

Characterization of Peroxisome Proliferator–Activated Receptor α –Independent Effects of PPAR α Activators in the Rodent Liver: Di-(2-ethylhexyl) phthalate also Activates the Constitutive-Activated Receptor

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Peroxisome proliferator chemicals (PPC) are thought to mediate their effects in rodents on hepatocyte growth and liver cancer through the nuclear receptor peroxisome proliferator–activated receptor (PPAR) α . Recent studies indicate that the plasticizer di-(2-ethylhexyl) phthalate (DEHP) increased the incidence of liver tumors in PPAR α -null mice. We hypothesized that some PPC, including DEHP, induce transcriptional changes independent of PPAR α but dependent on other nuclear receptors, including the constitutive-activated receptor (CAR) that mediates phenobarbital (PB) effects on hepatocyte growth and liver tumor induction. To determine the potential role of CAR in mediating effects of PPC, a meta-analysis was performed on transcript profiles from published studies in which rats and mice were exposed to PPC and compared the profiles to those produced by exposure to PB. Valproic acid, clofibrate, and DEHP in rat liver and DEHP in mouse liver induced genes, including *Cyp2b* family members that are known to be regulated by CAR. Examination of transcript changes by Affymetrix ST 1.0 arrays and reverse transcription-PCR in the livers of DEHP-treated wild-type, PPAR α -null, and CAR-null mice demonstrated that (1) most (~94%) of the transcriptional changes induced by DEHP were PPAR α -dependent, (2) many PPAR α -independent genes overlapped with those regulated by PB, (3) induction of genes *Cyp2b10*, *Cyp3a11*, and *metallothionein-1* by DEHP was CAR dependent but PPAR α -independent, and (4) induction of a number of genes (*Cyp8b1*, *Gstm4*, and *Gstm7*) was independent of both CAR and PPAR α . Our results indicate that exposure to PPAR α activators including DEHP leads to activation of multiple nuclear receptors in the rodent liver.

Key Words: peroxisome proliferator–activated receptor α ; transcript profiling; liver cancer; di-(2-ethylhexyl) phthalate; constitutive-activated receptor; pregnane X receptor.

products, personal care products, automotive industries, and residential construction). Phthalate compounds have become ubiquitous environmental contaminants as they migrate out from PVC-containing items into food, air, dust, water, and soils (Clark *et al.*, 2003). Diet, particularly fatty food (e.g., fish, oils), is the main source of phthalate exposure in the general public (Clark *et al.*, 2003; Meek and Chan, 1994; Wormuth *et al.*, 2006). Other sources include blood storage bags (Labow *et al.*, 1986; Shintani, 1985) and kidney dialysis and blood transfusion equipment (Nassberger *et al.*, 1987; Sjoberg *et al.*, 1985). An increasing number of studies sampling human urine reveal the ubiquitous phthalate exposure of consumers in industrialized countries (Wormuth *et al.*, 2006). Concern over human exposure comes from the toxicological effects of phthalates, which include an increased incidence of liver tumors in mice and rats (Klaunig *et al.*, 2003) and irreversible changes in the development of the male reproductive tract (Corton and Lapinskas, 2005; Foster *et al.*, 2001).

Many phthalates belong to a large class of structurally diverse chemicals, including herbicides, industrial solvents, and pharmaceutical agents known as peroxisome proliferator chemicals (PPC). PPC refers to any chemical that when administered to rats and mice elicits a classic pleiotropic response in the liver, including a dramatic increase in the size and number of peroxisomes, induction of peroxisomal and microsomal fatty acid–oxidizing enzymes, and an increase in the size and number of hepatocytes (Moody *et al.*, 1991; Rao and Reddy, 1987). Sustained oral administration of most PPC at doses sufficient to induce moderate to marked peroxisome proliferation in rats and mice results in liver tumor formation (Reddy and Lalwani, 1983), most likely through a nongenotoxic mode of action (Klaunig *et al.*, 2003).

Phthalates, diesters of benzenedicarboxylic acid (phthalic acids), are high production volume chemicals used in numerous industrial applications (polyvinyl chloride [PVC]

Many of the effects of PPC are mediated by three members of the nuclear receptor superfamily called the peroxisome proliferator-activated receptors ([PPAR] α , β , and γ). The PPAR α subtype plays a dominant role in mediating the effects of hypolipidemic and xenobiotic PPC in the liver (Corton *et al.*, 2000; Klaunig *et al.*, 2003). Definitive evidence that PPAR α is involved in the short-term effects of PPC exposure comes from experiments performed in PPAR α -null mice (Lee *et al.*, 1995). PPAR α -null mice do not exhibit the classic short-term effects of PPC exposure, including peroxisome proliferation, hepatomegaly, induction of lipid-metabolizing enzymes such as *Cyp4a* family members, and increases in hepatocyte proliferation (summarized in Corton, 2009). The hypolipidemic agents WY-14,643 (WY) and bezafibrate increased liver tumors in wild-type but not in PPAR α -null mice (Hays *et al.*, 2005; Peters *et al.*, 1997). In contrast, exposure to one of the most commonly used phthalates in industry, di-(2-ethylhexyl) phthalate (DEHP), resulted in a low level of liver tumors in PPAR α -null but not in wild-type mice (Ito *et al.*, 2007). The lack of statistically significant increases in liver tumors in the wild-type mice may be due to the relatively low levels of DEHP in the diet fed to the mice in the Ito *et al.* study compared to other studies (Klaunig *et al.*, 2003). Additionally, PPAR α -null but not wild-type mice exhibited sustained increases in markers of oxidative stress after DEHP exposure (Ito *et al.*, 2007; Takashima *et al.*, 2008). These data demonstrate that PPAR α -independent biological events underlie the DEHP-induced mouse liver tumors in PPAR α -null mice.

PPC including phthalates may induce transcriptional effects in the liver through other nuclear receptors. These candidate nuclear receptors include constitutive activated androstane receptor (CAR) and pregnane X receptor (PXR), which regulate the expression of subsets of xenobiotic-metabolizing enzymes (XME) in response to exposure to drugs and environmental chemicals (Stanley *et al.*, 2006; Timsit and Negishi, 2007). Activators of CAR and PXR, such as phenobarbital (PB) and pregnenolone-16- α -carbonitrile (PCN), respectively, regulate an overlapping set of XMEs, including members of the *CYP2B* and *CYP3A* families and growth regulatory genes in the rat and mouse liver (Nelson *et al.*, 2006). Like PPC, exposure to CAR or PXR activators can lead to increases in liver weight and hepatocyte hyperplasia that are abolished in mice nullizygous for the individual receptors (Chen *et al.*, 2003; Huang *et al.*, 2005; Staudinger *et al.*, 2001; Yamamoto *et al.*, 2004). Chronic exposure to the CAR activators PB or 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) leads to increases in liver cancer in wild-type but not in CAR-null mice (Huang *et al.*, 2005; Yamamoto *et al.*, 2004).

Evidence is accumulating that phthalate ester plasticizers activate CAR and PXR. In transactivation assays, monoethylhexyl phthalate (MEHP), the primary metabolite of DEHP, activated mouse and human PXR (Hurst and Waxman, 2004)

and mouse CAR (Baldwin and Roling, 2009). DEHP activated human PXR (Cooper *et al.*, 2008; Takeshita *et al.*, 2001) and a splicing variant of human CAR (CAR2) (DeKeyser *et al.*, 2009). DEHP also increased the protein expression of *Cyp3a11*, a PXR target in livers of both wild-type and PPAR α -null mice (Fan *et al.*, 2004). Di-n-butyl phthalate (DBP) activated CAR and PXR in transactivation assays (Wyde *et al.*, 2005). DBP increased the expression of *CYP2B1* and *CYP3A1* messenger RNA (mRNA) or protein in male and female rats (Fan *et al.*, 2004) as well as dams and gestation day 19 fetuses (Wyde *et al.*, 2005). In addition to phthalates, other PPC likely activate CAR or PXR. Exposure to PPAR α -null mice to perfluorooctanoic acid (PFOA) results in a transcript profile similar to that of PB (Rosen *et al.*, 2008a,b). Perfluorodecanoic acid was shown to regulate the CAR target gene and protein *Cyp2b10* in wild-type but not in CAR-null mice (Cheng and Klaassen, 2008). The ability of PPC including DEHP to activate gene expression through nuclear receptors other than PPAR α has not been comprehensively evaluated.

Toxicogenomic analyses in which global gene expression profiles of chemicals with different modes of action are directly compared can be invaluable to make predictions as to the pathway basis for toxicity. Given the evidence that DEHP increases liver tumors in the absence of PPAR α (Ito *et al.*, 2007) and indications that DEHP and other PPC can activate CAR and PXR, we compared the transcript profiles produced by structurally diverse PPC to known activators of CAR and PXR in rats and mice. We tested the role of PPAR α and CAR in mediating the effects of DEHP directly by examining responses in wild-type, PPAR α -null mice, and CAR-null mice. Overall, the data demonstrate that a number of PPC including DEHP exhibit transcriptional responses independent of PPAR α but dependent on CAR.

MATERIALS AND METHODS

Animal studies. The first study was carried out at U.S. Environmental Protection Agency (EPA), Research Triangle Park, NC, and utilized wild-type and PPAR α -null male mice 7.5 months \pm 2.5 weeks of age. PPAR α -null mice (129S4/SvJae-*Ppara*^{tm1Gonz}/J, stock #003580) and wild-type mice (129S1/SvImJ, stock #002448) were originally purchased from Jackson Laboratory (Bar Harbor, ME) and maintained as an inbred colony on the 129/Sv background at the U.S. EPA. PPAR α -null and wild-type male mice used in the study were housed one per cage and were allowed to acclimate for a period of 1 week prior to the start of the study. Food (LabDiet 5001; PMI Nutrition International, St Louis, MO) and municipal tap water were provided *ad libitum*. Municipal tap water was filtered through sand and charcoal to reduce organic material, remove chlorine, and most of the chloramine. Chlorine was added back to the filtered water to maintain bacteria-free drinking water for the animals. Animal facilities were controlled for temperature (20°C–24°C) and relative humidity (40–60%) and kept under a 12-h light-dark cycle. The mice were a kind gift from Dr. Barbara Abbott. Exposure studies were similar to those published earlier (Currie *et al.*, 2005) in which 1150 mg/kg gave robust transcriptional responses in wild-type mice. A dose of 200 mg/kg was selected as it was more similar to that used earlier (Ito *et al.*, 2007). Wild-type and

TABLE 1
Characteristics of the Studies Used in the Rat and Mouse Liver Microarray Analysis

Species	Reference	GEO accession number	Strain	Dose frequency	Chemical (dose) ^a	Time of treatment	Vehicle	Array Type	Replicates	Number of .cel files
Rat	Nelson <i>et al.</i> (2006)	GSE14712	SD	Once daily	PB (100) or PCN (100)	6 h, 1 day, 5 days	1% Tween 80 in 0.5% aqueous methylcellulose	RGU34A	5	80
Rat	Ellinger-Ziegelbauer <i>et al.</i> (2005)	GSE14712	SD (Wistar)	Once daily	WY (60)	1 day, 3 days, 7 days	Carboxymethyl-cellulose	RAE230A	3	12
Rat	Jolly <i>et al.</i> (2005)	GSE2303	SD	Once	DEHP (20,000) or VPA (2000)	4, 24, 48 h	Distilled water	U34A	3–5	16 (DEHP) and 15 (VPA)
Rat	Jolly <i>et al.</i> (2005)	GSE2303	SD	Once	CLO (1000)	4, 24, 48 h	0.9% saline	U34A	3–5	16
Mouse	www.nursa.org/10.1621/datasets.01003		Wild type and CAR-null	Once	TCPOBOP (3)	3 days	Corn oil	430_2	2	8
Mouse	www.nursa.org/10.1621/datasets.01003		Wild type and CAR-null	Once daily	PB (100)	3 days	Corn oil	430_2	2	8
Mouse	Sanderson <i>et al.</i> (2008)	GSE8292, GSE8295	SV129 and PPAR null	Once daily	WY ^b	6 h, 5 days		430_2	3–5 (6 h) and 4 (5 days)	17 (6 h) and 16 (5 days)
Mouse	Currie <i>et al.</i> (2005)		C57Bl/6	Once daily	DEHP (1150)	2, 8, 24 h, 3 days	Corn oil	430_2	3	24

Note. All exposure experiments were conducted on male rats or mice by gavage. SD, Sprague-Dawley.

^aAll doses are in milligrams per kilogram per day.

^b400 µl of a 10 mg/ml solution/day.

PPAR α -null mice were given one daily gavage dose of DEHP (200 or 1150 mg/kg/body weight per day) in corn oil or corn oil alone each day for 4 days.

The second study was carried out at the University of Kansas Medical Center (Kansas City, KS). Eight-week-old adult C57BL/6 mice were purchased from Jackson Laboratory to establish a breeding colony. Breeding pairs of CAR-null mice on the C57BL/6 background were obtained from Dr. Ivan Rusyn (University of North Carolina, Chapel Hill, NC), which were engineered by Tularik, Inc. (South San Francisco, CA), as described previously (Ueda *et al.*, 2002). CAR-null and C57BL/6 mice were administered DEHP identical to study 1.

Livers were removed 24 h after the last dose. Portions of the livers were rapidly snap frozen in liquid nitrogen and stored at -70°C until analysis. All animal studies were conducted under federal guidelines for the use and care of laboratory animals and were approved by Institutional Animal Care and Use Committees.

RNA isolation. Total RNA was isolated and purified from mouse livers according to the mirVana miRNA Isolation Kit (Ambion, Austin, TX), which isolates total RNA including microRNAs. The integrity of each RNA sample was determined using an Agilent 2100 Bioanalyzer (Agilent, Foster City, CA), and RNA quantity was determined using a Nanodrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE).

Microarray hybridization and analysis. Liver gene expression analysis was performed according to the Affymetrix-recommended protocol using Affymetrix Mouse ST v1.0 GeneChips containing probes for over 28,000 well-annotated genes. Total RNA (300 ng per sample) was labeled using the Affymetrix GeneChip WT cDNA Synthesis and Amplification kit protocol and hybridized to the arrays as described by the manufacturer (Affymetrix, Santa

Clara, CA). The complementary RNA hybridization cocktail was incubated overnight at 45°C while rotating in a hybridization oven. After 16 h of hybridization, the cocktail was removed and the arrays were washed and stained in an Affymetrix GeneChip fluidics station 450, according to the Affymetrix-recommended protocol. Arrays were scanned on an Affymetrix GeneChip scanner. Four mice per dose group were examined. A detailed description of the microarray experiment is available through Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information at <http://www.ncbi.nlm.nih.gov/geo/>, as accession number GSE18564. Data (.cel files) were analyzed and statistically filtered using X-Ray version 3.9934 software (www.biotiquesystems.com/Products-Solutions/XRAY/). Input files were normalized with full quantile normalization (Irizarry *et al.*, 2003). Statistically significant genes were identified using mixed model analysis of variance with a false discovery rate (Benjamini-Hochberg test) of $p \leq 0.05$. Fold-change values $< |\pm 1.3|$ were removed.

Reanalysis of published microarray data. A summary of the microarray studies reanalyzed is shown in Table 1. The doses selected in these studies would be expected to elicit close to a maximal transcriptional response. The raw data files analyzed in this project (.cel files from Affymetrix DNA chips) were either downloaded from GEO or communicated through the original authors. All the Affymetrix .cel files were first analyzed by Bioconductor SimpleAffy to assess data quality (Wilson and Miller, 2005). All .cel files passed this QC step. Data (.cel files) were background corrected and statistically filtered using Rosetta Resolver version 7.1 software (Rosetta Inpharmatics, Kirkland, WA). The background correction was done by Resolver's specific data processing pipeline (Affymetrix Rosetta-Intensity Profile Builder). Statistically significant genes were identified using one-way

ANOVA with a false discovery rate (Benjamini-Hochberg test) of ≤ 0.05 , followed by a *post hoc* test (Scheffe) for significance. Fold-change values $< |\pm 1.5|$ were removed. As most of the experiments in rats used the RG-U34A array, we compared profiles in the RG-U34A annotation file from Affymetrix (<http://www.affymetrix.com/analysis/index.affx>). We identified probe set IDs (a total of 8799) on the U34A chip that exhibited sequence similarity with those on the RAE230_2 chip using the “good match” comparison and then built fold-change values for those genes from the RAE230_2 chip, which were altered significantly. Signature genes were identified in the rat liver as those that were expressed in at least two of the three PB and PCN treatment groups (common genes), expressed in at least two of three PB groups but in only one or less of the PCN groups (CAR signature genes), or expressed in at least two of three PCN groups but in only one or less of the PB groups (PXR signature genes). Pearson’s correlation coefficients were generated in Excel (2003). CAR signature genes in the mouse liver were defined as those that were altered by both PB and TC in wild-type mice, and the fold changes were of greater magnitude than that in similarly treated CAR-null mice. Heat maps were generated using Eisen Lab Cluster and Treeview software (<http://rana.lbl.gov/EisenSoftware.htm>). A detailed description of each experiment is available through GEO at the National Center for Biotechnology Information at <http://www.ncbi.nlm.nih.gov/geo/>, as accession numbers indicated in Table 1.

Evaluation of selected genes by real-time reverse transcription-PCR. The levels of expression of selected genes were quantified using real-time reverse transcription-PCR (RT-PCR) analysis. Briefly, total RNA was reverse transcribed with murine leukemia virus reverse transcriptase and oligo(dT) primers. The forward and reverse primers for selected genes (Supplementary file 1) were designed using Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA). The SYBR green DNA PCR kit (Applied Biosystems) was used for real-time PCR analysis. The relative differences in expression between groups were expressed using cycle threshold (Ct) values as follows. The Ct values of the genes were first normalized with β -actin and glyceraldehyde 3-phosphate dehydrogenase of the same sample. Assuming that the Ct value is reflective of the initial starting copy and that there is 100% efficiency, a difference of one cycle is equivalent to a twofold difference in starting copy. Means and SE ($n = 4$) for RT-PCR data were calculated by Student’s *t*-test. The level of significance was set at $p \leq 0.05$.

RESULTS

Expression profiles were compared after exposure of rodents to a number of xenobiotics and drugs that activate well-characterized pathways of gene expression. The list of studies is shown in Table 1. To address whether CAR and/or PXR were activated upon exposure to DEHP and other PPC, the expression profiles induced by classical activators of CAR and PXR (PB and PCN, respectively) in the rat liver and activators of CAR in mouse liver (PB and TCPOBOP) were compared. PB and PCN transcript profiles in rat livers were compared to known activators of PPAR α , including two hypolipidemic compounds (WY and clofibrate [CLO]), an anti-epilepsy drug (valproic acid [VPA]) as well as DEHP. In mouse livers, PB, and TCPOBOP transcript profiles were compared to those induced by DEHP in wild-type mice and WY in both wild-type and PPAR α -null mice. WY is selective for the PPAR α subtype in liver; almost all genes regulated by WY were dependent on PPAR α in mice (Anderson *et al.*, 2004a,b; Rosen *et al.*, 2008a,b).

Similarities in the Transcriptional Profiles of PPAR α , CAR, and PXR Activators in the Rat Liver

The expression of *CYP* family members is often used as an indicator of xenobiotic activation of nuclear receptors. The expression of all *CYP* family members on the Affymetrix RU-U34A array that exhibited significant change(s) in at least two of the 18 treatment comparisons were compared (Supplementary file 2). Figure 1A shows the expression of *CYP* family members best known to be regulated by PPAR α , CAR, and PXR (*CYP4A*, *CYP2B*, and *CYP3A*, respectively). As expected,

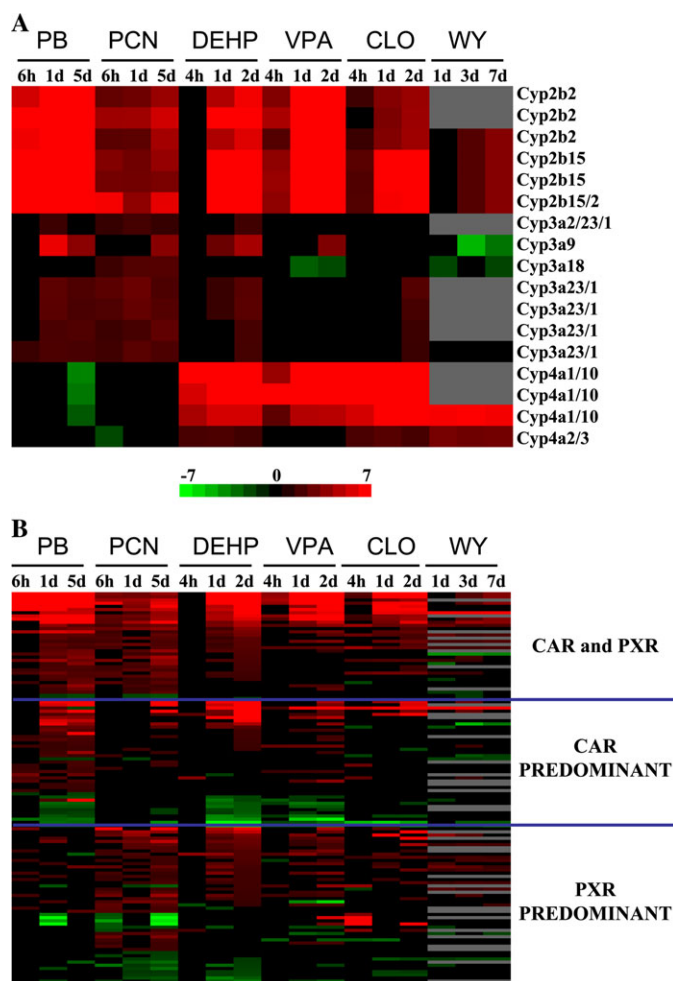


FIG. 1. Gene expression in rat liver after exposure to activators of CAR, PXR, and PPAR α . (A) Expression of *CYP* genes. *CYP2B*, *CYP3A*, and *CYP4A* family members significantly altered after chemical treatment in one or more of the comparisons were grouped by family. The profiles show that DEHP, VPA, and CLO exhibit increased expression of a number of *CYP* genes that overlap with PB and PCN (*CYP2B* and *CYP3A* family members) and WY (*CYP4A* family members). Genes indicated as pairs (e.g., *CYP4A1/10*) are interrogated by one Affymetrix probe set. (B) Comparison of genes regulated exclusively by PB, PCN, or both PB and PCN. Genes that were regulated by PB and/or PCN were identified, and their expression pattern was examined across all the treatment groups. The intensity scale indicates fold change due to chemical exposure relative to controls for A and B.

TABLE 2
Correlation Coefficients between Transcriptional Changes after Exposure to the Indicated Chemicals in the Rat Liver

	DEHP	VPA	CLO	WY	PCN
PB	0.66	0.72	0.51	0.04	0.41
PCN	0.46	0.42	0.41	0.15	

Note. Pearson's correlation coefficients were generated as described in the "Materials and Methods" section.

the PPC, DEHP, VPA, CLO, and WY but not PB and PCN exposure led to increased expression in *CYP4A* family members. DEHP, VPA, and CLO activated *CYP2B* family members that were also activated by PB and to a lesser extent by PCN. WY was a weak inducer of only *CYP2B15*. DEHP also activated *CYP3A* family members that were activated by both PB and PCN. CLO activated *CYP3A23/1* only at 2 days, and WY decreased the expression of *CYP3A9*, *CYP3A18*, and *CYP3A23/1*. Overall, the expression profiles indicate that DEHP, VPA, and CLO exhibit the ability to alter the expression of *CYP* genes in multiple families that are regulated by PPAR α and CAR.

The expression of CAR and/or PXR signature genes including *CYP* genes as detailed in the "Materials and Methods" section were compared to the expression of these genes after exposure to various PPAR α activators (Fig. 1B). Correlation coefficients were generated for these comparisons between 1-day treatment groups. DEHP, VPA, and CLO but not the WY treatment groups exhibited strong correlations with the PB and to a lesser extent the PCN groups (Table 2). DEHP, VPA, and CLO when compared to the PB group gave higher correlation coefficients than when PB was compared to the PCN group. These data indicate that under these exposure conditions, DEHP, VPA, and CLO but not WY treatments exhibit transcriptional similarities to classical CAR and PXR inducers.

Overlap in Genes Regulated by DEHP and Activators of CAR in Mouse Liver

To investigate the possibility that DEHP regulates gene expression similar to activators of CAR in mouse liver, the profiles from wild-type mice treated with DEHP were compared to those from livers of mice exposed to CAR activators. Wild-type and CAR-null mice were administered the CAR activators PB or TCPOBOP. As expected, DEHP and WY treatment in wild-type mice resulted in strong induction of *Cyp4a* family members (Fig. 2A). DEHP-treated wild-type mice also induced expression of *Cyp2b9* and *Cyp2b10* at 8 h and 3 days but not at 1 day. *CAR* and regulated genes such as *Cyp2b10* exhibit circadian regulation; *CAR* expression peaks near the time of the 8-h sacrifice (Zhang *et al.*, 2009). We

speculate that the relatively weak induction of *Cyp2b10* by DEHP in wild-type mice was enhanced at 8 h due to the increased expression of CAR. DEHP exposure led to inconsistent induction of *Cyp3a* family members. In contrast, WY treatment downregulated expression of *Cyp2b* and *Cyp3a* family members.

Genes attributed to CAR activation were identified (i.e., genes coordinately regulated by PB and TCPOBOP in a CAR-dependent manner), and of those 244 genes, the correlation between PB and DEHP or WY treatment groups was determined. DEHP treatment for 2 h, 8 h, 1, day, or 3 days gave correlation coefficients of 0.11, 0.83, 0.03, and 0.21, respectively, with genes altered by PB treatment. WY treatments at 6 h or 5 days gave coefficients of 0.05 and -0.19 , respectively, with genes altered by PB treatment. The expression of 43 genes that were expressed in at least two of the four DEHP treatment groups showed a high degree of concordance to expression of CAR signature genes especially the 8-h DEHP treatment group (Fig. 2B). WY but not DEHP treatment altered the expression of a number of genes opposite to that of PB and TCPOBOP, including *Cyp2b10*, *Gsta1/a2*, and *Ces6*. Overall, these results indicate that DEHP treatment induces a gene expression pattern that overlaps with CAR activators.

PPAR α Is Required for the Majority of Transcriptional Changes after DEHP Exposure

To uncover the gene regulatory effects that may contribute to liver tumor induction by DEHP, we exposed wild-type and PPAR α -null mice to 200 or 1150 mg/kg of DEHP once each day for 4 days. Liver to body weights increased only in the wild-type mice exposed to 1150 mg/kg/day but not to 200 mg/kg/day (Fig. 3A). Global gene expression changes were assessed using Affymetrix ST 1.0 mouse chips containing ~28,000 genes. There were 475 genes that exhibited altered expression by DEHP in wild-type mice of which only 27 (~6% of 475) were also differentially expressed in PPAR α -null mice after DEHP exposure (Fig. 3B, Supplementary file 3). All but 3 of the 27 genes exhibited similar regulation in the wild-type and PPAR α -null mice (Fig. 3C). The 27 genes included those involved in xenobiotic metabolism and cholesterol biosynthesis. In the absence of PPAR α , DEHP exposure altered an additional 163 genes (Fig. 3B). Thus, this analysis revealed that PPAR α controls the expression of most (~94%) of the genes regulated by DEHP in wild-type mice. The absence of PPAR α leads to the altered regulation of additional genes.

Characterization of the PPAR α -Independent Genes Regulated by DEHP

The functional categories of DEHP-regulated genes between wild-type and PPAR α -null mice were compared. Ingenuity Pathways Analysis showed statistical significance of altered changes in groups of genes involved in fatty acid metabolism,

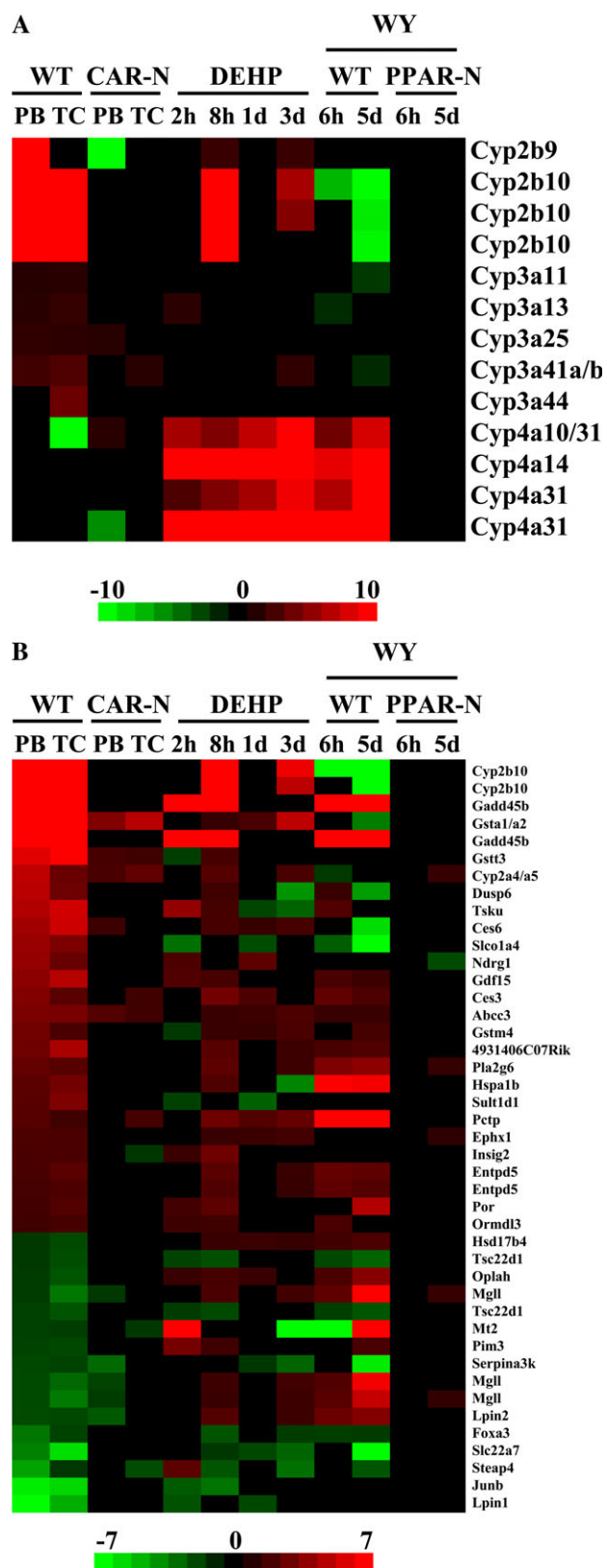


FIG. 2. Gene expression in mouse liver after exposure to activators of CAR, PXR, and PPAR α . (A) Expression of *Cyp* genes. *Cyp2b*, *Cyp3a*, and *Cyp4a* family members that were significantly altered after chemical treatment

tryptophan metabolism, valine, leucine and isoleucine degradation, butanoate metabolism, propanoate metabolism, and β -alanine metabolism in wild-type mice and to a lesser extent in PPAR α -null mice, whereas xenobiotic metabolism signaling, metabolism of xenobiotics by cytochrome P450, and biosynthesis of steroids were unaffected by genotype. The previous analysis of genes regulated by PFOA in PPAR α -null mice demonstrated that many lipid-metabolizing genes, especially those involved in fatty acid β -oxidation, were similarly regulated in PFOA-treated wild-type and PPAR α -null mice (Rosen *et al.*, 2008a,b). In contrast, the consistent upregulation of these genes by DEHP was PPAR α dependent, with the exception of *Elovl3* that was altered only in PPAR α -null mice (Fig. 4A).

Many genes involved in cholesterol biosynthesis were uniformly upregulated by DEHP in PPAR α -null mice (Fig. 4B). Given the observation of a similar induction of cholesterol biosynthetic genes after exposure to a RXR pan-agonist (AGN194,204; AGN) (Anderson *et al.*, 2004a), the expression of these genes was compared between DEHP and AGN. Many of these cholesterol biosynthetic genes were regulated by DEHP and AGN in a PPAR α -independent manner, including 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (*Hmgcs1*), mevalonate (diphospho) decarboxylase (*Mvd*), farnesyl diphosphate farnesyl transferase 1 (*Fdft1*), isopentenyl-diphosphate delta isomerase (*Idi1*), and sterol-C4-methyl oxidase-like (*Sc4mol*). These results indicate that DEHP regulates many of the cholesterol biosynthetic genes in a manner similar to an RXR agonist.

As shown previously for PFOA, DEHP altered the expression of many XMEs independently of PPAR α . Most of these XMEs were regulated exclusively in PPAR α -null mice (Fig. 4C). The expression of all DEHP-regulated PPAR α -independent genes were compared to those regulated by the two CAR activators, PB or TCPOBOP (TC), from a previous study (www.nursa.org/10.1621/datasets.01003). Very little concordance was observed except for a number of XMEs that were almost uniformly upregulated (Fig. 4C, arrowheads). Additional non-XME genes also exhibited concordance including hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 5 (*Hsd3b5*), NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2 (*Ndufb2*), and programmed cell death 6 (*Pdcd6*), which were downregulated by DEHP

in one or more of the comparisons were grouped by family. Genes regulated by both PB and TCPOBOP were identified and their expression was compared to the same genes after four different times of DEHP exposure in wild-type mice or WY treatment for 6 h or 5 days in wild-type and PPAR α -null mice. The profiles show that DEHP exhibits increased expression of a number of *Cyp* genes that overlap with PB and PCN (*Cyp2b* and *Cyp3a* family members) and WY (*Cyp4a* family members). The changes due to WY were PPAR α dependent. (B) Comparison of CAR genes to those regulated by DEHP and WY. The intensity scale indicates fold change due to chemical exposure relative to controls.

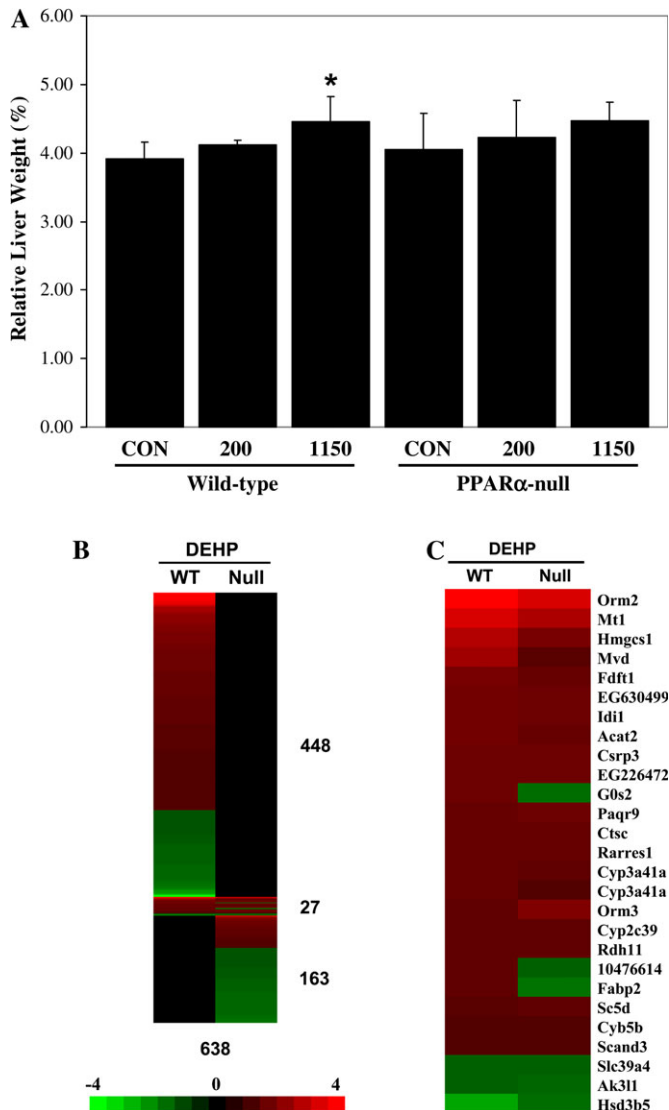


FIG. 3. Identification of PPAR α -dependent and -independent genes regulated by DEHP in the mouse liver. (A) Liver to body weights in wild-type and PPAR α -null mice after exposure to DEHP. (B) Genes regulated by DEHP in wild-type and PPAR α -null mice. Significantly altered genes were identified as described in the “Materials and Methods” section. The genes were rank ordered based on their fold change after exposure to DEHP in wild-type or PPAR α -null mice. There were a total of 475 and 190 genes that were significantly altered by DEHP in wild-type (WT) or PPAR α -null (Null) mice, respectively. Of these, 448 and 163 genes were uniquely altered in wild-type or PPAR α -null mice, respectively. Twenty-seven genes exhibited altered expression in both mouse strains. (C) Genes regulated by DEHP in both wild-type and PPAR α -null mice. The intensity scale in B indicates fold change due to DEHP exposure relative to controls in B and C.

and either PB or TCPOBOP (data not shown). It cannot be ruled out that the lack of concordance is due to the comparison of results between two microarray platforms, i.e., mouse 430_2 versus mouse ST v1.0. Many of the XMEs were upregulated by DEHP in PPAR α -null but not in wild-type mice and by PB and

TC in a CAR-dependent manner, indicating that at least a subset of the XMEs regulated by DEHP are CAR dependent.

CAR-Dependent Transcriptional Changes Induced by DEHP

To directly test the hypothesis that a subset of DEHP-regulated genes are mediated by CAR, we exposed wild-type and CAR-null mice once each day to 200 or 1150 mg/kg/day DEHP for 4 days. Induction of liver weights was observed in the wild-type mice at both doses and the CAR-null mice at the highest dose (Fig. 5A). RT-PCR analysis of gene expression of PPAR α and CAR target genes revealed genes that can be divided into three classes, based on PPAR α - and/or CAR-dependent induction of expression. DEHP induction of acyl-Coenzyme A oxidase 1, palmitoyl (*Acox1*) exhibited partial dependence of induction on PPAR α . Induction in wild-type mice at the highest dose was approximately fivefold compared to approximately twofold in PPAR α -null mice. Partial induction of *Acox1* and other genes involved in fatty acid catabolism was observed in PPAR α -null mice by PFOA (Rosen *et al.*, 2008a,b) and was attributed to minor activation of other PPAR subtypes. *Acox1* induction was not affected by CAR genotype (Fig. 5B). A number of genes including *Cyp2b10*, *Cyp3a11*, *Cyp3a41a*, and metallothionein 1 (*Mt1*) were induced by DEHP that was dependent on CAR but not on PPAR α (Fig. 5C). *Cyp2b10* and *Cyp3a11* are known targets of CAR from previous studies (Maglich *et al.*, 2002). Metallothionein protein levels were increased by DEHP but not by PB in mouse liver (Waalkes and Ward, 1989). The expression of the *CAR* gene itself was increased by DEHP in PPAR α -null but not in wild-type mice. A number of putative CAR and PXR targets exhibited PPAR α - and CAR-independent induction, including *Cyp8b1*, *Gstm4*, and *Gstm7* (Fig. 5D). These results indicate that DEHP requires CAR for induction of a subset of genes.

DISCUSSION

DEHP Induces Gene Expression through Multiple Nuclear Receptors

A feature of PPAR α activators is their structural heterogeneity, leading to the hypothesis that a subset of these compounds could interact with other nuclear receptors. Consistent with this, the present study identified PPAR α and CAR as targets of DEHP indirectly through meta-analysis of transcript profiles of livers from rats treated with nuclear receptor activators and directly through transcriptional analysis in wild-type mice and mice nullizygous for these nuclear receptors. Microarray analysis of PPC-treated rats showed an overlap in the profiles of DEHP-, VPA-, and CLO-treated rats with classical activators of CAR and to a lesser extent an activator of PXR. The overlapping genes included *CYP* gene families that are often considered signature genes for nuclear receptor activation. Consistent with these findings, VPA was

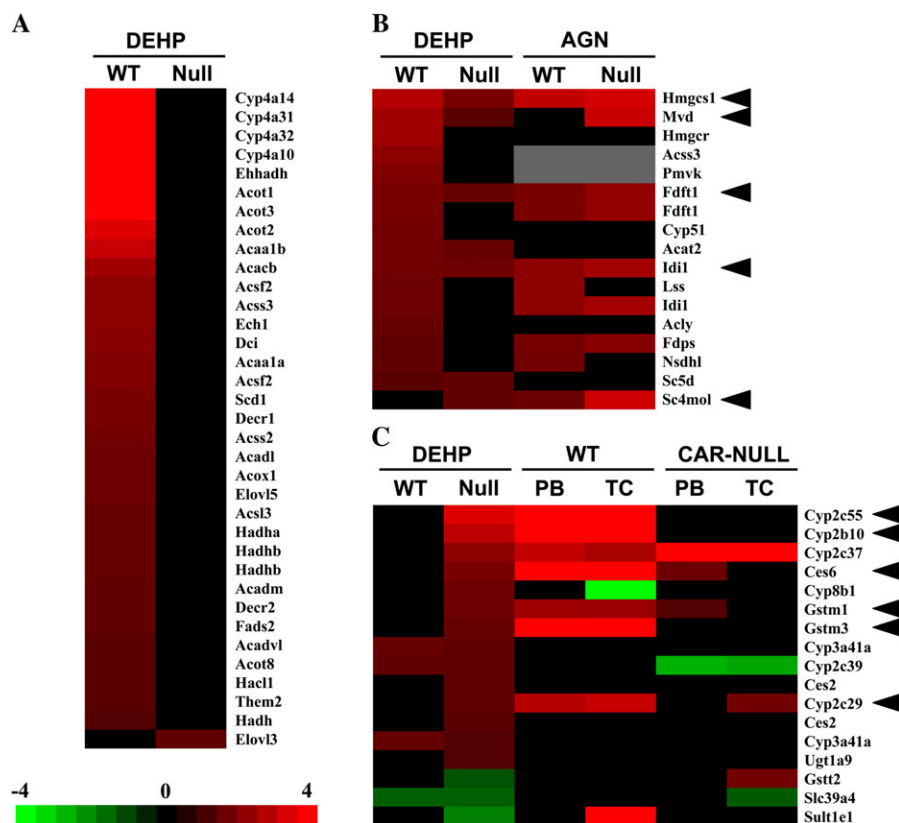


FIG. 4. Functional analysis of DEHP-regulated genes. (A) Fatty acid catabolism and synthesis genes. All but *Elov13* were regulated by DEHP in a PPAR α -dependent manner. (B) Cholesterol biosynthetic genes. Genes involved in cholesterol biosynthesis altered by DEHP in wild-type or PPAR α -null mice were compared to those regulated by the RXR pan-agonist AGN194,204 (AGN) in wild-type and PPAR α -null mice. Many of the genes were regulated by DEHP or AGN independently of PPAR α (arrowheads). (C) Xenobiotic metabolism genes. XME genes that exhibited altered regulation in PPAR α -null mice were rank ordered and compared to the regulation by PB or TCPOBOP (TC) in wild-type and CAR-null mice. Arrowheads indicate genes that exhibit increased expression by DEHP in PPAR α -null mice and by both PB and TC in wild-type but not in CAR-null mice. Genes represented more than once were identified through the analysis of different probe sets. The intensity scale in A indicates fold change due to DEHP exposure relative to controls in A–C.

recently shown to activate CAR and PXR in transactivation assays (Cervený *et al.*, 2007). In contrast to the other PPC, WY treatment in rats resulted in little or no overlap with genes induced by CAR and PXR activators, providing evidence that WY is more PPAR α -specific. A comparison of DEHP-treated wild-type and PPAR α -null mice revealed that PPAR α is required for ~94% of all transcriptional changes in wild-type mice. The PPAR α -dependent genes were the typical target genes of PPC identified in many other studies and included those involved in fatty acid β -oxidation (Anderson *et al.*, 2004a,b; Wong and Gill, 2002; Woods *et al.*, 2007). The remaining 6% of the genes were dominated by those involved in xenobiotic metabolism, which are known target genes of CAR or PXR (Stanley *et al.*, 2006; Timsit and Negishi, 2007), and cholesterol biosynthesis, which are regulated by a number of transcription factors, including RXR (Anderson *et al.*, 2004a). XMEs, including *Cyp2b10*, *Cyp3a11*, and *Cyp3a41a* as well as *Mtl*, were induced by DEHP that was partially or completely dependent on CAR but not on PPAR α . *Cyp2b10* is primarily a CAR target gene but can be induced by PXR

activation (Maglich *et al.*, 2002). In the present study, DEHP induced *Cyp2b10* in CAR-null mice to low levels possibly through PXR activation. The results of the present study are consistent with a recent study in which an array of 320 nuclear receptor-targeted genes was used to show that in mouse liver, most DEHP-regulated genes were PPAR α -dependent and more specifically the induction of *Cyp2b10* was PPAR α -independent (Eveillard *et al.*, 2009). The identification of *Cyp8b1*, *Gstm4*, and *Gstm7* as genes regulated by DEHP independent of PPAR α and CAR indicates the existence of a class of XMEs possibly regulated by PXR or the oxidant-activated transcription factor nuclear factor erythroid 2-related factor 2.

Other environmentally relevant PPC, such as PFOA (Rosen *et al.*, 2008a,b) and PFOS (Rosen, Corton, Schmid, Zehr, Das, Abbott, and Lau, in preparation), exhibit similar transcriptional features as DEHP. PPAR α is required for 85 and 92% of the genes regulated by PFOA and PFOS, respectively. WY, originally developed as a hypolipidemic drug, requires PPAR α for > 99% of the transcriptional changes in wild-type mice (Rosen *et al.*, 2008a,b). The activation of CAR and/or PXR

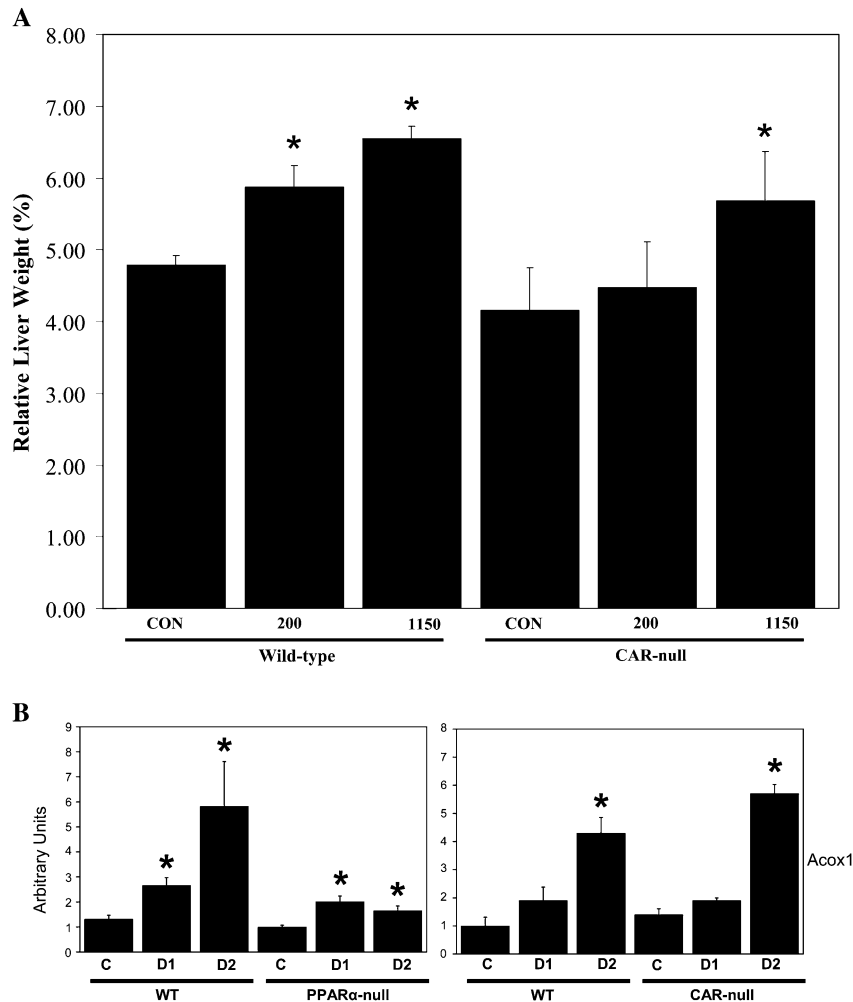


FIG. 5. DEHP responses in wild-type and CAR-null mice. Wild-type and CAR-null mice were treated with DEHP (200 [day 1] or 1150 mg/kg [day 2]) by gavage for 4 days. (A) Liver to body weights. (B–D) Gene expression in the livers of DEHP-treated wild-type, PPAR α -null, and CAR-null mice determined by RT-PCR. (B) Induction of *Acox1* by DEHP is partially dependent on PPAR α but not on CAR. (C) Induction of *Cyp2b10*, *Cyp3a11*, *Cyp3a41a*, and *Mt1* is dependent on CAR but not on PPAR α . The expression of the *CAR* gene is also shown. (D) PPAR α - and CAR-independent induction of *Cyp8b1*, *Gstm4*, and *Gstm7* by DEHP. There were four animals per treatment group, and each sample was analyzed in duplicate. Variability is expressed as SEM. Means and SE ($n = 4$) for RT-PCR data were calculated by Student's *t*-test. The level of significance was set at $p \leq 0.05$. *Indicates statistically significant change relative to control animals of the same genotype.

in vivo by DEHP was not unexpected because DEHP or its proximate metabolite MEHP *trans*-activate CAR or PXR *in vitro* (Baldwin and Roling, 2009; Cooper *et al.*, 2008; DeKeyser *et al.*, 2009; Hurst and Waxman, 2004; Takeshita *et al.*, 2001). Taken together, the present results provide direct evidence that DEHP, and likely other PPC, regulate gene expression in liver through PPAR α and CAR. Similar experiments in wild-type and PXR-null mice will be useful to determine the extent of involvement of PXR.

Interactions between PPAR α and CAR

Functional antagonism between transcription factors involved in opposing biological functions is a common molecular mechanism for regulation of gene expression. There is a growing body of evidence that PPAR α and CAR have

antagonist properties. In the absence of PPAR α , CAR expression levels were increased (Martin *et al.*, 2007) and PB-induced hyperplasia was greater when compared to that in wild-type mice (Columbano *et al.*, 2001). The transcript profiles of DEHP-treated mice in the present study showed that the mRNA of a number of XMEs that are known CAR target genes were induced to a greater extent by DEHP in PPAR α -null mice than in wild-type mice. Using RT-PCR *Cyp2b10* and *Mt1* as well as the *CAR* gene itself were induced more in PPAR α -null mice than in wild-type mice. An increase in CAR regulation of gene expression in the absence of PPAR α was also observed in a microarray comparison between wild-type and PPAR α -null mice after PFOA (Rosen *et al.*, 2008a,b) and PFOS (Rosen, Corton, Schmid, Zehr, Das, Abbott and Lau, in preparation) treatments. In the reverse situation, absence of

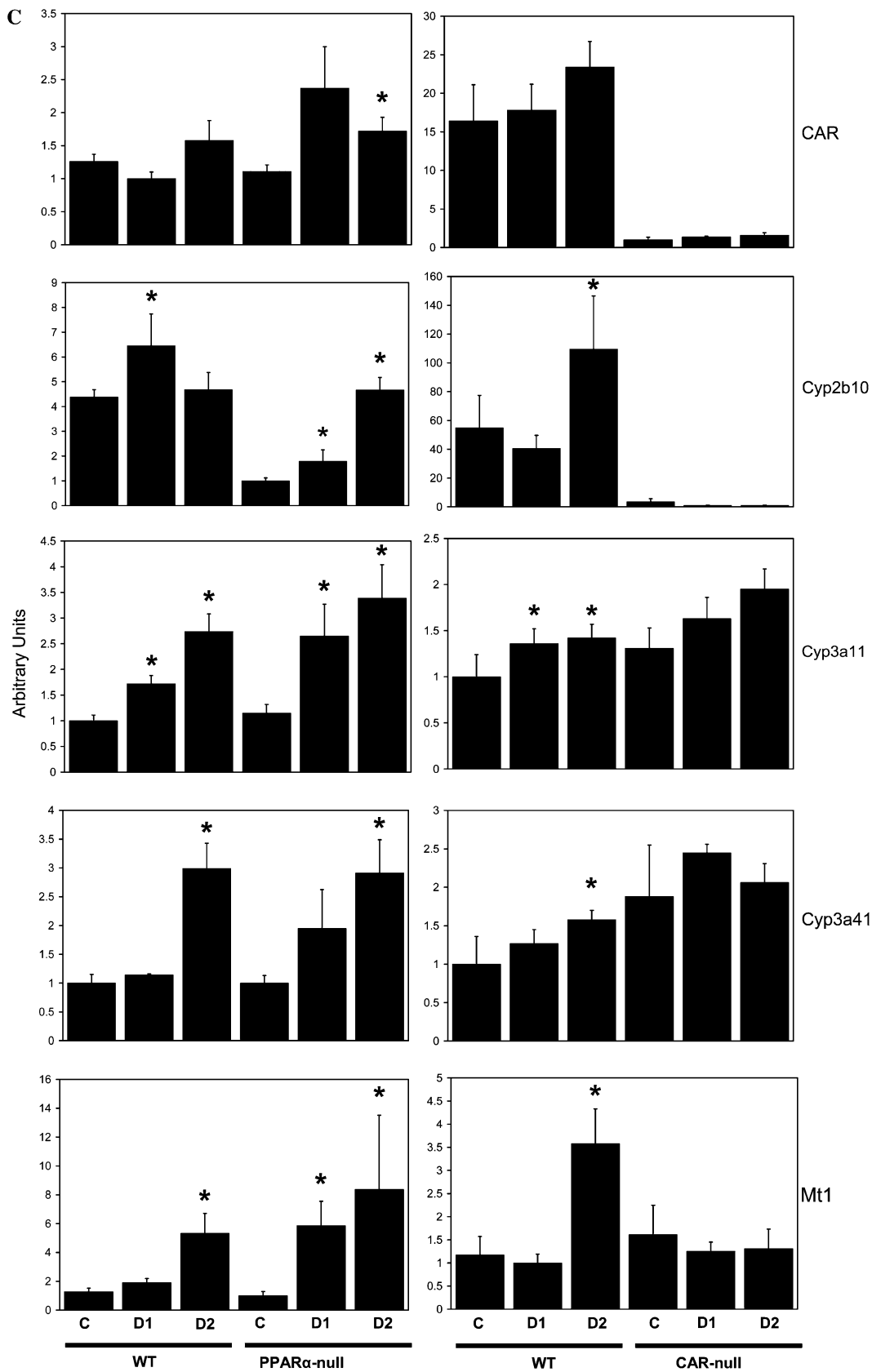


FIG. 5. Continued

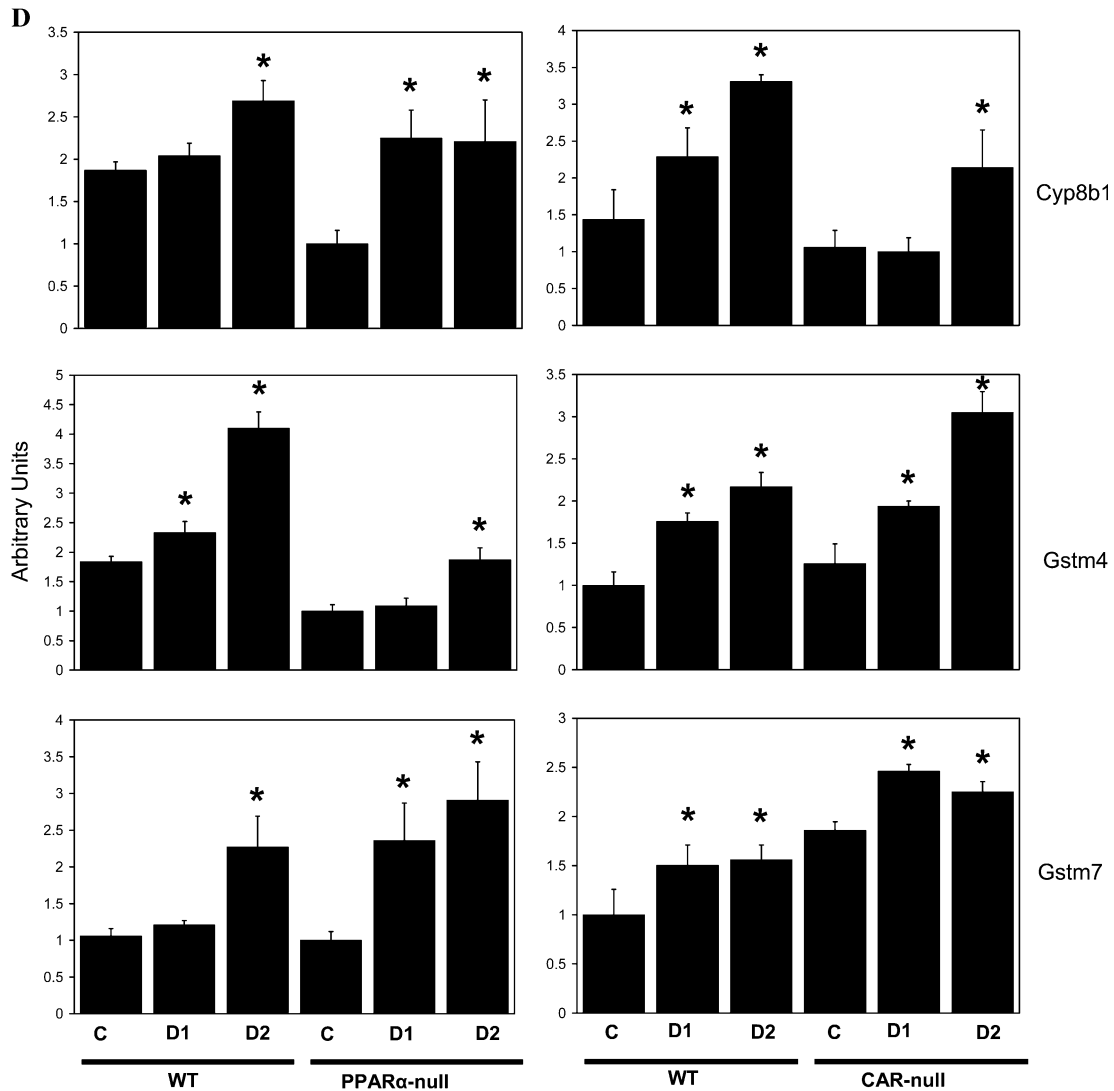


FIG. 5. Continued

CAR may lead to increased induction of PPAR α -dependent responses. CAR-null mice exhibited increased hepatic fatty acid β -oxidation typically controlled by PPAR α ; treatment of wild-type mice with TCPOBOP decreased expression of PPAR α mRNA as well as PPAR α target genes in liver (Maglich *et al.*, 2009).

Functional antagonism between PPAR α and CAR can occur at a number of levels. PPAR α agonists may act as CAR antagonists, much like the CAR reverse agonist androstanol; exposure to WY or ciprofibrate leads to CAR nuclear localization but not target gene induction (Guo *et al.*, 2007). PPAR α and CAR may also compete for a number of shared coactivators, including PPAR-binding protein, PRIC320, and PGC1 α (Corton and Brown-Borg, 2005; Jia *et al.*, 2005; Mäkinen *et al.*, 2002; Surapureddi *et al.*, 2006), but apparently not the shared heterodimer partner, RXR (Guo *et al.*, 2007).

Furthermore, CAR-RXR heterodimers from mice and humans can bind and *trans*-activate at a PPAR-RXR DNA-binding site, and in humans, this transactivation does not require the CAR DNA-binding domain (Guo *et al.*, 2007; Stoner *et al.*, 2007). Further work is required to clarify the functional significance of PPAR α and CAR antagonistic effects.

Mode of Action of Liver Tumor Induction by DEHP

Chronic exposure to DEHP resulted in a low level of liver tumors in PPAR α -null mice but in not wild-type mice (Ito *et al.*, 2007). DEHP induction of tumors in PPAR α -null mice is in part the basis for an argument against a PPAR α mode of action in wild-type mice (Guyton *et al.*, 2009). However, there are a number of findings that argue against this view. First, the transcript profile comparison in the present study showed that the vast majority of genes altered by DEHP in wild-type mice

were not similarly altered in PPAR α -null mice. Thus, the DEHP-induced tumors in wild-type mice could only be PPAR α independent if the ~6% of the PPAR α -independent gene changes were responsible for the tumors, an unlikely scenario given the magnitude of the PPAR α -dependent effects. Second, wild-type and PPAR α -null mice exhibited differences in DEHP-induced carcinogenesis. DEHP did not induce equivalent levels of tumors in the wild-type and PPAR α -null mice; there were no statistically significant increases in liver tumors in the wild-type mice under these exposure conditions (200 ppm). DEHP increased the expression of growth control genes in PPAR α -null mice but not in wild-type mice at equivalent doses (Ito *et al.*, 2007). Transcript profiling and RT-PCR showed highly dissimilar changes in gene expression in the liver tumors from the wild-type and PPAR α -null mice, indicating different molecular mechanisms of their origins (Takashima *et al.*, 2008). Lastly, in the absence of PPAR α , DEHP altered a unique set of genes not similarly altered in wild-type mice (Fig. 3B). Some of these genes are known targets of CAR (Fig. 4C). Taken together, these data indicate that although DEHP can induce marginal increases in liver tumors in PPAR α -null mice, the mode of action is different from that in wild-type mice. The transcriptional responses induced by DEHP in wild-type and nullizygous mouse strains indicates that CAR is important in the induction of tumors in PPAR α -null mice.

CAR and Endocrine Modulation by DEHP

Activation of CAR may lead to a number of extrahepatic effects due to CAR-dependent regulation of genes that metabolize thyroid and steroid hormones. CAR controls phase II genes uridine 5'-diphosphate-glucuronosyltransferases (UGT) and sulfotransferases (SULT) involved in the metabolism of thyroid hormones (Klaassen and Hood, 2001; Kretschmer and Baldwin, 2005). PB increases metabolism of thyroid hormones and leads to a decrease in thyroid hormone levels followed by compensatory increases in thyroid stimulating hormone, thyroid epithelial hyperplasia, and tumors (Klaassen and Hood, 2001; McClain *et al.*, 1988; Qatanani *et al.*, 2005). CAR activation by DEHP is consistent with increases in thyroid hyperplasia and hypertrophy observed in rats (Elcombe *et al.*, 2002; Price *et al.*, 1988). The indication that increased exposure to DEHP metabolites was associated with decreases in thyroid hormone in men (Meeker *et al.*, 2007) indicates that the relationships between DEHP exposure, CAR activation, and alteration in thyroid hormone metabolism should be further characterized.

A growing number of endocrine disrupting chemicals including pesticides activate PXR and sometimes CAR resulting in increases in the expression of steroid hydroxylases, including *CYP2B* and *CYP3A* family members, potentially perturbing normal steroid metabolism (Kretschmer and Baldwin, 2005). DEHP activates the CAR-regulated gene *CYP2B6* in human primary hepatocytes (Eveillard *et al.*, 2009;

Goyak *et al.*, 2008). PB exhibits classical antiandrogen effects in developing rats, including reduced ano-genital distance and delayed testicular descent (Gupta *et al.*, 1980) overlapping with the male reproductive tract defects induced by active phthalates (summarized in Corton and Lapinskas, 2005). Whether there are increases in fetal liver steroid hydroxylases after exposure to CAR inducers has not been adequately explored for linkage to decreases in testosterone levels. In humans, an association has been noted between treatment of pregnant mothers with CAR activators, PB or phenytoin, and a higher risk for developmental abnormalities, such as undescended testes and genital malformations in male offspring (Dessens *et al.*, 2001). Workers exposed to high levels of DEHP and DBP had associated decreases in serum-free testosterone (Pan *et al.*, 2006). Additional studies are required to determine the significance of endocrine disruption by DEHP through different nuclear receptors, including CAR.

Conclusions

The present analysis of gene expression in wild-type and PPAR α -null mice demonstrate that like other PPAR α activators, including WY, PFOA, and PFOS, DEHP transcriptional responses are overwhelmingly dependent on PPAR α . A minor number of genes in wild-type mice including *Cyp2b10* are dependent on CAR for activation. In the absence of PPAR α , CAR-dependent effects are likely important. The chronic activation of the CAR mode of action in the PPAR α -null mice likely underlies the low level of liver tumor induced by DEHP in these mice.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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