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Hepatic Lipid Accumulation and Nrf2 Expression following Perinatal and Peripubertal Exposure to Bisphenol A in a Mouse Model of Nonalcoholic Liver Disease

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Background: Exposure to chemicals during critical windows of development may re-program liver for increased risk of nonalcoholic fatty liver disease (NAFLD). Bisphenol A (BPA), a plastics component, has been described to impart adverse effects during gestational and lactational exposure. Our work has pointed to nuclear factor E2-related factor 2 (Nrf2) being a modulator of hepatic lipid accumulation in models of NAFLD.

Objectives: To determine if chemical exposure can prime liver for steatosis via modulation of NRF2 and epigenetic mechanisms.

Methods: Utilizing BPA as a model exposure, pregnant CD-1 mice were administered 25 μg/kg/day BPA via osmotic minipumps from gestational day 8 through postnatal day (PND)16. The offspring were weaned on PND21 and exposed to same dose of BPA via their drinking water through PND35. Tissues were collected from pups at week 5 (W5), and their littermates at week 39 (W39).

Results: BPA increased hepatic lipid content concomitant with increased Nrf2 and pro-lipogenic enzyme expression at W5 and W39 in female offspring. BPA exposure increased Nrf2 binding to a putative antioxidant response element consensus sequence in the sterol regulatory-element binding protein-1c (*Srebp-1c*) promoter. Known Nrf2 activators increased *SREBP-1C* promoter reporter activity in HepG2 cells. Methylated DNA immunoprecipitation-PCR and pyrosequencing revealed that developmental BPA exposure induced hypomethylation of the *Nrf2* and *Srebp-1c* promoters in livers of W5 mice, which was more prominent in W39 mice than in others.

Conclusion: Exposure to a xenobiotic during early development induced persistent fat accumulation via hypomethylation of lipogenic genes. Moreover, increased Nrf2 recruitment to the *Srebp-1c* promoter in livers of BPA-exposed mice was observed. Overall, the underlying mechanisms described a broader impact beyond BPA exposure and can be applied to understand other models of NAFLD. https://doi.org/10.1289/EHP664

Introduction

The prevalence of nonalcoholic fatty liver disease (NAFLD) has increased from 3.9% in 1988–1994 to 10.7% in 2007–2010 (Bedogni et al. 2014). There is evidence that, in addition to accepted factors such as obesity, energy imbalance, and sedentary lifestyle (Li et al. 2002), critical windows of development may prime or reprogram the liver for increased risk of disease, such as NAFLD. Multiple classes of chemicals of environmental exposures, including pesticides, insecticides, and polychlorinated biphenyls, are potential modifiers of fat metabolism in liver, and such exposures are suspected to increase the risk for developing NAFLD (Al-Eryani et al. 2014). These exposures can be tools to better elucidate mechanisms by which hepatic lipid deposition occurs. In this study, bisphenol A (BPA), a plastics component used in manufacturing of polycarbonate and epoxy resins found in plastic bottles, food containers, metal cans, and thermal receipts was utilized to identify underlying epigenetic mechanisms of steatosis.

In rodents, perinatal BPA exposure increased hepatic lipid content and lipogenic gene expression, along with disturbances in adipokines and insulin signaling in adolescent and adult female offspring (Ben-Jonathan et al. 2009; Alonso-Magdalena et al. 2010; Angle et al. 2013). Epigenetic mechanisms, such as DNA methylation and histone modifications, contribute to NAFLD (Pogribny et al. 2009; Lee et al. 2014). DNA-methylation patterns and lipogenic gene expression have been correlated in liver biopsy tissues from NAFLD patients (Sookoian et al. 2010). The mechanism by which early-life BPA exposure induces lipogenic genes, such as sterol regulatory element binding protein-1c (*Srebp-1c*), in rodents has not been elucidated, but induction could occur through promoter hypomethylation (Wei et al. 2014).

Nuclear factor E2 related factor 2 (Nrf2) functions primarily as an antioxidant defense of the cell. Recently, Nrf2 has been linked to adipose differentiation and lipid homeostasis as reviewed by (Schneider and Chan 2013). Previous work has suggested that *NRF2* expression aids in hepatic lipid accumulation. In leptin-deficient mice, constitutive activation of Nrf2, via Kelch-like ECH-associated protein 1 (Keap1) knockdown (KD), enhanced hepatic steatosis (Xu et al. 2012). Also, hepatic lipid deposition and glucose tolerance was worsened in Keap1KD mice fed a long-term high-fat-diet– challenge (More et al. 2013). In rodent preadipocyte experiments, Nrf2 transcriptionally regulated Peroxisome proliferator-activated receptor gamma (*Ppar*-γ) and CCAAT/enhancer-binding protein (*Cebp*-β) to enhance adipocyte differentiation and consequently lipid synthesis (Pi et al. 2010).

Herein, we utilized BPA as a tool to uncover novel methylation changes associated with hepatic steatosis in the *Srebp-1c* and *Nrf2* promoters. First, we hypothesized that perinatal-peripubertal (PNPP) BPA exposure induces hypomethylation of CpG sites in promoters of lipogenic genes [e.g., *Srebp-1c* and fatty acid synthase (*Fas*)] early in development that persists into adulthood. Second, we hypothesized that BPA exposure would induce *Nrf2* expression in association with lipogenic gene expression (i.e., *Srebp-1c*). PNPP BPA exposure increased hepatic lipid deposition in conjunction with lipogenic enzyme expression. We identified novel sites in the *Fas, Srebp-1c*, and *Nrf2* genes that

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were hypothmethylated in conjunction with steatosis. Moreover, recruitment of Nrf2 to the *Srebp-1c* promoter increased in livers of BPA-exposed mice. Moreover, tissues from PNPP BPA-exposed male mice were also studied alongside tissues of female mice. In accordance with previous findings (Rubin et al. 2016), which suggest sex-specific effects of BPA PNPP exposure, we also observed prominent effects in females as opposed to males.

Overall, the underlying mechanisms described have a broader impact beyond BPA exposure and can be applied to understand more general mechanisms contributing to hepatic steatosis.

Materials and Methods

Animals and BPA Administration

CD-1 male and female mice (10 – 12 week old) were purchased (Charles River Laboratories) and maintained in temperature- and light-controlled (14/10-h light/dark cycle) conditions at the Tufts University Human Nutrition and Research Center Animal Facility. All experimental procedures were approved by the Tufts University New England Medical Center Institutional Animal Care and Use Committee. All animals were treated humanely and with regard for alleviation of suffering. The food (Harlan Teklad Rodents Diets® 2018 chow, Harlan Laboratories), polysulfone cages, and water were assessed for estrogenicity, and the levels were found to be negligible. Mice were paired for mating, and females were monitored daily for evidence of vaginal plugs. Upon confirmation of a plug (day 1 of pregnancy), females were housed individually. On gestational day (GD) 8, dams were implanted subcutaneously with Alzet osmotic minipumps (Alza Corp.) designed to deliver vehicle alone (50% DMSO in water) or BPA (25 μg/kg bw/day) based on mother's body weight on GD 8 for a period of 4 weeks, and therefore exposure of the dams continued through postnatal day (PND) 16. On PND21, litters were weaned, and BPA exposure was continued in the offspring of the BPA treatment group via administration in the drinking water. BPA exposure to the weanlings continued through PND35 (W5) and thus encompassed the prepubertal and peripubertal period. Schematic for number of animals in this study is described in Figure S1. The study included $n = 5$ dams per treatment group (vehicle or BPA) and one litter per dam. Pups were culled to 8 pups per litter (4 males and 4 females) the day after birth (PND2). Two female mice were chosen arbitrarily per dam for W5 studies ($n = 10$), and one female per dam was chosen for W39 studies ($n = 5$). Another female pup was subjected to the high-fat-diet study, and the data from that work will be published as a part of an independent manuscript. One male pup per dam was also studied at W39 ($n = 5$), and data are provided in Figure S2. Tissues collected from both W5 and W39 littermates were stored in −80°C for further analysis.

The rationale for delivering BPA via osmotic minipump was to decrease variability in exposure using glass drinking bottles that are subject to release of water due to mouse activity. Other studies that have evaluated BPA effects have utilized minipumps, such as perinatal exposure to BPA via subcutaneous minipump to assess fertility and fecundity in female mice (Cabaton et al. 2011), as well as disturbed global metabolism (Cabaton et al. 2013). With respect to environmental chemical exposure, conducting the study at a low dose was imperative to maintain human exposure relevance. The Environmental Protection Agency and U.S. Food and Drug Administration published tolerable daily intake (TDI) value for BPA as 50 μg/kg, and cutoff dose for low-dose effects is 5 mg/kg/day as a, regardless of the route or duration of exposure (Melnick et al. 2002). The exposure level (50 μg/kg) chosen in this study is below TDI and Low Observable Adverse Effect Level (LOAEL) levels. More important, published studies suggest that a dose of 400 μg/kg bw/day in mice resulted in serum levels (0.5 ng/ml) of unconjugated BPA, which is within the range of or, in some cases, lower than levels measured in human serum after environmental exposure (2 ng/ml) (Taylor et al. 2011). Following exposure to 25 μg/kg/day BPA as described here, circulating levels of unconjugated BPA in dams and pups were found to be below the detectability of the assay (<0.6–0.7ng/ml) (unpublished data). As hepatic lipid content and gene expression was our primary end point, we surveyed the livers first for those changes both in male and female. Based on our analysis that showed changes in female livers, we decided to continue with the analysis of tissues from females. Data from males is depicted in Supplementary Material (Figure S2).

Hepatic Lipid Content

Lipids were extracted from liver tissue by methanol-chloroform as described (More et al. 2013). Triglyceride quantification was performed using a kit from Pointe Scientific Inc. Oil Red O (ORO) staining was performed as described (More et al. 2013).

RNA Isolation and Quantitative Real-time PCR

Total RNA isolation and quantitative real-time PCR was performed as described earlier (Xu et al. 2012). Target gene expression was calculated from Cp value, and normalized to expression of housekeeping gene β2-microglobulin (*B2M*) to get relative expression. Primers are listed in Table S1.

Relative Protein Expression by Western Blot

Total protein extraction and Western blot were performed as described earlier (More et al. 2013). β-Actin was used as a housekeeping loading control. Antibody source and conditions are detailed in Table S2.

Glutathione (GSH) Quantification Assay

Reduced GSH was determined using a GSH-Glo® kit (Promega). Briefly, liver tissues (∼20 mg) were extracted with PBS containing 2 mM EDTA and subjected to the assay.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays using PNPP BPA-treated liver tissues were performed according to the ChIP-IT® Express Kit (Active Motif) with modifications. Briefly, liver tissue homogenate was fixed by 37% formaldehyde followed by sonication to obtain 200–800 bp fragments. Sheared chromatin was incubated with ChIP validated rabbit anti-Nrf2 antibody (C-20, Santa Cruz Biotechnologies) or anti-rabbit IgG antibody overnight at 4°C. Antibody-bound chromatin was eluted, and purified DNA fragments were analyzed by end-point PCR or real-time PCR using primers that covered the putative antioxidant response element (ARE) sequences in the mouse *Srebp-1c* promoter (Table S3). Results are represented as agarose gel scans (end-point PCR) as well as fold enrichment (qPCR).

Methylation Analysis

For all DNA methylation analyses, DNeasy® Blood & Tissue Kit (Qiagen) was used to isolate genomic DNA from liver tissues.

Global DNA Methylation

Genomic DNA from liver tissues was isolated by DNeasy® Blood & Tissue Kit (Qiagen). Global DNA methylation was assessed by determination of 5-methylcytosine (5-mC) using ELISA-based MethylFlash™ Methylated DNA Quantification Kit

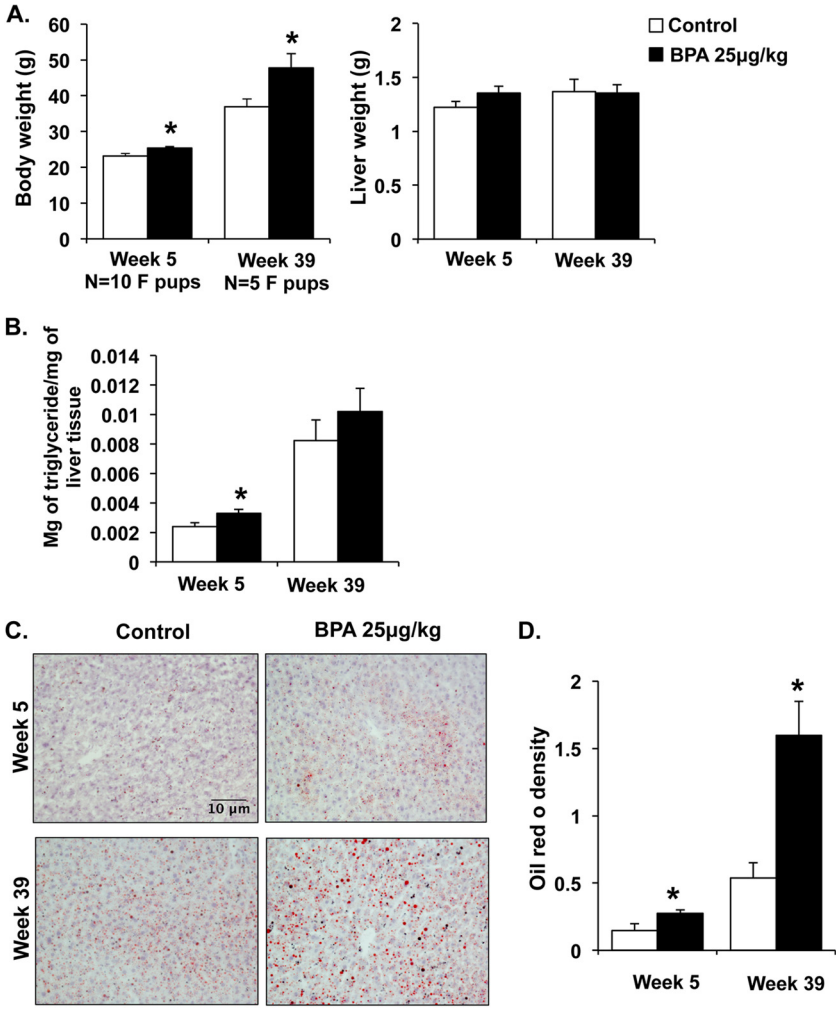

Figure 1. Effects of PNPP BPA exposure on body weight and hepatic lipid accumulation in female CD-1 mice. *A*) Body weight and liver weight. Asterisk (*) represents significant difference in weights between BPA treated and vehicle treated animals of same age ($p \leq 0.05$). *B*) Hepatic triglyceride quantification. *C*) Oil Red O staining of lipids in the liver tissue. Representative images are displayed in 200X magnification. *D*) Quantification of oil red staining density from all the samples.

(Epigentek) following the manufacturer's instructions. Briefly, 100 ng of genomic DNA was loaded into each well and absorbance was read on a microplate reader at 450 nm. The amount of 5-mC of total DNA was calculated as outlined by the manufacturer.

Methylated DNA Immunoprecipitation (MeDIP)

MeDIP assays were performed as described (Guerrero-Bosagna et al. 2010) with genomic DNA and 5-mC monoclonal antibody or anti-rabbit IgG antibodies (Cell Signaling Tech). Methylated enriched DNA was amplified by real-time PCR by using primers that covers CpG sites of *Srebp-1c*, *Fas*, and *Nrf2* promoters (Table S4). Methylation status of these promoters was plotted as fold enrichment.

Pyrosequencing

Quantitative DNA methylation bisulfite sequencing analysis and primer design at CpG sites was performed by using PyroMark® MD technology (Qiagen). Genomic DNA was bisulfite converted using the EZ DNA Methylation™ Kit (Zymo Research), and amplification of *Nrf2* gene regions of interest was performed using the PyroMark® PCR Kit (Qiagen), forward primers (50 pmol), and biotinylated reverse primers (50 pmol). The PCR conditions, forward, reverse, and sequencing primers and biotin labeling are shown in Table S5. The PyroMark® MD Pyrosequencer platform (Qiagen) was utilized according to the manufacturer's protocol. Percent of methylated cells at each CpG site was computed by Pyro Q-CpG Software™, and the software also includes internal quality-control checks (e.g., bisulfite conversion check). Percent DNA methylation at each CpG was standardized to batch (e.g., experimental plate) by calculating a standard curve from controls from 0% to 100% methylation status ran with each 96-well plate (EpigenDx).

Histone Extraction for Acetyl Histone Western Blot

Histone proteins were extracted from liver tissues using a total-histone-extraction kit (Epigentek), according to the instructions provided by the manufacturer. Then, the protein concentration of the histone proteins was measured by DC assay. Relative protein expression of total histone H3 and H3K9 was assessed by Western blot by using specific antibodies.

MicroRNA Quantification

Total RNA from W5 and W39 liver tissues after PNPP BPA exposure was isolated using TRIzol™ reagent (Invitrogen™, Thremo Fisher Scientific). In addition, 600 ng total RNA was used for miRNA cDNA preparation using RT^2™ miRNA first strand kit (Qiagen), and the expression of mature miRNAs was measured by RT-PCR by using specific primers for miR-34a, miR-122, and miR-370 (SA Biosciences). The miRNA expression was

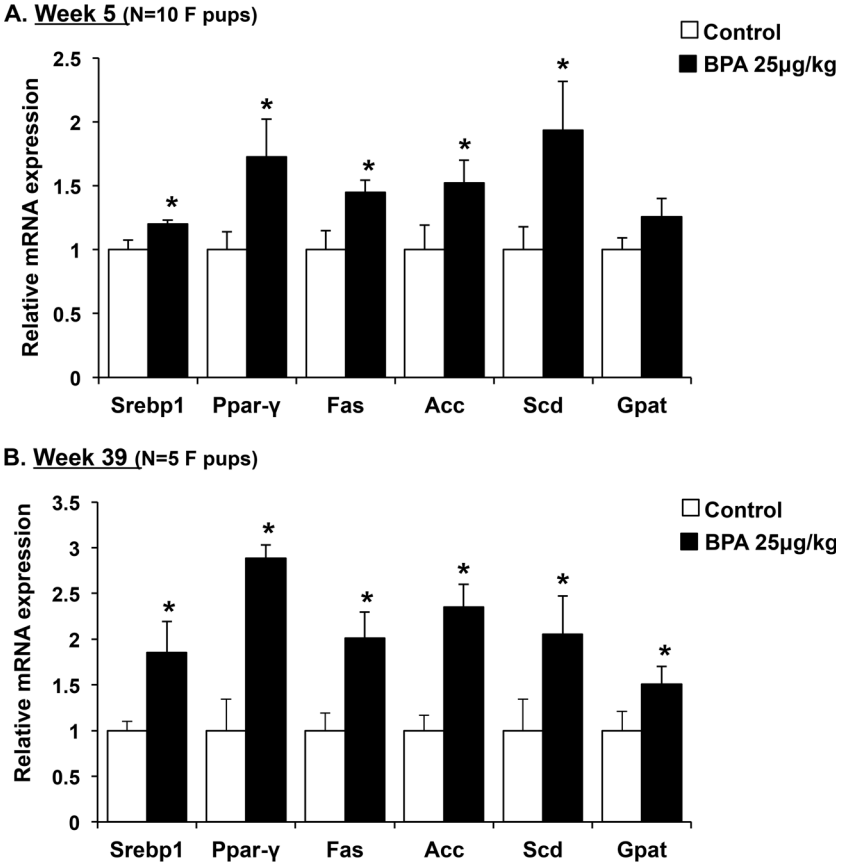

Figure 2. Effects of PNPP BPA exposure on mRNA expression of lipogenic targets in livers of female CD-1 mice (*A*.W5; *B*. W39). (*) represents significant difference in weights between BPA treated and vehicle treated animals of same age ($p \leq 0.05$).

normalized to expression level of housekeeping miR-U6 (SA Biosciences).

Transient Transfection Reporter Gene Assay

The human *SREBP-1C* promoter of 1565 bp corresponding to the 5′ upstream region cloned in pGL3-basic luciferase vector was donated by Dr. Marta Casado (Instituto de Biomedicina de Valencia, Valencia, Spain). The *NRF2* ORF cloned into EV26 was donated by Dr. Jose Manautou (University of Connecticut, Storrs, Storrs, Connecticut). Co-transfections in HepG2 cells were performed using Lipofectamine 3,000 (Life Technologies). Cells were treated with oleanolic acid (50 µM) and sulforaphane (10 µM) for 12 hours. Luciferase activity was measured using a Dual-Luciferase® Reporter Assay System (Promega).

Protocol for Cryopreserved Human Hepatocyte

Single-donor cryopreserved human hepatocytes (male, 56 years of age) were obtained from Xenotech LLC (Lenexa; Catalog no. H1000.H15+, Lot No. HC5-25). Hepatocytes were thawed and cultured following Xenotech protocol. Briefly, cells were thawed in pre-warmed thawing media (supplemented DMEM without Pen/Strep and isotonic Percoll™). Hepatocytes were pelleted by centrifugation at $100 \times g$ for 5 min at 4°C, resuspended in hepatocyte plating (seeding) medium (supplemented DMEM without Pen/Strep). The cell suspension was quantified for viability and cell concentration was based on Trypan Blue (Millipore Sigma) exclusion. Cell concentration of the hepatocyte suspension was adjusted to 1.4×10^6 per mL in plating (seeding) media. A volume of 330 µL and 1.6 mL of the cell suspension were added to each well of 24-well plate and 6-well plate respectively. Seeded plates were kept under an atmosphere of 95% air and 5% CO_2 at 37°C. After 3 hrs, plating medium was replaced with hepatocyte culture medium (serum-free supplemented MCM^+ (Modified Chee's Medium), spiked with Matrigel (diluted to 0.25 mL/mL in hepatocyte culture media) to overlay the cells. After 24 hrs, cells were treated with BPA (100 nM) and vehicle (DMSO–01% v/v). Treatments were continued for 72 hrs; every 24 hrs, media was removed and replaced by fresh media containing treatments. After treatment, cells were washed twice with saline and harvested to isolate RNA, lipids, and ORO staining.

Statistical Analysis

Data are represented as average ±SEM (W5, $n = 10$/group; W39, $n = 5$/group). All statistical analyses were performed by Student's *t*-test. Asterisk (*) indicates statistically significant difference between the control and BPA treated group ($p < 0.05$).

Results

Effects of PNPP BPA Exposure on Body Weight and Hepatic Lipid Accumulation in Female Offspring

BPA exposure increased body weight by 5% at W5, and increased body weight by 15% at W39 (Figure 1A), but liver weight was similar between treatment groups (Figure 1A). BPA exposure also increased hepatic triglyceride content at W5 by 33%, but not at W39 (Figure 1B). ORO staining revealed BPA increased hepatic lipid deposition at W5 and W39 (Figures 1C & 1D) females. Livers from male offspring were also analyzed. Both W5 and W39 PNPP BPA-exposed males and control males had similar body weights. Liver triglycerides and mRNA

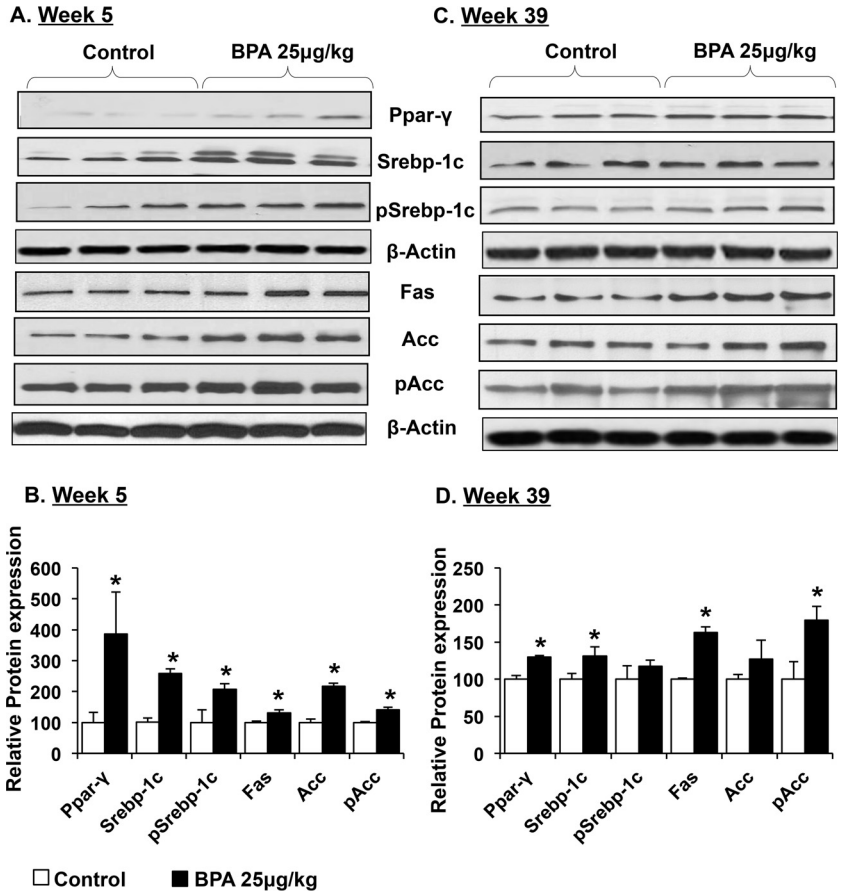

Figure 3. Effects of PNPP BPA exposure on protein expression of lipogenic transcription factors and enzymes in livers of female CD-1 mice. (*A*: W5 blots, *B*: quantification of W5 blots, *C*: W39 blots, *D*: quantification of W39 blots). Nuclear proteins were detected for expression using specific antibodies for peroxisome proliferator activated receptor gamma (Ppar-γ), sterol regulatory element binding protein-1c (Srebp-1c), and phosphorylated Srebp-1c. Fatty acid synthase (Fas), acetyl-CoA carboxylase (Acc) and phosphorylated Acc were quantified from total protein lysates by Western blot using specific antibodies. The mean blot intensity is presented as percent expression. (*) represents significant difference in weights between BPA-treated and vehicle-treated animals of same age ($p \leq 0.05$).

expression of lipogenic genes were also similar between PNPP BPA-treated males and controls at W39 (Figure S2). Therefore, female mice were chosen to characterize further.

Effects of PNPP BPA Exposure on Hepatic Lipogenic Gene Expression in Female Mice at Puberty and Adulthood

Hepatic mRNA expression of transcription factors (*Srebp-1c*, *Ppar-γ*) and enzymes acetyl-CoA carboxylase (*Acc-1*), *Fas*, stearoyl-CoA desaturase-1 (*Scd-1*), and glycerol 3 phosphate acetyl transferase (*Gpat*) was measured. At W5, lipogenic targets were induced by BPA by 15 – 30 %. At W39, BPA exposure approximately doubled *Ppar-γ*, *Fas*, *Acc-1*, and *Gpat* mRNA expression (Figures 2A, 2B). Along with lipogenesis, lipid oxidation and transport targets were also measured on mRNA level. No consistent changes were observed for β-oxidation or fatty acid transport proteins with PNPP BPA exposure (Figures S3, S4). Increased protein expression for multiple lipogenic targets was also observed at W5 (Figures 3A, 3B) and W39 (Figures 3C, 3D), with *Ppar-γ*, *Srebp-1c*, and *Fas* protein levels in liver being increased at both W5 and W39.

Effects of PNPP BPA Exposure on the Nrf2 Signaling Pathway

BPA exposure increased Nrf2 and glutamate cysteine Ligase (Gclc) mRNA and protein expression at W5 and W39 (Figures 4A–D). GSH content was increased (W5) or remained unchanged (W39) after BPA exposure, suggesting the absence of significant oxidative stress (Figures 4E, F). In addition, liver expression of inflammation markers was similar between control and BPA-exposed mice (Figure S5).

Effects of PNPP BPA Exposure on Promoter Methylation in Target Genes

The effect of BPA on gene-specific promoter methylation was determined using methylated-DNA immunoprecipitation (MeDIP). Promoter sequences for lipogenic targets were analyzed for presence of CpG sites on Methprimer (University of California), and these sites were checked for methylation status. In W5 mice, BPA decreased methylation in regions upstream of the translational start site. BPA-treated groups showed less enrichment of 5-mC at the *Srebp-1c* (−231 to −346 and −1325 to −1456), and at the *Fas* (−306 to −472 and −654 to −832) promoters, and at one region of the *Nrf2* promoter (−1059 to −1168) (Figure 5). In W39 livers, BPA was associated hypomethylation at multiple regions. BPA exposure decreased methylation in the *Srebp-1c* promoter at −231 to −346 and −1325 to −1456. Moreover, pyrosequencing of the *Nrf2* promoter (−1405 to −1088) containing 4 CpG sites (CpG #1, 2, 3, and 4, respectively at −1132, −1144, −1147, −1157) revealed that in W5, CpG #1 site had less methylation in BPA-treated mice compared to controls. No change in three other CpG sites was observed. At W39, lower percent methylation of CpG #4 was detected in BPA-exposed mice. We investigated whether the observed changes in DNA

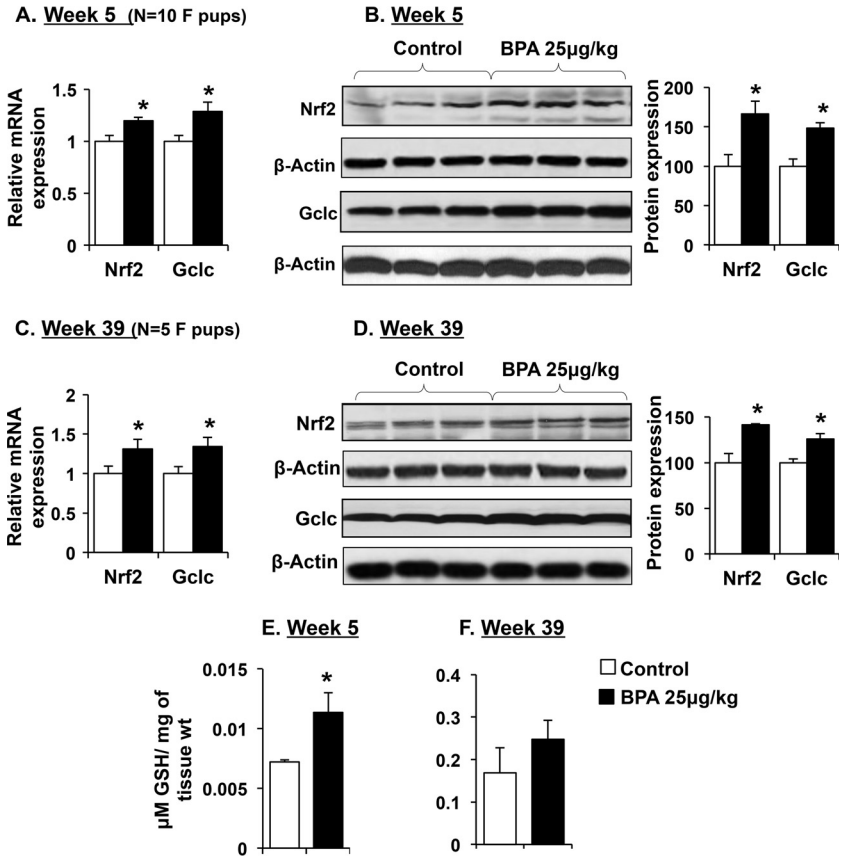

Figure 4. Effects of PNPP BPA exposure on Nrf2 signaling in livers of female CD-1 mice. *A*) *Nrf2* and its target gene glutamate cysteine ligase (*Gclc*) expression in livers of W5 animals. Messenger RNA expression was quantified using real time polymerase chain reaction (RT-PCR). *B*) Protein expression of nuclear Nrf2 and Gclc by Western blot for W5 animals and blot intensity quantification. *C*) *Nrf2* and *Gclc* mRNA expression in W39 animals. *D*) Protein expression of nuclear Nrf2 and Gclc in W39 animals and blot intensity quantification. *E* & *F*) Glutathione (GSH) content in W5 and W39 animals. (*) represents significant difference in weights between BPA treated and vehicle treated animals of same age ($p \leq 0.05$).

methylation were accompanied by changes in expression of known DNA methyl transferase (*Dnmt*). Based on ELISA, we demonstrated no change in global methylation upon BPA exposure at W5 and genome-wide hypermethylation at week 39 (Figure S6A). Furthermore, *Dnmt1*, *Dntm3a*, and *Dnmt3b* mRNA expression was not significantly altered with BPA exposure (Figure S6B).

Effects of PNPP BPA Exposure on Nrf2 Recruitment to the Srebp-1c Promoter

In W5 mice, BPA increased recruitment of Nrf2 to a putative ARE binding site at −120 to −130 upstream of translational start site in mouse *Srebp-1c* gene (depicted in Figure 6A) in comparison with negative control (IgG) depicted transcriptional regulation of *Srebp-1c* by Nrf2 upon BPA treatment. Interestingly, this transcriptional regulation of lipogenic proteins by Nrf2 was persistent with BPA in W39 mice as well (Figure 6B). BPA exposure caused higher binding of Nrf2 on *Srebp-1c*, at the same region upstream of translation start site in W39 mice as in W5 mice. Real-time PCR results also showed higher enrichment of Nrf2 on *Srebp-1c* promoter upon BPA treatment in both W5 and W39 mice (Figure 6C).

Effect of NRF2 Over-expression on Transactivation of Human SREBP-1C Promoter

Co-transfection with human *NRF2* increased transactivation of a human *SREBP-1C* luciferase reporter construct containing a putative ARE (Figure 7A). Furthermore, treatment of HepG2 cells with known NRF2 activators, sulforaphane, and oleanolic acid enhanced transactivation of the *SREBP-1C* promoter, supporting the presence of functional ARE in the *SREBP-1C* promoter (Figure 7B).

Discussion

Our previous work demonstrated induction of the Nrf2-signaling pathway in models of hepatic steatosis (Xu et al. 2012; More et al. 2013). A principle objective of this work was to address epigenetic mechanisms underlying hepatic steatosis caused by early-life exposure, as well as developmental induction of the Nrf2 pathway. Based on previous rodent studies with BPA exposure being linked to increased hepatic steatosis (Strakovsky et al. 2015), we chose a developmental exposure paradigm of BPA to investigate Nrf2-mediated mechanisms in an environmental origin of NAFLD. This work describes new findings to the field, such as Nrf2 induction in a model of developmentally induced steatosis, the presence of a putative ARE in the *Srebp-1c* promoter, and *Srebp-1c* activation via Nrf2. We observed persistent effects of BPA-induced steatosis and epigenetic modification that include DNA methylation of *Nrf2* and lipogenic genes, which brings to light the notion that DNA hypomethylation can persist in sites of the liver genome well after exposure stops.

Timing of exposure to potential endocrine disruptors like BPA is a critical determinant for disease susceptibility (Chapin et al. 2008; Marmugi et al. 2012). Fetal development is considered to be very sensitive for any kind of external stress, including

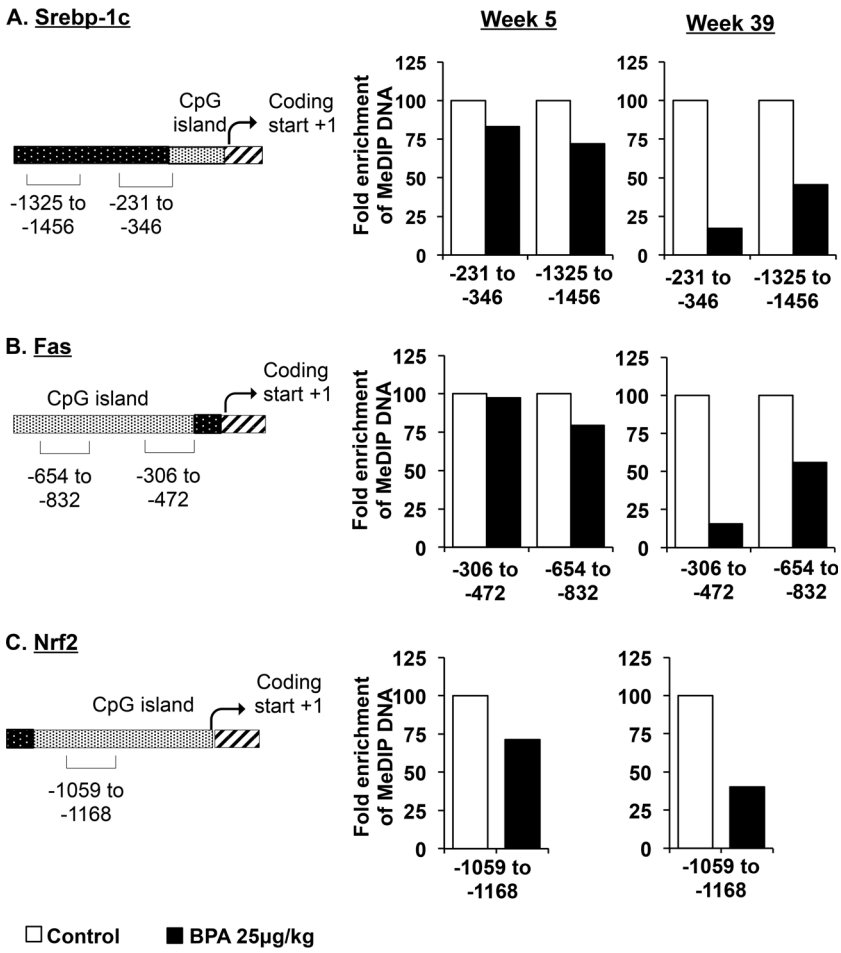

Figure 5. Effect of PNPP BPA exposure on *Srebp-1c*, *Fas*, and *Nrf2* promoter methylation. CpG sites in promoter sequences of *Srebp-1c*, *Fas*, and *Nrf2* (*A*, *B*, and *C*, respectively) up to 2 kb upstream of translational start site were analyzed to check methylation effects of PNPP BPA. Results are plotted as fold enrichment.

exposure to environmental chemicals. Chemical exposure during a vulnerable window of development may lead to increased incidence of diseases later in life. This hypothesis is referred to as "fetal basis of adult diseases," which states that any effect on an embryo can remain persistent for life (Heindel and vom Saal 2009). We observed that PNPP BPA exposure induced hepatic steatosis without increase in liver weight, which has been noted before (Wei et al. 2014; Marmugi et al. 2012). Furthermore, PNPP BPA effect observed was prevalent in female offspring, which is consistent with a recent pediatric study in which higher urinary BPA levels were observed in females in comparison with observations in males in association with NAFLD (Khalil et al. 2014). We observed that increased lipid level was associated with induction of mRNA and protein expression of enzymes and transcription factors that induce *de novo* lipogenesis (DNL). Interestingly, fatty-acid oxidation targets were not changed in liver for either age (Figure S3, S4). Some reported findings suggest repressed fatty-acid oxidation by BPA. This difference in results could be attributed to differences in species (rat vs. mice), exposure mode (osmotic pump vs. oral gavage), or dose (25 μg/kg/day in our study vs. approximately 100 μg/kg/day in other research results (Strakovsky et al. 2015).

Typically, obesity and insulin-resistance precipitate hepatic steatosis. Insulin resistance can be a cause or consequence of hepatic steatosis, and DNL is under regulation of insulin action in liver (Ress and Kaser 2016). In this study, steatosis was observed in the absence of overt weight gain. Additionally, AKT phosphorylation and expression was similar in control and BPA-exposed livers, ruling out insulin-signaling changes as a cause for steatosis. Similar AMPK phosphorylation patterns further suggest that BPA-mediated steatosis did not occur via decreased AMPK activity (Figure S7, S8). Additionally, mRNAs for inflammatory markers were not induced, suggesting that steatosis was not caused by oxidative stress, and induction of *Nrf2* was likely not due to inflammation (Figure S5). Together, these data argue for more direct mechanisms in the regulation of *Fas*, *Srebp-1c*, and *Nrf2*, such as promoter hypomethylation.

Epigenetic reprogramming and modification of genes has been one of the proposed mechanisms for fetal or developmental origin of adulthood and chronic diseases. The early developmental stage of embryo is a sensitive window for alteration in epigenotype or genotype as adaptive response to any xenobiotics exposure. Based on the 'fetal plasticity' theory (Heindel and vom Saal 2009), any alteration in epigenotype or genotype in early stages may result in lasting effects, possibly increasing risk of adulthood diseases (Grun et al. 2006; Faulk et al. 2014). Here, we observed alteration of epigenome in W5 mouse livers, and even though effects were persistent in W39 mice, the site-specific differences in methylation were evident (Table 1). These data suggest that some sites on the DNA might be persistently "marked," whereas others are more "plastic." This observation is highly relevant to the field of epigenetics and supports other

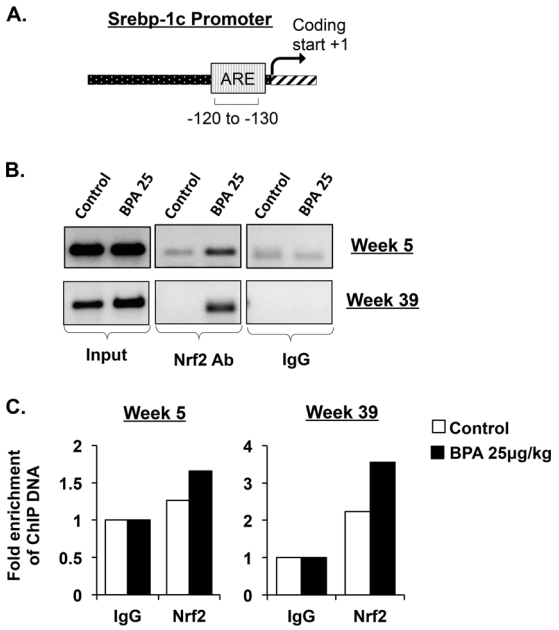

Figure 6. Nrf2 recruitment to the *Srebp-1c* promoter by chromatin immunoprecipitation (ChIP). *A*) Schematic of Nrf2 recruitment on promoter of *Srebp-1c*. *B*) End-point PCR. *C*) Real-time PCR amplification by using primers as enlisted in supplementary Table S3, which covers putative ARE consensus sequence on promoter of *Srebp-1c*. Results are plotted as fold enrichment in comparison with negative control.

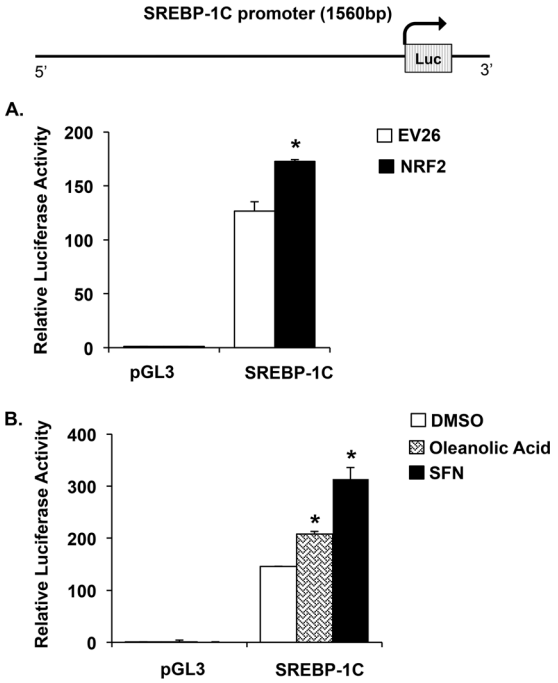

Figure 7. *NRF2* mediated transactivation of human *SREBP-1C in vitro*. *A*) *NRF2* expression plasmid were transiently co-transfected with the *SREBP-1C* promoter luciferase reporter constructs (−1.5kb) or pGL3 basic, in HepG2 cells. Luciferase activity was measured as relative firefly/renilla luciferase and was recorded as relative light units data are presented as mean fold change ± SEM. *B*) *SREBP-1C* promoter luciferase reporter constructs or pGL3 basic were transiently transfected into HepG2 cell. After 24 hrs of transfection, HepG2 cell were treated with the oleanolic acid (50uM) and sulforaphane (10 μM) along with DMSO (0.05%) for 12hrs. Luciferase activity was measured using a commercial kit.

observations of plasticity in methylation or epigenetic drift with aging (Tang et al. 2012; Teschendorff et al. 2013). Interestingly, the site-specific methylation changes that we demonstrated were not accompanied by global DNA hypomethylation (Figure S6). Such discrepancies in the global vs. site-specific epigenetic changes are not uncommon, as BPA has been described to alter gene-specific methylation without affecting *Dnmt*s expression (Bromer et al. 2010). The precise mechanism by which BPA exerts gene-specific hypomethylation remains unknown. As histone and microRNA modifications have been linked to NAFLD (Aagaard-Tillery et al. 2008; Panera et al. 2014) as well as BPA exposure (Strakovsky et al. 2015; Kovanecz et al. 2014), preliminary analysis of these mechanisms was performed. Liver acetylated histone levels appeared unaltered with BPA exposure (Figure S9). With BPA exposure, mi34a and mi122 mRNA levels showed an increasing trend in W5 mice, but not in W39 mice (Figure S10). We suggest detailed analysis of these epigenetic changes to rule out the possibility of their involvement in BPA induced steatosis.

Because W5 is when the exposures ended and steatosis was observed, we evaluated whether direct BPA exposure could cause lipid accumulation in hepatocytes, and we treated primary human hepatocytes and HepG2 cells with BPA (Figure S11 and S12). Lack of BPA-induced steatosis or NRF2 activation in primary human hepatocytes further supported our hypothesis that BPA-induced NAFLD is primarily via developmental exposure and not due to direct exposure to BPA. Moreover, *SREBP-1C* promoter reporter activity remained unchanged with BPA exposure, in *SREBP-1C* as well as *SREBP*-1*C* + *NRF*2 co-transfection in HepG2 cells (Figure S12). Perhaps direct exposure of the mature hepatocyte to BPA elicits different cellular transcriptional responses in comparison with the endoderm, hepatopancreatic progenitor cells, or hepatoblast during development. The studies herein point to the importance of the developmental window, and this window is difficult to recapitulate utilizing cell-based models.

BPA-mediated induction of lipid synthesis enzyme expression has been demonstrated before (Marmugi et al. 2012), however the upstream mechanism by which BPA imparts this up-regulation remains relatively unknown. One potential mechanism could be via up-regulation of Nrf2 expression and regulation of lipogenic genes (Xu et al. 2012; More et al. 2013). Although most commonly studied for countering oxidative stress, Nrf2 can be an upstream regulator of adipogenesis through *Ppar*-γ and *Cebp*-β transcriptional regulation in rodent pre-adipocytes (Pi et al. 2010). PNPP BPA-exposure enhanced nuclear localization concomitant with Nrf2 binding to an undescribed new putative ARE sequence on the *Srebp-1c* promoter. This increase in Nrf2 signaling was likely not a result of oxidative stress, as GSH levels in the liver tissues were not depleted, and inflammation was not

Table 1. Site-specific methylation (%) of 4 CpG sites for NRF2 promoter upon PNPP BPA exposure in W5 ($n = 10$ F pups) and W39 ($n = 5$ F pups) age mice.

	CpG #1	CpG #2	CpG #3	CpG #4
Week 5				
($N = 10$ F pups)				
Control	20.47 ± 2.44	7.46 ± 1.62	2.39 ± 0.90	8.16 ± 1.88
BPA	11.61 ± 1.74*	6.52 ± 1.47	5.16 ± 2.24	10.84 ± 2.67
Week 39				
($N = 5$ F pups)				
Control	13.26 ± 0.61	6.44 ± 1.23	4.38 ± 1.74	19.03 ± 0.9
BPA	15.70 ± 3.30	6.49 ± 0.95	2.44 ± 1.26	7.64 ± 2.42*

Note: Values were adjusted to a standard curve of control DNA (ranging between 0 and 100%) run for each CpG site on the corresponding experimental plate for each sample. Asterisk (*) represents significant difference in expression between BPA treated and control animals ($p \leq 0.05$).

observed (Figure S5). *Nrf2* transfection in HepG2 cells, as well as oleanolic acid and sulforaphane treatment, increased *SREBP-1C* promoter reporter activity. These findings highly suggest a novel function for Nrf2 as transcriptional regulator of hepatic DNL.

Our findings could be applicable to other chemicals that have estrogenic properties, such as BPS and BPF, which possess potency and efficacy similar to those of BPA with regard to hormone-disruptive properties *in vitro* and *in vivo* (Rochester and Bolden 2015), as well as potentially to other chemicals known to cause lipid accumulation in liver via developmental exposure (i.e., tributyltin) (Chamorro-Garcia et al. 2013).

Conclusion

Overall, the present study is an exploration into mechanisms of early-life exposure and development of hepatic steatosis in adulthood. Novel hypomethylated sites in the *Fas*, *Srebp-1c*, and *Nrf2* genes were identified in conjunction with steatosis; however, exploration into other epigenetic mechanisms is needed. Nrf2 recruitment to the *Srebp-1c* promoter in livers of BPA-exposed mice was uncovered, which is a new finding for this important transcription factor. The mechanisms described herein are broad and can be applied to other environmental exposure and hepatic steatosis models.

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