

Neonatal Activation of the Xenobiotic-Sensors PXR and CAR Results in Acute and Persistent Down-regulation of PPAR α -Signaling in Mouse Liver

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ABSTRACT

Safety concerns have emerged regarding the potential long-lasting effects due to developmental exposure to xenobiotics. The pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are critical xenobiotic-sensing nuclear receptors that are highly expressed in liver. The goal of this study was to test our hypothesis that neonatal exposure to PXR- or CAR-activators not only acutely but also persistently regulates the expression of drug-processing genes (DPGs). A single dose of the PXR-ligand PCN (75 mg/kg), CAR-ligand TCPOBOP (3 mg/kg), or vehicle (corn oil) was administered intraperitoneally to 3-day-old neonatal wild-type mice. Livers were collected 24 h post-dose or from adult mice at 60 days of age, and global gene expression of these mice was determined using Affymetrix Mouse Transcriptome Assay 1.0. In neonatal liver, PCN up-regulated 464 and down-regulated 449 genes, whereas TCPOBOP up-regulated 308 and down-regulated 112 genes. In adult liver, there were 15 persistently up-regulated and 22 persistently down-regulated genes following neonatal exposure to PCN, as well as 130 persistently up-regulated and 18 persistently down-regulated genes following neonatal exposure to TCPOBOP. Neonatal exposure to both PCN and TCPOBOP persistently down-regulated multiple Cyp4a members, which are prototypical-target genes of the lipid-sensor PPAR α , and this correlated with decreased PPAR α -binding to the Cyp4a gene loci. RT-qPCR, western blotting, and enzyme activity assays in livers of wild-type, PXR-null, and CAR-null mice confirmed that the persistent down-regulation of Cyp4a was PXR and CAR dependent. In conclusion, neonatal exposure to PXR- and CAR-activators both acutely and persistently regulates critical genes involved in xenobiotic and lipid metabolism in liver.

Key words: neonatal exposure; PXR; CAR; PPAR α ; drug-processing genes.

Liver is the major organ for xenobiotics biotransformation and nutrient homeostasis. Many Phase-I and Phase-II drug-metabolizing enzymes, as well as transporters (together called “drug-processing genes” [DPGs]) are highly expressed in liver (Fu et al., 2016; Parkinson et al., 2013). The expression of DPGs is transcriptionally regulated by nuclear receptors, such as the pregnane X receptor (PXR/Nr1i2) and constitutive androstane receptor (CAR/Nr1i3), which are the major xenobiotic sensors that are activated by a wide range of natural and synthetic

compounds (Aleksunes and Klaassen, 2012; Hernandez et al., 2009; Kliewer et al., 2002; Moore et al., 2003). In liver, PXR and CAR trans-activate a large battery of genes involved in xenobiotic biotransformation, including their prototypical target genes CYP3A/Cyp3a and CYP2B/Cyp2b, respectively (Aleksunes and Klaassen, 2012; Gao and Xie, 2010; Salem et al., 2013; Topletz et al., 2013). There are species differences in PXR and CAR between mice and humans. For example, the pregnenolone-16 α -carbonitrile (PCN) is a prototypical ligand for PXR in mice,

whereas the antibiotic drug rifampicin is a specific activator for PXR in humans. The mouse CAR is activated by the chemical 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP), whereas the human CAR is activated by CITCO (Romano et al., 1986; Swales and Negishi, 2004). In addition to modulate xenobiotic metabolism, PXR and CAR are also involved in the regulation of glucose, fatty acid and lipid metabolism (Gao and Xie, 2010; Wada et al., 2009). However, relatively less is known regarding the exact molecular target(s) that these xenobiotic sensors interact with in intermediary metabolism. The peroxisome proliferator-activated receptor (PPAR α) is an important lipid-sensing nuclear receptor that is known to regulate the expression of numerous genes involved in lipid and glucose metabolism (Kassam et al., 2000). The prototypical target genes of PPAR α are the CYP4A family members, which are important for the fatty acid oxidation in the endoplasmic reticulum (Sanderson et al., 2009). An absence of PPAR α in mice increased lipids and blood pressure, decreased insulin sensitivity, and promoted atherosclerosis (Tordjman et al., 2001).

During liver development, many DPGs display distinct ontogenic expression patterns. For example, CYP1A2 enzyme activity in 1-year-old human is only 50% of adults (Hines and McCarver, 2002). CYP2A6 and CYP2B6 activities are not detectable in humans before birth, but are markedly increased after birth (Hines and McCarver, 2002). The CYP3A7 enzyme is highly expressed in fetal liver microsomes but decreases rapidly during the first week after birth. Conversely, CYP3A4 is minimally expressed before birth but increases rapidly thereafter and reaches 50% of adult levels between 6 and 12 months of age (Hines, 2013; Hines and McCarver, 2002). Regarding transporters, hepatic expression of MRP2, OATP1B1 and OATP1B3 in all pediatric age groups is significantly lower than in adults, whereas MDR1 mRNA expression in fetuses, neonates, and infants is lower than in adults (Mooij et al., 2014). In mouse livers, this distinct ontogenic expression pattern of DPGs has also been characterized (Cui et al., 2012; Lu et al., 2013; Peng et al., 2012, 2013). The unique ontogenic expression of DPGs leads to altered pharmacokinetics and toxicokinetics of chemicals in newborns and children, making them at a much higher risk of adverse drug reactions as compared to adults.

Neonatal exposure to drugs or environmental chemicals have been associated with increased risks of various diseases such as cancer, diabetes, and obesity in adults (Blake et al., 2005; Jirtle and Skinner, 2007). We have recently demonstrated in mouse liver that the inducibility of DPGs at various ages is age-specific following acute exposure to PXR and CAR ligands (Li et al., 2015); however, very little is known on a genomic scale regarding the potential long-lasting effects following the neonatal exposure to xenobiotics that are PXR or CAR activators. It was thought that xenobiotic exposure at neonatal ages may persistently alter the chromatin epigenetic architecture and subsequently alter the expression of genes that are involved in xenobiotic biotransformation and other critical metabolic processes (Chen et al., 2012; Tien et al., 2015). However, a systematic investigation regarding the effect of neonatal CAR activation on expression of DPGs in liver is lacking; there is also little information regarding the potential effect of neonatal activation of PXR on expression of DPGs in liver during and beyond the neonatal period. Therefore, the goal of the present study was to test the hypothesis that neonatal exposure to PXR- and CAR-activators not only produces acute but also persistent changes in the expression of genes involved in xenobiotic and/or intermediary metabolism.

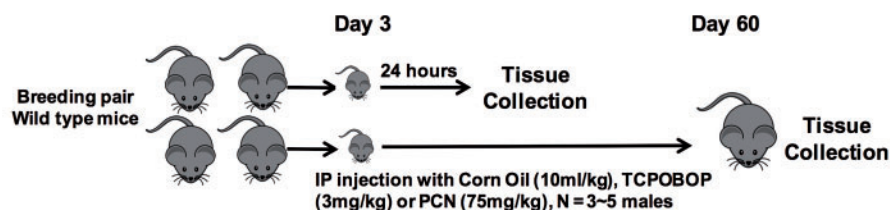
MATERIALS AND METHODS

Chemicals. The 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) and pregnenolone-16 α -carbonitrile (PCN) were purchased from Sigma-Aldrich (St Louis, Missouri).

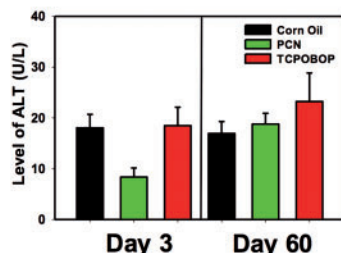
Animals. Eight-week-old wild-type (WT) C57BL/6J mouse breeders were purchased from Jackson Laboratories (Bar Harbor, Maine). Breeder pairs of PXR-null mice on the C57BL/6 background were kindly provided by Dr. Frank Gonzalez (National Cancer Institute, Bethesda, Maryland). Breeder pairs of the CAR-null mice in the C57BL/6 background were obtained from Amgen (Thousand Oaks, California). The PXR-null and CAR-null mice were described previously (Aleksunes and Klaassen, 2012), and were backcrossed 10 generations in the C57BL/6 background. Mice were housed according to the American Animal Association Laboratory Animal Care Guidelines, and were bred under standard conditions at the University of Washington. All animals were given *ad libitum* access to water and irradiated Picolab Rodent Diet 20 number 5053 (PMI Nutrition International, Brentwood, Missouri). At 3 days of age, the offspring of WT mice were intraperitoneally injected with a single dose of the prototypical PXR activator PCN (75 mg/kg), the CAR activator TCPOBOP (3 mg/kg), or vehicle (corn oil, 10 ml/kg); the offspring of PXR-null mice were injected with PCN or corn oil; and the offspring of CAR-null mice were injected with TCPOBOP or corn oil, following the same dosing regimen, as described in Figure 1A. A single dose of PCN (75 mg/kg) was selected and TCPOBOP (3 mg/kg) was selected as the model ligands for PXR and CAR activation in 3-day-old mice because this dose has been used in newborn and adult mice in other publications (Chen et al., 2012; Cui et al., 2009; Diwan et al., 1992; Ghaffari et al., 2011; Lickteig et al., 2016; Romano et al., 1986; Wagner et al., 2005; Wei et al., 2000). Newborn mice from different litters were randomly assigned to different treatment groups. For each treatment group, the neonatal mice from at least 3 litters were collected. Livers were collected 24 h post-dose (day 3) or from 60-day-old adult mice (day 60), immediately frozen in liquid nitrogen, and stored at -80°C . The 60-day age was selected based on the previous findings of the ontogeny of drug-processing genes, when the liver has grown to a mature metabolic organ (Peng et al., 2012; Tien et al., 2015). To avoid the complication of estrous cycles in females, only male pups were used in the present study ($n = 3-5$ per group). All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington.

Total RNA isolation. Total RNA was extracted from frozen livers using RNeasy lysis reagent (Qiagen, Crawfordsville, Indiana) according to the protocol of the manufacturer. For the newborn mice (day 3), total RNA was extracted using the whole liver, whereas for the adult mice (day 60), total RNA was extracted from a randomly selected section of liver (around 30–50 mg of frozen liver). RNA concentration was quantified using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, Delaware) at 260 nm. The integrity of each RNA sample was evaluated by formaldehyde agarose gel electrophoresis to visualize the 18S and 28S rRNA bands. For the microarray study, the RNA quality was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, California), and the samples with RNA Integrity Number (RIN) above 8.0 were used.

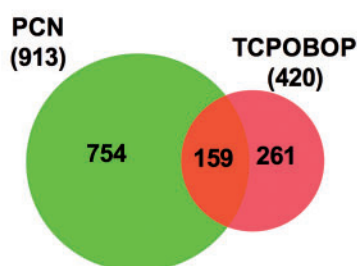
A. Study design



B. Serum ALT



C. Day 3



D. Day 60

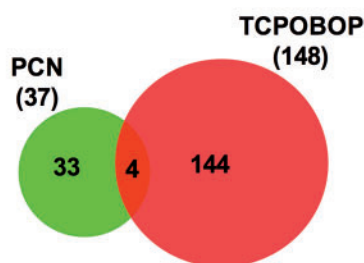


FIG. 1. A, Study design. B, Serum alanine aminotransferase (ALT) level in the blood of wild-type (WT) mice (at both day 3 and day 60) after neonatal exposure to corn oil, 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) or pregnenolone-16 α -carbonitrile (PCN). C, The Venn diagram of genes that were commonly or uniquely differentially expressed by PCN or TCPOBOP at 3-day of age in the livers of WT mice. D, The Venn diagram of genes that were commonly or uniquely differentially expressed by PCN or TCPOBOP at 60-day of age in the livers WT mice (differentially expressed genes are defined as >2-fold change and adjusted $P < 0.05$).

Microarray and data analysis. Gene expression profiling in livers of 3- and 60-day-old WT mice that were treated with corn oil, PCN, or TCPOBOP at 3 days of age were performed by the Functional Genomics Core Facility at the University of Washington using the Affymetrix Mouse Transcriptome Array 1.0. Three biological replicates per group were hybridized to individual arrays. The raw fluorescence data in CEL files were processed for quality assessment using the Affymetrix Expression Console™ Software 1.4 incorporating the Robust Multi-array Average (RMA) normalization algorithm. According to the quality control metrics, the fluorescence signal intensity across the probes for each array exhibited similar distributions. The log-transformed expression levels of each gene in each array to its median value across all the arrays were below 0.5, indicating the absence of outliers in the dataset. The area-under-the-curve of positive versus negative controls was above 0.8 for all arrays, which reflect the robustness of the array quantification.

The CHP files generated from Affymetrix Expression Console were analyzed using the Transcriptome Analysis Console (TAC) 3.0 to determine the differential expression, based on the adjusted ANOVA P -value (< 0.05) and a fold-change > 2 between the comparison groups. Genes that were differentially expressed were plotted in a Venn diagram, to identify the common and uniquely expressed genes for each treatment (PCN or TCPOBOP) at either day 3 or day 60. A 2-way hierarchical clustering dendrogram (Ward's minimum variance method, distance scale) was conducted on the DPGs that were differentially expressed in the PCN- or TCPOBOP-treated group compared with the corn oil-treated group of the same age, using JMP 12.0 software (SAS Institute, Cary, North Carolina). Functional and pathway analysis of differentially expressed genes was performed using the Core Analysis feature of Ingenuity Pathway Analysis (IPA, Ingenuity Systems), and the top signaling networks were determined.

RT-qPCR quantification of DPGs. DPGs that were differentially regulated by PCN or TCPOBOP at either day 3 or day 60 were confirmed by RT-qPCR in both the WT and the receptor (PXR or CAR) knockout mice. The total RNAs of male mouse livers from WT, PXR-null, or CAR-null mice collected at day 3 and day 60 ($n=3-5$ per group) were reverse-transcribed into cDNAs using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, California). The resulting cDNA products were amplified by qPCR, using the Sso Advanced Universal SYBR Green Supermix in a Bio-Rad CFX384 Real-Time PCR Detection System (Bio-Rad, Hercules, California). The primers for all qPCR reactions were synthesized by Integrity DNA Technologies (Coralville, Iowa), and primer sequences are shown in [Supplemental Table 3](#). Data are expressed as % of the expression of the housekeeping gene β -actin.

Western blotting. The liver homogenates were prepared using sucrose-tris buffer (250 mM sucrose, 10 mM Tris base, pH 7.5) with protease inhibitors. The crude membranes were prepared as described previously (Aleksunes et al., 2006), from WT, PXR-null, and CAR-null mice at 60-day of age. The protein concentrations were determined using the Qubit Protein Assay Kit (Thermo Fisher Scientific, Grand Island, New York) according to the instructions of the manufacturer. The samples (50 μ g of protein) were subjected to polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked in phosphate-buffered saline with 0.05% Tween 20 (PBST) with 5% non-fat dry milk for 1 h and incubated overnight with one of the following primary antibodies diluted in PBST with 1% non-fat dry milk: (1) rabbit anti-mouse Cyp2b10 (AB9916, 1:5000, EMD Millipore, Billerica, Massachusetts); (2) goat anti-mouse Cyp4a14 (SC-46087, 1:500, Santa Cruz, Dallas, Texas). Primary antibody against mouse β -actin (ab8227, 1:1000) and Histone H3 (ab72079, 1:500) were purchased from Abcam (Cambridge, Massachusetts) and used as loading controls. Membranes were then incubated with a horseradish peroxidase (HRP)-linked species-appropriate secondary antibody at 1:2000 (Sigma Aldrich, St. Louis, Missouri). Proteins were detected using the Novex ECL Chemiluminescent Substrate Reagent Kit (Life Technologies, Carlsbad, California). Intensities of the protein bands were quantified using the Image J Software (National Institutes of Health, Bethesda, Maryland).

Enzyme activities. The P450 enzyme activities in liver microsomes of WT, PXR-null, and CAR-null mice at 60-day of age were quantified using the P450-Glo™ Screening system (Promega, Madison, Wisconsin). Briefly, the liver microsomes were isolated using differential centrifugation as described previously (Pelkonen et al., 1974). Frozen liver samples were weighed and transferred to a Teflon pestle/glass homogenizer with 5 volumes (w/v) ST buffer (250 mM sucrose, 10 mM Tris base, pH 7.5) containing protease inhibitor cocktail (1:100). The homogenates were centrifuged at 10 000 g for 10 min at 4 °C. The supernatants were transferred to clean centrifuge tubes and centrifuged at 100 000 g for 60 min at 4 °C. The pellets were washed with 1 ml ST buffer, and dissolved in 100 μ l of ST buffer with protease inhibitor. P450 reactions (50 μ l) were performed in white opaque 96-well plates. Briefly, a luminogenic P450-Glo™ substrate (3 μ M Luciferin-2B6 for CYP2B/Cyp2b, and 50 μ M Luciferin-4A for CYP4A/Cyp4a) was incubated at 37 °C with 10 μ g of liver microsomal protein, or negative control membrane (devoid of CYP activity) for 10 min. The reactions were initiated by adding the NADPH-regeneration system and incubating for 10 min. At the

end of the incubation, an equal volume of the luciferin detection reagent (50 μ l) was added at room temperature, and luminescence was quantified 20 min later using a Glomax™ 96 Microplate Luminometer (Promega Corporation, Madison, Wisconsin). The magnitude of the luminescent signal is dependent on and directly proportional to the amount of luciferin product generated by the P450 reaction.

Serum alanine aminotransferase, serum, and hepatic triglyceride quantification. Serum samples were analyzed by standard enzymatic-colorimetric assays using alanine aminotransferase (ALT) and triglyceride (TG) kits according to the protocols of the manufacturer (Pointe Scientific, Canton, MI). The absorbance was quantified spectrophotometrically at wavelengths of 340 and 540 nm, respectively. Hepatic TGs were extracted using 100 mg frozen livers from WT (corn oil, TCPOBOP, PCN), CAR-null (corn oil, TCPOBOP), and PXR-null (corn oil, PCN) mice at 60-day of age. The liver tissues were homogenized in ice cold isopropanol, and then centrifuged at 2000 \times g for 10 min at 4 °C. The TG content was collected from the supernatant and quantified according to the manufacturer's protocols (Pointe Scientific, Canton, MI).

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed using approximately 200 mg of frozen livers from WT mice (corn oil, TCPOBOP, PCN) at 60-day of age, using the MAGnify Chromatin Immunoprecipitation System (Life Technologies, Grand Island, New York) with minor modifications. Briefly, livers were finely minced into less than 1 mm³ cubes using razor blades in cold 1 \times Dulbecco's Phosphate-Buffered Saline (D-PBS) in a sterile 10 cm culture dish on ice, and transferred into an ice-cold Dounce homogenizer (VWR International, Radnor, Pennsylvania) to further grind the liver into a homogenous solution with a glass pestle. Samples were subjected to cross-linking using freshly prepared formaldehyde (a final concentration of 1%), and were rotated for 20 min at room temperature using an ELMI Intelli Mixer (ELMI Company, Riga, Latvia). The cross-linking was quenched with glycine (a final concentration of 0.125 M) with rotation for 5 min at room temperature, followed by centrifugation to collect the pellets. The pellets were washed with cold D-PBS, and re-suspended using cold ChIP lysis buffer with a protease inhibitor cocktail (Sigma Aldrich, St. Louis, Missouri), rotated at 4 °C for 15 min, and centrifuged to obtain the pellets. The pellets were re-suspended in ChIP nuclear lysis buffer with protease inhibitors, and incubated on ice for 15 min. Chromatin was then fragmented into 300–500 bp average size-range using a Bioruptor UCD200 connected to a water-cooling system (Diagenode, Denville, New Jersey). The sonication condition was 10 \times (30 s on + 30 s off) at 4 °C, and was repeated after 10 min cool-down, at the highest intensity. The fragment size was confirmed by electrophoresis. ChIP-grade antibodies, namely NB600-636 (Novus Biologicals, Littleton, Colorado) for PPAR α and MMS-126R for RNA polymerase II (Covance, Emeryville, California) were used for immunoprecipitation. An IgG antibody (ab18413, Abcam, Cambridge, Massachusetts) was used as a negative control. The immunoprecipitation procedures are described in detail per the protocol of the manufacturer (MAGnify Chromatin Immunoprecipitation System, Life Technologies, Grand Island, New York).

ChIP-qPCR primer design and qPCR reactions. Positive PPAR α -genomic DNA binding sites were obtained by re-analyzing the ChIP-Seq data in control WT male mouse livers from a previous

TABLE 1. Genes Differentially Expressed by Neonatal Exposure to PCN or TCPOBOP in the Livers of 3-day-old and 60-day-old WT Mice (with >2-Fold Change and Adjusted $P < 0.05$)

Age (Days)	Comparison	Number of Genes Up-regulated	Number of Genes Down-regulated	Total Number of Gene Change
3	PCN vs CO	464	449	913
3	TCPOBOP vs CO	308	112	420
60	PCN vs CO	15	22	37
60	TCPOBOP vs CO	130	18	148

publication (Lee et al., 2014). Nuclear-receptor enrichment peaks were visualized by the Integrated Genome Viewer (IGV) (Robinson et al., 2011). The qPCR primers were designed around the targeted motifs using NCBI Primer Design Tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), and their specificities were confirmed using UCSC BLAT (<https://genome.ucsc.edu/cgi-bin/hgBlat?command=start>). The promoter sequences of Cyp4a clusters were retrieved using the Mammalian Promoter Database (MPromDb, Gupta et al., 2011). The qPCR primers for RNA-Pol-II were designed using the queried promoter sequences. Real-time qPCR reactions of the ChIP DNA were performed using SsoAdvanced Universal SYBR Green Supermix in a Bio-Rad CFX384 Real-Time PCR Detection System (Bio-Rad, Hercules, California). The qPCR primer sequences, targeted genomic regions, as well as putative motifs have been described in a previous publication (Selwyn et al., 2015).

Statistical analysis. Data are presented as mean \pm SEM. Differences among multiple groups were determined using an analysis of variance (ANOVA) followed by Duncan's Post Hoc test ($P < .05$).

RESULTS

Global Changes in Gene Expression Profiles Following Neonatal PXR or CAR Activation

As described in the study design (Figure 1A), to determine the acute and the potentially persistent alterations in the expression of hepatic transcriptome following neonatal PXR or CAR activation, a single dose of corn oil (CO), PCN or TCPOBOP was intraperitoneally injected to mice at 3-day of age, and the livers were collected 24 h post-dose (day 3) or from adult mice at 60 days of age (day 60). The serum ALT levels were within the normal range for all groups, indicating that there were no apparent hepatic injuries (Figure 1B).

The differential gene expression was determined using the Affymetrix Mouse Transcriptome 1.0 Array. A total of 65 956 transcripts were detected in the microarray analysis. As compared with the vehicle-treated group of the same age, neonatal exposure to PCN altered the expression of 913 genes in WT mouse livers at day 3, whereas neonatal exposure to TCPOBOP altered the expression of 420 genes (Figure 1C). Among the differentially regulated genes 24 h after exposure to PCN or TCPOBOP, 159 genes were co-regulated by both PCN and TCPOBOP; whereas 754 genes were uniquely regulated by PCN, and 261 genes were uniquely regulated by TCPOBOP (Figure 1C). Interestingly, at day 60, there were 37 and 148 genes of which the differential regulation persisted following neonatal exposure to PCN and TCPOBOP, respectively. Among these persistently regulated genes in adult liver, there were 4 genes that were co-regulated by neonatal exposure to both PCN and

TCPOBOP, whereas 33 genes were uniquely regulated by PCN, and 144 genes were uniquely regulated by TCPOBOP (Figure 1D). Together these data indicated that neonatal exposure to PCN or TCPOBOP produced not only acute but also persistent changes in the gene expression of mouse livers during development.

In livers of 3-day-old mice, there were 464 up-regulated and 449 down-regulated genes 24 h after PCN exposure (Table 1). Ingenuity Pathway Analysis (IPA) identified that at day 3, the differentially regulated genes 24 h after PCN exposure were mainly related to (1) drug metabolism, lipid metabolism, small molecule biochemistry; (2) energy production, lipid metabolism, small molecule biochemistry; (3) endocrine system development and function, small molecule biochemistry, and lipid metabolism; (4) carbohydrate metabolism; and (5) RNA post-transcriptional modification, cell-to-cell signaling and interaction, skeletal and muscular system development, and function (Supplemental Table 1). In contrast, in livers of 60-day-old adult mice, there were 15 persistently up-regulated genes and 22 persistently down-regulated genes following neonatal exposure to PCN (Table 1). According to the IPA analysis, the majority of these genes were involved in the following IPA networks: (1) connective tissue disorders, inflammatory disease, skeletal, and muscular disorders; (2) cancer, organismal injury and abnormalities, reproductive system diseases; (3) hematological system development and function, inflammatory response, tissue morphology; (4) hereditary disorder, neurological disease, cancer; and (5) cell cycle, cell death and survival, cellular assembly and organization (Supplemental Table 1). The IPA Top Tox (Toxicological) lists of PCN exposure revealed several events persistently occurring throughout development, such as fatty acid metabolism, cytochrome P450 panel, and lipopolysaccharide (LPS)/interleukin-1 (IL-1) mediated inhibition of retinoid X receptor (RXR) function. PCN-regulated gene alteration was also linked to Nrf2-mediated oxidative stress response at day 3, whereas at day 60, it appeared to be highly correlated with acute renal failure panel (Supplemental Table 2).

In livers of 3-day-old mice, there were 308 up-regulated genes and 112 down-regulated genes 24 h after TCPOBOP exposure. Ingenuity pathway analysis identified that the top differentially regulated genes 24 h after TCPOBOP exposure were involved in (1) drug metabolism, lipid metabolism, small molecule biochemistry; (2) molecular transport; (3) endocrine system development and function; and (4) cellular development, cellular growth and proliferation, digestive system development, and function (Supplemental Table 1). The Top Tox lists of TCPOBOP exposure shared a great similarity with those shown in the PCN-treated group in 3-day-old mouse livers (Supplemental Table 2). In contrast, in livers of 60-day-old adult mice, there were 130 persistently up-regulated genes and 18 persistently down-regulated genes following neonatal exposure to TCPOBOP (Table 1). The differentially regulated genes at day 60 were highly related to (1) gene expression, lipid metabolism,

small molecular biochemistry; and (2) cancer, hematological disease, and immunological disease (Supplemental Table 1). The Top Tox lists of TCPOBOP exposure at day 60 included glutathione depletion-hepatocellular hypertrophy, cytochrome P450 panel, Nrf2-mediated oxidative stress response, and LPS/IL-1 mediated inhibition of RXR function (Supplemental Table 2). Data are available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.0k90t>

Comparative Analysis of Gene Expression Between PCN and TCPOBOP

Considering that PXR and CAR have been shown to share some common target genes in xenobiotic and drug metabolism, it was of interest to identify those genes that were co-regulated by both TCPOBOP and PCN in the same or opposite direction (referred as “common”), and the genes that were regulated by PCN or TCPOBOP only (referred as “unique”). In each category, the top 10 up-regulated or down-regulated genes are listed in Table 2 (day 3) and Table 3 (day 60) according to their fold change (using PCN-induced fold change for the common genes).

By comparing the differentially expressed genes between PCN and TCPOBOP at day 3, we identified 159 common genes, of which 85 genes were up-regulated and 74 genes were down-regulated by both PCN and TCPOBOP, whereas the genes *Cyp2c54* and zinc finger imprinted 1 (*Zim1*) were up-regulated by TCPOBOP but down-regulated by PCN (Table 2). Of the 85 commonly up-regulated genes, cytochrome P450s (*Cyp2b10* and *Cyp2c55*), glutathione S-transferase (*Gstm3*), indolethylamine N-methyltransferase (*Inmt*), and hepatic hydroxysteroid sulfotransferase (*Sult2a5*) are responsible for xenobiotic and steroid metabolism, whereas others are widely associated with cell proliferation (transmembrane glycoprotein Prominin 1 (*prom1*)) (Takenobu et al., 2011), cell signaling (sulfatase 2 (*Sulf2*)) (Rosen and Lemjabbar-Alaoui, 2010), glucocorticoids transportation (serine peptidase inhibitor member 6 (*Serpina6*)), skeletal homeostasis (proteoglycan 4 (*Prg4*)) (Novince et al., 2012), as well as RNA transcription and splicing (Schlafen 9 (*Slfn9*)) (Mavrommatis et al., 2013) (Table 2). In contrast, neonatal exposure to PCN and TCPOBOP also down-regulated a series of genes, such as *Cyp4a* family members (*Cyp4a10*, *Cyp4a31*, and *Cyp4a32*), the Major urinary protein (*Mup*) family members (*Mup1*, *Mup2*, *Mup7*, *Mup10*, *Mup12*, and *Mup19*), which regulate glucose and energy metabolism (Zhou et al., 2009), as well as the prokineticin 1 (*Prok1*), which modulates cell proliferation and differentiation in immune system (Ngan et al., 2007) (Table 2).

We also identified 754 unique genes that were only regulated by PCN (including 379 up-regulated and 375 down-regulated genes), and 261 unique genes that were only regulated by TCPOBOP (including 221 up-regulated genes and 40 down-regulated genes) in neonatal mouse livers (Table 2). IPA analysis of the 754 PCN uniquely-regulated genes revealed several important networks. The top differentially regulated networks include (1) lipid metabolism, small molecule biochemistry, and drug metabolism (Figure 2); (2) energy production, lipid metabolism, and small molecule biochemistry (Supplemental Figure 1A); (3) cardiovascular disease, hematological disease, and hereditary disorder (Supplemental Figure 1B); (4) cell cycle, cellular assembly and organization, DNA replication, recombination, and repair (data not shown); and (5) inflammatory response, infectious diseases, carbohydrate metabolism (data not shown). As shown in Figure 2, the PXR-target gene *Cyp3a* family member (*Cyp3a25*) was significantly up-regulated (5.71-

fold increase in microarray data) following PCN-induced PXR activation, which is consistent with previous findings (Down et al., 2007). Along with the decreased expression of the transcription factor CAR (NR1I3), the *Cyp2b13/Cyp2b9*, *Cyp2f1* and *Gsta4* genes were all down-regulated by PCN in neonatal mouse livers. The PCN-mediated down-regulation was also observed for *Cyp2c40*, *Cyp2c44*, *Cyp2c70* and *Cyp2e1*, but not for *Cyp2a4/2a5* (which is the mouse homolog of CYP2A6) that appeared to be up-regulated by PCN. The expression of *Cyp4a14*, *Cyp4a22*, and *Cyp4f12* were down-regulated after neonatal exposure to PCN (Figure 2). Although the ALT and Glutamate Pyruvate Transaminase 2 (*GPT2*) genes were up-regulated by PCN, the serum ALT levels were within the normal range for all groups as shown in Figure 1B. Moreover, neonatal exposure to PCN also altered the expression of genes involved in bile acid metabolism, which was evidenced by the reduced expression of *Cyp7a1* that encodes the rate-limiting enzyme in bile acid synthesis (Figure 2). In addition, neonatal exposure to PCN also altered the expression of genes involved in the signaling pathways that may influence TG, cholesterol, glucose and energy homeostasis, as well as other physiological functions. For example, PCN uniquely up-regulated genes involved in microsomal TG transfer [*Mttp*], bile acid conjugation reaction (bile acyl-CoA: amino acid N-acyltransferase [*BAAT*]), cholesterol esterification (sterol O-acyltransferase [*SOAT2*]), protein folding and modification (chaperonin containing TCP 1 [*CCT3*, *CCT5*, *CCT7*]), arginine methyltransferases [*PRMT1*, *PRMT3*]), tubulins [*TUBB2A*, *TUBB6*]), as well as cell-cycle progression (G0/G1 switch gene [*GOS2*]) (Supplemental Figs. 1A and B). In contrast, neonatal exposure to PCN down-regulated a variety of genes related to fatty acid oxidation (long-chain and medium-chain acyl-CoA dehydrogenase [*ACADL* and *ACADM*]), TGs breakdown (lipoprotein lipase [*LPL*]), high-density lipoprotein formation (lecithin-cholesterol acyltransferase [*LCAT*]), growth hormone signaling pathway (apolipoproteins [*APOA5*, *Apoc1*]), growth hormone receptor [*GHR*]), hydroxysteroid dehydrogenase [*HSD11B1*]), coagulation (clotting factors [*F5*, *F9*, *F11*]), vitamin K epoxide reductase complex [*VKORC1*]), glucose metabolism (pyruvate dehydrogenase kinase [*PDK4*]), one cut homeobox 1 [*ONECUT1*]), as well as tryptophan metabolism (tryptophan 2, 3-dioxygenase [*TOD2*]) (Supplemental Figs. 1A and B).

In comparison with PCN, neonatal exposure to TCPOBOP produced less uniquely regulated genes at day 3 (Table 2). IPA analysis of the 261 unique genes revealed the following top networks: (1) energy production, lipid metabolism, small molecule biochemistry (Figure 3); (2) endocrine system development and function, lipid metabolism, small molecule biochemistry (Supplemental Figure 2A); (3) drug metabolism, glutathione depletion in liver, liver hyperbilirubinemia (Supplemental Figure 2B); (4) lipid metabolism, small molecule biochemistry, digestive system development, and function (data not shown); and (5) cellular growth and proliferation, cellular development, tissue development (data not shown). As shown in Figure 3, the CAR-target genes, Aldehyde dehydrogenase (*Aldh1a1*) and P450 oxidoreductase (*Por*), were both up-regulated by TCPOBOP as expected (Aleksunes and Klaassen, 2012; Finn et al., 2009). The up-regulated genes were also involved in bile acid metabolism (*Cyp8b1*), pyruvate metabolism (serine dehydratase [*SDS*]), energy production (ethylmalonic encephalopathy 1 [*ETHE1*]), glutathione reduction [*Gsta3*]), fatty acid oxidation (carnitine palmitoyltransferase [*CPT2*]) and transport (ATP-binding cassette member 3 [*Abcd3*]), glucose transport (solute carrier family members *Slc22a1*, *Slc2a1*), NF- κ B-related immune response (toll-like receptor 5 [*Tlr5*]), bacteria growth inhibition (lipocalin

TABLE 2. Top 10 Genes that were Uniquely Regulated by PCN, Uniquely Regulated by TCPOBOP, or Regulated by both PCN and TCPOBOP at 3-Day of Age in the Livers of WT Mice (with >2-Fold Change and Adjusted P <0.05)

Comparison Group	Regulation	Gene Symbol	Description
PCN vs CO vs CO (common)	Up-regulated	Cyp2c55	Cytochrome P450, family 2, subfamily c, polypeptide 55
		Gstm3	Glutathione S-transferase, mu 3
		Prom1	Prominin 1
		Cyp2b10	Cytochrome P450, family 2, subfamily b, polypeptide 10
		Inmt	Indolethylamine N-methyltransferase
		Serpina6	Serine (or cysteine) peptidase inhibitor, clade A, member 6
		Prg4	Proteoglycan 4
		Sult2a5	Sulfotransferase family 2A, member 5
		Sulf2	Sulfatase 2
		Slfn9	Schlafen 9
	Down-regulated	Mup2; Mup1	Major urinary protein 2; major urinary protein 1
		Mup7	Major urinary protein 7
		Mup10; Mup19	Major urinary protein 10; major urinary protein 19
		Mup1	Major urinary protein 1
		Cyp4a32	Cytochrome P450, family 4, subfamily a, polypeptide 32
		Prok1	Prokineticin 1
		Mup19; Mup16; Mup9	Major urinary protein 19; major urinary protein 16; major urinary protein 9
		Mup19; Mup13	Major urinary protein 19; major urinary protein 13
		Cyp4a10; Cyp4a31	Cytochrome P450, family 4, subfamily a, polypeptide 10 or 31
		Mup12	Major urinary protein 12
Up-regulated by TCPOBOP, down- regulated by PCN	Cyp2c54	Cytochrome P450, family 2, subfamily c, polypeptide 54	
	Zim1	Zinc finger, imprinted 1	
PCN vs CO (unique)	Up-regulated	Ces2a	Carboxylesterase 2A; carboxylesterase 6
		Cyp2a5; Cyp2a4	Cytochrome P450, family 2, subfamily a, polypeptide 5 or 4
		Sult2a2	Sulfotransferase family 2A
		G0s2	G0/G1 switch gene 2
		Akr1b7	Aldo-keto reductase family 1, member B7
		Cyp2a4	Cytochrome P450, family 2, subfamily a, polypeptide 4
		Il13ra1	Interleukin 13 receptor, alpha 1
		Gpt2	Glutamic pyruvate transaminase 2
		Pbk	PDZ binding kinase
		Baat	Bile acid-Coenzyme A: amino acid N-acyltransferase
	Down-regulated	Mbl2	Mannose-binding lectin (protein C) 2
		Apol7a	Apolipoprotein L 7a
		Qpct	Glutaminyl-peptide cyclotransferase (glutaminyl cyclase)
		Tdo2	Tryptophan 2,3-dioxygenase
		Acot3	Acyl-CoA thioesterase 3
		Etnpp1	Ethanolamine phosphate phosphorylase
		Hsd17b11	Hydroxysteroid (17-beta) dehydrogenase 11
		Ugt3a2	UDP glycosyltransferases 3 family, polypeptide A2
		Cyp2f2	Cytochrome P450, family 2, subfamily f, polypeptide 2
		Cyp4a14	Cytochrome P450, family 4, subfamily a, polypeptide 14
TCPOBOP vs CO (unique)	Up-regulated	Cyp2c50	Cytochrome P450, family 2, subfamily c, polypeptide 50
		Ugt2b36	UDP glucuronosyltransferase 2 family, polypeptide B36
		Pnliprp1	Pancreatic lipase related protein 1
		A2m	Alpha-2-macroglobulin
		Ces1g	Carboxylesterase 1G; carboxylesterase 1
		Dio1	Deiodinase, iodothyronine, type I
		Tmem86b	Transmembrane protein 86B
		Cyp8b1	Cytochrome P450, family 8, subfamily b, polypeptide 1
		Sult1d1	Sulfotransferase family 1D, member 1
		Orm1	Orosomucoid 1
	Down-regulated	Gzma	Granzyme A
		Krt23	Keratin 23
		Inhbe	Inhibin beta E
		Ak4	Adenylate kinase 4; adenylate kinase 3 alpha-like 1
		Csad	Cysteine sulfinic acid decarboxylase
		Dak	Dihydroxyacetone kinase 2 homolog (yeast)
		Hsd17b6	Hydroxysteroid (17-beta) dehydrogenase 6
		Tff3	Trefoil factor 3, intestinal
		Hamp2	Hepcidin antimicrobial peptide 2
		Hamp	Hepcidin antimicrobial peptide

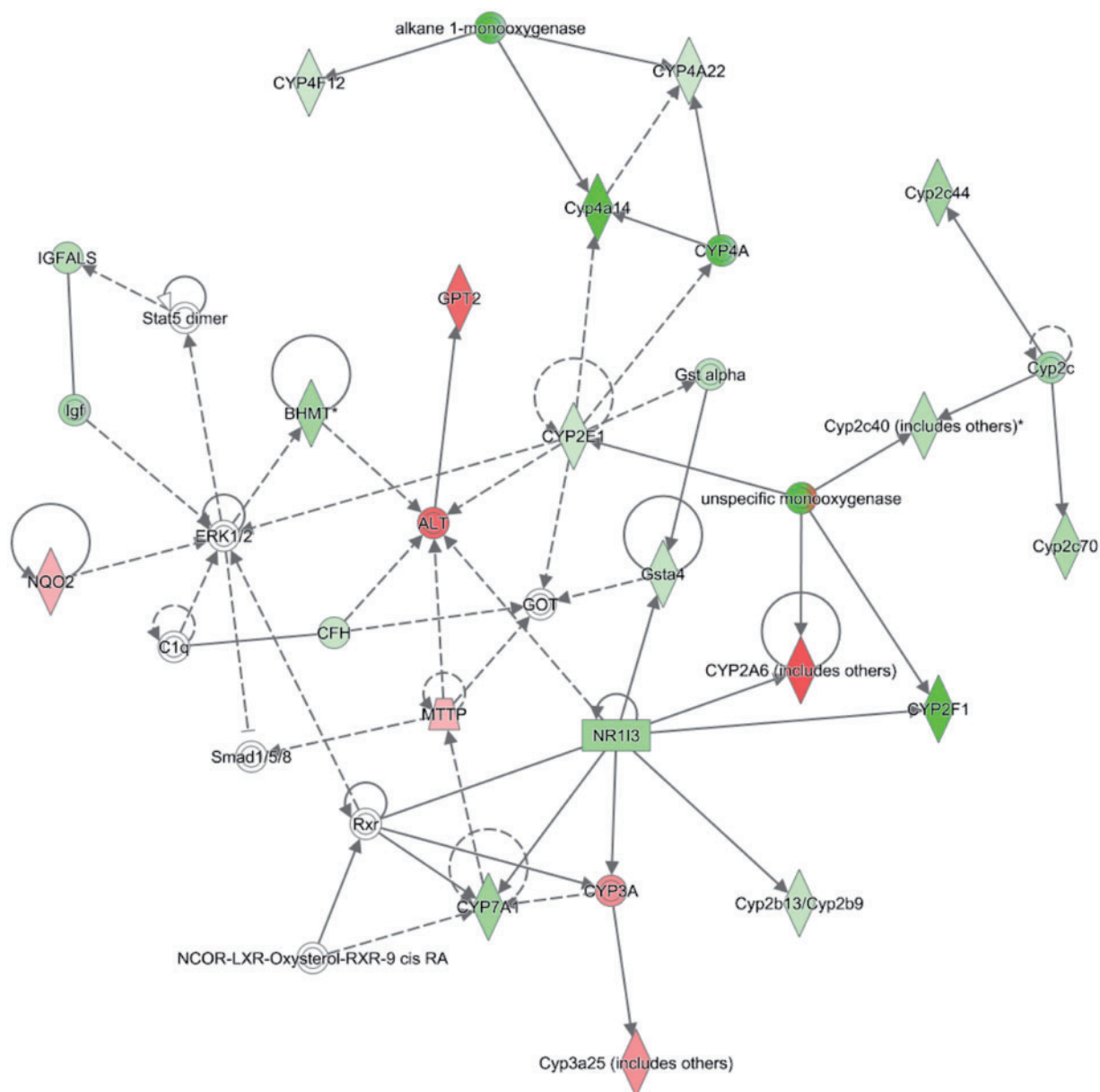
TABLE 3. Gene Expression Changes that were Uniquely Regulated by PCN, Uniquely Regulated by TCPOBOP, or Regulated by Both PCN and TCPOBOP at 60-Day of Age in the Livers of WT Mice (with >2-Fold Change and Adjusted $P < 0.05$)

Comparison Group	Regulation	Gene Symbol	Description	
PCN vs CO CO (common)	Up-regulated	<i>Cyp2b13</i>	Cytochrome P450, family 2, subfamily b, polypeptide 13	
	Down-regulated	<i>Cyp4a10</i> ; <i>Cyp4a31</i>	Cytochrome P450, family 4, subfamily a, polypeptide 10 or 31	
	Up-regulated by TCPOBOP, down-regulated by PCN	<i>Afp</i> <i>Serpinb1a</i>	Alpha fetoprotein Serine (or cysteine) peptidase inhibitor, clade B, member 1a	
PCN vs CO (Unique)	Up-regulated	<i>Cxcl1</i>	Chemokine (C-X-C motif) ligand 1	
		<i>Mir378c</i>	microRNA mir-378c	
		<i>Saa2</i>	Serum amyloid A 2	
		<i>Saa1</i>	Serum amyloid A 1	
		<i>Zbtb16</i>	Zinc finger and BTB domain containing 16	
		<i>Steap4</i>	STEAP family member 4	
		<i>Dbp</i>	D site albumin promoter binding protein	
	Down-regulated	<i>Asns</i>	Asparagine synthetase	
		<i>Irs2</i>	Insulin receptor substrate 2	
		<i>Cyp4a31</i>	Cytochrome P450, family 4, subfamily a, polypeptide 31	
		<i>Igfbp1</i>	Insulin-like growth factor binding protein 1	
		<i>Osgin1</i>	Oxidative stress induced growth inhibitor 1	
		<i>Igfbp2</i>	Insulin-like growth factor binding protein 2	
		<i>Npas2</i>	Neuronal PAS domain protein 2	
		<i>Cyp4a32</i>	Cytochrome P450, family 4, subfamily a, polypeptide 32	
		<i>Arntl</i>	Aryl hydrocarbon receptor nuclear translocator-like	
		<i>Cyp4a14</i>	Cytochrome P450, family 4, subfamily a, polypeptide 14	
TCPOBOP vs CO (Unique)	Up-regulated	<i>Cyp2b10</i>	Cytochrome P450, family 2, subfamily b, polypeptide 10	
		<i>Cyp2c55</i>	Cytochrome P450, family 2, subfamily c, polypeptide 55	
		<i>Cyp2c29</i>	cytochrome P450, family 2, subfamily c, polypeptide 29	
		<i>Gna14</i>	Guanine nucleotide binding protein, alpha 14	
		<i>Cyp26a1</i>	Cytochrome P450, family 26, subfamily a, polypeptide 1	
		<i>Orm3</i>	Orosomucoid 3	
		<i>Gstm3</i>	Glutathione S-transferase, mu 3	
		<i>Dhrs9</i>	Dehydrogenase/reductase (SDR family) member 9	
		<i>Sult5a1</i>	Sulfotransferase family 5A, member 1	
		<i>Mup-ps16</i>	Major urinary protein, pseudogene 16	
		<i>AF357426</i>	snoRNA AF357426	
		<i>2310067E19Rik</i>	RIKEN cDNA 2310067E19 gene	
		<i>Prss8</i>	Protease, serine 8 (prostatic)	
		<i>Tmeff2</i>	Transmembrane protein with EGF-like and two follistatin-like domains 2	
		<i>Cyp2c66</i>	Cytochrome P450, family 2, subfamily c, polypeptide 66	
		<i>Slc3a1</i>	Solute carrier family 3, member 1	
		<i>Mir493</i>	microRNA 493	
		<i>Klk1b4</i>	Kallikrein 1-related peptidase b4	
		<i>Cyp2c65</i>	Cytochrome P450, family 2, subfamily c, Polypeptide 65	
		<i>Prg4</i>	Proteoglycan 4	
		<i>Kcnk1</i>	Potassium channel, subfamily K, member 1	
		<i>Ccl9</i>	Chemokine (C-C motif) ligand 9	
		<i>DQ267102</i>	snoRNA DQ267102	
		<i>Rarres1</i>	Retinoic acid receptor responder (tazarotene induced) 1	
		Down-regulated	<i>Snord14e</i>	Small nucleolar RNA, C/D box 14E

[*Lcn2*]), post-transcriptional regulation (ring finger protein [*Zfp36*]), methylation (thiopurine methyltransferase [*Tpmt*]), and thyroid hormone activation/deactivation (deiodinase [*DIO1*]) (Figure 3). However, the expression of *CPT1B*, which functions as the rate-controlling enzyme in the oxidation of fatty acid, appeared to be down-regulated by TCPOBOP in neonatal mouse livers (Figure 3). The other networks revealed more DPGs that were elevated 24 h after TCPOBOP exposure, including carboxylesterases (*Ces1e*, *Ces1g*), xanthine dehydrogenase (*Xdh*), flavin containing monooxygenase (*Fmo5*), glutathione S-transferases (*Gsta3*, *Gstt1*), UDP glucuronosyltransferases (*Ugt2b35/36* [mouse

ortholog of human *UGT2B7*], *Ugt2b37/38* [mouse ortholog of human *UGT2B28*]), and transporters (*Slc22a7*, *Slc23a1*); whereas the hepcidin antimicrobial peptidase (*Hamp*), which is involved in the maintenance of iron homeostasis, was down-regulated by TCPOBOP at day 3 (Supplemental Figs. 2A and B).

Interestingly, there were some genes persistently differentially expressed after neonatal exposure to PCN or TCPOBOP in 60-day-old adult mouse livers (Table 3). By comparing the differentially expressed genes between PCN and TCPOBOP at day 60, we identified 4 common genes, including the up-regulated *Cyp2b13*, down-regulated *Cyp4a* (*Cyp4a10* or *Cyp4a31*), and



Lipid Metabolism, Small Molecule Biochemistry, Drug Metabolism








-  Ligand-dependent Nuclear Receptor
-  Enzyme
-  Transporter
-  Group or Complex
-  Transmembrane receptor
-  Transcriptional regulator
-  Other

FIG. 2. Ingenuity pathway analysis (IPA) identified networks associated with gene expression changes that were uniquely regulated by pregnenolone-16 α -carbonitrile (PCN) compared with corn oil at 3-day of age in livers of wild-type (WT) mice (>2 -fold change, adjusted $P < 0.05$). More networks are shown in Supplemental Figure 1. Genes or gene products are represented as nodes. The intensity of the node color indicates the degree of up-regulation (red) or down-regulation (green). The edges (lines and arrows between nodes) represent direct (solid lines) and indirect (dashed lines) interactions between molecules as supported by information in the Ingenuity knowledge base. Node shapes represent functional classes of gene products.

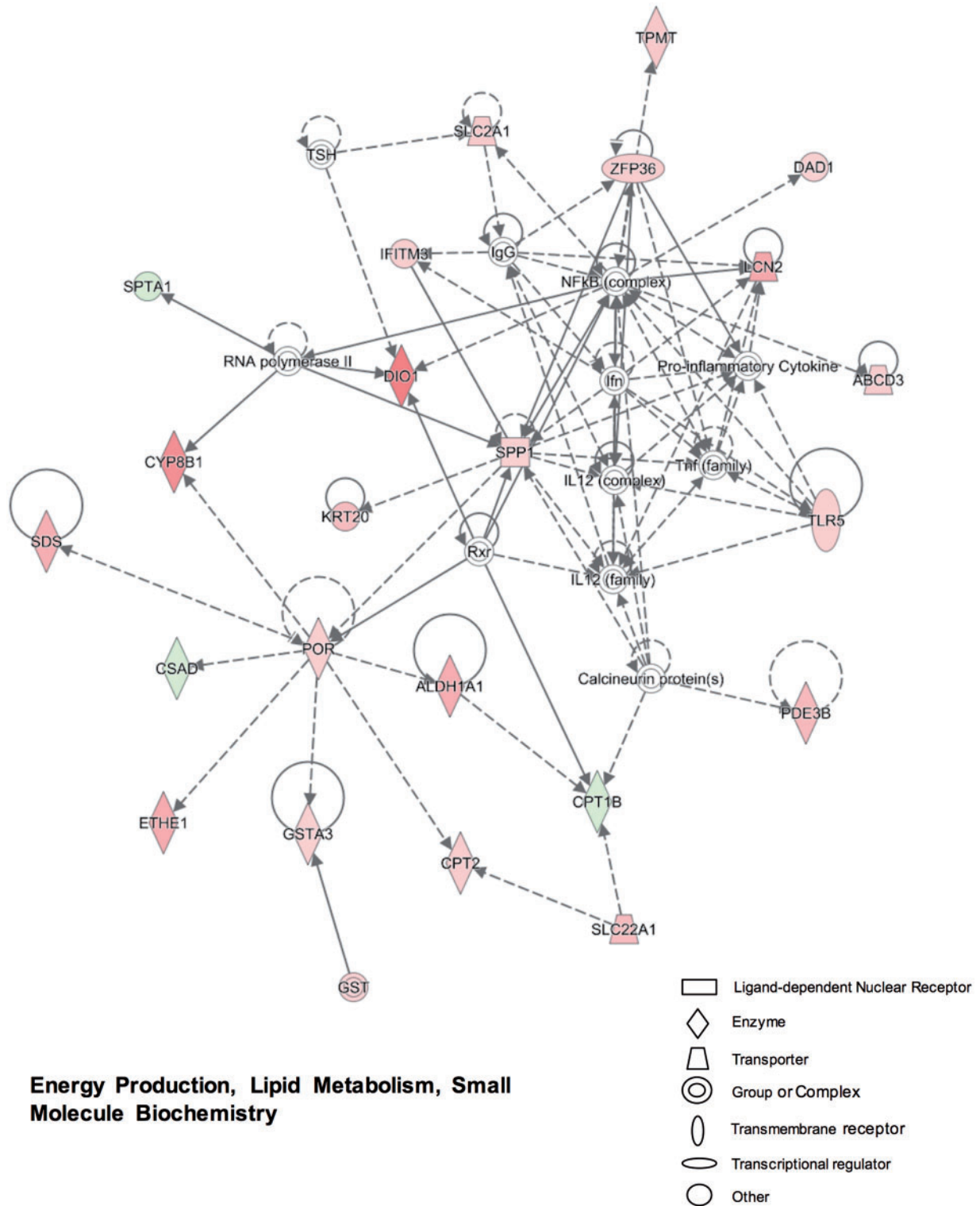


FIG. 3. Ingenuity pathway analysis (IPA) identified networks associated with gene expression changes that were uniquely regulated by 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) compared with corn oil at 3-day of age in livers of wild-type (WT) mice (>2-fold change, adjusted $P < 0.05$). More networks are shown in Supplemental Figure 2. Genes or gene products are represented as nodes. The intensity of the node color indicates the degree of up-regulation (red) or down-regulation (green). The edges (lines and arrows between nodes) represent direct (solid lines) and indirect (dashed lines) interactions between molecules as supported by information in the Ingenuity knowledge base. Node shapes represent functional classes of gene products.

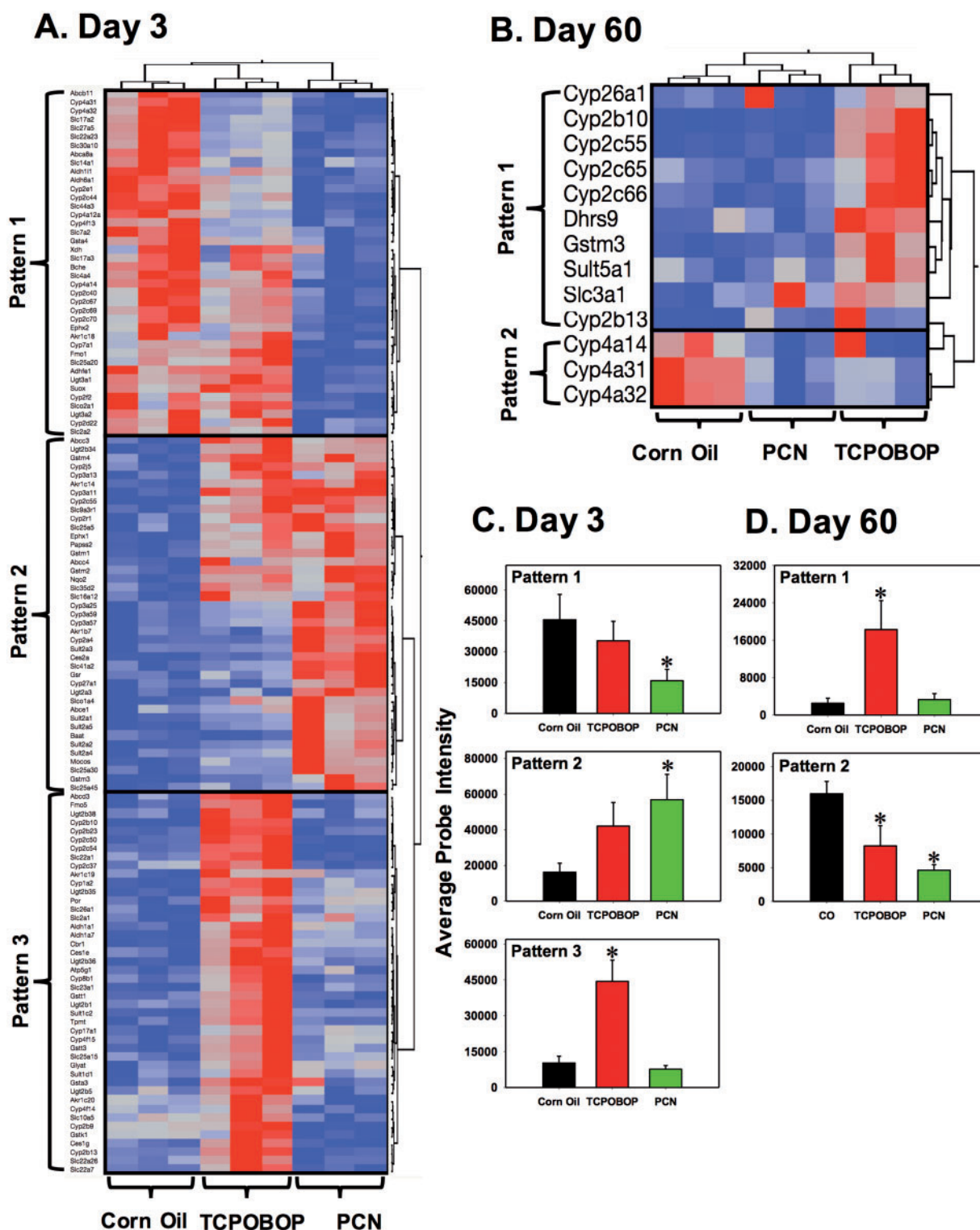


FIG. 4. Two-way hierarchical clustering dendrogram of drug-processing genes (DPGs) that were differentially expressed by pregnenolone-16 α -carbonitrile (PCN) or 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) at day 3, A, or day 60, B, in the livers of wild-type (WT) mice. Three biological replicates (shown as columns) were tested for each group. Each row represents a different gene. A two-way hierarchical clustering dendrogram was generated of the differentially regulated DPGs by either PCN or TCPOBOP (fold-change > 2, $P < 0.05$) using the standardized means and Ward's minimal variance. Distances between genes reflect the significance of associations. Red represents relatively higher and blue represents relatively lower expression. The expression profiles of differentially expressed drug-processing genes (DPGs) were categorized to 3 major patterns of interest at day 3, and two patterns at day 60.

alpha fetoprotein (*Afp*), as well as the serine peptidase inhibitor (*Serp1b1a*), which was up-regulated by TCPOBOP but down-regulated by PCN (Table 3). As for the unique genes, neonatal exposure to PCN persistently up-regulated the expression of chemokine ligand 1 (*Cxcl1*) and serum amyloids (*Saa1*, *Saa2*), but down-regulated *Cyp4a* (*Cyp4a14*, *Cyp4a31*, *Cyp4a32*), insulin-like growth factor binding proteins (*Igf1p1*, *Igf1p2*) and oxidative stress induced growth inhibitor (*Osgin1*); whereas neonatal exposure to TCPOBOP persistently up-regulated the expression of *Cyp2b10*, *Cyp2c55*, *Cyp2c29*, but down-regulated the non-coding small nucleolar RNA (*Snord14e*), which is required for early cleavage of precursor rRNAs (Table 3).

Hierarchical Clustering of DPGs

Because PXR and CAR are well-known xenobiotic-sensing nuclear receptors in the liver, the acute and persistent changes in the DPGs following neonatal activation of these receptors were examined in detail. DPGs that were differentially expressed in at least one experimental condition included 125 DPGs at day 3 (phase I: 60, phase II: 30, transporter: 35) and 13 DPGs at day 60 (phase I: 10, phase II: 2, transporter: 1). Two-way hierarchical clustering dendrograms were plotted as shown in Figure 4A (day 3) and Figure 4B (day 60).

The differentially expressed DPGs in the livers of 3-day-old mice were partitioned into 3 distinct clusters: pattern 1 included 40 genes that were down-regulated by PCN; pattern 2 included 41 genes that were up-regulated preferably by PCN (and to a lesser extent by TCPOBOP); pattern 3 included 44 genes that were up-regulated by TCPOBOP (Figure 4A). The average probe intensities for each pattern were plotted as shown in Figure 4C. On an average, genes in pattern 1 were down-regulated 65% by PCN, whereas genes in pattern 2 were up-regulated by PCN with a 3.5-fold change. In pattern 3, genes were markedly up-regulated by TCPOBOP but not PCN (Figure 4C).

The 13 differentially regulated DPGs in the livers of 60-day-old mice were divided into two clusters (Figure 4B). Pattern 1 included *Cyp26a1*, *Cyp2b10*, *Cyp2c55*, *Cyp2c65*, *Cyp2c66*, *Dhrs9*, *Gstm3*, *Sult5a1*, *Slc3a1*, and *Cyp2b13* and they were in general up-regulated by neonatal exposure to TCPOBOP; pattern 2 included *Cyp4a14*, *4a31*, and *4a32* and they were down-regulated by PCN and TCPOBOP (Figure 4B). The average probe intensities of genes in each pattern were plotted as shown in Figure 4D. Overall, genes in pattern 1 were increased 6.3-fold by TCPOBOP, whereas the genes in pattern 2 were down-regulated by both TCPOBOP and PCN with 48.4% and 71.1% decrease, respectively (Figure 4D).

Verification of Microarray Data in WT and receptor-Knockout Mouse Livers Using RT-qPCR and Western Blotting Analysis

Representative DPGs that were differentially regulated by neonatal exposure to PCN or TCPOBOP from microarray were validated as shown in Figures 5 and 6. To rule out the off-target effects of the chemicals, the differentially expressed genes were also examined in livers of PXR-null and CAR-null mice as well. In general, the RT-qPCR data were highly consistent with the microarray data in WT livers.

Regarding the PXR effect, at day 3, *Cyp1a2* mRNA was up-regulated by PCN (5.33-fold from microarray) (Table 4). Using RT-qPCR, it was found that *Cyp1a2* was also increased in WT mouse livers in a PXR-dependent manner, because such up-regulation disappeared in livers of PXR-null mice (Figure 5A).

Similarly, the mRNAs of *Cyp2a4*, *3a11*, *3a25*, *Aldh1a7*, *Ugt2b34*, *Sult2a1*, *Papss2*, *Gstm1*, *Slco1a4*, *Abcc3*, and *Abcc4* were all up-regulated by PCN in a PXR-dependent manner; whereas the mRNAs of *Cyp4a14*, *4a31*, and *4a32* were all down-regulated by PCN in a PXR-dependent manner in livers of 3-day-old mice (Figure 5A). At day 60, *Cyp2b13* mRNA was up-regulated by neonatal exposure to PCN in a PXR-dependent manner, whereas *Cyp4a14*, *4a31*, and *4a32* were down-regulated by neonatal exposure to PCN in a PXR-dependent manner (Figure 5B). The protein expression of *Cyp4a14* in livers of 60-day-old mice was quantified by western blotting analysis as shown in Figure 5C. Consistent with the mRNA data, *Cyp4a14* protein was also persistently down-regulated by neonatal exposure to PCN in a PXR-dependent manner. Similarly, the *Cyp4a* enzyme activity was decreased by neonatal exposure to PCN in a PXR-dependent manner (Figure 5D). However, the *Cyp2b* enzyme activity was not changed by PCN in either WT or PXR-null mouse livers (Figure 5D), even though *Cyp2b13* mRNA was persistently up-regulated in 60-day-old WT mouse livers by neonatal PCN exposure (Figure 5B). This is likely due to the fact that other more abundantly expressed *Cyp2bs* remained unchanged by neonatal PCN exposure in adult livers.

As expected, microarray analysis showed that the prototypical CAR-target gene *Cyp2b10* was markedly increased by TCPOBOP in livers of 3-day-old WT mice (421.6-fold from microarray) (Table 5). A similar pattern of increased *Cyp2b10* was observed using RT-qPCR in WT mice but not in CAR-null mice (Figure 6A). The mRNAs of *Cyp1a2*, *Cyp2c55*, *Cyp3a11*, *Aldh1a7*, *Ugt2b34-36*, *Gstm1*, *Gstm3*, *Gstm4*, *Gstt1*, and *Slco1a4* were all up-regulated by TCPOBOP in a CAR-dependent manner; whereas *Cyp4a31* and *4a32* mRNAs were down-regulated by TCPOBOP in a CAR-dependent manner (Figure 6A). At 60 days after neonatal exposure, consistent with a previous report (Chen et al., 2012), *Cyp2b10* mRNA was persistently up-regulated by neonatal TCPOBOP exposure in a CAR-dependent manner; similarly, the mRNAs of *Cyp2c55* and *Sult5a1* were also persistently up-regulated by neonatal TCPOBOP exposure in a CAR-dependent manner. *Gstm3* mRNA was persistently up-regulated by neonatal TCPOBOP exposure not only in livers of adult WT mice but also in livers of adult CAR-null mice, indicating that there may be an off-target effect of the chemical (Figure 6B). Microarray analysis showed that *Cyp4a31* mRNA was down-regulated by neonatal TCPOBOP exposure; whereas RT-qPCR showed that it tended to be decreased by TCPOBOP (although a statistical significance was not achieved) in livers of WT mice, but it was markedly increased in livers of CAR-null mice. Similar findings were observed for *Cyp4a14* mRNA (Figure 6B). Western blotting analysis showed that *Cyp2b10* protein was persistently up-regulated by neonatal TCPOBOP exposure in livers of 60-day-old mice in a CAR-dependent manner. *Cyp4a14* protein was persistently down-regulated by neonatal TCPOBOP exposure in livers of 60-day-old mice in a CAR-dependent manner, but it was not up-regulated in livers of CAR-null mice (likely due to post-transcriptional modifications of the *Cyp4a14* transcript). The overall hepatic *Cyp2b* enzyme activity was increased by neonatal TCPOBOP exposure in 60-day-old mice in a CAR-dependent manner. However, the overall hepatic *Cyp4a* enzyme activity was actually moderately increased by neonatal TCPOBOP exposure in 60-day-old mice in a CAR-dependent manner. Because the substrate for *Cyp4a* enzyme activity assay was not designed specifically to detect the *Cyp4a14* isoform, we could not directly compare it with its protein and mRNA alteration (Figure 6D). For both the protein and the enzyme activity of *Cyp4a*, there appeared to be a constitutive increase in livers of

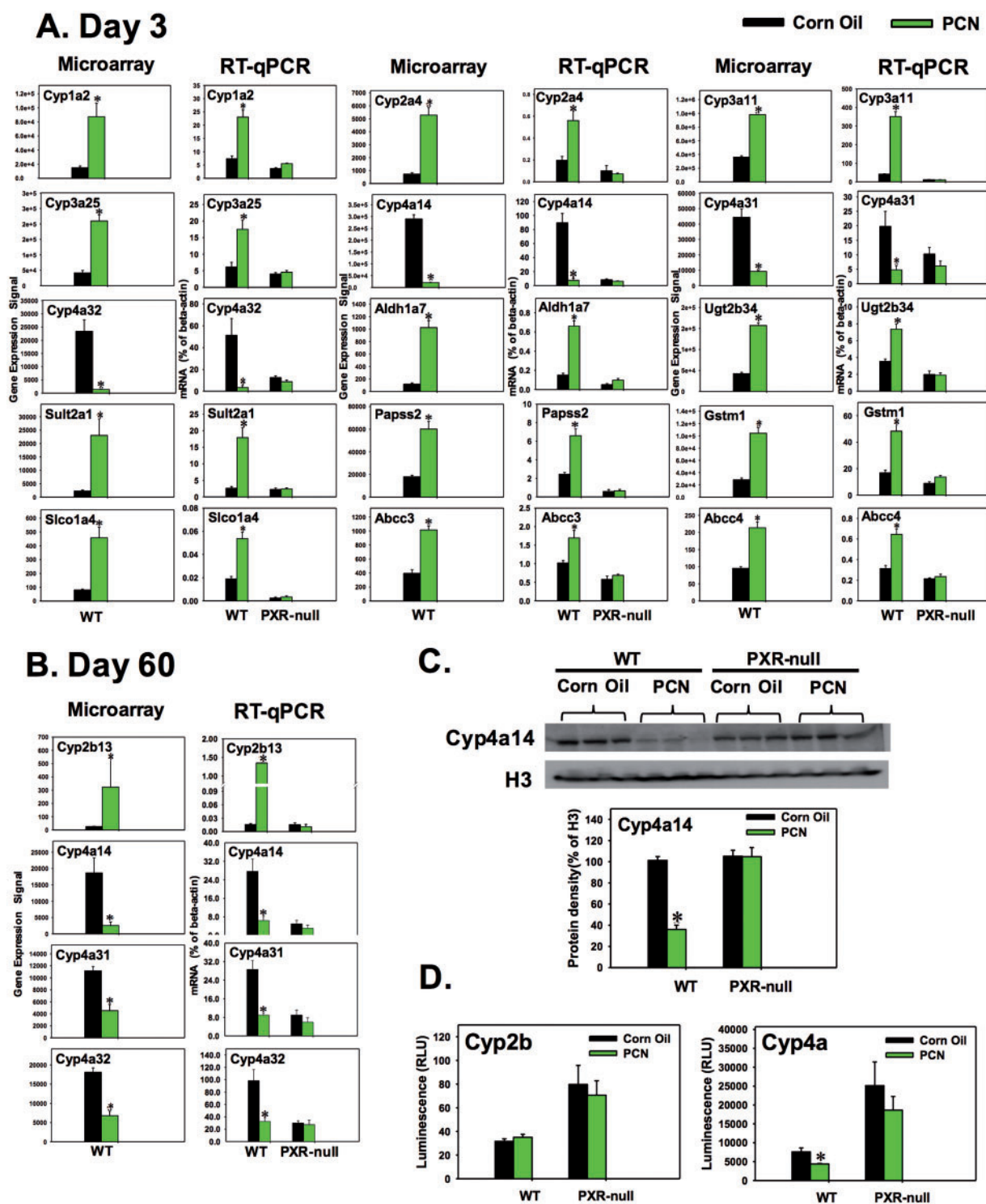


FIG. 5. Neonatal pregnane X receptor (PXR) activation caused acute (day 3) and persistent changes (day 60) in the expression of drug-processing genes (DPGs) in the livers of wild-type (WT) mice but not pregnane X receptor knockout mice (PXR-null). The microarray data of the drug-processing genes (DPGs) that indicated up-regulated (eg fold-change > 2, $P < 0.05$) or down-regulated (eg fold-change < -2, $P < 0.05$) by pregnenolone-16 α -carbonitrile (PCN) at day 3, A, or day 60, B, in livers of wild-type (WT) mice were further validated by RT-qPCR in both WT and PXR-null mice. C, Western blot analysis for protein expression of Cyp4a14 in 60-day-old WT and PXR-null mouse livers. D, Cyp4a enzyme activity in 60-day-old WT and PXR-null mouse liver microsomes using the Promega P450-Glo™ CYP4A Luciferin-4A Assay. Asterisk (*) indicates statistically significant differences between PCN and control group ($P < 0.05$) by Student's t-test.

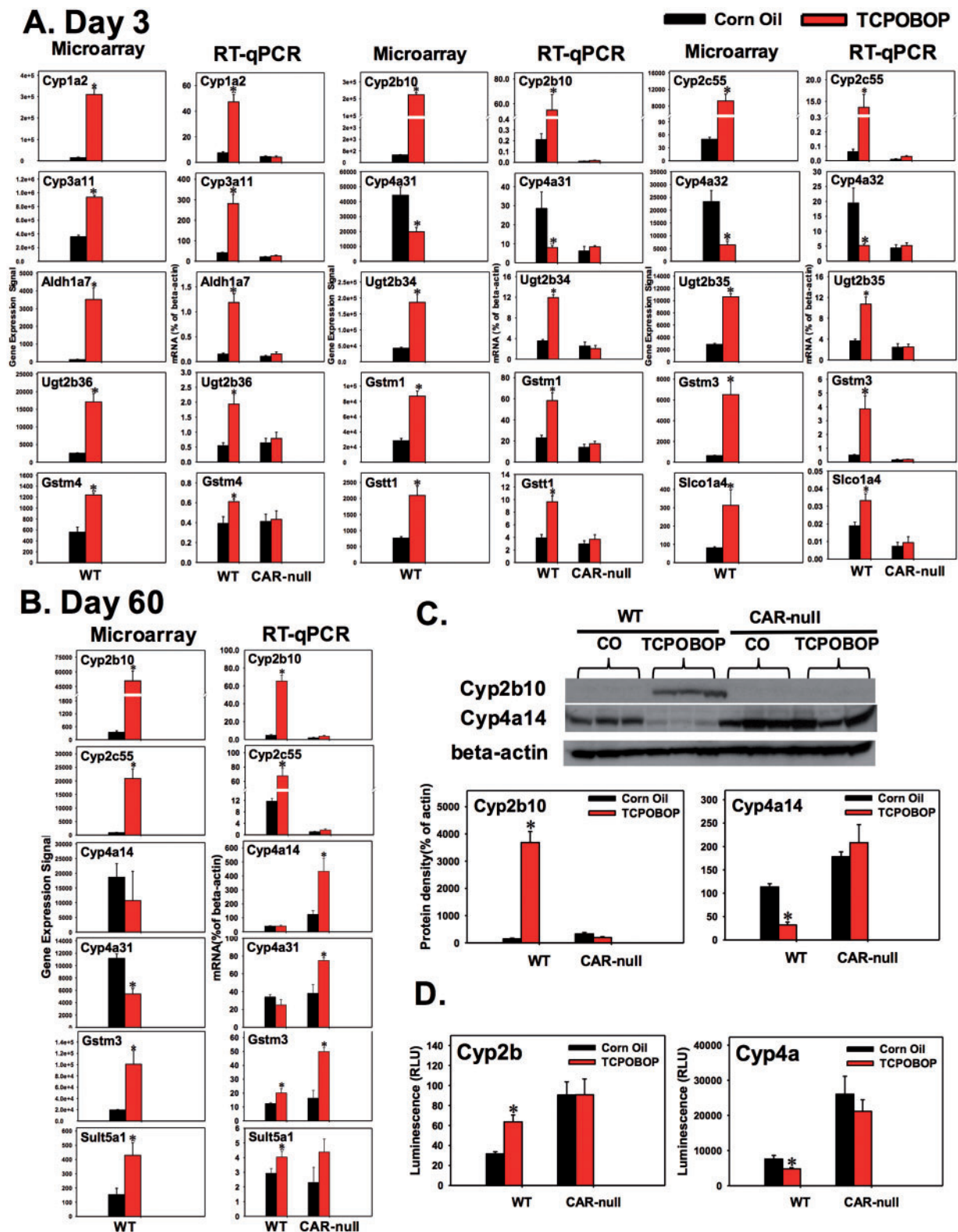


FIG. 6. Neonatal constitutive androstane receptor (CAR) activation caused acute (day 3) and persistent changes (day 60) in the expression of drug-processing genes (DPGs) in the livers of wild-type (WT) mice but not constitutive androstane receptor knockout mice (CAR-null). The microarray data of the DPGs that indicated up-regulated (eg fold change > 2 , $P < 0.05$) or down-regulated (eg fold change < -2 , $P < 0.05$) by 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) at day 3, A, or day 60, B, in livers of WT mice were further validated by RT-qPCR in both WT and CAR-null mice. C, Western blot analysis for protein expression of Cyp2b10 and Cyp4a14 in 60-day-old WT and CAR-null mouse livers. D, Cyp2b and Cyp4a enzyme activity in 60-day-old mouse liver microsomes using the Promega P450-Glo™ CYP2B and CYP4A Assay. Asterisk (*) indicates statistically significant differences between TCPOBOP and control group ($P < 0.05$) by Student's t-test.

TABLE 4. Differentially Regulated DPGs by PCN at 3- or 60-Day of Age in the Livers of WT Mice. The Fold-Change Refers to the Ratio of the Expression Values of the PCN Treatment Over the Corn Oil Treated Averaged at the Same Age

Day 3 PCN vs Day 3 Corn Oil				
Gene Symbol	Description	Phase	Fold-Change	ANOVA p-value
Cyp2c55	Cytochrome P450, family 2, subfamily c, polypeptide 55	Phase I	202.03	0.000002
Gstm3	Glutathione S-transferase, mu 3	Phase II	78.83	0.000392
Cyp2b10	Cytochrome P450, family 2, subfamily b, polypeptide 10	Phase I	16.27	0.001024
Sult2a5	Sulfotransferase family 2A, member 5	Phase II	10.44	0.002128
Ces2a	Carboxylesterase 2A	Phase I	10.1	0.000008
Cyp2a5; Cyp2a4	Cytochrome P450, family 2, subfamily a, polypeptide 5 or 4	Phase I	8.49	0.00039
Sult2a1	Sulfotransferase family 2A, member 1	Phase II	8.18	0.002287
Sult2a2	Sulfotransferase family 2A, member 2	Phase II	7.57	0.000634
Aldh1a7	Aldehyde dehydrogenase family 1, subfamily A7	Phase I	7.46	0.000415
Cyp3a59	Cytochrome P450, family 3, subfamily a, polypeptide 59	Phase I	6.83	0.001527
Akr1b7	Aldo-keto reductase family 1, member B7	Phase I	6.47	0.002692
Cyp2a4	Cytochrome P450, family 2, subfamily a, polypeptide 4	Phase I	6.02	0.000527
Cyp3a25	Cytochrome P450, family 3, subfamily a, polypeptide 25	Phase I	5.71	0.001775
Cyp1a2	Cytochrome P450, family 1, subfamily a, polypeptide 2	Phase I	5.33	0.003019
Slco1a4	Solute carrier organic anion transporter family, member 1a4	Transporters	4.76	0.000606
Baat	Bile acid-Coenzyme A: amino acid N-acyltransferase	Phase II	4.69	0.003927
Ephx1	Epoxide hydrolase 1, microsomal	Phase I	4.52	0.000624
Ugt2b34	UDP glucuronosyltransferase 2 family, polypeptide B34	Phase II	4.16	0.000102
Cyp2j5	Cytochrome P450, family 2, subfamily j, polypeptide 5	Phase I	3.81	0.006269
Sult2a4	Sulfotransferase family 2A, member 4	Phase II	3.74	0.00479
Sult2a3	Sulfotransferase family 2A, member 3	Phase II	3.65	0.002052
Cyp3a57	Cytochrome P450, family 3, subfamily a, polypeptide 57	Phase I	3.52	0.001399
Gsr	Glutathione reductase; glutathione reductase 1	Phase I	3.48	0.001057
Gstm1	Glutathione S-transferase, mu 1	Phase II	3.4	0.000689
Paps2	3-Phosphoadenosine 5-phosphosulfate synthase 2	Phase II	3.33	0.000854
Gadd45b	Growth arrest and DNA-damage-inducible 45 beta	Epi	2.95	0.002009
Akr1c14	Aldo-keto reductase family 1, member C14	Phase I	2.9	0.000391
Ugt2a3	UDP glucuronosyltransferase 2 family, polypeptide A3	Phase II	2.8	0.000404
Cyp3a11	Cytochrome P450, family 3, subfamily a, polypeptide 11	Phase I	2.77	0.000076
Gstm2	Glutathione S-transferase, mu 2	Phase II	2.76	0.009338
Sult1c2	Sulfotransferase family, cytosolic, 1C, member 2	Phase II	2.72	0.001618
Abcc3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	Transporters	2.62	0.002583
Slc25a45	Solute carrier family 25, member 45	Transporters	2.55	0.039597
Slc41a2	Solute carrier family 41, member 2	Transporters	2.53	0.000375
Gsta4	Glutathione S-transferase, alpha 4	Phase II	-2.51	0.009568
Suox	Sulfite oxidase	Phase I	-2.55	0.007792
Cyp2b9	Cytochrome P450, family 2, subfamily b, polypeptide 9	Phase I	-2.61	0.000017
Cyp2c40	Cytochrome P450, family 2, subfamily c, polypeptide 40	Phase I	-2.62	0.010139
Slc30a10	solute carrier family 30, member 10	Transporters	-2.63	0.002622
Cyp4f13	Cytochrome P450, family 4, subfamily f, polypeptide 13	Phase I	-2.88	0.003594
Slc17a3	Solute carrier family 17 (sodium phosphate), member 3	Transporters	-2.88	0.007564
Cyp2c69	Cytochrome P450, family 2, subfamily c, polypeptide 69	Phase I	-2.91	0.002362
Fmo1	Flavin containing monooxygenase 1	Phase I	-2.92	0.000406
Slc4a4	Solute carrier family 4 (anion exchanger), member 4	Transporters	-2.93	0.007225
Aldh11l1	Aldehyde dehydrogenase 1 family, member L1	Phase I	-2.94	0.000932
Slc2a2	Solute carrier family 2 (facilitated glucose transporter), member 2	Transporters	-2.99	0.018215
Aldh6a1	Aldehyde dehydrogenase family 6, subfamily A1	Phase I	-3.18	0.000758
Cyp2c67	Cytochrome P450, family 2, subfamily c, polypeptide 67	Phase I	-3.26	0.007684
Cyp2c54	Cytochrome P450, family 2, subfamily c, polypeptide 54	Phase I	-3.28	0.002595
Ephx2	Epoxide hydrolase 2, cytoplasmic	Phase I	-3.34	0.004101
Cyp2c70	Cytochrome P450, family 2, subfamily c, polypeptide 70	Phase I	-3.74	0.004401
Slc44a3	Solute carrier family 44, member 3	Transporters	-3.76	0.000014
Cyp2c44	Cytochrome P450, family 2, subfamily c, polypeptide 44	Phase I	-4.23	0.000018
Ugt3a1	UDP glycosyltransferases 3 family, polypeptide A1	Phase II	-4.3	0.000873
Abca8a	ATP-binding cassette, sub-family A (ABC1), member 8a	Transporters	-4.33	0.000211
Adhfe1	Alcohol dehydrogenase, iron containing, 1	Phase I	-4.4	0.003224
Cyp7a1	Cytochrome P450, family 7, subfamily a, polypeptide 1	Phase I	-4.6	0.006761
Nr1i3	Nuclear receptor subfamily 1, group I, member 3	Nuclear receptor	-4.8	0.000138
Slc27a5	Solute carrier family 27 (fatty acid transporter), member 5	Transporters	-4.85	0.00128
Cyp4a31	Cytochrome P450, family 4, subfamily a, polypeptide 31	Phase I	-4.91	0.000435
Slc17a2	Solute carrier family 17 (sodium phosphate), member 2	Transporters	-7.73	0.000744

Continued

TABLE 4. (continued)

Day 3 PCN vs Day 3 Corn Oil				
Gene Symbol	Description	Phase	Fold-Change	ANOVA p-value
Ugt3a2	UDP glycosyltransferases 3 family, polypeptide A2	Phase II	-11.5	0.000359
Cyp2f2	Cytochrome P450, family 2, subfamily f, polypeptide 2	Phase I	-11.51	0.002444
Cyp4a14	Cytochrome P450, family 4, subfamily a, polypeptide 14	Phase I	-14.75	0.000008
Cyp4a32	Cytochrome P450, family 4, subfamily a, polypeptide 32	Phase I	-20.05	0.000269
Cyp4a10; Cyp4a31	Cytochrome P450, family 4, subfamily a, polypeptide 10 or 31	Phase I	-29.63	0.000181
Day60 PCN vs Day60 Corn Oil				
Cyp2b13	Cytochrome P450, family 2, subfamily b, polypeptide 13	Phase I	4.4	0.032423
Cyp4a31	Cytochrome P450, family 4, subfamily a, polypeptide 31	Phase I	-2.37	0.020546
Cyp4a32	Cytochrome P450, family 4, subfamily a, polypeptide 32	Phase I	-2.6	0.005586
Cyp4a10; Cyp4a31	Cytochrome P450, family 4, subfamily a, polypeptide 10 or 31	Phase I	-2.92	0.018139
Cyp4a14	Cytochrome P450, family 4, subfamily a, polypeptide 14	Phase I	-7.92	0.023341

Note: Fold change is defined as a ratio. Positive, up-regulation; Negative, the inverse of ratio, down-regulation.

the CAR-null mice (Figs. 6C and D), suggesting that basal expression of CAR suppresses the expression of Cyp4a14 in control conditions.

PPAR α -DNA Binding to the Cyp4a Gene Loci

The present study identified a persistent decrease in the expression of Cyp4a genes following neonatal activation to PXR and CAR. Because Cyp4a genes are well-known prototypical target genes of PPAR α (Aleksunes and Klaassen, 2012), we hypothesized that PPAR α -DNA binding would be decreased in adult livers following neonatal exposure to PCN and TCPOBOP. Therefore, to determine the molecular mechanisms underlying the persistent down-regulation of the Cyp4a genes in adult mouse liver following neonatal exposure to PCN or TCPOBOP, ChIP was conducted in WT mouse livers at 60-day of age in 2 independent pull-downs using a ChIP-grade antibody against PPAR α as described in Materials and methods section. Due to lower sample yield, PPAR α ChIP was not performed at day 3 neonatal age. The PPAR α -DNA response elements of Cyp4a14, 4a31, and 4a32 were examined in detail, because their gene expression was down-regulated by PCN and/or TCPOBOP in a PXR/CAR-dependent manner. ChIP-qPCR demonstrated that there were constitutive PPAR α -bindings to all 3 Cyp4 genes under control conditions; however, neonatal PCN exposure appeared to decrease the PPAR α -DNA binding to the response elements of all Cyp4 genes (84.0% decrease for Cyp4a14, 73.1% decrease for Cyp4a31, and 66.9% decrease for Cyp4a32). Similarly, neonatal TCPOBOP exposure also appeared to decrease the PPAR α -DNA binding to the response elements of the Cyp4 genes (70.2% decrease for Cyp4a14, 56.8% decrease for Cyp4a31, and 54.3% decrease for Cyp4a32) (Figure 7A). In summary, neonatal exposure to PCN and TCPOBOP, which lead to PXR and CAR activation, appeared to persistently interfere with the constitutive PPAR α -DNA binding to some Cyp4 genes in adult liver, and this may contribute to the persistent down-regulation of the Cyp4 genes.

Serum and Hepatic TG Levels

Based on previous findings that activation of PPAR α could result in decreased circulating TG (Monsalve et al., 2013), we hypothesized that decreased PPAR α -signaling would raise the constitutive TG levels in circulation. Surprisingly, at day 3, the serum TG levels generally remained unchanged except for a slight

decrease by TCPOBOP in WT mice, and a moderate increase by PCN in PXR-null mice (Supplemental Figure 3A). At day 60, serum TG levels also generally remained unchanged, except for a moderate increase by PCN in WT mice (Supplemental Figure 3B). Consistently, we also did not observe any changes in hepatic TG levels by TCPOBOP or PCN in WT and knockout mouse livers at day 60 (Supplemental Figure 3C). Therefore, the xenobiotic-sensors PXR and CAR mediated down-regulation of PPAR α -DNA binding is likely regional (e.g. around the Cyp4a gene loci), and do not produce an overall alteration in TG synthesis/metabolism.

DISCUSSION

In this study, using mouse models, we have demonstrated that neonatal exposure to the PXR-activator PCN and the CAR-activator TCPOBOP not only produces acute but also persistent changes in the expression of genes involved in both xenobiotic and intermediary metabolism in a PXR- and CAR-dependent manner. In particular, we have demonstrated that neonatal activation of the xenobiotic-sensing nuclear receptors PXR and CAR may lead to decreased PPAR α -signaling in adult liver, suggesting that neonatal exposure to certain xenobiotics may lead to an adult-onset of certain idiosyncratic lipid disorders.

We have previously shown that the DPGs can be up-regulated in livers of newborn mice following an acute exposure to the PXR and CAR activators (Li et al., 2015). Consistent with our previous findings, in this study, we found both Cyp2b10 and Ugt2b35 were up-regulated following neonatal exposure to TCPOBOP in 3-day-old mouse livers. In addition, we found that TCPOBOP acutely up-regulated the mRNAs Cyp3a11, Aldh1a7, Ugt2b34, Papss2, Slco1a4, and Mrp3 in 3-day-old neonatal mouse livers (24 h after exposure). These additional genes were up-regulated in this study, but was not observed previously (Li et al., 2015). This is likely because a 10-fold higher dose of TCPOBOP was used in the present study. Regarding the effect of PXR activation on neonatal hepatic gene expression, we found that both Cyp3a11 and Cyp3a25 were up-regulated following neonatal exposure to PCN in 3-day-old mouse livers. A similar up-regulation was observed in 4-day-old livers of mice treated with the PXR activator dexamethasone (Down et al., 2007). The up-regulation of Gstm2, Gstm3, and Slco1a4 by PCN in neonatal mouse livers was also consistent with our prior publication (Li et al., 2015). In addition to the expected up-regulation of many well-characterized CAR- or PXR-targeted DPGs, our

TABLE 5. Differentially Regulated DPGs by TCPOBOP at 3- or 60-Day of Age in the Livers of WT Mice

Day 3 TCPOBOP vs Day 3 Corn Oil				
Gene Symbol	Description	Phase	Fold-Change	ANOVA p-value
Cyp2b10	Cytochrome P450, family 2, subfamily b, polypeptide 10	Phase I	412.63	1.37E-07
Cyp2c55	Cytochrome P450, family 2, subfamily c, polypeptide 55	Phase I	162.12	0.000013
Aldh1a7	Aldehyde dehydrogenase family 1, subfamily A7	Phase I	30.56	0.000225
Cyp1a2	Cytochrome P450, family 1, subfamily a, polypeptide 2	Phase I	20.82	0.000081
Cyp2c50	Cytochrome P450, family 2, subfamily c, polypeptide 50	Phase I	11.17	0.000482
Sult1c2	Sulfotransferase family, cytosolic, 1C, member 2	Phase II	10.37	0.000403
Cyp2c54	Cytochrome P450, family 2, subfamily c, polypeptide 54	Phase I	9.14	0.000063
Gstt3	Glutathione S-transferase, theta 3	Phase II	8.42	0.000583
Gstm3	Glutathione S-transferase, mu 3	Phase II	8.41	0.000189
Ugt2b36	UDP glucuronosyltransferase 2 family, polypeptide B36	Phase II	8.07	0.000309
Ces1g	Carboxylesterase 1G; carboxylesterase 1	Phase I	6.73	0.00248
Cyp8b1	Cytochrome P450, family 8, subfamily b, polypeptide 1	Phase I	5.72	0.009413
Sult1d1	Sulfotransferase family 1D, member 1	Phase II	5.21	0.040664
Slc23a1	Solute carrier family 23 (nucleobase transporters), member 1	Transporters	4.77	0.003867
Cbr1	Carbonyl reductase 1	Phase II	4.49	0.000364
Cyp2j5	Cytochrome P450, family 2, subfamily j, polypeptide 5	Phase I	4.4	0.008509
Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	Phase I	4.4	0.010497
Cyp2b13	Cytochrome P450, family 2, subfamily b, polypeptide 13	Phase I	4.33	0.00022
Ugt2b34	UDP glucuronosyltransferase 2 family, polypeptide B34	Phase II	4.15	0.000553
Ephx1	Epoxide hydrolase 1, microsomal	Phase I	3.92	0.000384
Slco1a4	Solute carrier organic anion transporter family, member 1a4	Transporters	3.83	0.01557
Aldh1a1	Aldehyde dehydrogenase family 1, subfamily A1	Phase I	3.8	0.002794
Ugt2b35	UDP glucuronosyltransferase 2 family, polypeptide B35	Phase II	3.8	0.000055
Ugt2b1	UDP glucuronosyltransferase 2 family, polypeptide B1	Phase II	3.73	0.00348
Abcc3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	Transporters	3.45	0.000975
Fmo5	Flavin containing monooxygenase 5	Phase I	3.36	0.000213
Slc26a1	Solute carrier family 26 (sulfate transporter), member 1	Transporters	3.36	0.00246
Slc22a7	Solute carrier family 22 (organic anion transporter), member 7	Transporters	3.26	0.006992
Cyp2b23	Cytochrome P450, family 2, subfamily b, polypeptide 23	Phase I	3.06	0.000016
Slc22a1	Solute carrier family 22 (organic cation transporter), member 1	Transporters	3.05	0.000654
Cyp3a13	Cytochrome P450, family 3, subfamily a, polypeptide 13	Phase I	3	0.006573
Glyat	Glycine-N-acyltransferase	Phase II	3	0.010934
Sult2a5	sulfotransferase family 2A, member 5	Phase II	2.99	0.001624
Gstm1	Glutathione S-transferase, mu 1	Phase II	2.87	0.00101
Gstt1	Glutathione S-transferase, theta 1	Phase II	2.87	0.002713
Paps2	3-Phosphoadenosine 5-phosphosulfate synthase 2	Phase II	2.85	0.000886
Cyp4f15	Cytochrome P450, family 4, subfamily f, polypeptide 15	Phase I	2.84	0.003176
Ces1e	Carboxylesterase 1E	Phase I	2.76	0.002784
Cyp3a11	Cytochrome P450, family 3, subfamily a, polypeptide 11	Phase I	2.71	0.000137
Ugt2b5	UDP glucuronosyltransferase 2 family, polypeptide B5	Phase II	2.6	0.029453
Ahr	Aryl-hydrocarbon receptor	Transcription factor	2.56	0.000065
Gstm4	Glutathione S-transferase, mu 4	Phase II	2.54	0.006722
Cyp4a31	Cytochrome P450, family 4, subfamily a, polypeptide 31	Phase I	-2.65	0.013086
Cyp4a10; Cyp4a31	Cytochrome P450, family 4, subfamily a, polypeptide 10	Phase I	-5.18	0.008392
Cyp4a32	Cytochrome P450, family 4, subfamily a, polypeptide 32	Phase I	-5.44	0.011237
Day 60 TCPOBOP vs Day 60 Corn Oil				
Cyp2b10	Cytochrome P450, family 2, subfamily b, polypeptide 10	Phase I	131.29	0.000069
Cyp2c55	Cytochrome P450, family 2, subfamily c, polypeptide 55	Phase I	28.43	0.0002
Cyp2b13	Cytochrome P450, family 2, subfamily b, polypeptide 13	Phase I	6.76	0.042971
Cyp2c29	Cytochrome P450, family 2, subfamily c, polypeptide 29	Phase I	5.76	0.000109
Cyp26a1	Cytochrome P450, family 26, subfamily a, polypeptide 1	Phase I	4.27	0.016422
Gstm3	Glutathione S-transferase, mu 3	Phase II	3.93	0.001592
Dhrs9	Dehydrogenase/reductase (SDR family) member 9	Phase I	3.4	0.022735
Sult5a1	Sulfotransferase family 5A, member 1	Phase II	2.96	0.039028
Cyp2c66	Cytochrome P450, family 2, subfamily c, polypeptide 66	Phase I	2.42	0.033951
Slc3a1	Solute carrier family 3, member 1	Transporters	2.37	0.009555
Cyp2c65	Cytochrome P450, family 2, subfamily c, polypeptide 65	Phase I	2.19	0.044539
Cyp4a10; Cyp4a31	Cytochrome P450, family 4, subfamily a, polypeptide 10 or 31	Phase I	-2.39	0.033451

Note: The fold-change refers to the ratio of the expression values of the TCPOBOP treatment over the corn oil treated averaged at the same age (fold change is defined as a ratio. Positive, up-regulation; negative, the inverse of ratio, down-regulation).

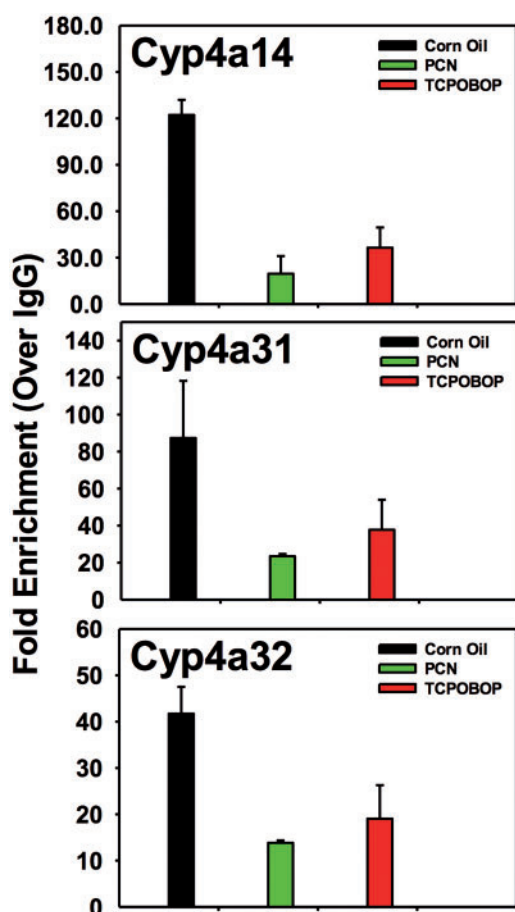


FIG. 7. Chromatin immunoprecipitation (ChIP)-qPCR of the DNA binding fold enrichment for PPAR α and RNA Pol II to Cyp4a gene loci in 60-day-old wild-type (WT), pregnane X receptor knockout mouse (PXR-null) and constitutive androstane receptor knockout mouse (CAR-null) livers. ChIP assays were performed using specific antibodies against peroxisome proliferator-activated receptor alpha (PPAR α), RNA polymerase II (RNA Pol II), and Immunoglobulin G (IgG). Data were first normalized to genomic DNA input, and then expressed as fold-enrichment over IgG control.

microarray results also revealed many other genes that are commonly or uniquely regulated by TCPOBOP or PCN in the neonatal mouse livers. For example, this study was among the first to identify that neonatal exposure to TCPOBOP and PCN both up-regulated the prototypical aryl hydrocarbon receptor (AhR)-target gene Cyp1a2 in the neonatal livers of wild-type mice, but not the CAR and PXR receptor-knockout mice. In addition, neonatal exposure to TCPOBOP or PCN both down-regulated the PPAR α -target genes Cyp4a in 3-day-old neonatal mouse livers. Therefore, there is apparent cross-talk among the xenobiotic-sensing nuclear receptors (PXR and CAR) and other nuclear receptors (such as AhR and PPAR α) following xenobiotic insult.

The effect of activation of the xenobiotic-sensors PXR and CAR on the expression of many genes in adult liver has been studied extensively (Aleksunes and Klaassen, 2012; Fisher et al., 2008), but there are fewer studies regarding the effect of exposure of young animals to PXR/CAR activators on gene expression profiles later in life. It was reported that CAR activation by an indirect activator, phenobarbital, at early life caused persistent up-regulation of Cyp2b10, Cyp2c29, and Cyp3a11 in adult mouse livers (Tien et al., 2015). Neonatal activation of CAR by TCPOBOP during neonatal age also persistently induced the

expression of Cyp2b10 and Cyp2c37 throughout the life of exposed mice (Chen et al., 2012). Consistent with previous findings, the present study has confirmed that Cyp2b10 and Cyp2c29 are both up-regulated in neonatal and adult mouse livers after neonatal exposure to TCPOBOP. However, the present microarray results did not show any significant changes in Cyp3a11 expression in adult mouse livers after neonatal exposure to TCPOBOP (Chen et al., 2012). The discrepancy of Cyp3a11 is likely due to the different CAR activators that were administered (phenobarbital vs TCPOBOP). It is possible that phenobarbital can persistently activate or down-regulate additional signaling pathways besides CAR (Tien et al., 2015).

The present study has also provided new information on a transcriptome-wide scale regarding the persistent regulation of many other DPGs by neonatal CAR activation, including a persistent up-regulation of Cyp2c55, Cyp2b13, Cyp26a1, Dhrr9, Sult5a1, Slc3a1, Cyp2c65, Cyp2c66, and Gstm3, but a persistent down-regulation of Cyp4a10 and Cyp4a31 in adult liver in a CAR-dependent manner (Table 5). In addition to the CAR-signaling, the present study is among the first to unveil the effect of neonatal PXR activation on the acute and persistent regulation of DPGs on a transcriptomic scale. Similar to CAR, neonatal activation of PXR also resulted in a number of genes differentially expressed in liver during and beyond the neonatal period (Table 4). Unexpectedly, neonatal up-regulation of PXR also leads to a persistent up-regulation of Cyp2b13/2b9, which are well-known CAR-target genes, in adult mouse liver. Therefore, there are potential interactions between these receptors and/or down-stream signaling molecules during liver development.

PPAR α stimulates gene transcription by binding to peroxisome proliferator response elements (PPRE) in the promoter of its target genes, which are involved in lipid metabolism, fatty acid oxidation, and glucose homeostasis (Zandbergen and Plutsky, 2007). Activation of PPAR α by its activator clofibrate up-regulates the expression of CYP4A genes (Maglich et al., 2009). Interestingly, the present study has found that neonatal exposure to PCN and TCPOBOP not only acutely but also persistently down-regulates the Cyp4a gene in mouse livers, at the mRNA, protein and/or enzyme activity levels in a PXR and CAR-dependent manner. Consistent with the gene expression results, ChIP assay results also showed decreased PPAR α -DNA binding to the response elements of all Cyp4 genes in adult mouse livers after neonatal exposure to PCN and TCPOBOP, indicating there might be cross-talk between xenobiotic sensors and lipid sensor during and beyond the neonatal period. Many studies have demonstrated the role of CAR in xenobiotic metabolism, but CAR has also been implicated in the down-regulation of many critical genes involved in bile acid synthesis and fatty acid beta-oxidation (Miao et al., 2006). It has been reported that TCPOBOP-activated CAR decreases the expression of PPAR α mRNA as well as Cyp4a14 in livers of adult wild-type mice, whereas the PPAR α activity increased in the absence of CAR, and an increased hepatic fatty acid oxidation was detected in the CAR $^{-/-}$ mice (Maglich et al., 2009). Similar to the CAR-signaling, the activation of PXR also reduced expression of PPAR α mRNA in the adult mouse liver after administration of the PXR ligand PCN (Inoue et al., 2008). As shown in Figure 5, when PCN was administered to mice at 3-days of age, Cyp4a14, Cyp4a31 and Cyp4a32 are down-regulated at both day 3 and day 60 only in WT mice, but not in PXR-null mice. However, the basal Cyp4a expression was not further up-regulated in livers of control PXR-null mice, indicating that the basal expression of Cyp4a is not regulated by PXR. Cyp4a genes are prototypical

targets of PPAR α , thus PPAR α is likely responsible for the constitutive expression of Cyp4a genes in liver. The mechanism regarding how neonatal CAR and PXR activation modulate PPAR α activity during development is still unclear and needs to be further investigated in future studies.

In addition to the xenobiotic biotransformation pathways and the PPAR α -signaling, neonatal exposure to PCN and CAR also regulates other critical genes involved in the endobiotic signaling. For example, neonatal activation of PXR down-regulated the mRNA expression of Cyp7a1, which is rate-limiting enzyme in the synthesis of bile acids, as well as the nuclear receptor small heterodimer partner (SHP/NR0B2) in neonatal mouse livers (Figure 2 and Supplemental Figure 1). SHP plays an essential role in maintaining cholesterol and bile acid homeostasis by inhibiting cholesterol conversion to bile acids (Miao et al., 2009). Our results were consistent with other studies that showed rifampicin-induced PXR activation could inhibit both CYP7A1 and SHP gene transcription in primary human hepatocyte and HepG2 cells (Li and Chiang, 2006). Future studies will be performed to reveal the mechanisms underlying the effect of neonatal PXR activation on cholesterol and bile acid homeostasis.

Regarding the tissue-specific effects due to PXR- and CAR-depletion in PXR-null and CAR-null mice, it has been shown before that CAR is predominantly expressed in liver, gallbladder, and small intestine, whereas PXR is predominantly expressed in liver, gallbladder, and both the small and large intestine. These 2 receptors are minimally expressed in other organs (Bookout et al., 2006). Therefore, the depletion of PXR and CAR is expected to affect mainly the liver and the GI tract. It is possible that the absence of PXR and CAR in intestine may produce non-hepatic effects, such as changes in the disposition of microbial metabolite and nutritional factors that may influence the hepatic expression of certain genes. Future studies using tissue-specific PXR- and CAR-null mice will address this issue. Because our wild-type control mice and PXR and CAR null mice are not siblings, comparisons between the lines of mice are limited. We instead have focused the interpretation of our results and discussion on the response of these different lines to the PXR and CAR activators.

Regarding the pharmacokinetics of TCPOBOP or PCN, we speculate that at day 3, there will be significant amount of PCN and TCPOBOP present in liver, whereas 60 days later, the chemicals will be eliminated from the body. This expectation is based on previous studies in the literature. Specifically, the pharmacokinetics of TCPOBOP has been examined in Sprague Dawley rats and mice, and the results have demonstrated that this chemical has a long biological half-life and is stored primarily in the fat as the parent compound. The fat levels of TCPOBOP decrease slowly over a 30-day period (Poland et al., 1981), thus we do not expect a significant amount of TCPOBOP present in the liver 60 days after the initial exposure. For PCN, currently there are no publications regarding the clearance of this chemical. However, it has been shown that the half-life of pregnenolone, which is a structurally similar compound to PCN, is 4.4min in guinea pigs (Weinand et al., 1966). Therefore, we speculate that PCN will likely have a short half-life and minimal levels will be present in day 60 liver samples. It has been shown before that neonatal activation of CAR mediated persistent increase in certain P450s is due to epigenetic memory (Chen et al., 2012). It is possible that neonatal activation of PXR mediated persistent changes in certain genes is also due to epigenetic memory, and this hypothesis will be tested in future studies.

In conclusion, the present study has identified that neonatal exposure to PXR and CAR activators leads to both acute and persistent changes in the hepatic expression of genes involved in drug and lipid metabolism, suggesting that it is important to understand the connections between neonatal exposure to PXR and CAR activators and the adult onset of PPAR α -related human liver diseases.

SUPPLEMENTARY DATA

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

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