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Developmental Bisphenol A (BPA) Exposure Leads to Sex-specific Modification of Hepatic Gene Expression and Epigenome at Birth that May Exacerbate High-fat Diet-induced Hepatic Steatosis

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Abstract

Developmental bisphenol A (BPA) exposure increases adulthood hepatic steatosis with reduced mitochondrial function. To investigate potential epigenetic mechanisms behind developmental BPA-induced hepatic steatosis, pregnant Sprague-Dawley rats were dosed with vehicle (oil) or BPA (100 µg/kg/day) from gestational day 6 until postnatal day (PND) 21. After weaning, offspring were either challenged with a high-fat (HF; 45% fat) or remained on a control (C) diet until PND110. From PND60 to 90, both BPA and HF diet increased the fat/lean ratio in males only, and the combination of BPA and HF diet appeared to cause the highest ratio. On PND110, Oil-HF, BPA-C, and BPA-HF males had higher hepatic lipid accumulation than Oil-C, with microvesicular steatosis being marked in the BPA-HF group. Furthermore, on PND1, BPA increased and modified hepatic triglycerides (TG) and free fatty acid (FFA) composition in males only. In PND1 males, BPA increased hepatic expression of FFA uptake gene *Fat/Cd36*, and decreased the expression of TG synthesis- and β -oxidation-related genes (*Dgat*, *Agpat6*, *Cebpa*, *Cebp β* , *Pck1*, *Acox1*, *Cpt1a*, *Cybb*). BPA altered DNA methylation, histone marks (H3Ac, H4Ac, H3Me2K4, H3Me3K36), and decreased the binding of several transcription factors (Pol II, C/EBP β , SREBP1) within the male *Cpt1a* gene, the key β -oxidation enzyme. In PND1 females, BPA only increased the expression of genes involved in FFA uptake and TG synthesis (*Lpl*, *Fasn*, and *Dgat*). These data suggest that developmental BPA exposure alters and reprograms hepatic β -

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oxidation capacity in males, potentially thorough the epigenetic regulation of genes, and further alters the response to a HF diet.

Keywords

Bisphenol A (BPA); adiposity; endocrine disrupting chemical (EDC); NAFLD; methylation; histones

INTRODUCTION

Various dietary, behavioral, and genetic factors contribute to the increased incidence of metabolic syndrome worldwide, and recent data suggest that environmental endocrine disrupting chemicals (EDCs) may also be important factors in the growing epidemic of obesity and metabolic disorders. Although a number of EDCs have been shown to disrupt normal reproductive outcomes (Unuvar and Buyukgebiz, 2012), more recently several EDCs, including the ubiquitous plasticizer bisphenol A (BPA), were shown to alter metabolic processes and have been associated with obesity and adulthood chronic diseases (Olsen *et al.*, 2012; Trasande *et al.*, 2012).

The Developmental Origins of Health and Disease Hypothesis suggests that exposure to *in utero* dietary or environmental stressors can program the adulthood phenotype, and this has been confirmed in humans and experimental animal models (Barker, 2004; Hanley *et al.*, 2010; Tamashiro and Moran, 2010). In animals, maternal dietary manipulations during pregnancy have been shown to contribute to the development of non-alcoholic fatty liver disease (NAFLD) in offspring (Aagaard-Tillery *et al.*, 2008; McCurdy *et al.*, 2009), and recent studies suggest that prenatal low-dose BPA exposure can also modify markers of hepatic energy metabolism at weaning (Somm *et al.*, 2009) and lead to steatosis in adult animals (Jiang *et al.*, 2014). While the precise mechanisms behind these observations are not completely understood, in cell models, BPA's effect on hepatocyte metabolism has been proposed to occur through endoplasmic reticulum stress (Asahi *et al.*, 2010), the production of reactive oxygen species (Huc *et al.*, 2012), and decreased fatty acid β -oxidation (Grasselli *et al.*, 2013).

Carnitine palmitoyltransferase 1a (CPT1A) in the liver is the regulatory enzyme for the transport of long-chain fatty acids into the mitochondria for subsequent β -oxidation (Nakamura *et al.*, 2014). In humans, CPT1A deficiency causes hepatomegaly, hepatic steatosis, and metabolic perturbations (Bennett and Santani, 1993), while in rats, *Cpt1a* expression was shown to be down-regulated during steatosis in male rats consuming a high-fat diet (Xie *et al.*, 2010), while a moderate overexpression of *Cpt1a* in obese male rats increased hepatic fatty acid β -oxidation and decreased hepatic triglyceride accumulation (Stefanovic-Racic *et al.*, 2008). *Cpt1a* expression has been shown to be regulated by the binding of transcription factors (including C/EBP β and SREBP1 (Attia *et al.*, 2011; Thakran *et al.*, 2013) in cell culture, as well as with altered DNA methylation in humans (Frazier-Wood *et al.*, 2014; Irvin *et al.*, 2014). However, despite the critical regulatory role of this gene in hepatic energy metabolism and lipid accumulation, and the proposed role of BPA exposure in NAFLD development, no studies have specifically assessed the gene's potential

epigenetic regulation in response to developmental BPA exposure. Therefore, the present study first determined the effect of prenatal exposure to BPA, with or without a post-weaning HF dietary challenge, on adult liver pathophysiology. We then investigated whether the adulthood phenotypes observed were associated with neonatal changes in hepatic lipid metabolism and fatty acid homeostasis – at the biochemical, genetic, and epigenetic levels, with a special focus on *Cpt1a*. Furthermore, because BPA is known to affect males and females differently, and because NAFLD development has been shown to be sex-specific (Lazo *et al.*, 2013), we also considered offspring's sex in our analyses.

METHODS

Dosing of BPA and Animal Experimental Design during Gestation and Weaning

Timed-pregnant Sprague-Dawley rats (n=6/treatment group) were obtained from Charles River Laboratories (Wilmington, MA, USA) on gestational day 2, individually housed in ventilated cages in a temperature-controlled environment, and fed a modified AIN-93G diet and water *ad libitum*. Beginning on gestational day 6 until postnatal day (PND) 21, dams were randomized into two groups, and daily orally dosed using a 1 mL Eppendorf pipette with either vehicle control (tocopherol-stripped corn oil) or BPA (100 µg/kg/day BW). This dose has been shown to affect metabolism (including increased body weight and altered glucose homeostasis) and reproductive outcomes (including altered estrous cyclicity and hormone levels) in previous rodent models of perinatal exposure (Rubin *et al.*, 2001; Liu *et al.*, 2013). A relatively similar perinatal BPA exposure dose (50 µg/kg/day BW) led to steatosis in adult offspring (Jiang *et al.*, 2014; Wei *et al.*, 2014), and adult exposure to BPA at 50 and 500 µg/kg/day BW increased hepatic triglycerides in mice (Marmugi *et al.*, 2012). Immediately after birth, litter sex distributions and offspring body weight were recorded, male and female pups (n=6, one of each sex from each dam) were removed for sampling, and the remainder of the pups were completely mixed within each treatment group, and 4 male and 4 female pups were randomly selected and placed back with each dam within the respective control or BPA-treated groups for cross-fostering. PND1 livers were snap-frozen in liquid nitrogen and stored at –80°C for all subsequent biochemical and molecular analyses.

Animal Experimental Design after Weaning Using a High-fat Dietary Challenge

On PND22, male and female pups were once again randomized into new cages (within treatment groups), with 2–3 male or female offspring per cage, and separated into two dietary groups: control (C: 64% carbohydrate, 20% protein, and 16% fat) or high-fat diet (HF: 35% carbohydrate, 20% protein, and 45% fat) (Table 1), which yielded 4 experimental post-weaning groups: Oil-C, Oil-HF, BPA-C, and BPA-HF. All animals were single-housed after the onset of puberty until the end of the study (PND110), when tissues were collected and either snap-frozen in liquid nitrogen and stored at –80°C or fixed in 10% formalin for histopathology analysis.

Body Weight, Cumulative Energy Intake, and Body Composition throughout the Study

Body weight and food intake were measured every 4 days beginning on PND22. Energy intake after weaning was calculated by weighing pellets remaining on the fourth day,

subtracting from initial food supplied, and multiplying daily calculated intake by the caloric composition of each diet (4.0 kcal/g in control and 4.8 kcal/g in HF). Body composition was measured on PND1, at weaning on PND21, after the onset of puberty on PND60, and on PND90 using the EchoMRI-700 Body Composition Analyzer (Echo Medical Systems, Houston, TX), which allows for measurement of fat and lean mass in conscious and unrestrained animals. On PND1 and PND21, male and female pups were scanned as a litter, and on PND60 and PND90 offspring were scanned individually.

Liver Histopathology and Staining in Adult Offspring

At the end of the study (PND110), liver samples from adult animals were fixed in 10% formalin, embedded in paraffin, and 3 μ m histological slides were prepared and stained with hematoxylin and eosin. Formalin-fixed tissues were embedded in OCT, and slides were prepared with a cryostat and stained with Oil-Red-O. Histopathological evaluation was performed by a board-certified pathologist. Samples were first assigned a score for overall hepatic vacuolation severity, and then independently graded for either macro- or micro-vacuolation. Pathological findings were graded from 1 (minimal) to 5 (severe). For all analyses, the pathologist was blind to the treatment, and reported all information using a numerical code.

Hepatic Triglyceride (TG) Content, Free Fatty Acid (FFA) Content, and FFA Composition in PND1 Offspring

Hepatic lipids were extracted and purified from frozen livers from 6 male and 6 female PND1 pups from each maternal treatment group (oil or BPA) using the Bligh & Dyer method (Bligh and Dyer, 1959). A portion of the resulting chloroform layer was used for TG quantification. Briefly, this chloroform aliquot was dried under nitrogen and re-suspended in a 1:1:3 solution of TritonX100-Methanol-Tert-Butanol. This dilution of lipids was analyzed for TG content as previously reported (Miao *et al.*, 2004).

The remainder of the chloroform phase was utilized for FFA gas chromatographic (GC) analysis. Fatty acid methyl esters (FAMES) were prepared by derivatization of chloroform extracts in 3 N methanolic HCl (95°C, 40 min), using 17:0 (heptadecanoic acid) as the internal standard. Samples were dried under nitrogen, re-suspended in hexane, and resulting FAMES were injected onto a 30 m $\times$ 0.32 mm DB-225 column with 0.25 μ m film thickness, with helium as the carrier gas and using a 5890 series II gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector. GC was programmed with starting temperature of 180°C (5 min), ramping at 6°C/min increments to final temperature 220°C (held 15 min). Peaksimple software (SRI Instruments, Torrance, CA) was used for peak integration and analysis. Total FFAs were calculated as the sum of all identified peaks using several commercially available FAME standards (PUFA-2, Cat# 47015-U; PUFA-3, Cat# 47085-U; and Low Erucic Rapeseed Oil, Cat# O7756, Sigma-Aldrich, St. Louis, MO). Long-chain FFAs were defined as those having tails with 14–21 carbons, and very long-chain FFAs as those having tails with 22–26 carbons.

Hepatic RNA Isolation and mRNA Expression Analysis in PND1 Offspring

To investigate the mRNA expression of hepatic lipid metabolism-associated genes, frozen livers from 6 male and 6 female PND1 pups from each maternal treatment group (oil or BPA; same as those used for FFA analysis) was ground in liquid nitrogen with mortar and pestle. Total RNA isolation, cDNA synthesis and real-time PCR were performed as previously described (Strakovsky and Pan, 2011). A serial dilution was used to create a standard curve for quantification and a dissociation curve was analyzed following each reaction. All primers for real-time PCR analysis (Table 2) were designed using the VectorNTIR® software (Life Technologies™, Grand Island, NY), analyzed using BLAST, and synthesized by IDT (Coralville, IA). All mRNA data were normalized to the housekeeping gene encoding ribosomal protein L7a (*rpl7a*), the expression of which did not differ by treatment group.

Genomic DNA Isolation and Methylation Analysis of *Cpt1a* Using Methylation Sensitive PCR (MSP) in PND1 Offspring

To determine whether BPA affected the methylation status of *Cpt1a*, the gene encoding the regulatory and rate-limiting step of hepatic β -oxidation, MSP was performed as previously reported (Strakovsky *et al.*, 2014a) to assess the methylation status of six sites surrounding and spanning the *Cpt1a* promoter (approximately 3 kB upstream and 2 kB downstream of the transcription start site). MSP analysis provides a valid and reliable approach for comparing relative changes in DNA methylation between treatment groups within relatively targeted regions of the DNA. Briefly, frozen livers from 6 male and 6 female PND1 pups from each maternal treatment group (oil or BPA; same as those used for FFA and mRNA analyses) were ground in liquid nitrogen with mortar and pestle. Genomic DNA (gDNA) was isolated using the GenElute Mammalian Genomic DNA Purification Kit (Sigma-Aldrich) per manufacturer's instruction with an additional initial spin-down to remove the lipid layer. gDNA (20 ng/ μ L in a 20 μ L reaction volume) was bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA), following the manufacturer's instructions. Real-time PCR was performed using 20 ng gDNA as the template, SYBR Green PCR Master Mix (Quanta Biosciences, Gaithersburg, MD), and 5 μ M of each forward and reverse primer (Table 2) designed using the methprimer website (<http://www.urogene.org/methprimer/index.html>) (Li and Dahiya, 2002) to specifically amplify either the methylated or unmethylated templates in the 7300 Real-Time PCR System (Applied Biosystem) as for mRNA analysis, but using 45 cycles. A serial dilution from a reaction combining one sample from each experimental group was used to create an internal standard curve for quantification. Data are presented as the ratio of methylated values divided by the sum of methylated and unmethylated values.

Hepatic Chromatin Immunoprecipitation (ChIP) Analysis of the *Cpt1a* gene in PND1 Offspring

To investigate whether BPA had an effect on histone marks and transcription factor binding within the *Cpt1a* gene, ChIP analysis was performed following a modified protocol (Chen *et al.*, 2004) as previously described (Strakovsky *et al.*, 2011), but using magnetic beads in the current study. ChIP was performed using frozen livers from 6 male and 6 female PND1 pups

from each maternal treatment group (oil or BPA; same as those used for FFA, mRNA, and MSP analyses), and examined the regions approximately corresponding to those regions also assessed by MSP. Briefly, 100 mg of frozen liver samples were ground using a mortar and pestle with liquid nitrogen and washed with PBS. The samples were resuspended in PBS and cross-linked in 1% formaldehyde for 10 min at room temperature. After centrifugation, the pellets were resuspended in nuclei swelling buffer containing protease inhibitor and phosphorylation inhibitor. The nuclei were lysed in SDS buffer containing protease and phosphorylation inhibitors. The chromatin was sonicated (Fisher Scientific model 100 Sonic Dismembrator, Pittsburgh, PA) on ice with 5 bursts for 40 s at power setting 5 with 2 min cooling intervals between each burst. After removal of the cell debris by centrifugation at 13000 rpm in 4 °C for 10 min, sheared chromatin was diluted to 10 mL in ChIP Dilution Buffer. Then, 1 mL of the diluted lysate was incubated overnight on a hematology mixer (Model 346, Fisher Scientific) with 2 µg of each primary antibody at 4 °C (Table 3). Each antibody was first bound to Dynabeads® Protein G beads (Life Technologies) by incubating 1 µg of antibody with 450 µg beads (15 mL of the suspension provided by the manufacturer) at room temperature in PBS-T (PBS with 0.02% Tween 20) for 1 hr and washed to remove any unbound antibody, and 750 µL of sheared chromatin was added to the beads and incubated at room temperature for 2 h to form the chromatin-antibody-bead complex. Normal rabbit IgG was used as the negative control, and supernatant from the normal rabbit IgG was also saved as the input for normalization of the data. Pellets containing the immunoprecipitated complexes were washed sequentially with 1 mL of low salt solution, high salt solution, and LiCl solution, and twice with TE (pH 8.0). Antibody/protein/DNA complexes were eluted from the magnetic beads by adding twice 250 µL of elution buffer followed by shaking at 37°C for 15 min at 300 rpm and flash spinning at room temperature. The combined supernatants were incubated at 65°C for 5 hr with 20 µL 5 mol/L NaCl and 1 µg of RNase A (Qiagen, Hilden, Germany) to reverse the formaldehyde cross-linking and release the DNA fragments. Samples were then treated with proteinase K (Sigma) at 37°C for 1 hr to remove any protein. DNA was purified with a DNA miniprep system (Qiagen). 5 µL of immunoprecipitated DNA was used for the real-time PCR reaction (primers are listed in Table 2).

We evaluated several histone modifications and the binding of hepatic lipid metabolism-related transcription factors (C/EBPβ and SREBP1) within regions approximately corresponding to regions of the DNA also evaluated for changes to the methylation status of *Cpt1a*. Specifically, we assessed the acetylation status of both histone H3 and histone H4 (H3Ac and H4Ac), the di-methylation of histone H3, lysine 4 (H3Me2K4), and the tri-methylation of histone H3, lysine 36 (H3Me3K27). We also evaluated the tri-methylation of histone H3, lysine 9 and 27 (H3Me3K9 and H3Me3K27), but did not observe significant binding (when compared to IgG; data not shown). The binding of RNA Polymerase II (CTD4H8; Pol II) was assessed as a marker of active gene transcription.

Statistical Analysis

Adulthood body weight, energy intake, and hepatic vacuolation were analyzed using two-way analysis of variance (ANOVA) with interactions to determine the overall effects of post-weaning diet, prenatal BPA exposure, and the interaction between the two main factors.

When an interaction was present, a post-hoc Tukey test was used to further determine differences between groups in $n=5$ male or female animals per group. Daily energy intake was summed to show overall intake from PND69-97. The PND60-90 fat/lean mass ratio was analyzed using two-way repeated-measures ANOVA to determine the overall effects of post-weaning diet, prenatal BPA exposure, and the interaction between the two main factors. When an interaction was present, a post-hoc Tukey test was used to determine differences between groups. The fat/lean mass ratio at birth and weaning ($n=5$ litters), hepatic TG content, FFA content, and FFA distribution, as well as gene expression, MSP results, and ChIP results in PND1 livers ($n=6$ male or female pups) was analyzed using Student *t*-test in Excel. All other analyses were performed in SAS (Chicago, IL). Differences were considered significant at $P<0.05$.

RESULTS

Body Weight and Energy Intake in Adult Offspring and Body Composition throughout the Study

BPA did not significantly alter maternal weight gain, maternal body composition at delivery or at weaning, litter size, sex distribution, or birth weights in either male or female offspring (data not shown). In males, HF diet significantly ($P<0.05$) increased both body weight (Figure 1A), and cumulative energy intake (Figure 1B) from PND69-97, while BPA only trended to increase body weight ($P=0.17$) and energy intake ($P=0.11$). BPA had no effect on body composition (fat/lean mass ratio) at birth and weaning in males, but during the post-weaning to adulthood period, both HF diet ($P<0.001$) and BPA ($P<0.0001$) increased the fat/lean mass ratio. While there was no statistically significant interaction between the factors, the BPA-HF group appeared to have the highest fat/lean mass ratio when compared to all other groups (Figure 1C). In females, neither BPA nor a HF diet affected body weight (Figure 1D), cumulative energy intake (Figure 1E), or body composition throughout the study (Figure 1F).

Liver Histopathology and Lipid Accumulation in Adult Offspring

In PND110 males, both HF diet ($P<0.01$) and BPA ($P<0.01$) increased overall hepatocellular vacuolation, and there was a significant interaction between the factors ($P<0.01$), where Oil-C males had no vacuolation, and overall vacuolation was increased by a HF diet in Oil-dosed animals, whereas all BPA-exposed animals had increased vacuolation, regardless of diet, when compared to Oil-C (Figure 2A and representative images of H&E and ORO in Figure 2E). In males, both HF diet ($P<0.05$) and BPA ($P<0.05$) increased macro-vacuolation, and there was a significant interaction between the factors ($P<0.01$), where macro-vacuolation was more prominent in BPA-C males when compared to Oil-HF and almost absent in the BPA-HF group (Figure 2B and representative images in Figure 2E). Both HF diet ($P<0.01$) and BPA ($P<0.001$) increased micro-vacuolation, with no interaction between the factors (Figure 2C and representative images in Figure 2E). Also, in males, both HF diet ($P<0.001$) and BPA ($P<0.0001$) increased the ratio of micro/macro-vacuolation, and there was a significant interaction between the factors ($P<0.05$), where the ratio was increased in Oil-HF and BPA-HF males when compared with Oil-C, but BPA-HF males had the highest ratio when compared to all groups (Figure 2D and representative images in

Figure 2E). In females, neither BPA nor the HF diet had a significant effect on overall vacuolation (Figure 2F), macro-vacuolation (Figure 2G), micro-vacuolation (Figure 2H), or the ratio of micro/macro-vacuolation (Figure 2I, with representative images in Figure 2J).

Hepatic TG and FFA Amount, and FFA Composition in PND1 Offspring

On PND1, BPA significantly increased the amount of both hepatic TG (Figure 3A) and FFAs (Figure 3B) in males. In contrast, BPA had no effect on either TGs (Figure 3C) or FFAs (Figure 3D) in females.

Also on PND1, as a % of total FFAs (which was increased in males), BPA significantly increased the % of monounsaturated long-chain, and decreased the % of very-long chain FFAs, as well as the ratio of very-long chain/long-chain FFAs (Table 4, * $P<0.05$ or ** $P<0.01$). BPA had no significant effect on FFA composition or distribution in PND1 female liver (Table 4).

Hepatic mRNA Expression in PND1 Offspring

In PND1 males, BPA increased the expression of *Fat/Cd36*, decreased the expression of TG synthesis-related genes, *Dgat* and *Agpat6* ($P<0.05$), but had no effect on *Fabp1*, *Lpl*, or *Fasn*. BPA in males also decreased the expression of genes directly or indirectly associated with hepatic mitochondrial β -oxidation, including *Cebpa*, *Cebp β* , *Pck1*, *Acox1*, *Cybb* ($P<0.05$), and *Cpt1a* ($P<0.01$), the gene encoding the rate-limiting step of hepatic fatty acid β -oxidation, but had no effect on *Cyba* (Figure 4A). In females, BPA increased the expression of fatty acid uptake and synthesis genes, including *Lpl*, *Fasn*, and *Dgat* ($P<0.05$), but had no effect on any hepatic β -oxidation-related genes (Figure 4B).

We also analyzed several key DNA and histone modifiers to examine how their mRNA expression correlates with DNA methylation or histone modifications. In PND1 males, BPA had no effect on the expression of *Dnmt1*, *Dnmt3a*, *Hdac1*, *Km2b*, trended to increase *Hdac3* ($P=0.086$) and decrease *Ash1l* ($P=0.086$), and significantly decreased the expression of *Dnmt3b* and *Kmt2c* ($P<0.05$; Figure 5). In females, BPA had no effect on the expression of *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Hdac1*, *Hdac3*, *Ash1l*, *Kmt2b*, and significantly increased the expression of *Kmt2c* ($P<0.05$; Supplementary Figure 1).

Hepatic Methylation of the *Cpt1a* Gene in PND1 Offspring

We focused our methylation analysis within approximately 5 kB spanning the *Cpt1a* transcription start site (TSS) (Figure 6A or Supplementary Figure 2A, upper panels). In PND1 males, BPA increased methylation at a region approximately 1 kB upstream of the TSS (–1148; $P<0.05$), and decreased methylation in a region approximately 250 bp upstream of the TSS (–297) ($P<0.005$; Figure 6A, **lower panel**). In females, BPA had no effect on DNA methylation within any of the regions tested (Supplementary Figure 2A, lower panel).

Histone Modifications and Transcription Factor Binding within the *Cpt1a* Gene in PND1 Offspring

In PND1 males, BPA decreased H3Me2K4 and H3Me3K36 within the region approximately 2.7 kB upstream of the TSS (–2747). At approximately 1 kB upstream of the TSS (–1050), BPA decreased H3Me2K4. Within the region approximately 400 bp upstream of the TSS (–401), BPA decreased Pol II, H3Me2K4, H3Me3K36, and C/EBP β , but increased H4Ac ($P<0.05$), and within the region approximately 300 bp upstream of the TSS (–321), BPA decreased H3Me2K4 and increased H4Ac ($P<0.05$). Within the region spanning the TSS (–31), BPA decreased Pol II (trend, $P=0.08$), H3Ac, H3Me2K4, and H3Me3K36, and increased H4Ac ($P<0.05$). BPA decreased Pol II, H3Ac, H4ac, H3Me2K4, C/EBP β , and SREBP1 within the region approximately 1.3 kB downstream of the TSS ($P<0.05$; Figure 6B).

In females, BPA decreased H3Ac at two of the regions tested (–1050 and –401; $P<0.05$) and also decreased H3Me3K36 at two of the regions tested (–401 and –31; $P<0.05$). However, it had no effect on H3Me2K4, or the binding of Pol II, C/EBP β , and SREBP1 (Supplementary Figure 2B).

DISCUSSION

The current study demonstrates that early-life BPA exposure is associated with the sex-specific epigenetic regulation of *Cpt1a*, the key enzyme in hepatic fatty acid β -oxidation. We observed that the inhibition of hepatic *Cpt1a* and other fatty acid β -oxidation genes at birth was associated with steatosis in adult males. Furthermore, early-life BPA exposure disrupted hepatic lipid handling and appeared to further exacerbate the negative long-term consequences of a post-weaning HF diet, thus contributing to the adulthood NAFLD phenotype. These effects were associated with changes to DNA methylation as well as with covalent changes to histone tails within the *Cpt1a* gene.

Sex differences in adiposity and NAFLD development in response to BPA

In the current study, we observed metabolic and hepatic dysregulations in response to BPA and a HF diet in male, but not female offspring. The baseline measurements for numerous outcomes (including hepatic mRNA expression, fat/lean mass ratio, and hepatic TG/FFA content) in the current study were different between males and females. The prevalence of NAFLD in humans has been shown to be sex-specific, with higher rates in males than females in the 1988–1994 NHANES survey (Lazo *et al.*, 2013). There has also been a larger increase in prevalence in males than in females from the 1988–1994 survey to the 2007–2010 survey (Welsh *et al.*, 2013). These sex differences have been attributed to innate differences in hepatic metabolism between males and females, which likely include differences in hepatic clearance of toxic substances. A study in BPA-treated rats showed that females had lower serum BPA concentrations than males, and a higher activity of UGT2B1, an isoform of a UDP-glucuronosyltransferase involved in BPA glucuronidation and clearance (Takeuchi *et al.*, 2004). In the current study, males, but not females, were sensitive to the effects of prenatal BPA exposure, which could potentially be related to differences in BPA clearance during the exposure window. Although BPA is a known endocrine-disruptor,

potentially acting through estrogen receptors (ERs), whether these outcomes are related to ER signaling in liver is not clear. A study in a hepatic cell line not expressing ER α and having very low expression of ER β showed that BPA was able to induce lipid accumulation and decrease lipid secretion, potentially through PI3K/Akt signaling (Grasselli *et al.*, 2013). Additional studies will be needed to establish the precise signaling pathways and mechanistic basis behind these observations in cell culture and our *in vivo* observations in males in response to BPA.

BPA exposure is associated with disrupted hepatic lipid metabolism

A previous study in Sprague-Dawley rats demonstrated that females perinatally exposed to BPA in the drinking water had increased adiposity on PND21 and an increase in hepatic expression of *Fasn*, *Acc*, and *Srebp1c* (Somm *et al.*, 2009). In our study, we also observed that females had increased *Fasn* expression on PND1, without evidence of TG or FFA accumulation at birth, and without hepatic steatosis in adulthood. Hepatic lipids were not measured or examined in the previous study, making it difficult to compare their study and ours. Additionally, both males and females in the previous study had increased body weights at birth, which was not observed in our study. This may be due to differences in exposure mode (in drinking water vs. as an oral gavage) or dose (100 $\mu\text{g/kg/day}$ in our study vs. approximated 70 $\mu\text{g/kg/day}$ in the previous study). Furthermore, a recent study in male Wistar rats showed that gestational and lactational BPA exposure (40 $\mu\text{g/kg/day}$) increased adulthood steatosis, together with reduced mitochondrial function, and was associated with increased TG and reactive oxygen species, and reduced ATP production in adults (15 and 26 weeks). NAFLD is known to be associated with mitochondrial damage, which has been shown to be both the upstream cause (by decreasing fatty acid β -oxidation capacity) and downstream consequence (through excessive ROS production) of steatosis. In the previous manuscript, the authors concluded that because the decrease in several mitochondrial respiratory complexes at postnatal week 3 preceded hepatic lipid accumulation at postnatal weeks 15 and 26, prenatal BPA's effect on mitochondrial function likely contributed to the increased steatosis observed in adult animals (Jiang *et al.*, 2014). While we observed the increase in both TG and FFA at birth with a concurrent decrease in genes associated with hepatic β -oxidation in response to BPA, based on the previous study by Jiang *et al.*, and the numerous reports of drug- and toxin-induced hepatic steatosis (mediated through the inhibition of β -oxidation), our observations support the notion that developmental BPA exposure targets mitochondrial β -oxidation, which contributes to lipid accumulation at birth and long-term steatosis. Furthermore, while we did not observe changes in maternal body composition or body weight in response to BPA, additional extensive studies are warranted to determine whether BPA-induced outcomes in neonatal liver are due to changes in maternal physiology or to the direct body burden of BPA on fetal tissues.

Interestingly, in addition to modifying overall TG and FFA content in PND1 male offspring, BPA also modified the types and classes of FFAs in liver. Specifically, BPA-exposed males had an increased amount of monounsaturated fatty acids and a decreased ratio of very long-chain to long-chain FFAs, a process partly regulated by β -oxidation, since the final step in the synthesis of 22:6 (docosahexaenoic acid, DHA) requires one round of peroxisomal β -oxidation (Barcelo-Coblijn and Murphy, 2009). Whether this occurred in response to the

direct action of BPA on fetal FFA supply, or whether these changes in β -oxidation resulted in altered FFA metabolism will need to be further investigated.

Perinatal exposure to BPA programs the hepatic response to a post-weaning HF diet in male offspring

The concept of “developmental programming” suggests that numerous *in utero* factors can program hepatic development and function (McMillen *et al.*, 2009), and these factors – including BPA exposure – could then govern the response to postnatal lifestyle factors, such as diet. In the current study, while all treatment groups had increased hepatic vacuolation and lipid accumulation when compared to Oil-C males, BPA-HF males had the highest ratio of micro/macro-vacuolation. Microvesicular steatosis is a hallmark sign of hepatic toxicity, associated with mitochondrial damage and decreased mitochondrial β -oxidation capacity (Fromenty and Pessayre, 1995), and is also the histological hallmark of acute metabolic perturbation in liver (Jaeschke, 2011). This appears to occur because hepatic β -oxidation capacity becomes overloaded, leading to a decrease in FFA esterification to TG, and this sudden increase in small vesicles prevents the standard coalescence of lipid droplets into storage macrovacuoles (Fromenty and Pessayre, 1995), which likely explains the observed “worst case scenario” observed in the males in our study who were first exposed to BPA *in utero* and then challenged with a HF diet. Recently, a study was published using Wistar rats exposed prenatally to BPA at 50 $\mu\text{g/kg/day}$, and consuming a post-weaning HF diet. Male rats (female data were not shown) consuming a HF diet presented with BPA-induced hepatic steatosis at 27 weeks (Wei *et al.*, 2014), and this was associated with a decrease in mitochondrial respiratory capacity and ATP production in the same model (Jiang *et al.*, 2014). The study by Wei *et al.* showed that in adult animals that had steatosis, *Cpt1a* was actually increased following developmental BPA exposures, and we also observed that BPA-exposed males in the current study had increased *Cpt1a* mRNA expression in adulthood (data not shown). Wei *et al.* proposed that this increase in adults was likely a compensatory mechanism, and that the early-life “first hit” in the progression of NAFLD would likely be associated with a decrease in *Cpt1a*, and therefore a decrease in fatty acid β -oxidation. We have confirmed this proposed “first hit” by showing that developmental BPA exposure was associated with a significant downregulation of *Cpt1a* expression in males at birth, together with a decrease in the expression of other fatty acid β -oxidation-related genes. As previously discussed, CPT1A inhibition is related to metabolic perturbation, so it is likely that both in our adult animals and in the study by Wei *et al.*, the compensatory response was still quite robust in young adults, but would decrease with age and as animals progressed into fibrosis or further into NAFLD. Together, these studies using 2 different strains of rats suggest that exposure to BPA during a critical developmental window programs a dysfunction in hepatic lipid metabolism and homeostasis (in males only, as shown in our study), which prevents the appropriate storage and processing of FFAs in response to a long-term diet high in lipids, likely favoring the development of NAFLD.

Cpt1a, the key regulatory enzyme of mitochondrial β -oxidation is epigenetically regulated in PND1 male offspring

Developmental programming of chronic diseases has been proposed to occur through epigenetic modification of genes (Maunakea *et al.*, 2010). Specifically, it is thought that

epigenetics serve as an adaptive mechanism during sensitive windows of development, and these early alterations in epigenotype and genotype increase the adulthood susceptibility to diseases, potentially by altering the response to postnatal cues. While several types of epigenetic modifications have been shown to interact to regulate gene expression, DNA methylation and the covalent modification of histone tails remain the most widely studied epigenetic regulators (Portela and Esteller, 2010). We and others have previously shown that maternal diet can alter the hepatic epigenome and metabolic phenotype in offspring (Strakovsky *et al.*, 2011; Strakovsky *et al.*, 2014b; Suter *et al.*, 2014), and a study in human fetal liver (Nahar *et al.*, 2014) and in mice (Anderson *et al.*, 2012) suggests that gestational BPA exposure is also associated with altered DNA methylation in offspring. However, no studies have specifically addressed the question of whether BPA exposure also alters the histone code in livers of neonates, and how these marks interact with DNA hypo- or hyper-methylation to regulate transcription.

In the current study, we observed several differentially methylated regions in male PND1 offspring within a ~5 kB region of the *Cpt1a* gene spanning the TSS. Promoter DNA methylation within CG-rich regions (CpG Islands) has been shown to be associated with programmed transcriptional regulation (Straussman *et al.*, 2009), and we observed that BPA decreased *Cpt1a* expression, and this was associated with CpG Island DNA hypermethylation within one of the regions tested in males. We also observed that H3Me2K4, a marker of transcriptional activation (Rivera *et al.*, 2014), was decreased within all regions tested in males, but not affected in females. Furthermore, while H3Me3K36, also a marker of actively transcribed genes, was decreased within several regions in males, it was also decreased in females – but without a concurrent change in the expression of *Cpt1a* in females. Our mRNA analysis of several DNA methylation-, histone acetylation- and histone methylation-related genes showed a significant decrease in the male mRNA expression of *Kmt2c*, which methylates and activates H3Me2K4, and this corresponded to a decrease in H3Me2K4 and decreased *Cpt1a* expression. In females, this gene was significantly upregulated, without a concurrent consequence for either its target histone modification or for *Cpt1a* expression. These data suggest that K4 dimethylation at histone H3 may be a unique regulatory mechanism for the transcription of *Cpt1a* in response to BPA.

Increasing evidence is beginning to suggest that the active transcriptional regulation of genes is a result of the interaction of numerous epigenetic modifications (Murr, 2010). In the current study, we focused our analyses on DNA methylation and histone modifications within specific regions of the chromatin, thus allowing us to assess any potential interactions between these events within the *Cpt1a* gene. While we did observe that BPA hypermethylated the DNA within one CpG island, which would typically be considered a marker of heterochromatin and transcriptional inhibition, we also observed significant hypomethylation of the DNA by BPA within a region located just outside of a CpG island (sometimes referred to as a “shore”), as well as a coordinated decrease in the mRNA expression of *Dnmt3b*, which is responsible for *de novo* DNA methylation. However, and perhaps more significantly, we assessed the potential interaction of DNA methylation within these regions with histone modifications. Interestingly, the substantial hypomethylation at the island “shore” was accompanied by relatively low binding (compared to other regions)

of H3Ac, H3Me2K4, H3Me3K36, C/EBP β , and SREBP1. Furthermore, this region of seemingly “naked chromatin” was surrounded by sites of BPA-induced decreases of histone marks that represent active transcription. The *Cpt1a* gene has been shown to have a TATA-less promoter (Rivera *et al.*, 2014) that is highly dependent on the binding of transcriptional regulators on the DNA (especially in CCAAT boxes located throughout the promoter and the remainder of the genome) (Mantovani, 1998; Steffen *et al.*, 1999), thus chromatin structure (through histones and methyl groups) likely drives and regulates the assembly of necessary transcriptional machinery. While we did not specifically address the precise mechanisms that regulate *Cpt1a* transcription, we report that the transcriptional inhibition of this critical gene by BPA in males is associated with changes to both DNA methylation, as well as the histone code. Furthermore, these marks were associated with the decreased binding of Pol II within several of the regions tested, as well as of both C/EBP β , and SREBP1 (which have been previously shown to regulate *Cpt1a* transcription *in vitro* (Attia *et al.*, 2011; Thakran *et al.*, 2013)) at the promoter, confirming the important role of epigenetics in establishing chromatin conformation and thus directing the binding of transcription factors.

In conclusion, we have demonstrated that the downregulation in *Cpt1a* mRNA expression in male, but not female offspring at birth in response to developmental BPA exposure is associated with susceptibility for adulthood hepatic steatosis, especially in response to a post-weaning HF diet. We also showed that this potentially occurs through the epigenetic regulation of the gene, with both DNA methylation and histone modifications likely playing an important role in regulating chromatin structure and gene expression. Future studies are critical to determine the basis for the observed sex differences in the hepatic response to BPA as well as to determine what role maternal physiology plays in the observed adulthood hepatic phenotype. Furthermore, more research in the field is needed to address the question of whether additional epigenetic mechanisms, other than DNA methylation and histone modifications, govern the dysregulated transcription of *Cpt1* (and other developmental genes) in response to early-life BPA exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations Used

Acx1

acyl-CoA oxidase 1, palmitoyl

<i>Agpat6</i>	1-acylglycerol-3-phosphate O-acyltransferase 6
<i>Ash1l</i>	absent, small, or homeotic-like 11
BPA	Bisphenol A
BW	Body weight
<i>Cebpa</i>	CCAAT/enhancer binding protein (C/EBP), alpha
<i>Cebpβ</i> or C/EBPβ	CCAAT/enhancer binding protein (C/EBP) beta
<i>Cpt1a</i>	carnitine palmitoyltransferase 1a
<i>Cyba</i>	cytochrome b-245, alpha polypeptide
<i>Cybb</i>	cytochrome b-245, beta polypeptide
<i>Dgat1</i>	diacylglycerol O-acyltransferase 1
<i>Dnmt1, Dnmt3a, or Dnmt3b</i>	DNA methyltransferase 1, 3a, or 3b
EDC	endocrine disrupting chemical
<i>Fabp1</i>	fatty acid binding protein 1
Fasn	fatty acid synthase
<i>Fat/Cd36</i>	fatty acid translocase/cluster determinant 36
FFA	free fatty acids
H3Ac or H4Ac	acetylated histone H3 or H4
H3Me2K4	di-methylated histone H3 at lysine residue 4
H3Me3K9 H3Me3K27, or H3Me3K36	tri-methylated histone H3 at lysine residue 9, 27, or 36
HF	high-fat
<i>Hdac1 or Hdac3</i>	histone deacetylase 1 or 3
<i>Kmt2b or Kmt2c</i>	lysine(K)-specific methyltransferase 2b or 2c
<i>Lpl</i>	lipoprotein lipase
NAFLD	non-alcoholic fatty liver disease
<i>Pck1</i>	phosphoenolpyruvate carboxykinase 1 (soluble)
PND	postnatal day
SREBP1	sterol regulatory element binding protein 1
TG	triglycerides
TSS	transcription start site

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Highlights

- Developmental BPA exposure exacerbates HF-diet induced steatosis in adult males
- Gestational BPA exposure increases hepatic lipid accumulation in neonatal males
- BPA decreases *Cpt1a* and other hepatic β -oxidation genes in neonatal males
- BPA alters neonatal male *Cpt1a* DNA methylation, histones, and transcription factors

FIGURE 1. Body weight on postnatal day (PND) 97 in male (A) and female (D) offspring, cumulative energy intake (kcal) from PND 69-97 in male (B) and female (E) offspring, and the fat/lean mass ratio at birth, weaning, and from PND60-90 in male (C) and female (F) offspring. Dams were dosed with BPA (100 µg/kg/day BW) or vehicle (oil) from gestational d6 until PND21, and offspring then consumed a post-weaning control (C) or high-fat (HF) diet until PND110. The ♀ symbol in Figure 1C is shown for comparison, and represents the mean fat/lean mass ratio in females on PND90. Values are means ± SEM. n=6 litters for birth and weaning body composition, and n=5 male or female offspring in puberty and adulthood. Bars with different letters differ at $P < 0.05$ if an interaction was present between the Diet and BPA factors using 2-way ANOVA.

FIGURE 2. Postnatal day (PND) 110 hepatic vacuolation scores in male (A) and female (F), macro-vacuolation in male (B) and female (G), micro-vacuolation in male (C) and female (H), ratio of micro/macro-vacuolation in male (D) and female (I) and representative images of vacuolation using hematoxylin & eosin (H&E) staining (top panels) and lipid accumulation using Oil-Red-O (ORO) staining (lower panels) in male (E) and female (F) offspring. Dams were dosed with BPA (100 µg/kg/day BW) or vehicle (oil) from gestational d6 until PND21, and offspring then consumed a post-weaning control (C) or high-fat (HF) diet until PND110. Findings were graded from 1 (minimal) to 5 (severe). Values are means ± SEM. n=5 male or female offspring. Bars with different letters differ at $P < 0.05$ if an interaction was present between the Diet and BPA factors using 2-way ANOVA.

FIGURE 3. Postnatal day (PND) 1 hepatic triglyceride (TG) content in male (A) and female (C) offspring, and free fatty acid (FFA) content in male (B) and female (D) offspring

Dams were dosed with BPA (100 µg/kg/day BW) or vehicle (oil) from gestational d6 until PND21. The data are shown relative to the PND1 female Oil group. The dashed lines in Figures 3A and 3B are shown for comparison, and represents the mean hepatic triglyceride or free fatty acid content in females on PND1. Values are means $\pm$ SEM. n=6 pups, with male or female offspring. *P<0.05.

MALES ON PND1

FEMALES ON PND1

FIGURE 4. Postnatal day (PND) 1 hepatic metabolism-related mRNA expression in male (A) and female (B) offspring

Dams were dosed with BPA (100 µg/kg/day BW) or vehicle (oil) from gestational d6 until PND21. All data were first normalized to the *L7a* housekeeping gene, and are shown relative to the PND1 female oil group. The dashed line in Figures 4A is shown for comparison, and represents the mean hepatic mRNA expression in females on PND1. Values are means ± SEM. n=6 pups, with male or female offspring. *P<0.05 and **P<0.01.

MALES ON PND1

FIGURE 5. Postnatal day (PND) 1 epigenetic modifier-related mRNA expression in male offspring

Dams were dosed with BPA (100 µg/kg/day BW) or vehicle (oil) from gestational d6 until PND21. All data were first normalized to the *L7a* housekeeping gene, and are shown relative to the PND1 female oil group. The dashed line is shown for comparison, and represents the mean hepatic mRNA expression in females on PND1. Values are means ± SEM. n=6 male pups. *P<0.05.

FIGURE 6. Postnatal day (PND) 1 hepatic *Cpt1a* methylation analysis (A; bottom panel) and chromatin immunoprecipitation analysis (B) of approximately 5 kB of the rat *Cpt1a* gene in male offspring

Six regions within the *Cpt1a* gene were selected for Methylation Sensitive PCR analysis (A, top panel, with CpG islands signified by light blue columns and individual CpGs by orange lines below), and six regions approximately corresponding to those selected for methylation analysis were selected for chromatin immunoprecipitation analysis. Red dashed lines represent regions of DNA where the pattern of histone marks and transcription factor binding reflects heterochromatin and potential regulatory regions for *Cpt1a* transcriptional inhibition. Dams were dosed with BPA (100 µg/kg/day BW) or vehicle (oil) from gestational d6 until PND21. Values are means ± SEM. n=6 male pups. **P*<0.05 for the ratio of (Methylated) to (Methylated+Unmethylated) for methylation analysis after normalization to an internal standard (representing the amount of methylation relative to total DNA input), and for the ratio of each immunoprecipitated protein after normalization to Input.

Table 1

Diet Composition.*

	<u>Control (C)</u>		<u>High-Fat (HF)</u>	
<u>%</u>	<u>Gram</u>	<u>kcal</u>	<u>Gram</u>	<u>kcal</u>
Protein	20	20	24	20
Carbohydrate	64	64	41	35
Fat	7	16	24	45
Kcal/g	4.0		4.8	
<u>Ingredients</u>				
Casein	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	397.5	1590	105	420
Maltodextrin	132	528	132	528
Sucrose	100	400	100	400
Cellulose	50	0	50	0
Soybean Oil	70	630	70	630
Lard	0	0	130	1170
Mineral Mix	35	0	35	0
Vitamin Mix	10	40	10	40
Choline Bitartrate	2.5	0	2.5	0

* Research Diets, Inc, New Brunswick, NJ

Table 2

Primer Information

Gene for mRNA Analysis	Forward Sequence and Transcript Location	Reverse Sequence and Transcript Location	Transcript ID
<i>Acox1</i>	GCCTTGTGTCCCTATCCGT (+433)	ACCGATATCCCCGACAGTGAT (+504)	ENSRNOT00000042372
<i>Agpat6</i>	TCTGCCACTCAGGATTGCTC (+1449)	GCAGGTATCCAACCACGGTA (+1525)	ENSRNOT00000024392
<i>Ash1l</i>	GGGGCGAAAACAAAACTGTC (+3404)	GCCGTTTGTTCAGCACAGCAA (+3481)	ENSRNOT00000027629
<i>Cebpa</i>	AGTCGGTGGATAAGAACAGCAACG (+821)	GCTGTTTGGCTTTATCTCGGCTC (+910)	ENSRNOT00000014517
<i>Cebpb</i>	AGAACGAGCGGCTGCAGAAGA (+1220)	GAACAAGTTCCGCAGCGTGC (+1287)	ENSRNOT00000072673
<i>Cpt1a</i>	GAGCGACTCTTCAATACTTCCC (+1027)	TGTGCCTGCTGTCCTTGATA (+1102)	ENSRNOT00000019652
<i>Cyba</i>	GGA CTC CCA TTG AGC CTA (+120)	GTT GGT AGG TGG CTG CTT (+190)	ENSRNOT00000017564
<i>Cybb</i>	GTG TGT CGG AAT CTC CTC (+211)	GGT TCC TGT CCA GTT GTC (+292)	ENSRNOT00000038994
<i>Dgat1</i>	TCAATCTGTGGTGCCGCCAG (+708)	CCCACTGACCTTCTCCCTGCA (+775)	ENSRNOT00000039795
<i>Dnmt1</i>	TCCTACGCCATGCCAGTTTG (+1713)	GAAGATGGGCGTCTCATCATC G (+1792)	ENSRNOT00000064932
<i>Dnmt3a</i>	GCCCATTTCGATCTGGTGATTG (+2255)	TCGTAAAGTCCCTTGCGGGC (+2333)	ENSRNOT00000047210
<i>Dnmt3b</i>	AATGCCTGCGGTACAGTGGTTTG (+1197)	AACAGACCCAGAGCCACCAGCT (+1274)	ENSRNOT00000015482
<i>Fabp1</i>	GAGCCAAGAGAACTTTGAGCCCTT (+77F)	CCTTGATGTCTTCCCTTTCTGGA (+156)	ENSRNOT00000008840
<i>Fasn</i>	CTTTGTGAGCCTCACCGCCAT (+1747)	ATGCCATCAGGTTTCAGCCCC (+1811)	NM_017332.1
<i>Fat/Cd36</i>	AGTGCTCTCCCTTGATTCTGC (+151)	GAGCCACAGTTCAGATCACA (+213)	ENSRNOT00000061687
<i>Hdac1</i>	GGAGGTGGCTATACCATC (+916)	AGGGATCTCTGTGTCCAG (+993)	ENSRNOT00000012854
<i>Hdac3</i>	CGTGGCGTATTTCTACGACCCC (+12)	GTTTCATCGGGTGTCCAGCTCC (+76)	ENSRNOT00000060417
<i>Kmt2b</i>	CTAGAATCAGGTCAGGGTCGTGGTC (+961)	GGTCCCCCTTCTGTTTCATCTCC (+1049)	ENSRNOT00000046359
<i>Kmt2c</i>	ACACACCGAACACCGTGAACATG (+1847)	CTGTGCTGTGTCTGGAGAATGA (+1941)	ENSRNOT00000071915
<i>L7a</i>	GAGGCCAAAAAGGTGGTCAATCC (+64)	CCTGCCCAATGCCGAAGTTCT (+127)	ENSRNOT00000006754
<i>Lpl</i>	GCCACTTCAACCACAGCAGCAA (+376)	GGGCACCCAACCTCTCATACATTCC (+455)	ENSRNOT00000016543
<i>Pck1</i>	AGGAGGAAGAAAGGTGGCACCAG (+90)	GGCAGAGAAGTCCAGACCATTATGC (+182)	ENSRNOT00000031586

Cpt1a MSP Analysis	Forward Sequence and Transcript Location	Reverse Sequence and Transcript Location	Transcript ID
Methylated	AAGGTTTCGCGGTTAAGGTTAC (−2815)	AACGTCCTACTTTACATCCGTC (−2736)	ENSRNOT00000019652
Unmethylated	GGTTTGTGGTTAAGGTTATGG (−2813)	AACATCCTACTTTACATCCATC (−2736)	
Methylated	GTTGGTATTATTTTGGGAGCG (−1148)	ATACCCTCTACTTCTCCAATACGC (−1061)	
Unmethylated	GTTGGTATTATTTTGGGAGTGT (−1148)	AAATACCCTCTACTTCTCCAATACA (−1059)	
Methylated	TTAGAGATTGTTTAAGGTCGCGT (−557)	TCACTCAAACCGAATCACGTA (−484)	
Unmethylated	TGTTTAGAGATTGTTTAAGGTTGTG (−560)	TCACTCAAACCAAATCACATA (−483)	
Methylated	TTTCGGGTAAATTTTCGTTC (−297)	CCTTAAACAACATCTAAATACACG (−238)	
Unmethylated	ATTTTGGGTAAATTTTGTGTTGG (−298)	CCTTAAACAACATCTAAATACACA (−238)	
Methylated	TTTTTAGTCGGTCGATTTCGA (−4)	CGAAACAAACACTATCCTCCG (+71)	
Unmethylated	TTTTTTAGTTGGTTGATTTTGA (−5)	AAAACAAACACTATCCTCCACA (+70)	
Methylated	AAATTTTACGATTTCGAGTTTGA (+1478)	AATTAAATACAGCTATACCTCGTA (+1564)	
Unmethylated	TTAAATTTTATGATTTGAGTTTGA (1476)	TATTTATAATTAAATACACACTATACCTCA (+1571)	

Cpt1a ChIP Analysis	Forward Sequence and Transcript Location	Reverse Sequence and Transcript Location	Transcript ID
	AAGCAGGACGCCAAGAAAAGAGG (–2747)	TCCCTAAAACCCCATCCTAACCC (–2681)	ENSRNOT00000019652
	ACCACGGCTCTTGATGTC (–1050)	GAGCATCCTTCACTGGGT (–989)	
	ACCAGGCTAGATACAATCAGG (–401)	ATGCACGGTACAGAAAGGA (–345)	
	TACAATCAGGCTCAGGACTCTC (–321)	GAATGCACGGTACAGAAAGG (–254)	
	CAGCAAGCATACATCACCACC (–31)	GTCCTCTGCTTTAGGTCCTCACT (+43)	
	GCAAAGATACATCCTCAGCTCAGG (+1315)	TTTAAGCCTAGTAGGCGGGTCA (+1393)	

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Table 3

Antibody Information

<u>Antibody</u>	<u>Species</u>	<u>Source</u>	<u>Catalog Number</u>
IgG	Rabbit	Santa Cruz	sc-2027
Pol II (CTD4H8)	Mouse	Millipore/Upstate	05-623
H3Ac	Rabbit	Millipore	06-599
H4Ac	Rabbit	Millipore/Upstate	06-866
H3K4Me2	Rabbit	Millipore/Upstate	07-030
H3K9Me3	Rabbit	Millipore/Upstate	07-442
H3K27Me3	Rabbit	Millipore/Upstate	07-449
H3K36Me3	Rabbit	Abcam	ab9050
C/EBP β	Rabbit	Santa Cruz	sc-150
SREBP-1	Rabbit	Santa Cruz	sc-366

Table 4**Fatty Acid Composition in Postnatal Day 1 Offspring Liver**

	Male		Female	
	Oil-Control	BPA	Oil-Control	BPA
Fatty Acid Composition (% of Total)				
Saturated	39.79 ± 1.75	39.13 ± 1.88	39.24 ± 1.23	41.16 ± 1.96
Monounsaturated	21.05 ± 0.29	23.69 ± 1.21 *	21.91 ± 0.82	22.82 ± 2.02
Polyunsaturated	39.16 ± 1.65	37.18 ± 2.29	38.85 ± 0.87	36.02 ± 2.68
Long-chain	85.92 ± 0.44	88.75 ± 0.43 **	87.67 ± 1.01	90.18 ± 1.07
Very long-chain	14.08 ± 0.44	11.25 ± 0.43 *	12.33 ± 1.01	9.82 ± 1.07
Very long-chain/Long-chain	0.16 ± 0.01	0.13 ± 0.01 **	0.13 ± 0.01	0.11 ± 0.01

* P<0.05.

** P<0.0;

Long chain: tails with 14–21 carbons, Very long-chain: tails with 22–26 carbons.