and AHR accessory proteins in the HaCaT shRNA cell lines normalized to ACTIN expression. Expression values are fold expression relative to the non-target cell line and represent mean ± SEM of $N = 3$ biological replicates. PC = positive control (lysate of COS-1 cells transfected with human PPARβ/δ expression vector). qPCR was performed using total RNA isolated from the HaCaT shRNA stable cell lines to quantify the mRNA expression of (**B**) *ANGPTL4* in response to the PPARβ/δ ligand GW0742 or (**C**) *CYP1A1* 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 (**D**) *CYP1A1* in response to 1 μM 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 *Cyp1a1*/*CYP1A1* mRNA in (**E**) primary keratinocytes or (**F**) HaCaT shRNA cell lines. Values were normalized to the respective genotype or stable cell line vehicle control and represent the mean ± SEM of $N = 4$ biological replicates. Values with different letters are significantly different than control ($P \leq 0.05$). *Significantly different than control ($P \leq 0.05$).

Bisulfite sequencing was performed to directly examine whether methylation at the mouse *Cyp1a1* promoter contributes to PPARβ/δ-dependent modulation of AHR signaling, in particular decreased occupancy of AHR on the *Cyp1a1* promoter observed in *Pparβ/δ*-null keratinocytes. A putative 933 basepair CpG island at approximately –1481 to –548 basepairs upstream of the *Cyp1a1* transcription start site was identified and examined (Supplementary Figure S2E, available at *Carcinogenesis* Online). Examination of the methylation map revealed that *Pparβ/δ*-null keratinocytes had more DNA methylation at the *Cyp1a1* promoter compared with wild-type cells (Supplementary Figure S2E, available at *Carcinogenesis* Online). Increased promoter methylation is known to repress gene expression (reviewed in ref. 55), and *Pparβ/δ*-null keratinocytes also exhibited reduced basal mRNA levels of *Cyp1a1* compared with wild-type keratinocytes (data not shown).

PPARβ/δ reduces complete skin carcinogenesis

Collectively, the former results suggest that PPARβ/δ could reduce PAH-induced initiation of DNA damage mediated by the AHR, and that *Pparβ/δ*-null mice would be resistant to PAH-induced skin cancer. This is paradoxical because previous studies showed that *Pparβ/δ*-null mice exhibit exacerbated skin tumorigenesis (6), and ligand activation of PPARβ/δ prevents chemically induced

skin tumorigenesis using a two-stage model (6,11,16–18), which requires application of both a chemical carcinogen and a tumor promoter. Since PPARβ/δ could influence both initiation and/or promotion, a complete carcinogenesis bioassay was performed (Supplementary Table 2, available at *Carcinogenesis* Online). This approach was used, rather than a two-stage bioassay, to reduce the impact of a tumor promoter coapplied with the chemical carcinogen because PPARβ/δ can modulate cell proliferation and differentiation in the two-stage model (9–15) and a complete carcinogen bioassay does not require coadministration of a tumor promoter. The mechanisms by which complete carcinogens cause tumorigenesis without administration of a tumor promoter and the precise molecular pathways that cause tumor promotion in this model are not entirely understood (56). However, the complete carcinogen bioassay is highly dependent on expression of PAH metabolizing enzymes including CYP1A1, to bioactivate the PAH throughout the assay (25–27). DMBA and B[*a*]P were used because each require AHR-dependent bioactivation (25–27) and to cause skin cancer, at least in part (25–27). In contrast, the mutagen MNNG was used as a control because it causes skin tumorigenesis independent of AHR signaling (57).

The incidence of lesions was higher in wild-type mice compared with *Pparβ/δ*-null and *Ahr*-null mice following treatment with DMBA from weeks 15 to 21 (Figure 6A). Tumor multiplicity was higher in wild-type mice compared with *Pparβ/δ*-null and *Ahr*-null mice following treatment with DMBA from weeks 17 to 27, or from weeks 19 to 27, respectively (Figure 6B). DMBA administration resulted in larger average tumor size in wild-type mice than *Pparβ/δ*-null mice for the final 2 weeks of the study (Figure 6C). The incidence of lesions was also higher in wild-type mice compared with *Pparβ/δ*-null mice following treatment with B[*a*]P from weeks 21 to 22, and completely absent in *Ahr*-null mice compared with other two genotypes (Figure 6D). Tumor multiplicity was higher in wild-type mice compared with *Pparβ/δ*-null mice following treatment with B[*a*]P from weeks 21 to 27, whereas *Ahr*-null mice had no tumors (Figure 6E). B[*a*]P administration resulted in larger average tumor size in *Pparβ/δ*-null mice compared with wild-type mice during the last 2 weeks, and was markedly higher in both wild-type and *Pparβ/δ*-null mice compared with *Ahr*-null mice, which were refractory to skin tumorigenesis by B[*a*]P (Figure 6F). In contrast, skin tumor incidence induced by MNNG was significantly higher in *Pparβ/δ*-null mice compared with wild-type mice during weeks 20–21 and 23–24 (Figure 6G). Tumor multiplicity induced by MNNG was lower in wild-type and *Ahr*-null mice compared with *Pparβ/δ*-null mice during the final 5 weeks (Figure 6H). No differences in average lesion size caused by MNNG were observed between the three genotypes (Figure 6I).

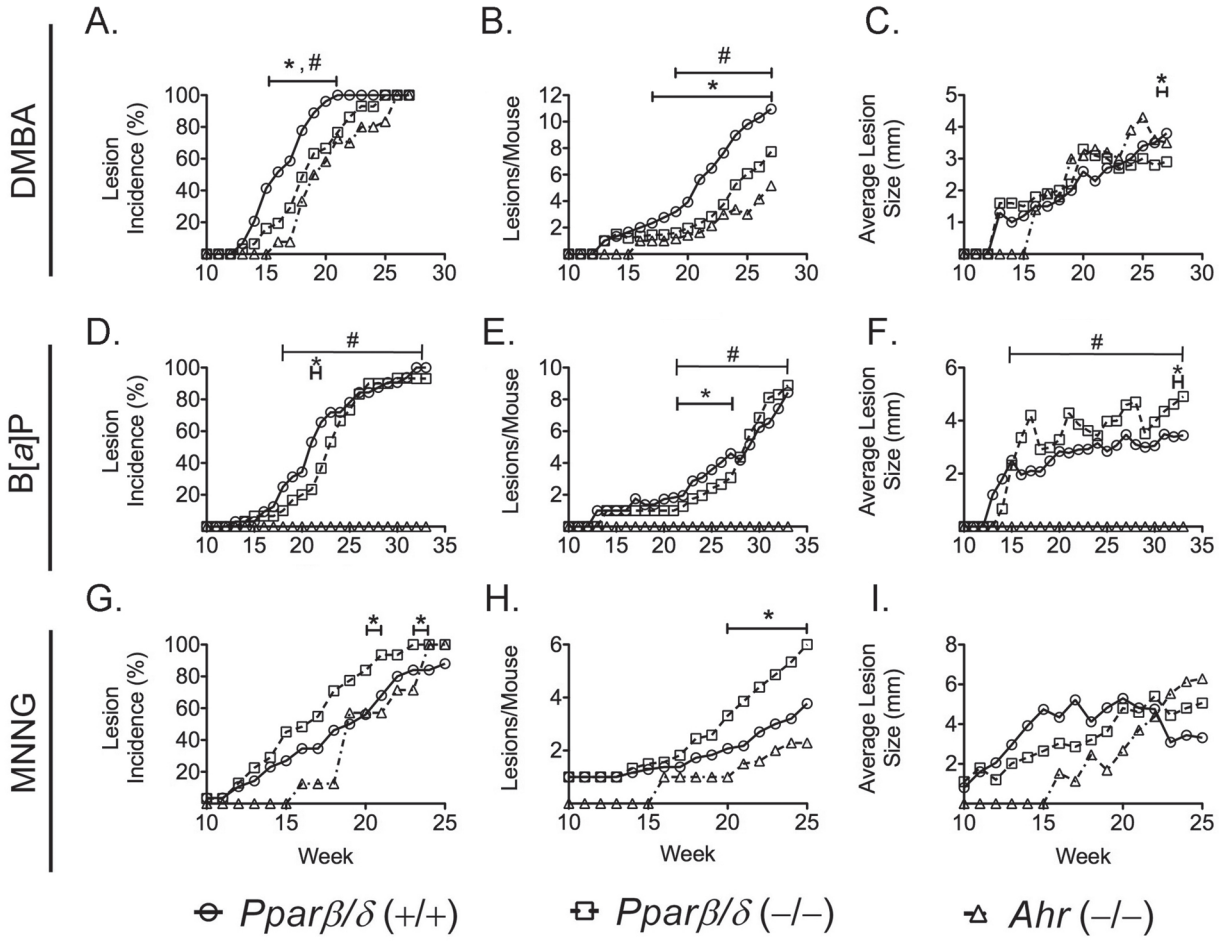

Fig. 6. Effect of PPARβ/δ on the outcome of complete carcinogenesis bioassays. Wild-type (+/+), *Pparβ/δ*-null (–/–) and *Ahr*-null (–/–) mice were topically treated weekly with 100 µg of PAH (DMBA or B[*a*]P) or 300 µg MNNG during a 25–33 week complete carcinogen bioassay. (**A**) Onset of lesion formation (the week when lesions were noted on any mouse within each group and treatment, as indicated by percentage of mice with visible lesion(s)) and incidence of lesions (the percentage of mice with visible lesion(s) on the indicated week), (**B**) lesion multiplicity (the average number of lesions per mouse) and (**C**) average lesion size in the complete carcinogen bioassay using DMBA. (**D**) Onset of lesion formation and incidence of lesions, (**E**) lesion multiplicity and (**F**) average lesion size in the complete carcinogen bioassay using B[*a*]P. (**G**) Onset of lesion formation and incidence of lesions, (**H**) lesion multiplicity and (**I**) average lesion size in the complete carcinogen bioassay using MNNG. *Significantly different between wild-type (+/+) and *Pparβ/δ*-null (–/–) mice ($P \leq 0.05$). #Significantly different between wild-type (+/+) and *Ahr*-null (–/–) mice ($P \leq 0.05$).

Discussion

Results from the present studies are the first to demonstrate that the lack of PPARβ/δ expression can reduce AHR-dependent signaling via decreased occupancy of the AHR on an AHR target gene, decreased expression of AHR-dependent target genes, decreased PAH-induced DNA adduct formation and decreased skin tumorigenicity in *Pparβ/δ*-null mice compared with wild-type. This is based on data obtained from two independent, complementary models: mouse wild-type and *Pparβ/δ*-null keratinocytes, and control and shPPARβ/δ HaCaT knockdown human keratinocytes. These effects are not due to altered expression of the AHR or AHR auxiliary protein, or altered ligand specificity for AHR. This PPARβ/δ-dependent influence on AHR signaling is unique to keratinocytes and conserved between mice and humans. Both of these models contain a 'rescue' component (i.e. the control cells express PPARβ/δ whereas the null cells and the shRNA cells exhibit complete ablation or markedly reduced expression of the receptor, respectively). However, additional experiments with alternative rescue approaches (i.e. reintroduce expression of PPARβ/δ in *Pparβ/δ*-null keratinocytes) would further strengthen the results from the present study.

The AHR is considered the master regulator of PAH-dependent tumorigenesis (reviewed in ref. 58) as *Ahr*-null mice are refractory to PAH-induced skin carcinogenesis (26,27). Identification of PPARβ/δ as a new modulator of AHR-dependent signaling is a novel finding. Although the AHR is essential for chemically induced tumorigenesis to bioactivate PAH (reviewed in ref. 58), increased PAH-induced tumorigenesis has also been found in phase II xenobiotic metabolizing enzyme null mice (reviewed in refs 59 and 60). Since the AHR regulates many phase I and II xenobiotic enzymes (47), results from the present studies support the hypothesis that the AHR is not the single master regulator of PAH-induced skin tumorigenesis and that PPARβ/δ that can also influence PAH-induced skin tumorigenesis. This is consistent with the results from studies showing that PAH, such as DMBA can cause some tumorigenesis in *Ahr*-null mice whereas *Ahr*-null mice are completely refractory to B[*a*]P-induced skin tumorigenesis (26,61). Results from the complete carcinogenesis studies indicate that PPARβ/δ can reduce PAH-induced skin cancer and the overall outcome depends on the AHR agonist used. This could be due to differences mediated by differential recruitment of coregulators to the AHR, that in turn influence expression of AHR target genes required for metabolic activation of chemical carcinogens. This hypothesis requires testing by further experimentation.

There is a complex regulatory network regulated by the AHR following agonist activation. This is illustrated by the fact that activation of the AHR by agonists not only increase expression of some phase I xenobiotic metabolizing enzymes (i.e. CYPs) (47), COX2 (62) and phase II xenobiotic metabolizing enzymes (i.e. GSTs, UGTs, NQO, etc.), but also increases oxidative stress (63), causing activation of NRF2 (64), which in turn coregulates expression of many phase II enzymes (GSTs, NQO, etc.) (64) and anti-inflammatory enzymes (i.e. HOX1) (65). Results from the present studies demonstrate that PPARβ/δ can influence some of these effects because PAH-induced expression of CYPs, COX2, NRF2, HOX1 and phase II xenobiotic metabolizing enzymes was reduced in the absence of PPARβ/δ expression compared with controls. This AHR/NRF2 pathway could be differentially influenced by various AHR agonists due to differences in the AHR agonists, differences in cofactor recruitment to the AHR and the relative contribution of NRF2-dependent signaling (48). Further studies are needed to examine this hypothesis. However, results from the present investigation also indicate that PPARβ/δ can impact the AHR/NFR2 axis, which contributes to the mechanisms by which PAH cause skin cancer.

Reduced occupancy of the AHR on a gene promoter following B[*a*]P treatment of *Pparβ/δ*-null keratinocytes compared with wild-type cells was correlated with minimal cytosine methylation in wild-type keratinocytes at the putative *Cyp1a1* CpG island. In contrast, increased methylation of the *Cyp1a1* promoter was found in *Pparβ/δ*-null keratinocytes. This difference could alter accessibility of the AHR to the *Cyp1a1* promoter and explain the decrease in CYP1A1 expression following treatment with PAH in *Pparβ/δ*-null keratinocytes. Recent studies in mouse and human cell lines have also shown that methylation of the *Cyp1a1* or *Cyp1b1* promoter causes decreased AHR-mediated transcriptional activity (66–70). Thus, it is of interest to note that when global methylation of chromatin was reduced by 5-Aza-dC in PPARβ/δ knockout or knockdown keratinocytes, B[a]P induced both CYP1A1 and CYP1B1 similarly compared with wild-type cells. Although B[*a*]P-induced expression of the two AHR target genes was relatively lower in the cells treated with 5-Aza-dC, compared with control, it is critical to note that 5-Aza-dC alters global methylation patterns. This could influence expression of multiple genes that in turn alter AHR-dependent activity. However, because reducing methylation by treating *Pparβ/δ*-null keratinocytes or shPPARβ/δ HaCaT cells with 5-Aza-dC caused a similar level of B[*a*]P-induced expression of two AHR target genes, these data support the hypothesis that PPARβ/δ-dependent alteration of DNA methylation of AHR target genes could explain how PPARβ/δ influences AHR-dependent signaling.

The complete chemical carcinogen bioassay was used in these studies to focus more on the potential role of PPARβ/δ to influence AHR-dependent signaling during chemical carcinogenesis and to minimize the influence of coadministering a tumor promoter because it is known that PPARβ/δ can inhibit proliferation of keratinocytes stimulated by phorbol ester (4–6), and thus influence the extent of tumor promotion. MNNG treatment was included in these studies because it is a direct mutagen that causes skin tumorigenesis independent of AHR signaling. Thus, it is of interest to note that in contrast to effects observed with B[*a*]P and DMBA, the tumor incidence and tumor multiplicity was greater in *Pparβ/δ*-null mice treated with the mutagen MNNG compared with wild-type and *Ahr*-null mice. This difference is consistent with the findings that PPARβ/δ inhibits cell proliferation in skin (1,3,4,6,9,10,12,16) and provides more support that PPARβ/δ can inhibit tumor promotion.

Combined, results from these studies demonstrate two distinct roles for PPARβ/δ in chemically induced skin cancer. Results from the complete carcinogen bioassay using either B[*a*]P or DMBA as the proximal carcinogen suggest that one plausible role is that PPARβ/δ is required for optimal AHR-dependent bioactivation of a chemical carcinogen in skin. Paradoxically, results from the complete carcinogen bioassay using MNNG as the proximal carcinogen that does not require AHR-dependent bioactivation suggest that PPARβ/δ can also have a different role and inhibit tumor promotion. The latter finding is consistent with previous studies showing PPARβ/δ-dependent inhibition of two-stage skin chemical carcinogenesis and malignant conversion (6,11,16,17). Future studies are needed to elucidate the mechanisms underlying these dual, opposing roles of PPARβ/δ in chemical skin carcinogenesis.

Supplementary material

Supplementary Materials and methods, Tables 1 and 2 and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/.

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