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Characterization of polybrominated diphenyl ether toxicity in Wistar Han rats and use of liver microarray data for predicting disease susceptibilities

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Abstract

The toxicity of polybrominated diphenyl ethers (PBDEs), flame retardant components, was characterized in offspring from Wistar Han dams exposed by gavage to a PBDE mixture (DE71) starting at gestation day 6 (GD 6) and continuing to weaning on postnatal day 21 (PND 21). Offspring from the dams began PBDE direct dosing by gavage at the same dose as their dam on PND 12 – PND 21, and then after weaning, 5 days/week for another 13 weeks. Liver samples were collected at PND 22 and week 13 for liver gene expression analysis (Affymetrix Rat Genome 230 2.0 Array). PBDE treatment induced 1,066 liver gene transcript changes in females and 1,200 transcriptional changes in males at PND 22 (false discovery rate (FDR) < 0.01), but only 263 liver transcriptional changes at 13 weeks in male rats (FDR < 0.05). No significant differences in dose response were found between male and female pups. Transcript changes at PND 22 coded for proteins in xenobiotic, sterol and lipid metabolism, and cell cycle regulation, and overlapped rodent liver transcript patterns after high fat diet or phenobarbital exposure. These findings, along with the observed PBDE-induced liver hypertrophy and vacuolization, suggests that long-term PBDE exposures have potential to modify cell functions that contribute to metabolic disease and/or cancer susceptibilities.

Keywords

polybrominated diphenyl ethers; liver toxicity; lipid metabolism

Introduction

Polybrominated diphenyl ether (PBDE) flame retardant exposures are widespread in the United States (Schechter *et al.* 2010). The PBDEs have physicochemical properties similar to those of other persistent organic chemicals; they are hydrophobic and lipophilic, have a low vapor pressure, and a high log K_{ow} (U. S. Environmental Protection Agency 2008a, b, c). There are up to 209 possible isomers of PBDEs. The most common PBDEs found in the environment include PBDE 47, 99, and 153 (U. S. Environmental Protection Agency 2010; World Health Organization 2003). The most prevalent PBDE congener found in human tissue is PBDE 47 (Petreas *et al.* 2003; Schechter, Colacino, Sjodin, Needham and Birnbaum

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2010; Sjodin *et al.* 2004). PBDE congeners show long-range atmospheric transport, environmental persistence and bioaccumulation in various species (including humans) and are considered persistent organic pollutants (POP) (World Health Organization 2003).

Environmental exposures come from the use of PBDEs as flame retardants found in polyester foams and other household products. Leaching of PBDEs from these foams deposited at waste dumps may result in the release PBDE isomers into the environment (water, fish, seals, humans) (Hale *et al.* 2003; Hale *et al.* 2002; Hale *et al.* 2001). The U. S. EPA has recently conducted an exposure assessment for PBDEs and reports that exposure in children (average PBDE intake, 47.2 ng/kg/day) is greater than in adults (average PBDE intake, 7.1 ng/kg/day) (U. S. Environmental Protection Agency 2010). PBDE exposure to the infant may occur from mother's milk (Schechter, Colacino, Sjodin, Needham and Birnbaum 2010; Schechter *et al.* 2006) and house dust (Frederiksen *et al.* 2010; Harrad *et al.* 2010; Johnson *et al.* 2010; Lorber 2008). PBDE exposure is associated with delayed time to pregnancy in women (Harley *et al.* 2010) and in altered learning parameters in children (Roze *et al.* 2009).

The European Union banned the use and sale of PBDEs in 2004 and the U. S. manufacturer of PBDEs voluntarily phased out its production in 2004 (U. S. Environmental Protection Agency 2009). Nevertheless, the potential for PBDE exposures remain a concern because of continued widespread occurrence of these chemicals in the environment, and the long half life of PBDEs in humans (up to 6 years) (U. S. Environmental Protection Agency 2010).

The biological effects of PBDEs in rodents include thyroid and liver toxicity, developmental neurotoxic effects, and reproductive toxicity (U. S. Environmental Protection Agency 2008a, b, c). PBDEs disrupt endocrine activity (Hamers *et al.* 2006; Legler and Brouwer 2003; Meerts *et al.* 2001), alter thyroid hormone levels (Darnerud *et al.* 2007; Ellis-Hutchings *et al.* 2006; Zhou *et al.* 2002), and induce liver cytochrome p450 levels (Birnbaum and Cohen Hubal 2006; Sanders *et al.* 2005; Zhou, Taylor, DeVito and Crofton 2002). PBDE-induced toxicities may increase the risk for the development of other disease (Ervin 2009); (Baker *et al.* 2007; Yanovski and Yanovski 2002). Exposures to PBDEs, nongenotoxic chemicals (Agency for Toxic Substances and Disease Registry 2004), may cause conditions characteristic of those seen in metabolic disease (Lim *et al.* 2008).

Many PBDE toxicities, including endocrine and liver toxicity, are similar across species (Harley, Marks, Chevrier, Bradman, Sjodin and Eskenazi 2010; Roze, Meijer, Bakker, Van Braeckel, Sauer and Bos 2009; U. S. Environmental Protection Agency 2008a, b, c, d). In this paper we analyze PBDE-induced rat liver toxicity and gene transcript changes to better understand the molecular basis for PBDE toxicities.

Methods and Materials

Chemical

DE-71 (technical pentabromodiphenyl oxide; CASs. No. 32534-81-9) was obtained from Great Lakes Chemical Corporation (West Lafayette, IN - Lot 25500A30A). Components of this mixture included primarily the tetra through penta PBDEs and a small component of

hexabromodiphenyl ethers. The molecular weight for tetrabrominated diphenyl ethers is 469.6, for pentabromated diphenyl ethers, 548.5, and for hexabromo diphenyl ethers, 627.4. The DE71 bulk chemical analysis (National Toxicology Program 2004; Sanders, Burka, Smith, Black, James and Cunningham 2005) showed the following composition: 2,2',4,4'-tetrabromodiphenyl ether- BDE-47 (36%), 2,2',4,4',5-pentabromodiphenyl ether - BDE-99 (42%); 2,2',4,4',6-pentabromodiphenyl ether - BDE-100 (10%), 2,2',4,4',5,5'-hexabromodiphenyl ether - BDE-153 (3%), 2,2',4,4',5,6'-hexabromodiphenyl ether - BDE-154 (4%), and 2,2',3,4,4'-pentabromodiphenyl ether - BDE-85 (2%). The remaining 3% consisted of several identified tri-heptaBDEs and some unidentified PBDEs (National Toxicology Program 2004). This flame retardant mixture also contained approximately 66 nanograms/gram of brominated dioxins or furans. Consequently, a dose of 50 mg/kg is a dose of approximately 3.3 nanograms/kg body weight of brominated dioxins or furans, a dose not expected to contribute to liver toxicity (National Toxicology Program 2004; Sanders, Burka, Smith, Black, James and Cunningham 2005).

Experimental Design

Time mated female Wistar/Han dams (CRL:WI (HAN)) were obtained from Charles River Laboratories (Raleigh, NC). Dams were dosed by gavage with 0 or 50 mg/kg DE71 in corn oil (dosing volume 5 ml/kg) from GD 6 through PND 21, 7 days per week. Pups were dosed by gavage at the same dose as their dam starting on PND 12 and continuing through PND 21 for 7 days per week until weaning. Weaning occurred on PND 21, which was designated as Day 1 of the 13-week portion of the study. On day one of the 13-week portion of the study dosing began on a 5 days/week schedule. Pups used for PND 22 toxicogenomic study were dosed through PND 21, the day prior to PND 22 liver sample collection. Gestational exposure at 50 mg/kg did not affect littering endpoints for number of dams delivering live litters, number of live or dead pups per litter, or body weights of pups during lactation (data not shown). The NIH 07 diet (Ziegler Brothers, Inc., Gardners, PA) was given to dams and pups throughout the pregnancy and gestation periods and until PND 22 at which time pups were given NTP-2000 diet (Ziegler Brothers, Inc., Gardners, PA). Tap water and NTP-2000 diet were available *ad libitum* (National Toxicology Program 2005a, b).

On PND 22, liver samples from five male and five female rat pups from the control and five male and five female rat pups from the 50 mg/kg group were collected for toxicogenomic analysis (each pup came from a different dam – total of ten dams/control group and ten dams/50 mg/kg group). Liver samples were collected from ten males and ten females from the control and ten males and ten females from the 50 mg/kg groups for histopathologic analysis (total of twenty dams/control group and twenty dams/50 mg/kg group). Five male rat liver samples were randomly selected from each group for the 13 week toxicogenomic analysis. No females were sampled for toxicogenomic analysis at 13 weeks due to possible interference of estrus cycling with interpretation of toxicogenomic findings at this timepoint.

The care of animals on this study was according to NIH procedures as described in the “The U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals”, available from the Office of Laboratory Animal Welfare, National Institutes of Health, Department of Health and Human Services, RKL1, Suite 360, MSC 7982, 6705 Rockledge

Drive, Bethesda, MD 20892-7982 or online at <http://grants.nih.gov/grants/olaw/olaw.htm#pol>". The study protocols were approved by the local animal care and use committee.

Necropsy and collection of liver samples for toxigenomic analysis

Animals at PND 22 and 13 weeks were euthanized with carbon dioxide. For liver collection, the abdominal cavity of each rat was opened, and the liver removed. A portion of the liver was minced on an ice-cold surface, and placed into RNeasy® (Ambion, Inc., Austin, TX) for incubation overnight at 4° C. The samples were then transferred to – 20° C for storage prior to RNA extraction.

Liver Histopathology

Sections of the livers from the week 13 male rats were fixed in 10% neutral buffer formalin, embedded in paraffin, sectioned at approximately 5 microns, and mounted on glass slides. After staining with hematoxylin and eosin, cover-slips were attached to the slides. Tissues were then evaluated microscopically at different magnifications by a board certified veterinary pathologist.

The grading criteria for hepatocellular hypertrophy was as follows: minimal severity was recorded when hepatocellular hypertrophy was observed but was present in <10% of the hepatocytes in the section; mild severity was recorded when 10% and <50% of the hepatocytes in the section were affected; moderate severity was recorded when 50% and <75% of the hepatocytes in the section were affected; and marked severity was recorded when 75% of the hepatocytes in the section were affected. Cytoplasmic vacuolization was graded independently of hepatocellular hypertrophy, and used the following grading scale: minimal severity was recorded when cytoplasmic vacuolization was observed but was present in <25% of the hepatocytes in the section; mild severity was recorded when 25% and <50% of the hepatocytes in the section were affected; moderate severity was recorded when 50% and <75% of the hepatocytes in the section were affected; and marked severity was recorded when 75% of the hepatocytes in the section were affected. Livers from control and treated rats were cryosectioned and stained with Oil Red O to identify the presence of lipids.

RNA preparation and microarray hybridization

A section of the liver was collected at necropsy and placed into RNeasy® (Ambion, Inc., Austin, TX). RNA was extracted from liver using the QIAGEN RNeasy® (QIAGEN, Valencia, CA). The RNA was analyzed for quantity and purity by UV analysis using the NanoDrop ND-1000 (NanoDropTechnologies, Wilmington, DE). Samples were concentrated using Microcon filters (Millipore, Billerica, MA). All samples were evaluated for RNA integrity by gel electrophoresis using the Flash Gel RNA cassette system (Lonza, Rockland, ME). Total RNA (50 ng) was used to synthesize double-stranded cDNA for each sample using Affymetrix GeneChipExpression 3' amplification two-cycle target labeling and control reagents (Affymetrix Inc. Santa Clara, CA). The cDNA served as a template to synthesize biotin-labeled antisense cRNA using an in vitro transcription (IVT) labeling kit. Labeled cRNA was fragmented and hybridized to the Affymetrix Rat Genome 230 2.0

Genechip® Array. Array hybridization, washing, and staining were performed according to the Affymetrix recommended protocol EuKGE_Ws2v5. The chips were scanned using an Affymetrix GeneChip Scanner 3000. Quality control measurements were evaluated to determine if the data derived from the arrays were of sufficient quality prior to comparisons for differential expression.

Microarray Data Analysis

Data normalization—Probe intensity data from all Rat Genome 230 version 2 Affymetrix GeneChip® arrays was read into the R software environment (<http://www.R-project.org>) directly from .CEL files using the R/affy package (Gautier *et al.* 2004). Probe-level data quality was assessed using image reconstruction, histograms of raw signal intensities, hierarchical clustering of samples and MvsA plots. Normalization was carried out using the robust multi-array average (RMA) method using all probe intensity data sets together (Irrizarry *et al.* 2003). Briefly, the RMA method adjusts the background of perfect match (PM) probes, applies a quantile normalization of the corrected PM values, and calculates final expression measures using the Tukey median polish algorithm.

Statistical assessment of differential gene expression

Analysis of variance (ANOVA) methods were used to statistically resolve gene expression differences using the R/maanova package (Wu *et al.* 2003). To find differentially expressed probe sets in response to dose, the data was subset into male pups, female pups, or male rats. Then the model,

$$Y_i = \mu + \text{DOSE} + \varepsilon_i \quad (1)$$

was used to fit the log-transformed gene expression measures Y_i , where μ is the mean for each array, DOSE is the dose effect (control, 50 mg/kg) and ε_i captures random error. To find sex-related effects in pups, the data was subset into 59 mg/kg dose or control pups and the model,

$$Y_i = \mu + \text{SEX} + \varepsilon_i \quad (2)$$

was used, where SEX is the sex effect (male, female). To find age-related effects in males, the model,

$$Y_i = \mu + \text{AGE} + \text{DOSE} + \text{AGE:DOSE} + \varepsilon_i \quad (3)$$

was used where AGE is the effect for age (PND 22, 13 weeks), DOSE is the dose effect (control, 50 mg/kg) and AGE:DOSE is the interaction effect between age and dose. Finally, to test for sex-dependent responses in dose response, the model,

$$Y_i = \mu + \text{SEX} + \text{DOSE} + \text{SEX:DOSE} + \varepsilon_i \quad (4)$$

was used where SEX:DOSE is the interaction effect between sex and dose. All statistical tests were performed using F_s , a modified F-statistic incorporating shrinkage estimates of variance components (Cui *et al.* 2005). P-values were calculated by permuting model residuals 1000 times and corrected using the p.adjust function in R using method = "BH".

Gene ontology (GO) analyses—Over-represented classifications of genes were determined from statistical outcomes by testing for association with ‘biological process’ GO terms. Enrichment of pathway members among differentially expressed probe sets was found using the one-tailed Fisher exact test for 2×2 contingency tables. Mappings between Affymetrix probe sets, Entrez gene identifiers and GO terms are based on the R/rat2302.db package (www.bioconductor.org).

Comparison of gene transcript expression to human disease—Gene lists and the normalized gene expression estimates (see Equation 1–Equation 4 above) were compared against results in the literature using the NextBio Platform (Kupershmidt *et al.* 2010). The NextBio Platform is a web-based tool to search, discover, and share knowledge using correlation analyses that are able to integrate data across various gene expression platforms and experiments for public and proprietary data. These studies include data sets which have been published and are available in GEO.

Using the NextBio datasets, we compared PND 22 male pup liver transcript data (~ 1200 differentially expressed transcripts; false discovery rate (FDR) < 0.01) to gene transcript patterns in other disease conditions. PBDE transcript patterns were compared to those in rats after phenobarbital exposure (Waterman *et al.* 2010). Because brominated chemicals induce colon cancers in rodents (Dunnick *et al.* 1997b), and to further examine the potential of brominated flame retardants to create colon disease susceptibilities we compared the PBDE transcript patterns to those in human colon cancer gene expression profiles (Ancona *et al.* 2006; Irizarry *et al.* 2009; Jorissen *et al.* 2008; Saaf *et al.* 2007).

Results

Polybrominated diphenyl ether exposures (DE71) at 50 mg/kg caused no treatment-related effects on survival of rats. Body weight of treated males was 7% greater than controls ($p = 0.1328$) and 14% lower than controls for females at week 13 ($p = 0.0015$) (Table 1). Treatment-related liver toxicity and increased liver weights occurred in both sexes (Table 1; Figure 1). At week 13, the liver toxicity consisted of hepatocyte hypertrophy characterized by enlarged hepatocytes with an increased amount of cytoplasm, enlarged nuclei, and pale eosinophilic and granular cytoplasm. The hepatocyte hypertrophy was primarily observed in the centrilobular and midzonal regions of the hepaticlobule (lesion not shown).

Hepatocyte cytoplasmic vacuolization was also observed at 13 weeks in treated rats and consisted of multiple, small, variably-sized vacuoles or large discrete, singular vacuoles within the cytoplasm of enlarged hepatocytes. Some hepatocytes contained a few scattered, small vacuoles within the cytoplasm and other hepatocytes were distended with numerous small vacuoles in the cytoplasm (microvesicular vacuolization). In addition, scattered large, generally singular but occasionally multiple, clear vacuoles, compatible with macrovesicular vacuolization, were also noted in association with the microvesicular cytoplasmic vacuolization (Figure 1). Hepatocellular cytoplasmic vacuolization was most prominent in the periportal regions. Positive staining with Oil Red O indicated the vesicles within the cells from treated rats contained lipid (Figure 2).

A total of 1,200 differentially expressed transcripts (DETs; control vs. treated animals) were found in male pups and 1,066 DETS were found in females, at PND 22 (FDR <0.01). The gene list size for 13 week male rats was too small to investigate at FDR < 0.01. However, at a FDR of 0.05 there were 263 DETs in male rats at week 13 (See Table 2 and Supplemental tables 1,2,3). Approximately 90 genes in common were found to be differentially expressed due to PBDE treatment (FDR <0.05) in PND 22 males, PND 22 females, and week 13 males (Figure 3; Supplement 4). Some of the transcripts that were upregulated at PND 22 and week 13 included *aldh1a1*, *cyp1a1*, *abcc3*, *zshx2*, *far4*, *cltb*, *abhd4*, *abhd4*, *ces2*, *zeint*, *fam134b*, and *vps26a*. However, at PND 22 there were many more cytochrome P450s upregulated than at week 13.

No sex-dependent differences in gene expression response to PBDEs were found at PND 22 (Table 2, FDR <0.01). Therefore, we used male PND 22 data to represent the PND 22 profile. With increasing time of PBDE exposures, an adaptive response to the PBDE liver toxicity was suggested by the larger number of DETs found at PND 22 (3,055 DETs FDR < 0.05) than at week 13. A total of 447 age-dependent changes in gene expression response to PBDEs were found here (FDR <0.05).

GO analysis (Supplement 5) indicated that gene transcript changes found at PND 22 (Supplement 1, 2) and week 13 (Supplement 3) are important in multiple cell functions, including oxidative and metabolic functions (Figure 4). In PND 22 male livers, there was upregulation of aldehyde dehydrogenases, aldo-keto reductases, glutathione S-transferases and carbonyl reductases (Table 3A). Alterations in transcripts for lipid metabolism were also significant at PND 22 (Table 3B). Many cytochrome P450 transcripts were increased in treated PND 22 pups (Table 3C). PBDE treatment induced alterations in cell cycle gene transcripts and in cancer disease pathways (Table 3D). Upregulation of the *Abcc3* transcript (functions in metabolite conjugation) and glutathione S-transferase alpha 4 (*Gsta4*) occurred at both PND 22 and week 13.

After scrutinizing the expression pattern of important genes (described above), PND 22 transcript data were subjected to a global bioinformatics analysis using NextBio software to compare the expression profiles obtained in this study to expression profiles found in the literature. PBDE liver gene transcript patterns correlated with other rodent studies on high fat diets including upregulation of *cyp2c7*, *ces2*, *lpl*, *cyp2c*, *cyp2b2* (Almon *et al.* 2009; van Erk 2011). PBDE transcript patterns correlated with liver transcript patterns after phenobarbital exposure (rats) (Supplement 6), with 67 upregulated transcripts and 164 downregulated transcripts that overlapped those in phenobarbital treated rats including upregulation (>4 fold) of transcripts for P450, chemokine, carboxyesterase, and growth arrest activity (*cyp1a1*, *cxcl9*, *ces2*, *zdhhc2*, *lpl*, *cyp2b2*, *rn71071*, *cyp1b1*, *egin3*, and *gadd45b*).

PBDE transcript patterns were similar to some of the changes found in human cancers including colon cancers (Supplement 7). Transcripts upregulated in human colon cancer and after PBDE treatment included *cyp2b6*, *fam134b*, *chek1*, *rcan2*, *ccng2*, *ccl5*, and *acacb*.

Discussion

In utero/postnatal exposure of Wistar Han rats starting on GD 6 (start of organogenesis) to a mixture of low molecular weight polybrominated diphenyl ethers (PBDEs) resulted in liver toxicity characterized by hepatocyte hypertrophy and hepatocyte cytoplasmic vacuolization. This liver toxicity in the Wistar Han rat is similar to that observed in the F344/N rat after PBDE exposures (Dunnick and Nyska 2009). Hepatocellular vacuolization, as seen in PBDE rat livers, is often indicative of accumulation of fat deposits (Eustis *et al.* 1990). Non-alcoholic fatty liver is a hepatic manifestation of the metabolic syndrome (Lucero *et al.* 2010), and a common cause of liver enzyme elevation in humans and in rats (Stickel and Hellerbrand 2010).

The occurrence of hepatocellular hypertrophy and vacuolization may predispose rats to liver cancer. However, not all exposures that result in these liver histopathologic features lead to liver cancer (Maronpot *et al.* 2010). From a review of NTP chemical study findings at 90-days, only 45% of rat studies with chemical-induced liver hypertrophy went on to be rat liver carcinogens in 2-year cancer studies (Allen *et al.* 2004). Thus, there is a need for additional biomarkers to predict disease.

PBDE induced liver gene transcript changes that modify cell pathways and functions that, along with the liver toxicity, may lead to disease susceptibilities. This includes alterations in lipid and metabolic pathways. For instance at PND 22, PBDE increased the transcript for lipoprotein lipase 7-fold versus that in untreated liver. An increase in lipoprotein lipase activity has been associated with diabetes and metabolic diseases (Lopaschuk *et al.* 2010). PBDE decreased the *cyp7a* transcript which codes for an enzyme critical in metabolism of cholesterol to bile acids and in controlling hepatic triglyceride levels (Lee *et al.* 2010). The PBDE transcript patterns reported here, support other findings which show that PBDE exposures lead to increased body weights in rodents and alterations in adipocyte metabolism (Hoppe and Carey 2007; Suvorov and Takser 2010).

Thyroid homeostasis is important in preventing metabolic, reproductive, developmental and cardiac disease (Choksi *et al.* 2003; Jahnke *et al.* 2004; Miller *et al.* 2009; Pedrelli *et al.* 2010). PBDE-induced alterations in thyroid hormone levels (Dunnick and Nyska 2009; Richardson *et al.* 2008) may affect mitochondria biogenesis (Leigh-Brown *et al.* 2010) or alter mitochondria based TCA cycle and lipid distribution (Harper and Seifert 2008). Liver gene transcript analysis in the current study supports a finding of altered lipid metabolism. A PBDE-induced effect on lipid metabolism along with PBDE-induced endocrine effects may create susceptibilities to metabolic diseases.

The PBDE induced liver toxicity was accompanied by increases in liver P450 transcripts including those for *cyp2a3a*, *cyp1a1*, *cyp1b1*, *cyp2c7*, *cyp2c13*, *cyt2c*, *cyp2b*, coding for enzymes critical in metabolism of chemicals, drugs, fatty acids, and steroids (Yang *et al.* 2010). Up regulation of these cytochrome P450 liver enzymes has been found with other chemicals causing fatty liver (steatosis) (Lee *et al.* 2008; Lee, Kim, Kim, Kang, Kong and Lee 2010), and is associated with the development of carcinogenic events (Rendic and Guengerich 2010). An adaptive response to the PBDE liver toxicity was suggested by fewer

significant liver gene transcript changes at week 13 than at PND 22, and induced liver transcripts for enzymes involved in decreasing aldehyde loads (Ellis 2007) and this may be part of an adaptive response to toxicity.

PBDE and dioxin exposures are both associated with increases in liver P450 enzyme levels (Boverhof *et al.* 2006a; Boverhof *et al.* 2006b; N'Jai *et al.* 2008; Szabo *et al.* 2009); (Sanders, Burka, Smith, Black, James and Cunningham 2005), and may involve activation of AH, PXR, or CAR receptors (Szabo, Richardson, Ross, Diliberto, Kodavanti and Birnbaum 2009). The PBDE mixture used in our studies contained a low level of brominated dioxins and furans that by themselves would not be expected to cause cancers (Dunnick and Nyska 2009), but in combination with the PBDEs may have synergistic carcinogenic effects. Whether that occurred in our studies would require further investigation.

Other studies report the PBDE exposures increase expression of genes coding for liver enzymes, including *cyp2B*, hepatic deiodinase 1 protein (D1) activities and *cyp2b1*, *d1*, and hepatic efflux transporter gene expression (*mdr1*, *mip2*, *mip3*) in rats at PND4, PND21, and PND60 (Szabo, Richardson, Ross, Diliberto, Kodavanti and Birnbaum 2009). Not all of these liver gene transcripts were upregulated in our PBDE studies, (although a different strain of rat (Long Evans rat) was used in the Szabo et al (2009) studies).

The liver gene expression profile generated by PBDE at PND 22 was similar to the transcriptional profile produced by phenobarbital in rodents ((Nesnow *et al.* 2009; Waterman, Currie, Cottrell, Dow, Wright, Waterfield and Griffin 2010), a chemical that induces liver tumors in rodents (Butler 1978; Imaida and Wang 1986; Rossi *et al.* 1977). The changes include transcript abundance increases for P450s, chemokines, carboxylesterases, and growth factor arrest (*gadd45b*) gene transcript levels, and were similar to liver gene transcript patterns found in human liver cancers (Frau *et al.* 2010; Tanaka and Arii 2010; Teoh 2009).

Liver genotoxic carcinogens are characterized by a specific liver fingerprint after 14 days or 13 weeks of exposure (upregulation of *mybl2*, *cyp1A1*, and *adam8* and downregulation of *wwox*, *fh1t*, *RGD1561899*) (Auerbach *et al.* 2009). This “genotoxic chemical” fingerprint was not seen with PBDE exposure, except for the upregulation of *cyp1A1*. Based on these findings, the PBDE liver toxic mechanisms appear to differ from those of genotoxic liver toxins.

PBDE liver transcript patterns include those found in metabolic disease conditions. Metabolic disease conditions may contribute to the development of cancer (e.g. colorectal or uterine cancer) or heart disease particularly when occurring in combination with other underlying genetic conditions or environmental exposures (Lopaschuk, Ussher, Folmes, Jaswal and Stanley 2010).

PBDE liver gene transcript patterns overlapped those seen in human cancer (colon) including upregulation of cyclins (Koliadi *et al.* 2010) and other cell cycle controls (Stephens *et al.* 2011). In other studies, we reported that brominated chemicals may have a particular propensity to cause intestinal tumors (Dunnick *et al.* 1997a). Bromide radical formation in the intestine resulting in DNA damage (Ballmaier and Epe 2006) is one

hypothesis for the cause of a brominated chemical carcinogenic response in the intestine, and additional studies would be needed to determine if this cancer occurs after long-term PBDE exposures.

PBDE treatment decreased expression of *cyp17a1*, a decrease that is associated with blocking androgen production (Hofland *et al.* 2010) and altering sex steroidogenesis (Canton *et al.* 2006). PBDE transcript patterns associated with alterations in the endocrine system are consistent with the ability of PBDEs to cause MCF7 cell proliferation (Darnerud 2008; Mercado-Feliciano and Bigsby 2008a, b; Song *et al.* 2008; Talsness *et al.* 2008). In addition, PBDE is an ER alpha and beta agonist in human T47D breast cancer cell lines (Meerts, Letcher, Hoving, Marsh, Bergman, Lemmen, van der Burg and Brouwer 2001).

PBDE and its metabolites (e. g. 6-OH-BDE47) may increase reactive oxygen species leading to lipid peroxidation, aldehyde formation and DNA adduct formation (An *et al.* 2011; Jin *et al.* 2010). For these reasons, PBDE exposures could contribute to the develop of carcinogenesis processes as suggested by our findings (Figure 5).

The liver gene transcript changes observed in this study suggest that PBDE exposures could lead to disease susceptibilities including those for metabolic/lipid disease (e.g. lipoprotein lipase (*lpl*)), cancer (e. g. cell cycle control transcripts (cyclins) or chemokine transcripts), and sex hormone synthesis (*cyp17*).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations in supplements

PMH	PND 22 PBDE treated male pups
PMC	PND 22 control male pups
PFH	PND 22 PBDE treated female pups
PFC	PND 22 control female pups
RMH	week 13 PBDE treated males
RMC	week 13 control males

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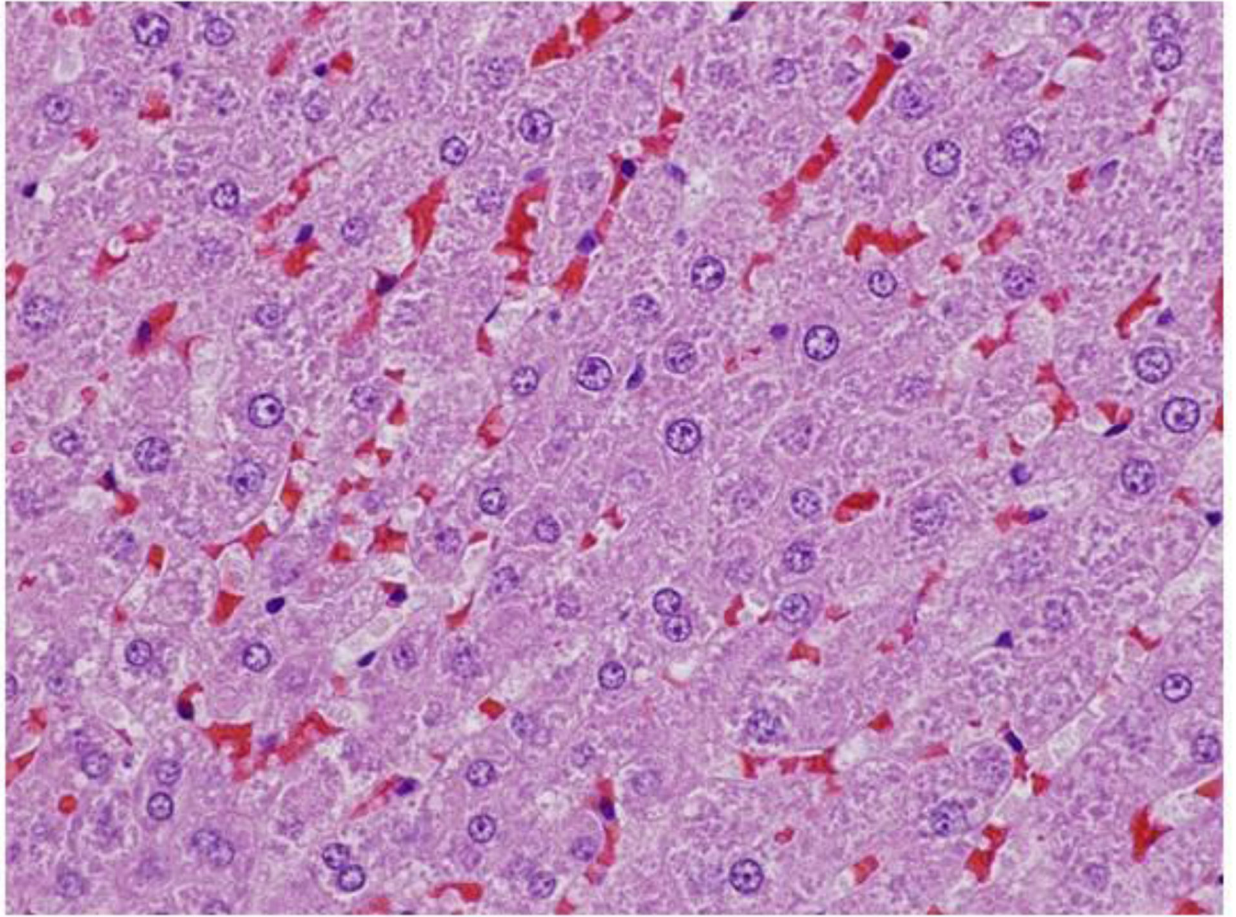
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A



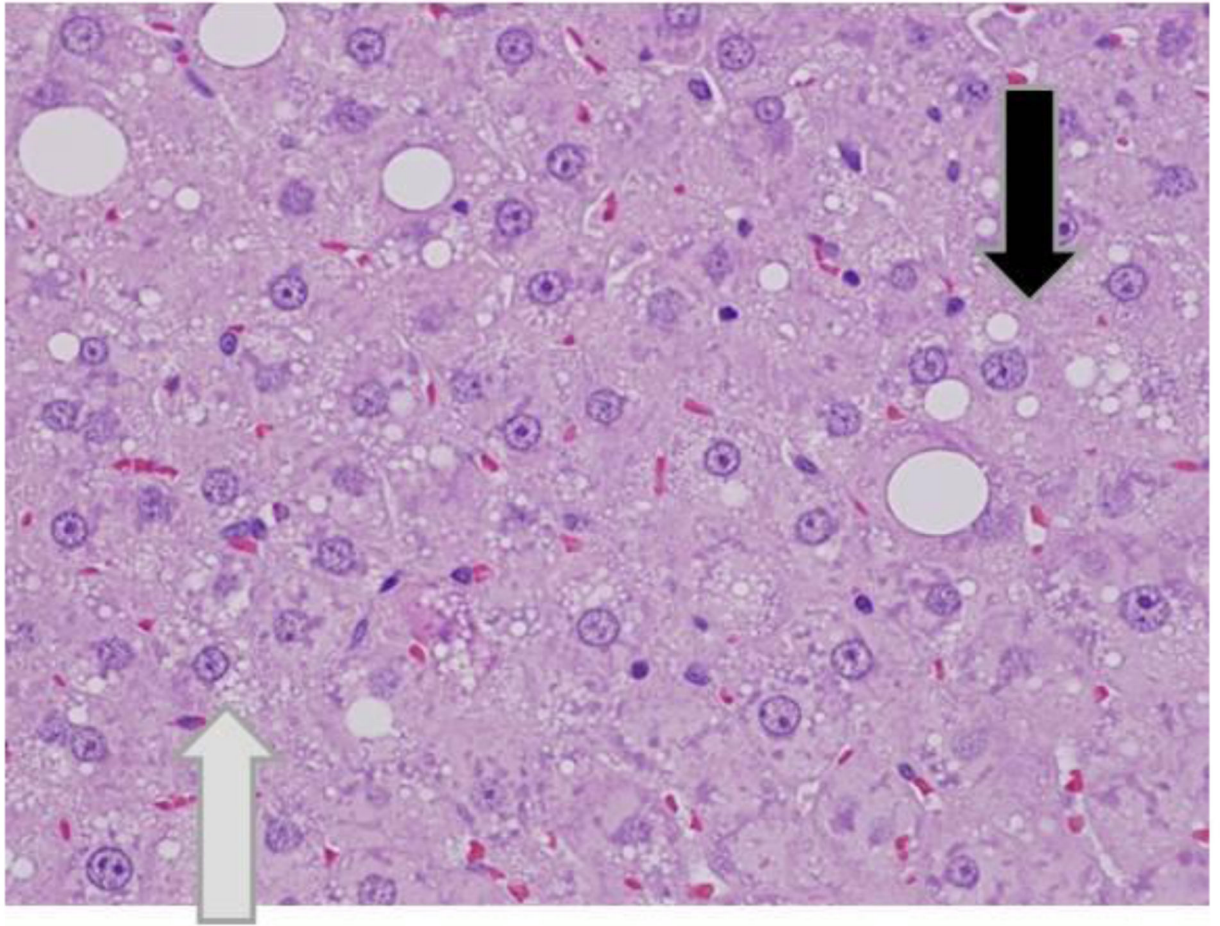
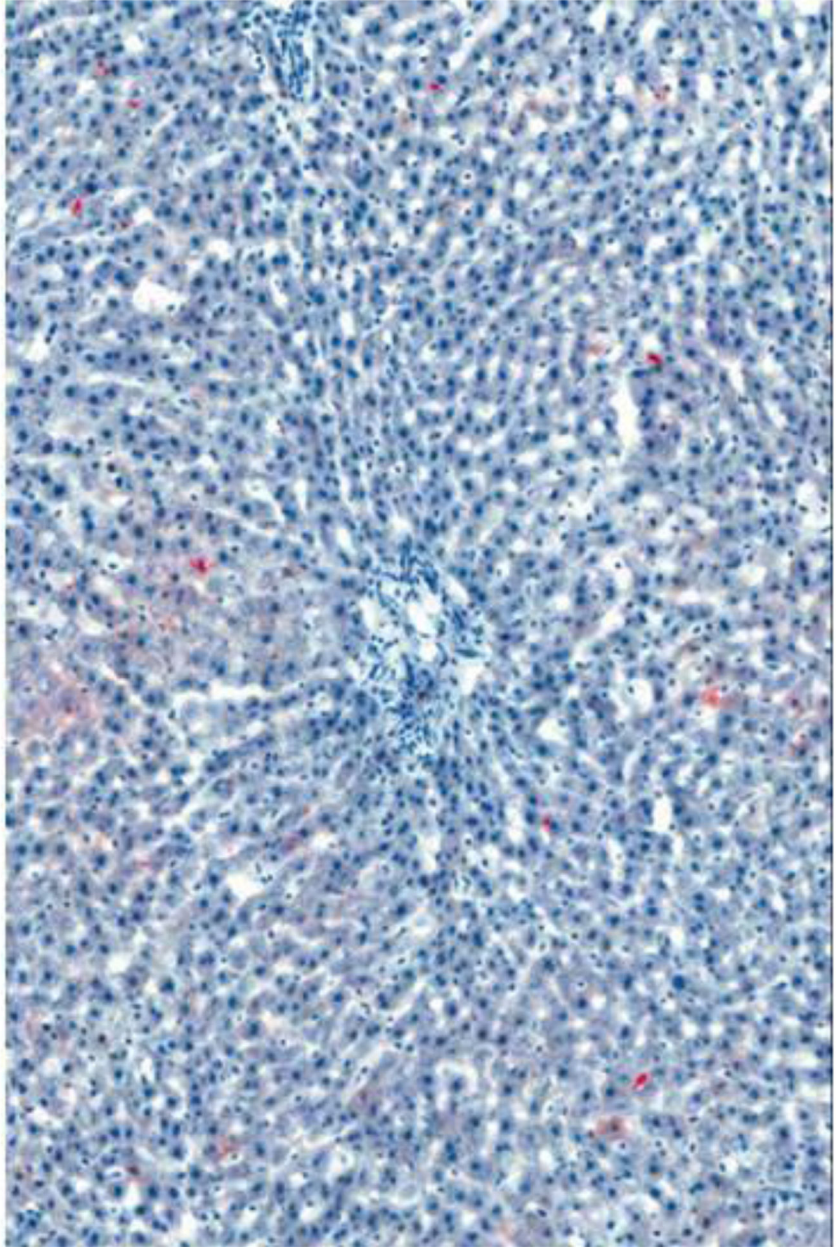
B

Figure 1.
Photomicrographs of control and PBDE treated livers at 13 weeks

- A. Male rat, control liver (H&E, 600X) – normal liver
- B. Male rat, 50 mg/kg (H&E, 600X) – microvesicular vacuolization of hepatocytes (blue arrow); macrovesicular vacuolization of hepatocytes (black arrow)

A



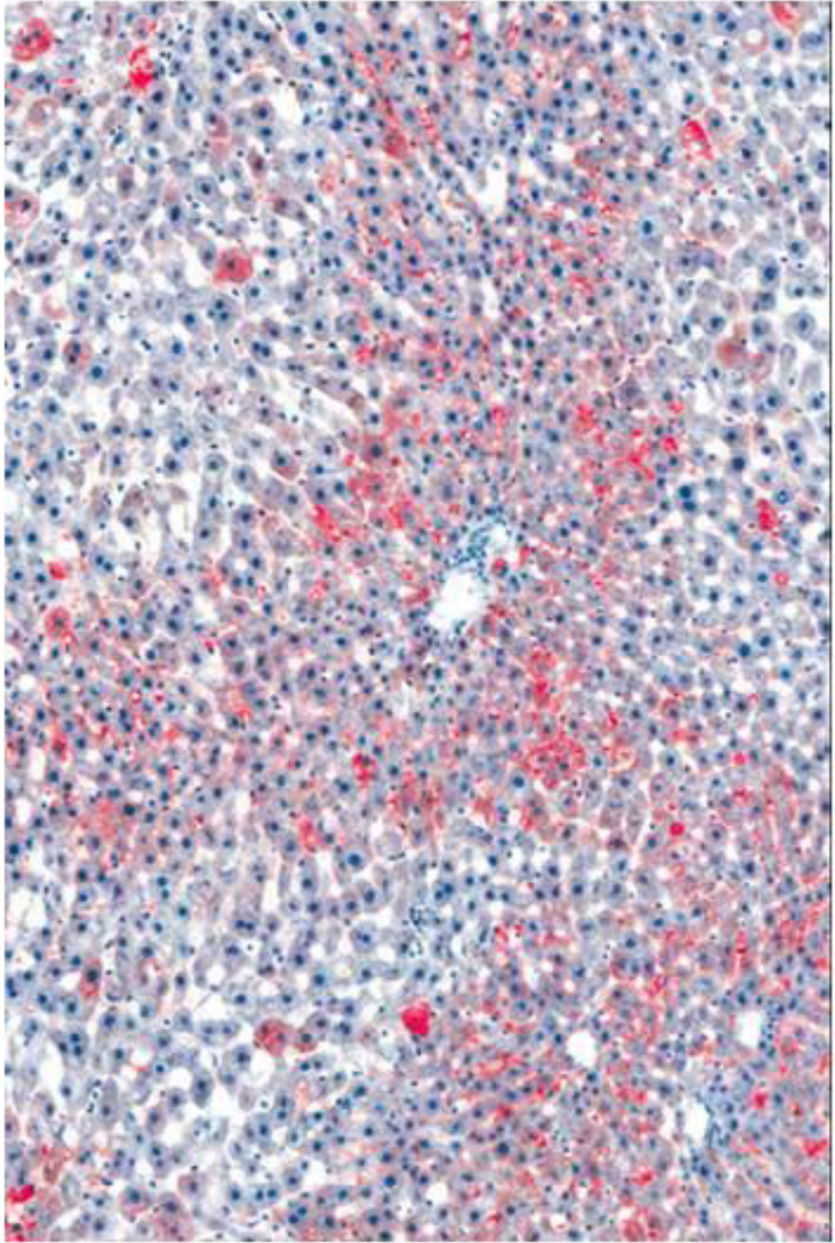
B

Figure 2. Fat accumulation in PBDE treated liver at 13 weeks (Oil Red O staining of frozen liver sections)

- A.** Male rat, control liver (10X) – normal liver
- B.** Male rat, 50 mg/kg (10X) - Increase in the amount of fat in the liver of the treated rat, as evidenced by increased red stain (10X)

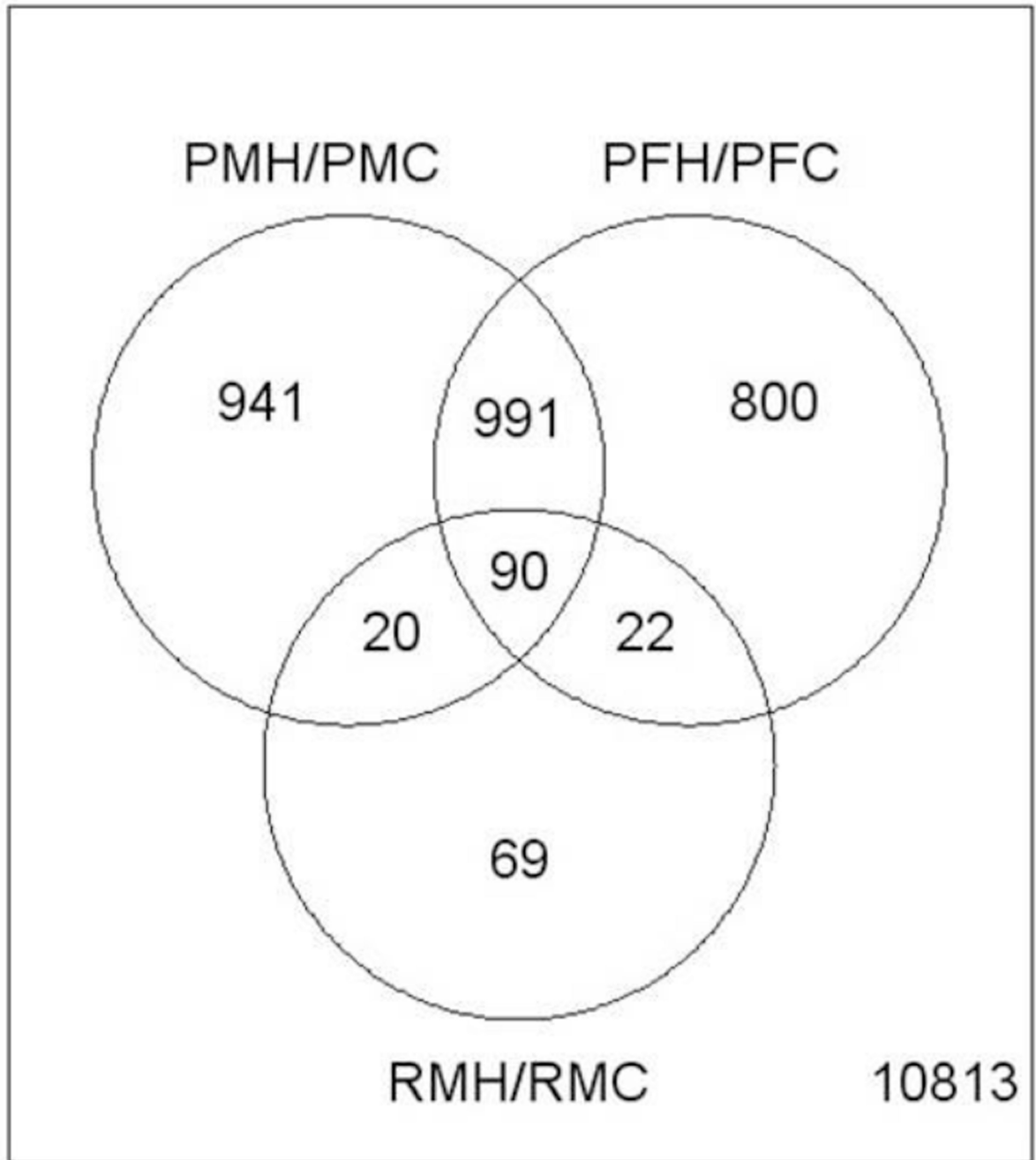


Figure 3. Venn diagram of overlapping significant gene transcripts at PND 22 (males (PMH/PMC) and females (PFH/PFC)) and week 13 (males (RMH/RMC))

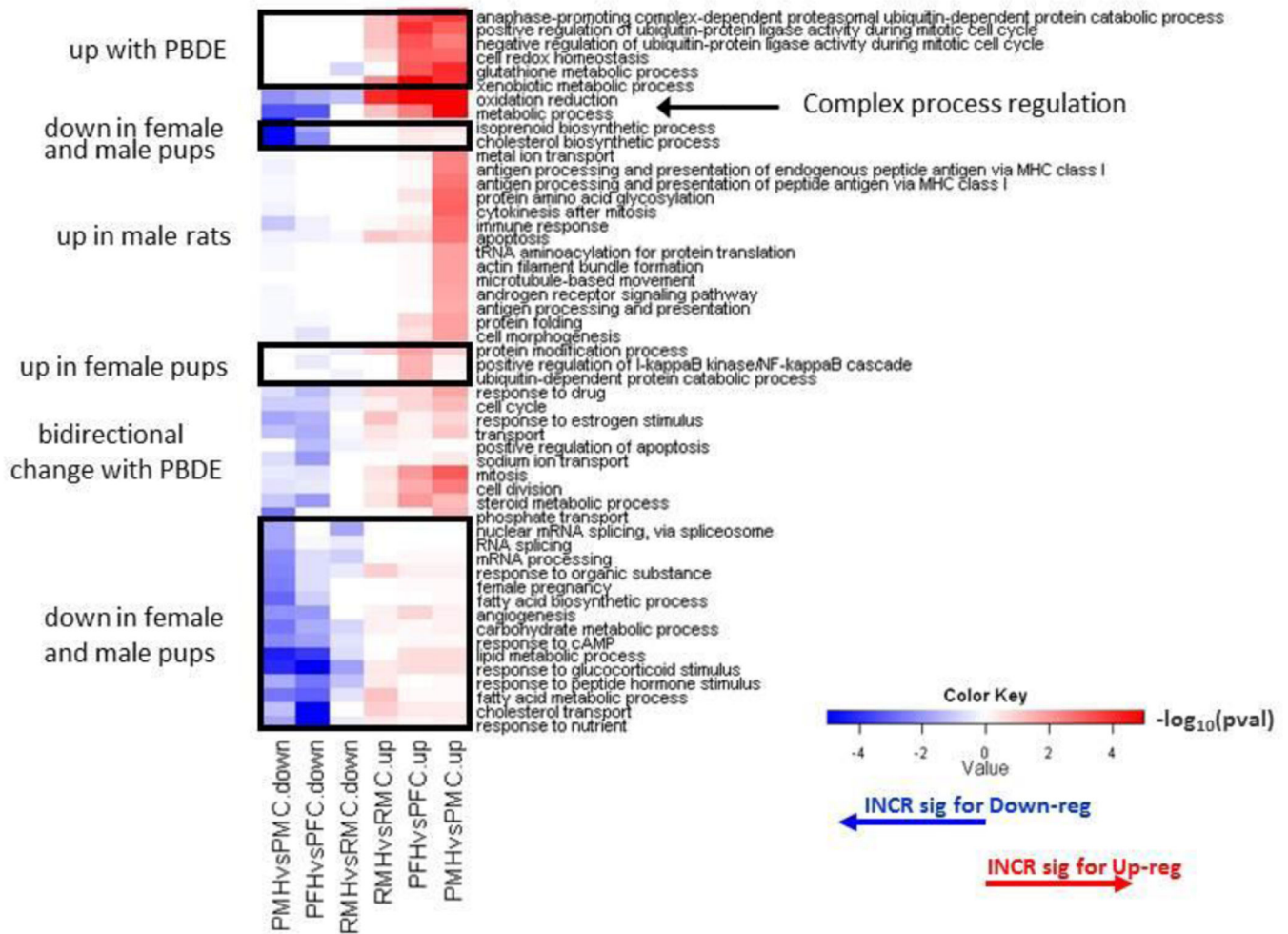


Figure 4. Clustered heat map of Biological Process Gene Ontology (GO) terms in upregulated (red) or downregulated (green) genes in comparison to PBDE treated (H) animals to controls (C). Differentially expressed genes for female and male pups (P) were determined by a false discovery rate (FDR) threshold of 0.01, while gene lists in 13 week old rats (R) were based on FDR < 0.05. Included processes have a p-value less than 0.05 and 5 differentially expressed genes in at least one comparison. Color key scale indicates the minus log₁₀ p-value of GO term significance. Comparisons of female pups (PFHvsPFC), male pups (PMHvsPMC), 13 week old rats (RMHvsRMC).

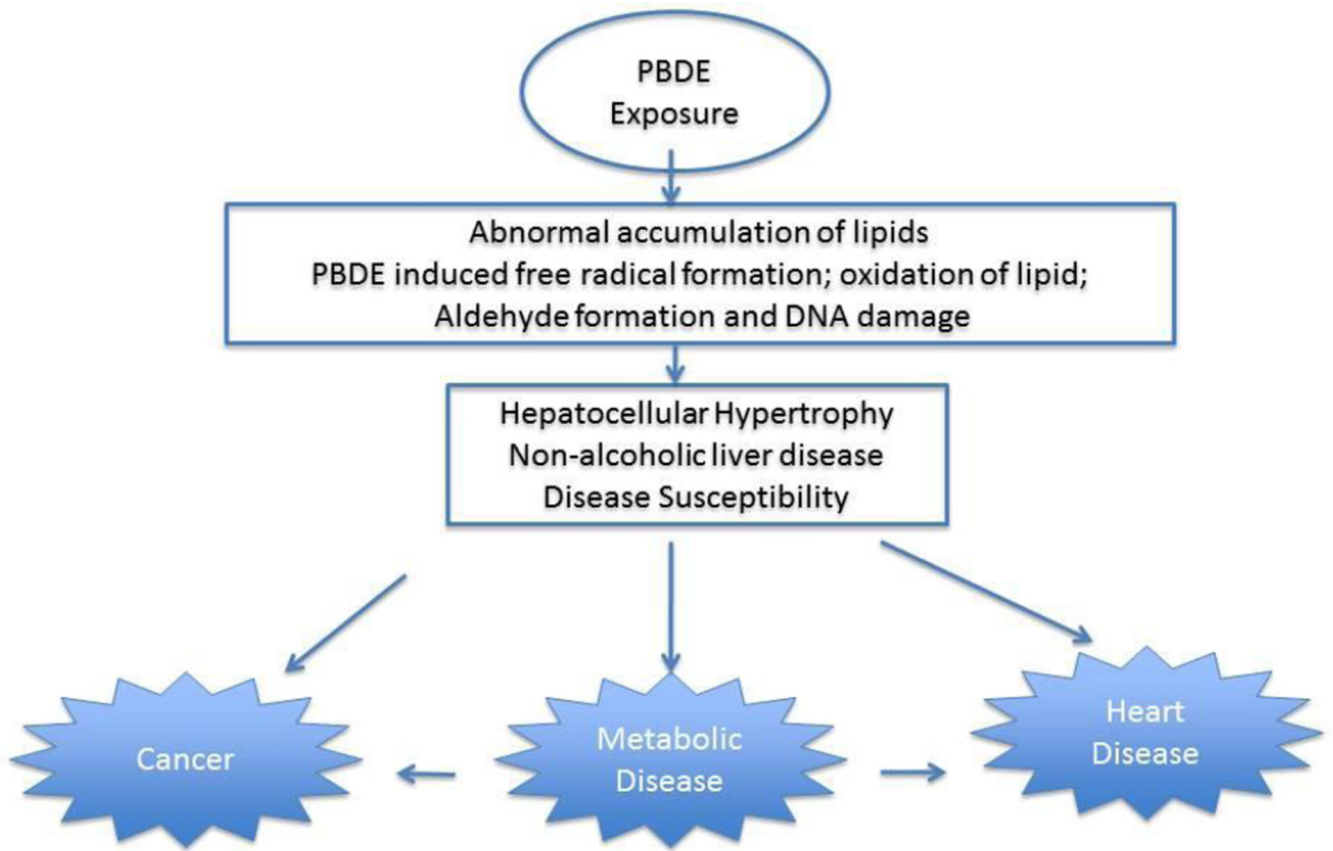


Figure 5.
Steps in PBDE toxicity process

Table 1

Treatment-related effects of polybrominated diphenyl ether, male rats, week 13

Dose group (mg/kg)	Males 0	Males 50	Females 0	Females 50
Hepatocyte hypertrophy	0/10	10/10 (3.1) ^a	0/10	10/10 (2.9)
Hepatocyte vacuolization	3/10 (1.0)	10/10 (1.2)	2/10 (1.0)	4/10 (1.0)
Thyroid gland Follicle hypertrophy	0/10	5/10 (1.2)	0/10	5/10 (1.2)
Mean body wt (gms)	403.4	431.2	245.6	213.3
Mean liver wt (gms)	13.7 ± 1.24	19.5 ± 2.39	7.94 ± 0.58	9.28 ± 1.35

^a Lesions severity grade

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Table 2

Number of PBDE-induced significant liver gene transcripts

	Gene List Sizes for Given False Discovery Rate						
	0.3	0.2	0.1	0.05	0.01	0.001	
PBDE male pup ^a vs. male pup control	9270	7015	4595	3055	1200	209	
PBDE female pup ^a vs. female pup control	8178	6264	4113	2832	1066	109	
PBDE male rat ^b vs. male rat control	3185	1748	611	263	22	0	
Male pup control ^a vs female pup control	312	140	25	15	7	7	
PBDE male pup ^a vs. PBDE female pups	39	26	19	11	9	5	
SEX-by-DOSE (pups)	12	8	0	0	0	0	
AGE-by-DOSE (males)	2923	1796	870	447	123	30	

Table 3Selected liver gene transcript changes in postnatal day 22 male pup liver^a

AffyProbe Set ID	Gene Symbol	GeneName	Fold Change Male pups	Fold change Female Pups
Up regulation of genes combating oxidative stress				
"1387958_at"	"Akr1c18"	"aldo-keto reductase family 1, member C18"	2.38	1.78
"1371997_at"	"Akr1c12"	"aldo-keto reductase family 1, member C-like 2"	1.34	1.24
"1398310_at"	"Akr1d1"	"aldo-keto reductase family 1, member D1 (delta 4-3-ketosteroid-5-beta-reductase)"	1.42	1.16
"1367843_at"	"Akr7a2"	"aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase)"	1.39	1.46
"1368121_at"	"Akr7a3"	"aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)"	3.04	2.40
"1387022_at"	"Aldh1a1"	"aldehyde dehydrogenase 1 family, member A1"	2.84	2.87
"1368718_at"	"Aldh1a7"	"aldehyde dehydrogenase family 1, subfamily A7"	2.78	2.65
"1368130_at"	"Aldh3a1"	"aldehyde dehydrogenase 3 family, member A1"	1.88	4.81
"1368037_at"	"Cbr1"	"carbonyl reductase 1"	1.43	1.52
"1367774_at"	"Gsta3"	"glutathione S-transferase A3"	1.30	1.22
"1372297_at"	"Gsta4"	"glutathione S-transferase alpha 4"	2.45	2.27
"1386985_at"	"Gstm1"	"glutathione S-transferase mu 1"	1.70	1.71
"1370952_at"	"Gstm2"	"glutathione S-transferase mu 2"	2.65	2.02
"1369921_at"	"Gstm3"	"glutathione S-transferase mu 3"	2.01	1.71
"1375909_at"	"Gstm4"	"glutathione S-transferase mu 4"	1.74	1.77
"1368354_at"	"Gstt1"	"glutathione S-transferase theta 1"	1.74	1.62
"1371942_at"	"Gstt3"	"glutathione S-transferase, theta 3"	1.96	1.73
"1372599_at"	"Mgst2"	"microsomal glutathione S-transferase 2"	1.45	1.46

AffyProbe Set ID	Gene Symbol	GeneName	Fold Change Male pups	Fold change Female Pups
Genes in lipid metabolism/ metabolic syndrome				
"1368126_at"	"Aacs"	"acetoacetyl-CoA synthetase"	-3.51	-2.70
"1369455_at"	"Abcg5"	"ATP-binding cassette, sub-family G (WHITE), member 5"	-4.57	-4.24
"1368718_at"	"Aldh1a7"	"aldehyde dehydrogenase family 1, subfamily A7"	2.78	2.65
"1369727_at"	"Apoa2"	"apolipoprotein A-II"	3.64	3.79
"1369011_at"	"Apoa5"	"apolipoprotein A-V"	-1.50	-1.60
"1393139_at"	"Apoc2"	"apolipoprotein C-II"	-1.23	-1.11
"1386901_at"	"Cd36"	"CD36 molecule (thrombospondin receptor)"	1.66	-1.06
"1367689_a_at"	"Cd36"	"CD36 molecule (thrombospondin receptor)"	1.26	-
"1384341_at"	"Faah"	"fatty acid amide hydrolase"	-1.52	-
"1393751_at"	"Fabp12"	"fatty acid binding protein 12"	-1.62	-
"1370281_at"	"Fabp5"	"fatty acid binding protein 5, epidermal"	-2.84	-
"1370024_at"	"Fabp7"	"fatty acid binding protein 7, brain"	-1.38	-
"1368453_at"	"Fads2"	"fatty acid desaturase 2"	-2.97	-1.94
"1372476_at"	"Fads3"	"fatty acid desaturase 3"	1.56	-
"1367707_at"	"Fasn"	"fatty acid synthase"	-2.99	-
"1367708_a_at"	"Fasn"	"fatty acid synthase"	-2.17	-
"1373491_at"	"Gba"	"glucosidase, beta, acid"	1.44	1.34
"1370200_at"	"Glud1"	"glutamate dehydrogenase 1"	-1.46	-
"1373222_at"	"Hexa"	"hexosaminidase A"	1.32	-
"1375852_at"	"Hmgcr"	"3-hydroxy-3-methylglutaryl-Coenzyme A reductase"	-1.70	-
"1387848_at"	"Hmgcr"	"3-hydroxy-3-methylglutaryl-Coenzyme A reductase"	-1.78	-
"1367648_at"	"Igfbp2"	"insulin-like growth factor binding protein 2"	-5.17	-2.51
"1367887_at"	"Lcat"	"lecithin cholesterol acyltransferase"	-1.14	-
"1381672_at"	"Ldlrap1"	"low density lipoprotein receptor adaptor protein 1"	1.10	-

AffyProbe Set ID	Gene Symbol	GeneName	Fold Change Male pups	Fold change Female Pups
"1386965_at"	"Lpl"	"lipoprotein lipase"	7.13	4.71
"1369150_at"	"Pdk4"	"pyruvate dehydrogenase kinase, isozyme 4"	1.88	-
"1386864_at"	"Pgam1"	"phosphoglycerate mutase 1 (brain)"	1.25	1.26
"1387803_at"	"Ppp2r2b"	"protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), beta isoform"	-4.83	-6.34
"1371143_at"	"Serpina7"	"serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antitrypsin), member 7"	-4.53	-4.44
"1387325_at"	"Slc27a5"	"solute carrier family 27 (fatty acid transporter), member 5"	-1.56	-1.49
"1369636_at"	"Sord"	"sorbitol Dehydrogenase"	1.29	-
"1394786_at"	"Sorl1"	"sortilin-related receptor, LDLR class A repeats-containing"	-1.44	-
"1393933_at"	"Sorl1"	"sortilin-related receptor, LDLR class A repeats-containing"	-1.44	-
"1371104_at"	"Srebf1"	"sterol regulatory element binding transcription factor 1"	-1.87	-
"1371979_at"	"Srebf2"	"sterol regulatory element binding transcription factor 2"	-1.31	-
"1367938_at"	"Ugdh"	"UDP-glucose dehydrogenase"	1.67	1.49
"1389611_at"	"Vldlr"	"very low density lipoprotein receptor"	-1.03	1.32
Cytochrome P450s				
1369136_at	Cyp2a3a	cytochrome P450, family 2, subfamily A, polypeptide 3a	37.40	6.84
1370269_at	Cyp1a1	cytochrome P450, family 1, subfamily a, polypeptide 1	26.68	39.63
1370241_at	Cyp2c7	cytochrome P450, family 2, subfamily c, polypeptide 7	23.68	9.17
1370495_s_at	Cyp2c13	cytochrome P450 2c13	21.10	-
1387328_at	Cyp2c	cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase)	6.99	2.45
1368990_at	Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1	4.95	10.96

AffyProbe Set ID	Gene Symbol	GeneName	Fold Change Male pups	Fold change Female Pups
1370387_at	Cyp3a9	cytochrome P450, family 3, subfamily a, polypeptide 9	2.95	-
1387243_at	Cyp1a2	cytochrome P450, family 1, subfamily a, polypeptide 2	2.24	2.13
1387118_at	Cyp3a23/3a1	cytochrome P450, family 3, subfamily a, polypeptide 23/polypeptide 1	1.97	2.03
1369424_at	Cyp2a2	cytochrome P450, subfamily 2A, polypeptide 1	1.77	-
1387511_at	Cyp2a1	cytochrome P450 IIA1 (hepatic steroid hydroxylase IIA1) gene	1.64	-
1368155_at	Cyp2c12	cytochrome P450, family 2, subfamily c, polypeptide 12	1.52	-
1387296_at	Cyp2j4	cytochrome P450, family 2, subfamily J, polypeptide 4	1.46	-
1387296_at	Cyp2j4	cytochrome P450, family 2, subfamily J, polypeptide 4	1.46	-
1387973_at	Cyp4f4	cytochrome P450, family 4, subfamily f, polypeptide 4	1.28	-
1371142_at	Cyp2g1	cytochrome P450, subfamily 2G, polypeptide 1	-1.03	-
1368458_at	Cyp7a1	cytochrome P450, family 7, subfamily a, polypeptide 1	-2.30	-
1368435_at	Cyp8b1	cytochrome P450, family 8, subfamily b, polypeptide 1	-2.93	-4.20
1387123_at	Cyp17a1	cytochrome P450, family 17, subfamily a, polypeptide 1	-6.16	-5.23
1371076_at	Cyp2b	cytochrome P450, family 2b	6.13	9.96
Cell cycle/cancer				
"1369698_at"	"Abcc3"	"ATP-binding cassette, sub-family C (CFTR/MRP), member 3"	3.23	3.23
"1379652_at"	"Ahctf1"	"AT hook containing transcription factor 1"	-1.54	-
"1379652_at"	"Ahctf1"	"AT hook containing transcription factor 1"	-1.54	-
"1369983_at"	"Ccl5"	"chemokine (C-C motif) ligand 5"	2.21	2.02
"1370345_at"	"Ccnb1"	"cyclin B1"	1.94	-

AffyProbe Set ID	Gene Symbol	GeneName	Fold Change Male pups	Fold change Female Pups
"1389566_at"	"Ccnb2"	"cyclin B2"	1.78	-
"1371643_at"	"Ccd1"	"cyclin D1"	-2.13	-
"1371150_at"	"Ccd1"	"cyclin D1"	-2.28	-1.94
"1383075_at"	"Ccd1"	"cyclin D1"	-2.33	-2.1
"1370810_at"	"Ccd2"	"cyclin D2"	-1.42	-
"1371953_at"	"Ccg2"	"cyclin G2"	2.33	-
"1380997_at"	"Ccnj"	"cyclin J"	-1.51	-
"1374220_at"	"Ccnj"	"cyclin Y"	1.33	-
"1372685_at"	"Cdkn3"	"cyclin-dependent kinase inhibitor 3"	1.79	-
"1387062_a_at"	"Chk1"	"CHK1 checkpoint homolog (S. pombe)"	2.90	3.89
"1367515_at"	"Cnot7"	"CCR4-NOT transcription complex, subunit 7"	-1.31	-
"1367515_at"	"Cnot7"	"CCR4-NOT transcription complex, subunit 7"	-1.31	-
"1368174_at"	"Egln3"	"EGL nine homolog 3 (C. elegans)"	4.43	4.89
"1373011_at"	"Fam134b"	"family with sequence similarity 134, member B"	4.34	3.87
"1368408_at"	"Grk5"	"G protein-coupled receptor kinase 5"	3.28	2.95
"1368549_at"	"Hbp1"	"HMG-box transcription factor 1"	1.30	-
"1368549_at"	"Hbp1"	"HMG-box transcription factor 1"	1.30	-
"1370728_at"	"Il13ra1"	"interleukin 13 receptor, alpha 1"	1.73	-
"1388711_at"	"Il13ra1"	"interleukin 13 receptor, alpha 1"	1.47	-
"1371170_a_at"	"Il1a"	"interleukin 1 alpha"	-1.37	-1.59
"1368592_at"	"Il1a"	"interleukin 1 alpha"	-1.75	-
"1387394_at"	"Il2rb"	"interleukin 2 receptor, beta"	1.50	-
"1387935_at"	"Il3ra"	"interleukin 3 receptor, alpha"	1.39	1.34
"1386987_at"	"Il6ra"	"interleukin 6 receptor, alpha"	-1.33	-
"1373379_at"	"Irak1"	"interleukin-1 receptor-associated kinase 1"	1.32	-
"1380336_at"	"Irak3"	"interleukin-1 receptor-associated kinase 3"	1.50	-
"1368915_at"	"Kmo"	"kynurenine 3-Monooxygenase (kynurenine 3-hydroxylase)"	-1.54	-0.02

AffyProbe Set ID	Gene Symbol	GeneName	Fold Change Male pups	Fold change Female Pups
"1371479_at"	"Mettl7a"	"methyltransferase like 7A"	1.90	1.54
"1384280_at"	"Nusap1"	"nucleolar and spindle associated protein 1"	1.59	-
"1392715_at"	"Ppargc1b"	"peroxisome proliferator-activated receptor gamma, coactivator 1 beta"	-1.82	-
"1370012_at"	"Ptgis"	"prostaglandin I2 (prostacyclin) synthase"	-3.10	-3.19
"1398814_at"	"Rab11a"	"RAB11a, member RAS oncogene family"	1.40	1.37
"1389066_at"	"Rcan2"	"regulator of calcineurin 2"	2.10	2.15
"1378605_at"	"Sigirr"	"single immunoglobulin and toll-interleukin 1 receptor (TIR) domain"	1.32	-
"1379275_at"	"Snx10"	"sorting nexin 10"	3.96	5.49
"1371104_at"	"Srebf1"	"sterol regulatory element binding transcription factor 1"	-1.87	-
"1388426_at"	"Srebf1"	"sterol regulatory element binding transcription factor 1"	-1.94	-
"1367941_at"	"Tfam"	"transcription factor A, mitochondrial"	1.04	-
"1376668_at"	"Ttc39a"	"tetratricopeptide repeat domain 39A"	8.84	14.87

^aFalse discovery rate < 0.01; p < .01 for all gene transcripts in this table (complete details in supplements 1 and 2). Most of these gene transcripts were not significantly changed in week13 male rat livers.