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Prenatal Exposure to Bisphenol A Disrupts Mouse Fetal Liver Maturation in a Sex-Specific Manner

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ABSTRACT

Bisphenol A (BPA) is one of the most prevalent endocrine disrupting chemicals in the environment. Developmental exposure to BPA is known to be associated with liver dysfunction and diseases, such as hepatic steatosis, liver tumors, metabolic syndrome, and altered hepatic gene expression, and DNA methylation profiles. However, the effects of BPA on rodent liver development are unknown. The present study was undertaken to address this important question using the mouse as an experimental model. Pregnant mice were exposed to BPA via diet from embryonic day 7.5 (E7.5) to E18.5. At E18.5, fetal livers were collected, and analyzed for changes in the expression of key hepatocyte maturation markers. We found the following significant alterations in BPA-exposed female but not male fetal livers: (a) levels of the mature hepatocyte markers, albumin and glycogen synthase proteins, were decreased (−65% and −40%, respectively); (b) levels of the immature hepatocyte marker, α-fetoprotein, were increased (+43%); (c) the level of C/EBP-α protein, the master transcription factor essential for hepatocyte maturation, was down-regulated (−50%); and (d) the level of PCNA protein (marker of proliferation) was elevated (+40%), while that of caspase-3 protein and activity (markers of apoptosis) was reduced (−40% and −55%, respectively), suggestive of a perturbed balance between cell proliferation and apoptosis in BPA-exposed female fetuses. Taken together, these findings demonstrate that prenatal exposure to BPA disrupts the mouse fetal liver maturation in a sex-specific manner, and suggest a fetal origin for BPA-induced hepatic dysfunction and diseases. J. Cell. Biochem. 117: 344–350, 2016. © 2015 Wiley Periodicals, Inc.

KEY WORDS: BISPHENOL A; ENDOCRINE DISRUPTING CHEMICAL; DEVELOPMENTAL EXPOSURE; LIVER MATURATION

Bisphenol A (BPA) is an endocrine-disrupting chemical (EDC) ubiquitously present in the environment. It is used extensively in the manufacture of consumer products including reusable water bottles, food containers, inner-linings of food cans, cardboards, computer keyboards, CDs, and cash register papers [Vandenberg et al., 2010; Rubin, 2011]. Thus, BPA has been detected in water as well as indoor and outdoor air samples [Vandenberg et al., 2010; Rubin, 2011]. In humans, exposure to BPA has fueled major public health concerns, as it has been linked to a wide range of metabolic, reproductive, cardiovascular, and neurological disorders as well as cancer [De Coster and van Larebeke, 2012; Rochester, 2013]. Of particular concern is exposure to BPA during pregnancy, a critical time during which key organs are undergoing growth and differentiation. Indeed, BPA has been detected in placental tissues, amniotic fluid, umbilical cord, and neonatal blood, as well as in maternal blood of pregnant women [Corbel et al., 2013; Gerona et al., 2013]. The developing fetus also possesses the ability to reactivate inactive BPA-glucuronide, which in combination with its limited drug-metabolizing system, further exacerbates fetal exposure to bioactive BPA [Nishikawa et al., 2010]. Previous animal studies have shown that exposure to BPA in utero alters the development of several fetal organs including the brain [Wolstenholme et al., 2012; Elsworth et al., 2013], heart [Chapalamadugu et al., 2014], mammary glands [Vandenberg et al., 2007; Tharp et al., 2012], uterus [Calhoun et al., 2014], ovaries [Susiarjo et al., 2007], testes [Horstman et al., 2012], and lungs [Hijazi et al., 2015]. However, to date, the effects of BPA exposure on fetal liver maturation remain largely unknown.

The liver is a key metabolic organ essential for maintaining overall homeostasis. The principal functions of the liver include metabolism, detoxification, glycogen storage, and production of proteins, bile, and cholesterol [Grijalva and Vakili, 2013]. Previous experimental animal studies have revealed that developmental exposure to BPA results in liver dysfunction and diseases, such as hepatic steatosis [Jiang et al., 2014; Wei et al., 2014], liver tumors [Weinhouse et al., 2014], and metabolic syndrome [Alonso-Magdalena et al., 2010; van Esterik et al., 2014] in adult animals. Furthermore, maternal BPA exposure has also

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been shown to induce mitochondria-mediated apoptosis [Xia et al., 2014], changes in gene expression [Garcia-Arevalo et al., 2014], and aberrant DNA methylation profiles [Ma et al., 2013] in the livers of adult mice. Given the robust and increasing evidence that adverse events in early-life permanently alter organ growth and function, ultimately leading to diseases in later life [Heindel and Vandenberg, 2015], the present study was designed to examine the fetal origins of BPA-induced liver dysfunction and diseases by determining the effects of prenatal exposure BPA on fetal liver development in the mouse.

MATERIALS AND METHODS

ANIMAL EXPERIMENTS

The use of animals in this study was approved by the Council on Animal Care at the University of Western Ontario, following the guidelines of the Canadian Council on Animal Care. Breeding pairs of adult C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed under standard conditions and provided with food and water ad libitum. Polystyrene cages were utilized in order to minimize background exposure to BPA beyond treatment regimen. Mice were maintained at humidity- and temperature-controlled rooms under a normal 12 h/12 h light–dark cycle. For experiments, 6–8-week-old female mice were placed overnight with males, and pregnancy was determined the next morning by the observation of a vaginal plug. Plugged females were separated from males, and gestational days were counted, with presence of a vaginal plug indicating embryonic day 0.5 (E0.5). Pregnant mice were randomly assigned to receive one of the following two diets: (1) control diet (phytoestrogen free food pellets supplemented with 7% corn oil; TD.120176, Harlan Teklad, Madison, WI), or (2) control diet supplemented with 25 mg BPA/kg diet (TD.120466, Harlan Teklad). Bisphenol A was purchased from Sigma–Aldrich Canada Ltd. (99+% purity; CAS 80-05-7; Oakville, ON). Although it is known that humans are exposed to BPA via oral and non-oral routes (e.g., sublingual and transdermal), for simplicity oral administration was chosen in the present study. Feeding was initiated at E7.5, subsequent to successful implantation and just prior to the onset of liver development, and resumed up until E18.5. At E18.5, animals were euthanized by CO_2 euthanasia, fetuses were recovered by caesarean section, and their weights recorded. In addition, maternal weight, pup number, and the number of reabsorption sites per uterine horn were noted. Fetal livers and fetal limbs were snap frozen in liquid nitrogen and stored at −80°C.

GENOTYPING PCR

Fetal sex was determined by standard PCR for the presence of the male-specific SRY gene. Briefly, DNA was isolated from fetal limb samples. The PCR reactions were carried out using the Platinum Taq DNA Polymerase Kit (cat. no. 10966-026, Invitrogen), with the primers SRY-F (GCA GGT GGA AAA GCC TTA CA) and SRY-R (AAG CTT TGC TGG TTT TTG G). PCR amplifications were carried out for 30 cycles (20 s at 95°C, 20 s at 55°C, and 35 s at 72°C) on the Eppendorf Mastercycler® Gradient PCR System (Eppendorf). PCR products were run on a 1% agarose gel, and fetal sex was determined to be male if a DNA product at 271 bp (indicative of the presence of the SRY gene) was observed.

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

Western blot analysis was conducted in order to assess protein expression levels, as described previously [Selvaratnam et al., 2013]. First, liver tissues were homogenized in 10 volumes of ice-cold 10 mM sodium phosphate buffer, pH 7.0, containing 0.25 M sucrose. Equal volumes of the homogenates were mixed with SDS gel loading buffer (50 mM Tris · HCl, pH 6.8, 2% wt/vol SDS, 10% vol/vol glycerol, 100 mM DTT, and 0.1% wt/vol bromophenol blue). Equal concentrations of this mixture were then subjected to a standard 10% SDS–PAGE. After electrophoresis, proteins were transferred to PVDF transfer membrane (Amersham Hybond-P, cat. no. RPN303F, GE Healthcare Lifesciences, Baie D'Urfe, QC) using a Bio-Rad Mini Transfer Apparatus. Nonspecific antibody binding was blocked with 5% wt/vol milk in TBST (0.1% vol/vol Tween-20 in TBS) for 1 h at room temperature. Membranes were then hybridized with primary antibody (albumin: cat. no. SAB2100098, 1:5,000 dilution, Sigma–Aldrich, Saint Louis, MO; glycogen synthase: cat. no. 3886, 1:1,000 dilution, Cell Signaling Technology, Beverly, MA; AFP: cat. no. sc-8108, 1:1,000 dilution, Santa Cruz Biotechnology, Dallas, TX; C/EBP-α: cat no. sc-61, 1:500 dilution, Santa Cruz Biotechnology; PCNA: cat. no. 2586, 1:1,000 dilution, Cell Signaling Technology; caspase-3: cat. no. 9662, 1:1,000 dilution, Cell Signaling Technology; GAPDH: cat. no. IMG-3073, 1:5,000, Imgenex) overnight at 4°C. The membrane underwent 3 × 10 min washes with TBST, and was then incubated with an anti-rabbit secondary antibody (cat. no. HAF008, 1:500 dilution, R & D Systems) or anti-mouse secondary antibody (cat. no. G-202-C, 1:10,000 dilution, Fisher Scientific) for 1 h at room temperature. Following another 3 × 10 min washes in TBST, proteins were detected using chemiluminescence (cat. no. WBLUR0500, Luminata Crescendo, Western HRP Substrate; Millipore, Etobicoke, ON). The membrane was viewed using the VersaDoc Imaging System (BioRad, UK). Densitometry was performed on the images and the level of various proteins expressed as percent of controls.

STATISTICAL ANALYSIS

Results are presented as mean ± SEM of four different litters. Livers from three pups were pooled per litter. Data were analyzed using Student's *t*-test. Significance was set at $P < 0.05$.

RESULTS

EFFECTS OF BPA ON ALBUMIN AND GS PROTEIN EXPRESSION

To determine whether prenatal exposure to BPA affected fetal liver maturation, we first examined albumin expression. We found that levels of albumin protein were significantly decreased in BPA-exposed female fetal livers when compared to controls (35% of control; Fig. 1A and C). In marked contrast, the abundance of albumin protein was not altered in the fetal livers of BPA-exposed males (Fig. 1B and D). We then determined the expression of glycogen synthase (GS), the rate-limiting enzyme of glycogen synthesis. We found that the level GS protein was significantly decreased in the fetal livers of female BPA-exposed mice when compared to controls (60% of control; Fig. 1E and G). However, there was no change in the level of GS protein in BPA-exposed male fetal livers (Fig. 1F and H).

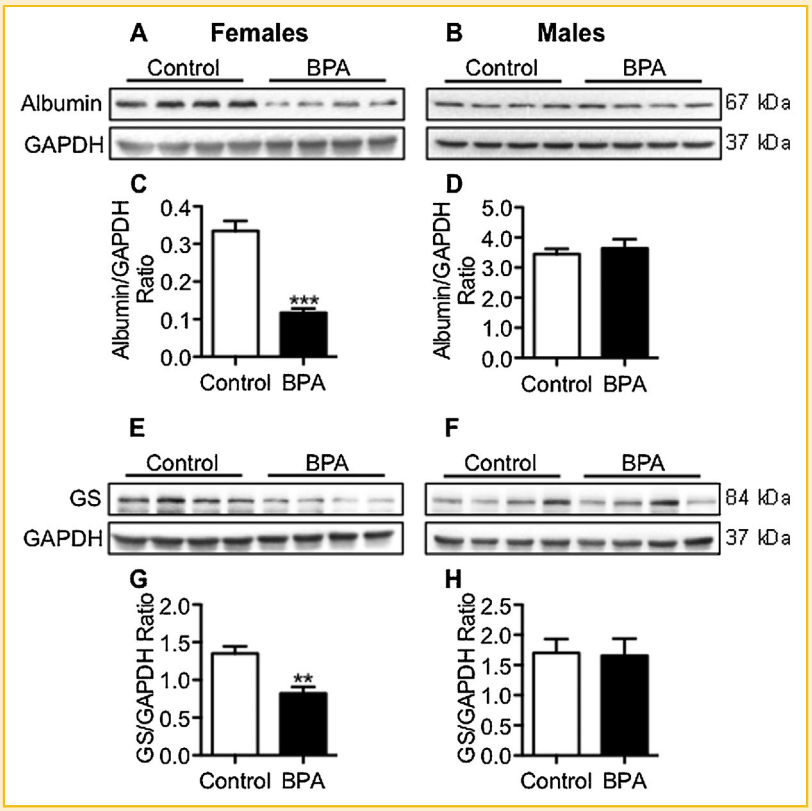

Fig. 1. Effects of BPA on albumin and GS protein expression. Levels of albumin protein (A–D) and GS protein (E–H) in female and male fetal livers were determined by Western blot analysis. Data are presented as means ± SEM (** $P < 0.01$, *** $P < 0.001$; n = 4 litters, livers from three pups were pooled per litter).

EFFECTS OF BPA ON AFP PROTEIN EXPRESSION

To gain further insight into the effects of BPA on fetal liver maturation, we examined changes in the expression of α-fetoprotein (AFP), a well-known marker of immature fetal hepatocytes [Hyatt et al., 2008]. We found that levels of AFP protein were significantly increased in BPA-exposed female fetal livers when compared to controls (143% of control; Fig. 2A and C). In contrast, AFP protein abundance was not changed in BPA-exposed male fetal livers (Fig. 2B and D).

EFFECTS OF BPA ON C/EBP-α PROTEIN EXPRESSION

Given that C/EBP-α is a master transcription factor essential for hepatocyte differentiation [Jochheim et al., 2004], we sought changes in the expression of this transcription factor following exposure to

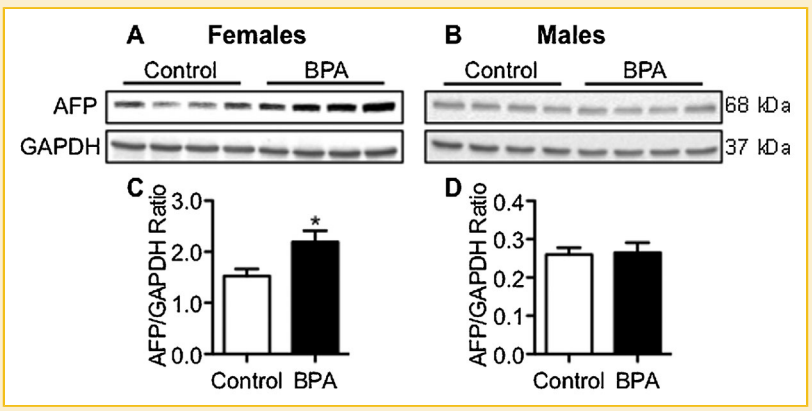

Fig. 2. Effects of BPA on AFP protein expression. Levels of AFP protein in female (A and C) and male (B and D) fetal livers were determined by Western blot analysis. Data are presented as means ± SEM (* $P < 0.05$; n = 4 litters, livers from three pups were pooled per litter).

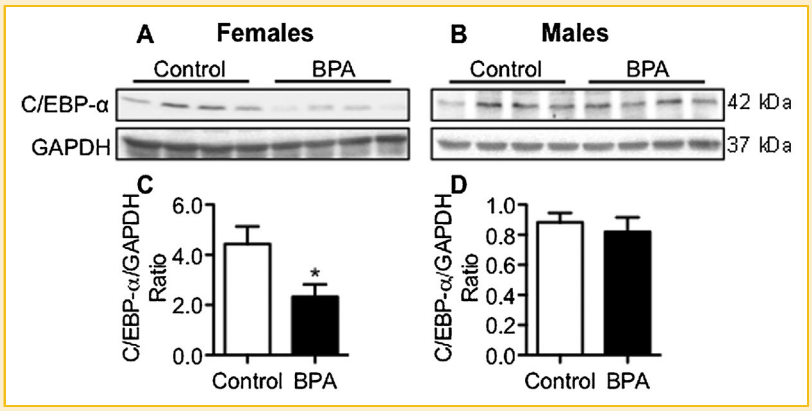

Fig. 3. Effects of BPA on C/EBP-α protein expression. Levels of C/EBP-α protein in female (A and C) and male (B and D) fetal livers were determined by Western blot analysis. Data are presented as means ± SEM (*$P < 0.05$; n = 4 litters, livers from three pups were pooled per litter).

BPA. We showed that levels of C/EBP-α protein were decreased by 50% in BPA-exposed female fetal livers when compared to controls (Fig. 3A and C). By contrast, the level of C/EBP-α protein in BPA-exposed male fetal livers was not changed (Fig. 3B and D).

EFFECTS OF BPA ON PCNA AND CASPASE-3 PROTEIN EXPRESSION

Normal organ growth and maturation depends critically on the right balance between cell proliferation and apoptosis [Her et al., 2006]. Thus, we sought to determine if this balance might be perturbed in BPA-exposed fetal livers. To do so, we first examined the proliferative status of the BPA-exposed fetal liver by analyzing the expression of PCNA, a universal marker of cell proliferation. We showed that levels of PCNA protein were up-regulated significantly in BPA-exposed female fetal livers when compared to controls (160% of control; Fig. 4A and C). In contrast, the level of PCNA protein was unchanged in BPA-exposed male fetal livers (Fig. 4B and D). We then examined the effect of BPA on the expression of caspase-3, a universal marker of apoptosis. We found that protein levels of both procaspase-3 (60% of control; Fig. 4E and G) and cleaved caspase-3 (45% of control; Fig. 4E and I) were significantly down-regulated in BPA-exposed female fetal livers when compared to controls. By contrast, no changes in the protein level of either procaspase-3 (Fig. 4F and H) or cleaved caspase-3 (Fig. 4F and J) were observed in livers of BPA-exposed male fetuses.

DISCUSSION

There is robust evidence that adverse events in early life can permanently alter organ growth and function, leading to a wide range of diseases later in life, including cardiovascular, metabolic, neurological, reproductive, and behavioral disorders as well as cancers [Heindel and Vandenberg, 2015]. Although developmental exposure to BPA has been shown to cause liver dysfunction and diseases, and elevated BPA concentrations have been associated with altered human fetal liver metabolism and function [Nahar et al., 2014,2015], the effects of BPA on rodent liver development had never been explored. In the present study, we addressed this important question, and demonstrate that in utero exposure to an environmentally relevant dosage of BPA via maternal diet disrupts female, but not male, fetal liver biochemical maturation in the mouse. Thus, our findings suggest a fetal origin for BPA-induced liver dysfunction and diseases. However, one caveat is that only one dose of BPA was used in this study. Furthermore, given that BPA is a well-known endocrine disruptor, it is important to note that the focus of the present study was on the adverse effects of BPA on fetal liver development and the underlying endocrine mechanisms were not addressed.

The dosage of BPA used (25 mg BPA/kg diet; equivalent to 5 mg/kg/day) in the present study was chosen based on our previous dose-dependent studies in which this dose was found to be effective in disrupting fetal lung maturation without altering fetal body weight, litter size, sex ratio, or resorption site number when examined at E18.5 [Hijazi et al., 2015]. This dosage is one tenth of the no observed adverse effects level (NOAEL) of 50 mg/kg/day for rodents, as defined by the US Environmental Protection Agency (EPA) [IRIS, 2012]. Importantly, maternal plasma levels of BPA have been measured to be 1.7 ng/ml [Hijazi et al., 2015], which is at the lower end range of 0.5–22.3 ng/ml reported previously in pregnant women of the US [Padmanabhan et al., 2008].

In the mouse, liver development begins at E9. At around E14, bipotential hepatoblasts begin to differentiate into either hepatocytes or bile duct epithelial cells [Crawford et al., 2010]. By E16, these two distinct cell types become irreversibly differentiated but continue to undergo maturation for several weeks after birth, at which time they are considered mature hepatocytes and cholangiocytes, respectively. In adults, hepatocytes are the main functional units of the liver accounting for nearly 80% of the total liver volume. The adult liver in mammals produces a myriad of proteins and enzymes that are crucial for maintaining homeostasis, the most abundant of which is albumin, constituting more than half of total plasma proteins. Albumin is first expressed in fetal hepatocytes at E12, and its expression increases progressively thereafter until it reaches maximal levels in the adult [Crawford et al., 2010]. Consequently, albumin is considered a hallmark of hepatocyte maturation [Cai et al., 2007]. As a first step in examining the effects of BPA on fetal liver maturation, we analyzed albumin protein expression. We found that levels of albumin protein were significantly reduced in BPA-exposed female fetal livers when

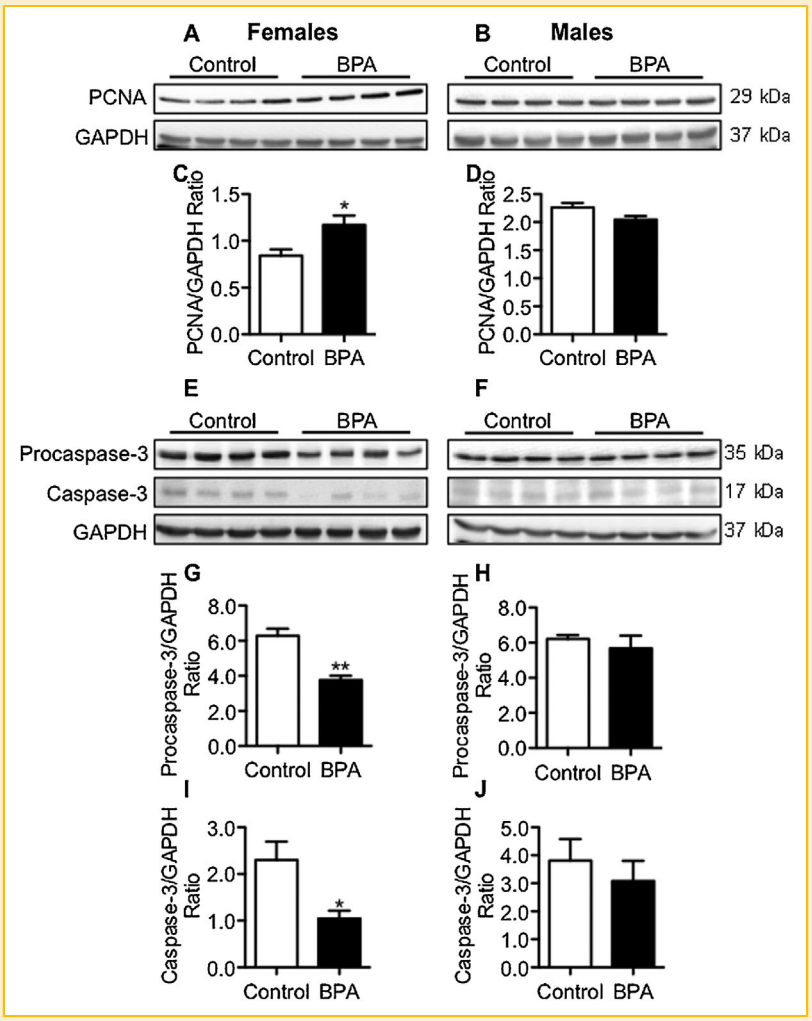

Fig. 4. Effects of BPA on PCNA and caspase-3 protein expression. Levels of PCNA protein (A–D) as well as procaspase-3 protein and cleaved caspase-3 protein (E–J) in female and male fetal livers were determined by Western blot analysis. Data are presented as means ± SEM ($^{*}P < 0.05$, $^{**}P < 0.01$; n = 4 litters, livers from three pups were pooled per litter).

compared to controls. In marked contrast, BPA had no effect on albumin protein expression in male fetal livers, demonstrating that BPA disrupts fetal liver maturation in a sex-specific manner.

Glycogen accumulation is another key feature of mature hepatocytes, and an important marker of hepatic maturation [Cai et al., 2007]. During most of gestation, hepatic glycogen store remains low as the fetus obtains an adequate supply of glucose from the mother via the placenta [Hay, 2006]. During late gestation, hepatocytes start to accumulate significant amounts of glycogen in order to prepare for the extrauterine survival at birth [Ward and Deshpande, 2005]. This surge in glycogen accumulation is accompanied by a corresponding increase in GS [Hsu et al., 1993], the rate-limiting enzyme of glycogen synthesis. In the present study, therefore, we examined GS protein expression and used it as a surrogate of glycogen accumulation. We showed that similar to its effects on albumin, prenatal BPA exposure significantly down-regulated fetal liver GS expression in females but not males. This finding further supports our conclusion that BPA disrupts fetal liver maturation in a sex-specific manner. Although glycogen content was not measured in the present study, previous studies have found that changes in fetal glycogen accumulation are directly correlated with alterations in GS expression [Tan et al., 2005; Sen et al., 2013].

To provide further insight into the effects of BPA on fetal liver maturation, we then examined the expression of α-fetoprotein (AFP), a marker of immature fetal hepatocytes [Hyatt et al., 2008]. AFP is the predominant fetal plasma protein produced by the fetal liver, and is considered the fetal equivalent of the adult serum albumin. During fetal development, the liver produces increasing amounts of AFP from E9 up to E15.5, at which time, AFP production begins to decline as the liver matures, ultimately becoming undetectable in the adult [Jochheim et al., 2004]. Here, we showed that in female livers, prenatal BPA exposure resulted in a significant increase in AFP protein expression at E18.5. In contrast, AFP protein levels in BPA-exposed male fetal livers were comparable to those of non-exposed controls. Taken together, the distinct changes in the expression of all three markers corroborate each other, and provide powerful evidence that prenatal exposure to BPA severely impairs fetal hepatic maturation only in females but not males.

C/EBP-α is a master transcription factor essential for hepatocyte maturation [Jochheim et al., 2004]. As liver undergoes maturation, C/EBP-α expression increases progressively until reaching a maximum in the adult [Jochheim et al., 2004]. Because of its temporal expression profile during liver development, C/EBP-α is also considered a differentiation marker of hepatocytes. Consequently, we determined whether prenatal BPA exposure affected the expression of this key transcription factor in fetal livers. We found that C/EBP-α protein levels were significantly decreased in BPA-exposed female fetal livers. Similar to the other markers of hepatic maturation, the level of C/EBP-α protein remained unaltered in BPA-exposed male fetal livers. The reduced hepatic C/EBP-α expression in BPA-exposed female fetal livers not only supports our conclusion that BPA disrupts fetal hepatic maturation in a sex-specific manner but also suggests that the altered expression of albumin, GS, and AFP is likely a result of the decreased C/EBP-α expression. Indeed, neonatal livers of C/EBP-α knockout mice exhibited properties characteristic of a dedifferentiated state, including decreased albumin expression, diminished glycogen stores and GS expression, as well as significantly elevated AFP levels [Wang et al., 1995]. Conversely, C/EBP-α knock-in mice deposited fetal hepatic glycogen earlier than their wild type littermates, and displayed a corresponding increase in GS expression [Tan et al., 2005]. In addition, Tan et al. [2007] found that albumin mRNA and protein levels were increased in an in vitro model of C/EBP-α knock-in hepatocytes.

A proper balance between cell proliferation and apoptosis is essential for organ development, including the liver [Her et al., 2006]. Thus, we also determined whether prenatal BPA exposure disrupted this balance in the fetal liver. First, we examined the proliferative status of the fetal liver by analyzing the expression of PCNA, a universal marker of cell proliferation. We found that levels of PCNA protein were significantly elevated in BPA-exposed female but not male fetal livers. Given that we revealed a significant decrease in C/EBP-α expression in female fetal livers, it is noteworthy that this transcription factor is known to exert powerful inhibitory effects on cell proliferation. For instance, hepatocyte proliferation is induced in C/EBP-α knockout mice [Timchenko et al., 1997] whereas hepatocyte C/EBP-α overexpression results in reduced proliferative activity [Johns, 1996]. Thus, the increased PCNA expression, and by inference hepatic proliferation, as observed in the present study may be a consequence of the decreased C/EBP-α.

In the present study, we determined whether prenatal BPA exposure also affected apoptosis in fetal livers by analyzing the expression of caspase-3, a universal apoptotic marker. Our results showed a significant decrease in levels of both pro- and cleaved (active) caspase-3 in BPA-exposed female fetal livers, suggesting that BPA reduced both the expression and activity of caspase-3. By contrast, neither pro- nor cleaved caspase-3 protein levels were altered in BPA-exposed male fetal livers.

Although the precise molecular mechanisms underlying the sex-specific effects in the present study remain largely unknown, it is possible that fetal sex steroid hormones may play a role. A more likely contributing factor may be sex chromosome complement, because chromosomal differences have been shown to dictate the responses of male and female cells to environmental stressors even before the production of fetal sex hormones [He et al., 2012]. It is also possible that these sex-specific effects could be mediated indirectly via differential effects of BPA on male and female placentas [Gabory et al., 2013], or via the sexually dimorphic regions of the brain that may be affected by BPA during fetal development [He et al., 2012]. Obviously, future studies will be required to determine the precise mechanisms underlying this phenomenon.

In conclusion, the present study demonstrates for the first time that prenatal exposure to an environmentally relevant dosage of BPA via maternal diet impairs female, but not male, fetal liver biochemical maturation in the mouse. Although the long-term consequences of the present findings remain to be determined, it is tempting to speculate that the disrupted biochemical hepatic maturation observed in the present study may result in permanent alterations in hepatic function, ultimately leading to hepatic dysfunction and diseases later in life. Obviously, this important question as well as the effects of BPA on fetal liver structural maturation and function warrant future investigation.

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