Sex-Specific Modulation of Fetal Adipogenesis by Gestational Bisphenol A and Bisphenol S Exposure

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The endocrine-disrupting chemical bisphenol A (BPA) increases adipose tissue mass *in vivo* and promotes adipogenesis *in vitro*; however, mechanisms explaining BPA's obesogenic effect remain unknown. We investigated the effects of gestational BPA and its analog, bisphenol S (BPS), exposure on the adipogenic differentiation ability of fetal preadipocytes and the role of endoplasmic reticulum stress in regulating this process. Pregnant sheep (n = 7 to 8 per group) mated to the same male were exposed to BPA or BPS from days 30 to 100 of gestation; pregnancies were terminated 20 days later. Adipose tissue was harvested and fetal preadipocytes isolated. Adipose tissue gene expression, adipocyte size, preadipocyte gene expression, adipogenic differentiation, and dynamic expression of genes involved in adipogenesis and endoplasmic reticulum stress were assessed. Gestational BPA enhanced adipogenic differentiation in female, but not male, preadipocytes. The unfolded protein response (UPR) pathway was upregulated in BPA-exposed female preadipocytes supportive of a higher endoplasmic reticulum stress. Increased expression of estradiol receptor 1 and glucocorticoid receptor in female preadipocytes suggests that this may be a potential cause behind the sex-specific effects observed upon BPA exposure. Gestational BPS affected adipogenic terminal differentiation gene expression in male preadipocytes, but not adipogenic differentiation potential. We demonstrate that gestational BPA exposure can modulate the differentiation ability of fetal preadipocytes. UPR upregulation in gestationally BPA-exposed female preadipocytes may contribute to the increased preadipocyte's adipogenic ability. The marked sex-specific effect of BPA highlights higher susceptibility of females to bisphenol A and potentially, a higher risk to develop obesity in adulthood. (***Endocrinology*** **158: 3844–3858, 2017)**

With obesity prevalence on the rise since the 1960s (1), more than one-third of the US population is currently overweight or obese (2). Importantly, obesity has been linked to a reduced life expectancy and comorbidities such as heart disease, type 2 diabetes, infertility, and cancer (3). Although genetic factors and a lack of energy balance are well-known factors contributing to the obesity epidemic (4), the upward trend suggests a multifactorial etiology, with social and environmental factors playing a role in this increased prevalence (4).

Endocrine-disrupting chemicals (EDCs) are compounds that can interfere with endocrine signaling pathways via receptor binding and/or epigenetic mechanisms, thus interfering with adipose tissue metabolism and contributing to the obesity epidemic (5). Obesogens are a class of EDC known to disrupt lipid metabolism, metabolic sensors, sex-steroid synthesis, and energy balance (6, 7). Bisphenol A (BPA), an EDC used in the manufacturing of plastics and epoxy resins, is considered an obesogen (6, 7). Epidemiological studies reveal a

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in USA

Received 6 July 2017. Accepted 11 September 2017.
First Published Online 14 September 2017

Abbreviations: *ADIPOQ*, adiponectin; ATF6, activating transcription factor 6; BPA, bisphenol A; BPS, bisphenol S; *CASP8*, caspase-8; cDNA, complementary DNA; *C/EBP*, CCAAT/enhancer-binding protein α; *CHOP10*, C/EBP homologous protein 10; *DLK1*, delta-like noncanonical Notch ligand 1; EDC, endocrine-disrupting chemical; ER, endoplasmic reticulum; *ERRα*, estrogen-related receptor α; *ESR1*, estrogen receptor 1; *ESR2*, estrogen receptor 2; *FABP4*, fatty acid–binding protein 4; *GLUT4*, glucose transporter type 4; *GR*, glucocorticoid receptor; *HSPA5*, heat shock protein family A member 5; *IRE1*, inositol-requiring protein 1; *LPL*, lipoprotein lipase; *MAPK*8, mitogen-activated protein kinase 8; mRNA, messenger RNA; MSC, mesenchymal stem cell; OD, optical density; ORO, Oil Red O; PCR, polymerase chain reaction; PERK, protein kinase–like ER kinase; PPARγ, peroxisome proliferator–activated receptor gamma; SE, standard error; *SOX6*, sex determining region-box 6; UPR, unfolded protein response; *XBP1*-s, spliced X-box binding protein 1.

positive association between prenatal BPA levels and obesity in children (8, 9) and adults (10–12). Although conflicting evidence is available (13, 14), BPA's obesogenic nature is further supported by *in vitro* studies (15, 16) and animal studies investigating the effects of pre-, peri-, and postnatal exposure to BPA on adipose tissue mass in the offspring (17–22). Our previous work has demonstrated that female sheep gestationally exposed to BPA develop insulin resistance, a disrupted adipokine profile in adipose tissue, adipocyte hypertrophy, and an increase in visceral to subcutaneous adipose tissue ratio upon a postnatal high-calorie diet (22). Additionally, previous work has shown sex-specific effects of perinatal BPA on adipose tissue deposition in animal studies, with females being more susceptible to BPA's effect (18, 19, 21). However, the mechanisms by which BPA increases body weight and adipose mass and its sex-specific effect have not been elucidated yet.

Adipogenesis is the complex process by which preadipocytes transition into lipid-filled, insulin-responsive adipocytes, and it is tightly regulated by transcription factors (23). The endoplasmic reticulum (ER) is a critical site of protein synthesis and lipid metabolism. ER stress is a homeostatic response that results in unfolded and/or misfolded proteins accumulation. To restore ER homeostasis, unfolded protein response (UPR), a highly conserved defense mechanism, is activated (24). Double-stranded RNA-dependent protein kinase–like ER kinase (PERK), inositol-requiring protein 1 (IRE1), and activating transcription factor 6 (ATF6) are the three main UPR pathways. All three play a role in the regulation of lipid metabolism via lipogenesis transcription factors modulation (25) and adipogenesis (26–28) and have been gaining attention because of their involvement in the pathogenesis of obesity (29). Importantly, BPA has been shown to affect ER stress response regulation in various organs (30–32); however, it remains unknown whether BPA can affect the ER stress response to modulate adipogenesis.

Current knowledge on adipocyte biology heavily relies on the use of the preadipocyte cell line 3T3-L1 (murine origin) and *in vivo* mouse studies. Although rodent species offer clear advantages for the study of adipogenesis (lower cost or easy access to genetic modification) (33), other species, such as the sheep, are also considered excellent models to study the relationship of sex steroids with obesity (34, 35) and offer several advantages critical for research that focus on the effects of gestational exposures on the progeny (36, 37). First, sheep, similar to humans and unlike rodents, are precocial species and thus the majority of their organs, including the adipose tissue, mature before birth (38). Sheep, as with humans, are monovulatory, which reduces potential confounding factors seen in litter-bearing species, such as the intrauterine fetal position phenomenon (39, 40). This becomes especially important when studying EDC with steroidal activity (41), such as bisphenols. Importantly, the sheep model has also been used to predict human fetal exposure to BPA (42). All the these points highlight the advantages of using sheep as an animal model in fetal adipocyte biology research (34).

In recent years, public pressure has led to shifting away from BPA in various consumer products. Attention has begun to focus on other bisphenolic chemicals that are also used in the manufacture of plastics (43, 44). Among these bisphenols, bisphenol S (BPS), an organic compound with a similar biochemical structure to that of BPA, has been widely used in beverage and food cans and in thermal receipt papers (45, 46) and can now be detected in up to 70% of human urine samples (43, 47) and fetal cord blood (48). Recent evidence has demonstrated that BPS may also act as an obesogen because it can induce adipogenesis *in vitro* (49). However, whether these effects are reproducible *in vivo* remains unknown.

Although prenatal exposure to BPA is associated with enhanced adipogenesis and increased adipose tissue accumulation in adulthood (17–22), to date, the mechanisms by which they occur remain unclear. The objectives of this study were to assess (1) whether gestational exposure to bisphenolic compounds BPA and/or BPS will affect the adipogenic ability of fetal preadipocytes, (2) whether these effects are sex-specific, and (3) the role of ER stress in modulating the adipogenic potential of gestationally exposed preadipocytes to these bisphenolic compounds. To address these questions, we have used a monovulatory, precocial species that has enabled us to investigate the effects of these EDC during adipose tissue fetal development in midpregnancy.

Materials and Methods

Animal experimentation

All procedures used in this study were approved by the Michigan State University Institutional Animal Care and Use Committee and are consistent with the National Research Council's Guide for the Care and Use of Laboratory Animals and the current Animal Welfare Act. This work meets the Animals in Research: Reporting *In Vivo* Experiments guidelines for reporting animal research (50). All reagents were from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. The study was conducted at the Michigan State University Sheep Research Facility (East Lansing, MI) using an in-house sheep flock of multiparous Polypay Dorsett crossbred. Healthy and primiparous female sheep were bred using a time-mated pregnancy strategy. Three vasectomized rams were raddled on their chests, and estrus was detected by the presence of paint on the backs of the females as a result of mounting. In the morning, all mounted females were moved with a fertile ram (a

single fertile ram was used to reduce confounding factors of paternal origin). Ram fertility was tested by a breeding soundness examination including inspection of the genital organs and assessment of sperm production and quality. Once mated, the females were randomly assigned to the treatment groups, ensuring similar body score condition and body weight across groups. Animals were fed a standard diet meeting National Research Council requirements, as previously described (51), and all feeding regimens met the nutrient requirements for sheep. Potential phytoestrogen load present in feed occurred across all groups. Because animals were kept group-housed to reduce isolation stress, all animals were exposed to the same phytoestrogen load from their food source.

Gestational BPA and BPS treatment consisted of daily subcutaneous injections of BPA (0.5 mg/kg/d; purity ≥99%, catalog no. 239658, Sigma-Aldrich) or BPS (purity: 99.7%; catalog no. 146915000, Acros Organics, Geel, Belgium) in corn oil from days 30 through 100 of gestation (term: ~147 days; Fig. 1A). Control mothers received corn oil only (vehicle). The internal dose of BPA achieved in umbilical arterial samples using the 0.5 mg/kg/d dose has already been published (52) and is targeted to produce maternal blood levels of BPA (~2.6 ng/mL) approaching the median concentration of BPA measured in maternal circulation in the United States (53). The same dose of BPS was used to match the BPA exposure dose used in the study. The number of breeders used was eight, eight, and seven, in control, BPA, and BPS groups, respectively. Breeding resulted in six control, six BPA, and four BPS female fetuses and four control, five BPA, and four BPS male fetuses. All pregnancies (n = 23) but six were singletons. Twinning was included in the statistical analysis as a covariate. To understand persistent effects of gestational bisphenol exposure, treatment was discontinued at gestational day 100. After a washout of 20 days, perirenal fetal adipose tissue (largest adipose depot at this fetal age) was harvested at day 120 of gestation upon pregnancy termination with a barbiturate overdose [intravenous pentobarbital sodium (Fatal Plus; Henry Schein, Melville, NY)]. A midline incision was performed, the uterus exposed, and the fetus quickly removed. Fetal body weight and biparietal diameter were assessed with calipers by a single operator. The perirenal adipose tissue was immediately harvested and either fixed in a 10% neutral buffered formalin solution for histological processing, flash frozen for gene expression studies, or freshly collected for cellular studies.

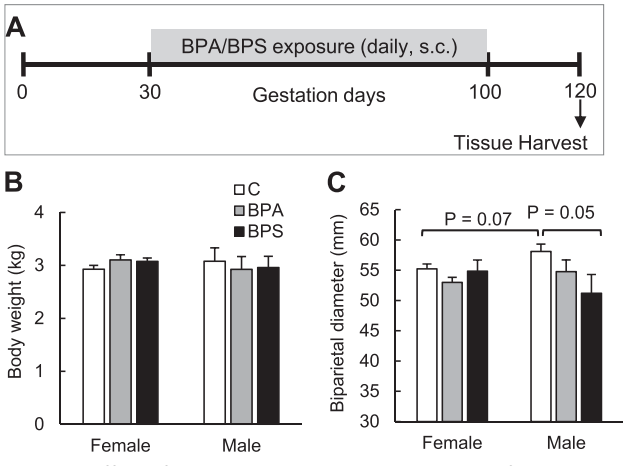

Figure 1. Effect of gestational BPA or BPS exposure on fetal body weight and biparietal diameter. (A) Experimental design. (B) Body weight and (C) biparietal diameter in females and males of control (open bars), BPA-exposed (gray bars), and BPS-exposed (closed bars) fetuses. n = 4 to 6 per group per sex. Different letters denote statistical differences among groups at $P < 0.05$. For biparietal diameter, a trend for treatment × sex effect (F, 2.874; $P = 0.078$) was observed. C, control; s.c., subcutaneous.

Tissue histology

Fixed fetal perirenal adipose tissue (n = 4 to 6 per group per sex) was embedded in paraffin. Sections (5 μm) were cut with a microtome, stained with hematoxylin and eosin, and mounted with acrytol mounting medium. To evaluate adipocyte size, three nonoverlapping images per section were taken using a bright-field microscope and the perimeter of 300 to 500 cells per animal measured.

Primary cultured cell isolation and proliferation

Fetal primary preadipocytes were isolated following our standardized and validated protocol (54), similar to that used in the isolation of human primary preadipocytes (55). In brief, freshly collected fetal perirenal adipose tissue (1 to 2 g) was placed in prewarmed Dulbecco's phosphate-buffered saline with antibiotic-antimycotic (Invitrogen, Carlsbad, CA). After removing any visible blood clots or connective tissue, the tissue was minced into small pieces. Collagenase-I (1 mg/mL) was used to digest the tissue for 40 minutes in a 37°C water bath, filtered through a mesh filter (250 μm), and centrifuged at 1200*g* for 5 minutes. The cell pellet was washed with fresh omental preadipocyte medium (Zen Bio, Research Triangle Park, Durham, NC) and seeded into six-well plates. After six days of culture, cells were frozen and stored in liquid nitrogen until further use. Primary cultured cells (n = 3 per group per sex) were harvested and trypsin-digested (0.05%) for subculture at 90% confluency. Proliferation ability for each cell lineage (passage 3) was assessed using a growth curve analysis. Cells were seeded at a density of 10,000 cells per well in 24-well plates and counted in hemocytometer chambers every 24 hours for eight days. Triplicate wells for each time point per primary cell culture were used.

Cell culture and adipocyte differentiation

Before differentiation induction, fetal preadipocytes (n = 3 primary cultured cells per group per sex; passage 3) were cultured in Dulbecco's modified Eagle medium/F12 (Invitrogen) supplemented with 1% penicillin-streptomycin (Invitrogen), 1% L-glutamine, 10 mM HEPES, and 10% fetal bovine serum (Fisher Scientific, Wilmington, NC) to confluency and allowed to grow for two additional days. Thereafter, adipocyte differentiation was induced as previously described (54). In brief, differentiation medium consisted of growth medium supplemented with 33 μM biotin, 17 mM pantothenate, 10 μg/mL insulin, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and the peroxisome proliferator–activated receptor gamma (PPARγ) agonist, rosiglitazone (20 μM). We have recently demonstrated that *in vitro* differentiation of sheep preadipocytes requires the supplementation of a PPARγ agonist (54), similar to human primary preadipocytes (56). Adipogenic differentiation was determined by Oil Red O (ORO) stain, as a marker of lipid accumulation as previously described (54). ORO absorbance at 500 nm was measured in a microplate reader (Gemini; Molecular

Devices, Sunnyvale, CA) and expressed as optical density (OD). ORO-positive area was also calculated using Fiji (imagej.nih.gov) (57). ORO-stained cells were further stained with 4′,6-diamidino-2-phenylindole to assess the number of cells. Nine fields were imaged and counted. No significant differences were observed in number of cells among treatment groups or sex (data not shown). Because phenol red may exert estrogenic action (58), a pilot study was conducted to assess if the presence of phenol red affected the adipogenic differentiation outcomes (Supplemental Fig. 1).

Quantitative real-time polymerase chain reaction

Total RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol from cell or adipose tissue. RNA quality and concentration were measured by Nanodrop (Thermo Fisher Scientific, Wilmington, NC). A total of 1 μg RNA (A260/A280: 2.0 ± 0.05, RNA concentration: 300 ± 50 ng/μL) was reverse transcribed into complementary DNA (cDNA) using a High Capacity cDNA Reverse Transcription Kit (Promega, Madison, WI) in 10-μL reaction volumes. Quantitative real-time polymerase chain reaction (qRT-PCR; ABI-Quant Studio 7 Flex Real-Time PCR System, Thermo Fisher, Carlsbad, CA) was performed to examine the messenger RNA (mRNA) expression of the genes encoding sheep adiponectin (*ADIPOQ*), caspase-8 (*CASP8*), CCAAT/enhancer-binding protein α (*C/EBPα*), C/EBP homologous protein 10 (*CHOP10*), delta-like noncanonical Notch ligand 1 (*DLK1*), estrogen receptor 1/2 (*ESR1/ESR2*), estrogen-related receptor α (*ERRα*), fatty acid–binding protein 4 (*FABP4*), glucocorticoid receptor (*GR*), glucose transporter type 4 (*GLUT4*), heat shock protein family A member 5 (*HSPA5*), *IRE1α*, leptin, lipoprotein lipase (*LPL*), mitogen-activated protein kinase 8 (*MAPK8*), *PPARγ* that detects both *PPARγ1* and *PPARγ2* transcripts, *PERK*, sterol regulatory element binding transcription factor 1 (*SREBF1*), sex determining region-box 6 (*SOX6*), spliced X-box binding protein 1 (*XBP1-s*), and Wnt family member 10B (*WNT10B*). Primer sequences are provided in Supplemental Table 1. mRNA levels encoding the indicated genes were normalized against *GAPDH* and presented as relative fold change to that of the control and calculated using the ΔΔCT method (59). Two additional housekeeping genes (*RPL27* and *β-ACTIN*) were used to validate this work (not shown). All experiments and quantitative real-time PCR was run in triplicate. cDNA amplification reaction (50 ng) consisted of template denaturation and polymerase activation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. Melt curve analyses were performed for all genes, and the specificity as well as integrity of the PCR products was confirmed by the presence of a single peak of melt curve and PCR products by agarose gel electrophoresis.

Statistical analysis

All data are presented as mean ± standard error (SE). Appropriate transformations were applied, as needed, to account for normality of data. Comparisons among the three treatment groups and two sexes were analyzed by two-way analysis of variance with Tukey *post hoc* tests with twinning as a covariate. Comparisons among groups without sex interaction were tested using a one-way analysis of variance with Tukey *post hoc* tests with twinning as a covariate or a *t* test between groups and within time points. All previously mentioned analyses were run using PASW Statistics for Windows, release 18.0.1. To assess the impact of gestational bisphenol exposure on adipocyte size, an empirical cumulative distribution function was also calculated for each measurement, and the difference among groups tested using a Kolmogorov-Smirnov test. The significance of the difference between the distributions of cell perimeter was tested using a permutation test with 10,000 iterations using R Statistical Computing, release 3.3.0. Differences were considered significant at $P < 0.05$.

Results

Gestational BPA and BPS effects on adipose tissue

Prenatal exposure to BPA or BPS did not affect fetal body weight in female or male (Fig. 1B) fetuses at gestational day 120 (before fast growth occurs in late gestation), but tended to lower biparietal diameter in male BPS-exposed fetuses ($P = 0.05$, Fig. 1C). Perirenal adipose tissue was not weighed to enable quick collection for cell culture work, but no gross differences were noticed among groups. Histological examination of the perirenal adipose pad revealed that gestational BPA or BPS did not significantly affect adipocyte size of unilocular adipocytes (white adipocytes), but cell size plot evidenced a left shift (smaller size) in BPA females (Fig. 2A) and both BPA and BPS males compared with their respective controls (Fig. 2B). No sex-specific differences were observed in adipocyte size. Gene expression in fetal adipose tissue revealed that no substantial differences were observed in BPA- or BPS-treated groups in adipogenesis initial (*DLK1*), early (*C/EBPα)*, and late (*LPL*, *FABP4*, *GLUT4)* stage marker genes expression in females or males compared with their respective control groups (Fig. 2C; Supplemental Fig. 2). *PPARγ* mRNA expression was significantly higher in fetal adipose tissue in BPA-exposed compared with BPS-exposed female fetuses (Fig. 2C). *ADIPOQ* expression was significantly lower in BPA-exposed females and BPS-exposed males compared with their respective controls (Fig. 2C). mRNA expression was also similar among groups in the cholesterol regulators *SREBF1*, leptin, (Supplemental Fig. 2) and steroid receptors (*ERRα*, *ESR1*, *ESR2*, and *GR*) (Fig. 2D).

Gestational BPA and BPS on fetal preadipocyte adipogenic differentiation and proliferation

We investigated the effects of prenatal BPA and BPS exposure on adipogenic differentiation by evaluating the differentiation ability of primary cultured fetal preadipocytes. After 8 days of adipogenic differentiation, regardless of treatment group, all female primary cells had higher differentiation ability compared with their male counterparts (Fig. 3C). Gestational BPA-exposed female preadipocytes had increased adipogenic differentiation compared with both control and BPS groups (Fig. 3A and 3B). This finding was supported by an

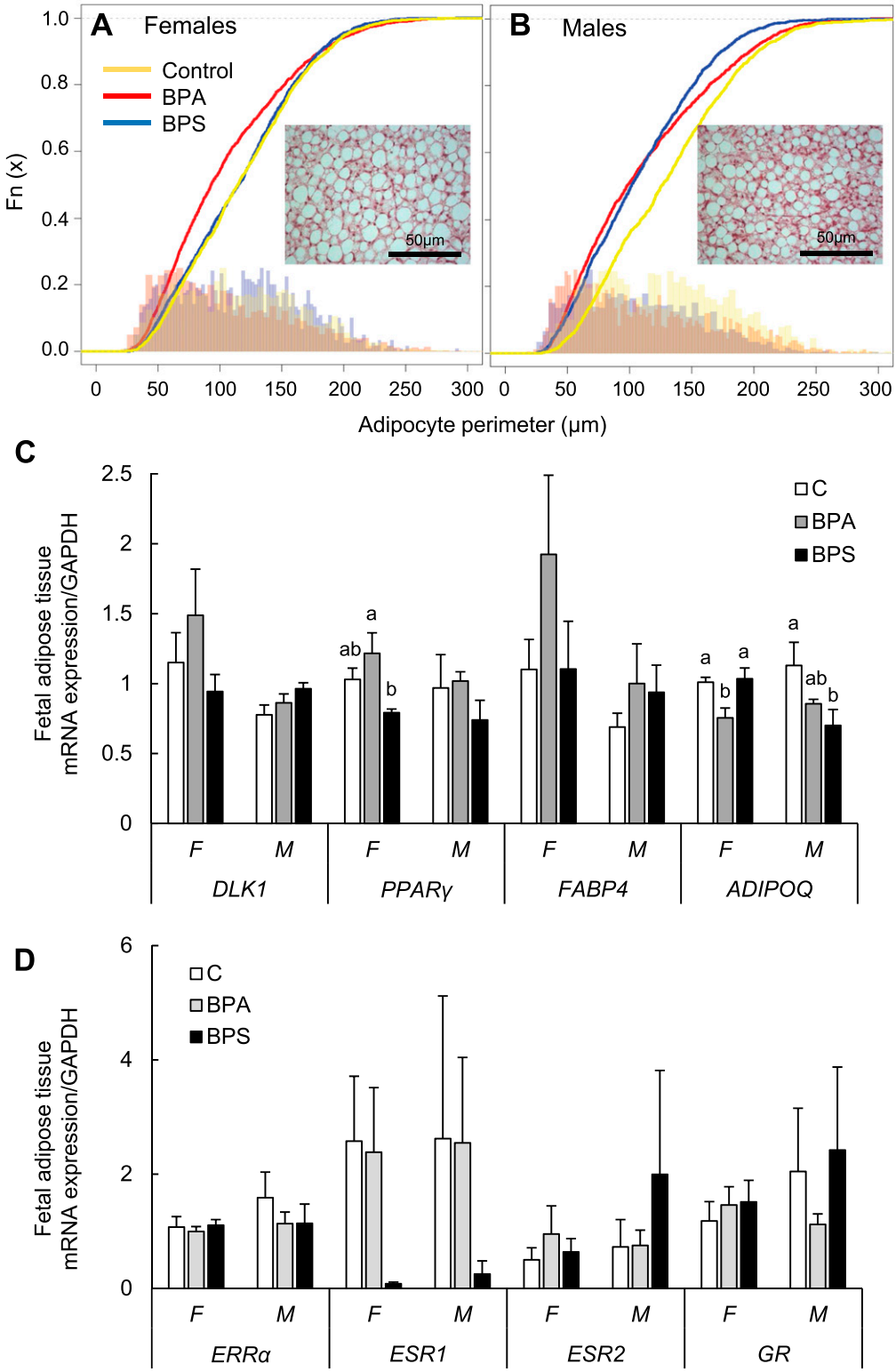

Figure 2. Effect of gestational BPA and BPS exposure on adipocyte size and mRNA expression (mean ± SE) in fetal perirenal adipose tissue. Adipocyte perimeter (μm) plots in (A) females and (B) males of control (yellow), BPA-exposed (red), and BPS-exposed (blue) fetuses. Representative fetal adipose tissue section (hematoxylin and eosin) from control female and male is shown within cell perimeter plots. (C, D) mRNA expression (mean ± SE) in fetal perirenal adipose tissue of preadipocyte (*DLK1*), early (*PPARγ*), and late (*FABP4*, *ADIPOQ*) adipocyte markers, and steroid receptors (*ERRα*, *ESR1*, *ESR2*, and *GR*) in females (F) and males (M) of control (open bars), BPA-exposed (gray bars), and BPS-exposed (closed bars) fetuses. Different letters denote differences among treatments within sex: a ≠ b, $P < 0.05$; a′ ≠ b′, $0.06 < P < 0.08$. n = 4 to 6 per group per sex. Horizontal bars denote differences between sexes within gene. For *PPARγ*, a treatment effect (F, 3.351; $P < 0.048$) was observed. For *ADIPOQ*, a treatment × sex effect (F, 4.107; $P < 0.033$) and a treatment effect (F, 5.262; $P < 0.015$) were observed. C, control; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

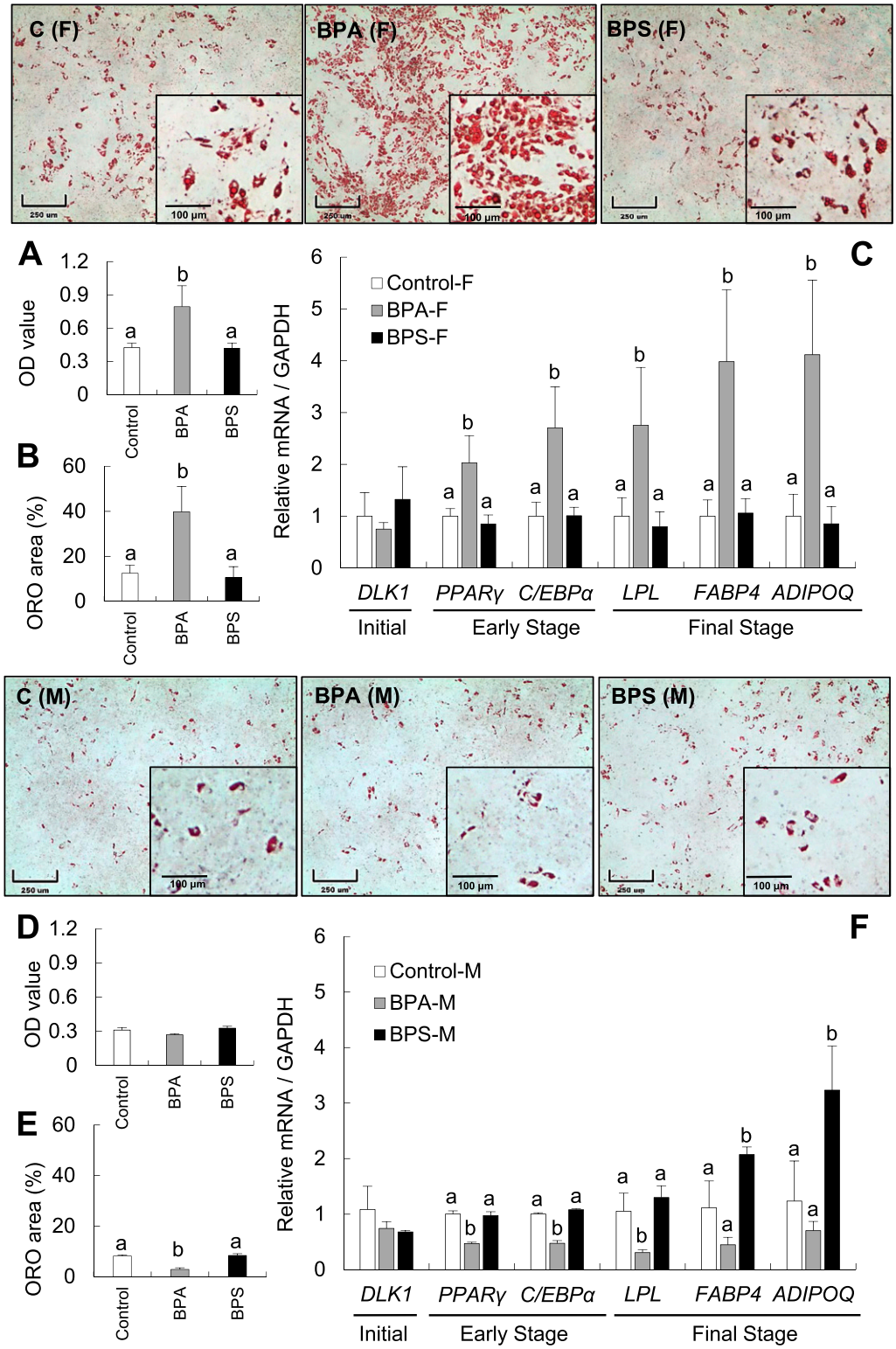

Figure 3. Effect of gestational BPA and BPS exposure on fetal adipogenic differentiation. Representative images of ORO-stained differentiated adipocytes (day 8) on females (top) and males (bottom) of control (left; C), BPA (middle), and BPS (right) gestationally exposed fetuses. Adipocyte differentiation quantification by OD value and ORO stain–positive area in control and gestationally BPA- and BPS-exposed (A, B) female and (D, E) male primary preadipocytes (n = 3 per group), respectively. (C, F) mRNA expression (mean ± SE) of preadipocyte (*DLK1*), early- (*PPARγ*, *C/EBPα*) and late- (*LPL*, *FABP4*, *ADIPOQ*) stage adipocyte markers at terminal differentiation in control (open), BPA (gray), and BPS (closed) (C) females and (F) males. Scale bar: 250 μm. Different letters denote statistical differences among treatment groups at $P < 0.05$. Given the large difference in adipogenic differentiation between sexes, gene expression normalization was performed relative to the control group of each sex.

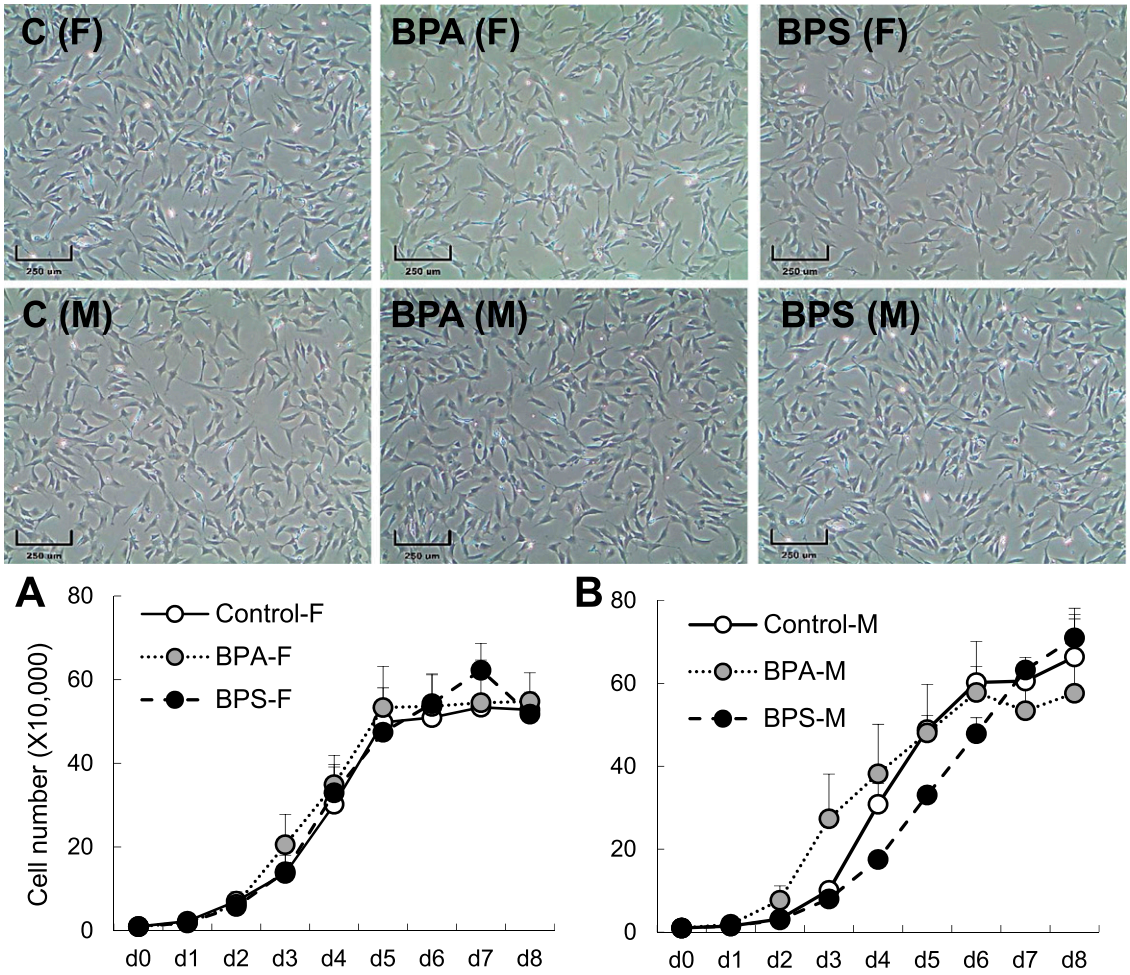

Figure 4. Effect of gestational BPA and BPS exposure on fetal preadipocyte proliferation. Representative images of preadipocytes at day (d) 0 of culture in control, BPA, and BPS gestationally exposed female (F; top) and male (M; bottom) cultured primary cells (n = 3 per group). Growth curves (mean ± SE) of (A) female and (B) male primary preadipocytes. Scale bar: 250 μm. C, control.

upregulation in gene expression of early (*PPARγ*, *C/EBPα*) and late (*LPL*, *FABP4*, *ADIPOQ*) adipogenic markers at terminal differentiation stage (day 8 of differentiation; Fig. 3C). Gestational BPS exposure had no effect on adipogenic ability or gene expression in female primary preadipocytes (Fig. 3C). Quantification of the adipogenic differentiation in gestationally BPA-exposed male preadipocytes revealed a substantial reduction when quantified using the ORO area method (Fig. 3E), but not when quantified using the OD value (Fig. 3D). This discrepancy is likely due to the lower sensitivity of the OD measurement in wells with very low differentiation ability. Gestational BPA reduced gene expression of early (*PPARγ*, *C/EBPα*) and late (*LPL*, *FABP4*, and *ADIPOQ*) adipogenic markers in male primary cells at differentiation day 8 (Fig. 3F). Gestational BPS did not affect adipogenic differentiation in male primary cells (Fig. 3D), but increased late (*FABP4* and *ADIPOQ*) adipogenic markers (Fig. 3F). To assess if differences observed in differentiation potential were driven by an increased preadipocyte proliferation ability, a cell proliferation assay was conducted. Preadipocyte proliferation rate was not different by sex or treatment after 8 days of culture (Fig. 4).

Gestational BPA and BPS on preadipocyte gene expression

To understand if the sex-specific difference in the adipogenic differentiation was partially due to differential expression in sex-steroid receptors, we evaluated gene expression on *GR*, *ESR1*, *ESR2*, and *ERRα*. To avoid the confounder of other cells present in the adipose tissue (endothelial, differentiated adipocytes, macrophages, or stromal cells), we investigated the steroid receptor expression in isolated preadipocytes. In fetal preadipocytes (Fig. 5A), *ESR1* and *GR* expression was higher in females than control ($P < 0.05$) and BPA ($P < 0.05$ and $P = 0.05$, respectively) groups compared with their male counterparts. *ESR2* expression was also higher in BPA females compared with their male counterparts ($P < 0.05$). The opposite expression pattern was observed in BPS-exposed male preadipocytes with higher *ESR1* (P =

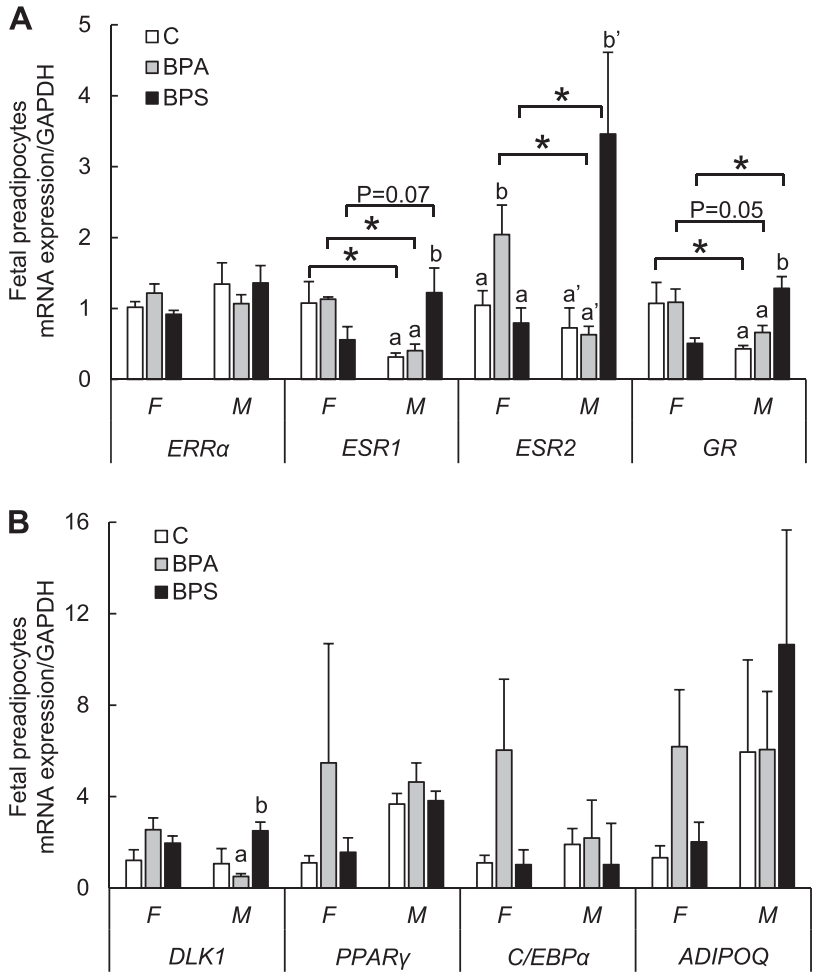

Figure 5. (A, B) Effect of gestational BPA exposure on fetal preadipocyte steroid receptor expression. mRNA expression (mean ± SE) of steroid receptor expression of *ESR1* and *ESR2*, *ERRα*, *GR*, preadipocyte *DLK1*, and early (*PPARγ*, *C/EBPα*) and late (*ADIPOQ*) adipocyte markers, in undifferentiated preadipocytes in females (F) and males (M) of control (open bars; C), BPA-exposed (gray bars), and BPS-exposed (closed bars) fetuses. n = 4 to 6 per group per sex. Treatment × sex effects were observed for *ESR1* (F, 6.686; $P < 0.05$), *ESR2* (F, 6.042; $P < 0.013$), *GR* (F, 12.092; $P < 0.001$), and *DLK1* (F, 4.827; $P < 0.03$). Treatment effect was observed for *DLK1* (F, 3.539; $P = 0.057$). *$P < 0.05$.

0.07), *ESR2* ($P < 0.05$), and *GR* ($P < 0.05$) expression compared with their female counterparts. In females, BPA-exposed preadipocytes had higher *ESR2* expression compared with control females ($P < 0.05$). In males, BPS-exposed preadipocytes had increased *ESR1*, *ESR2*, and *GR* ($P < 0.05$) expression compared with control males. BPA-exposed male preadipocytes had no differences in steroid receptor expression. Expression of *ERRα* was not significantly different among treatments or sex.

No important differences were observed in BPA- or BPS-treated groups in adipogenesis early (*C/EBPα* and *PPARγ)* and late (*ADIPOQ)* stage marker genes expression in female or male preadipocytes compared with their respective control groups (Fig. 5B). *DLK1* mRNA expression was higher in male preadipocytes in BPS- vs BPA-exposed fetuses (Fig. 5B).

Gestational BPA accelerates fetal adipogenic differentiation in females

To further explore the underlying mechanism of BPA on enhanced adipogenic differentiation, the remainder of the study focused on female preadipocytes. To test whether prenatal exposure to BPA affects the differentiation process, gene expression time course on differentiation days 0, 2, 4, 6, and 8 was evaluated in female preadipocytes (Fig. 6). *ZFP423*, a transcriptional regulator of PPARγ, and an indicator of the ability of adipogenic differentiation, was upregulated from day 0 to day 4 in prenatal BPA exposed female primary preadipocytes. Gene expression of *C/EBPα* and *PPARγ*, early adipogenic genes, were upregulated in gestationally BPA exposed preadipocytes after day 2 of differentiation. *FABP4*, *GLUT4*, and *SOX6* began to diverge later in the differentiation process (day 4) in gestationally BPA-exposed preadipocytes. Lower differentiation in control primary cells was not due to cellular apoptosis during differentiation, as shown by lack of change in apoptotic markers throughout differentiation (*CASP8*, *MAPK8*; data not shown).

Gestational BPA exposure modulates ER stress

Because ER stress can modulate adipogenesis, we tested if gestational BPA could trigger a cellular stress response leading to an increased UPR. *HSPA5* mRNA, induced upon an enhanced UPR, was upregulated in undifferentiated BPA-exposed female preadipocytes (Fig. 7). *IRE1α* and genes downstream of the IRE1α UPR pathway (*XBP1-s* and *WNT10B*) were also upregulated in undifferentiated BPA-exposed preadipocytes. Similarly, *PERK* and genes downstream of the PERK UPR pathway (*CHOP10*) were also upregulated in undifferentiated BPA exposed preadipocytes. Upon differentiation stimuli, and by day 2 and 4 of differentiation, *HSPA5* and all genes downstream of the UPR pathway, except for *CHOP10* were not different from control preadipocytes.

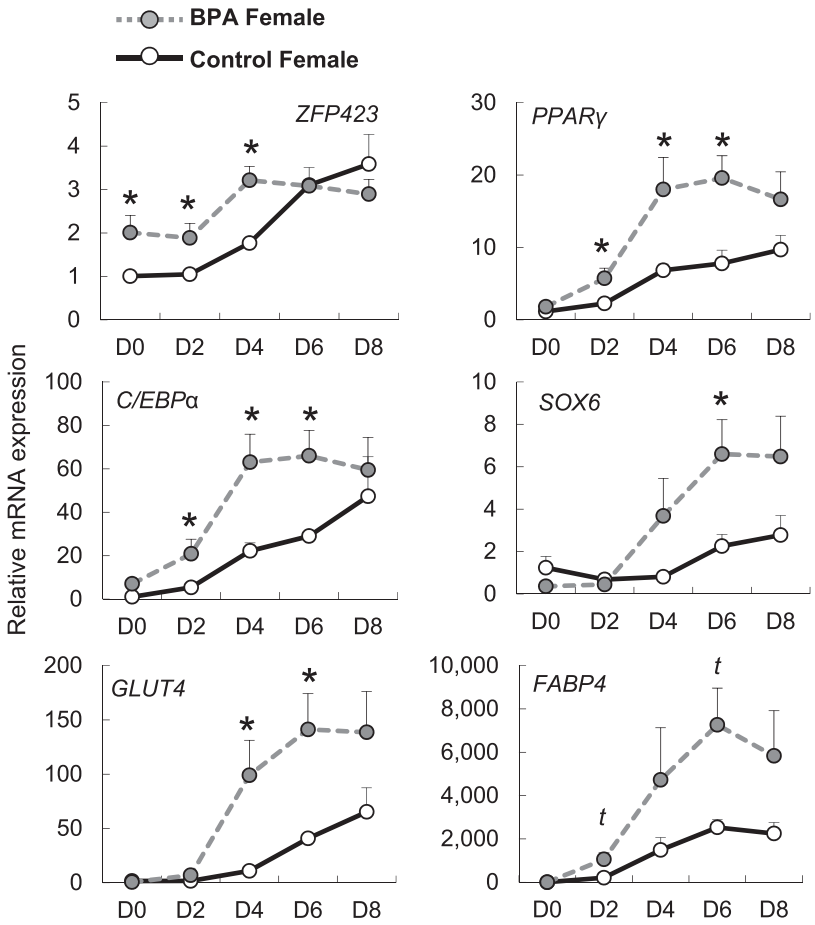

Figure 6. Effect of gestational BPA exposure on female adipogenic gene expression during adipogenic differentiation. mRNA (mean ± SE) expression of *ZFP423*, *C/EBPα*, *GLUT4*, *PPARγ*, *SOX6*, and *FABP4* at differentiation days (D) 0, 2, 4, 6, and 8 in control (solid black line) and BPA (dotted gray line) gestationally exposed female primary preadipocytes (n = 3 per group). *Significance at $P < 0.05$; *t*, statistical trend at $0.05 > P > 0.06$.

Discussion

Growing consensus supports the role of EDCs as a risk factor for metabolic diseases, including obesity (4). BPA is considered one of such EDCs with obesogenic ability (15, 16, 18, 19, 21) and can also result in programming of insulin resistance in rodents (20) and sheep (22). In this study, we have begun to dissect the mechanism by which BPA induces its obesogenic effects during fetal adipose tissue development. Our findings demonstrate that gestational exposure to BPA, at a relevant internal dose exposure [~2.5 ng/mL unconjugated BPA in fetal blood (52)] alters the differentiation ability of fetal preadipocytes. Increased adipogenesis was only observed in female derived BPA-exposed preadipocytes, supporting previously reported sex-specific effects of BPA in programming adipose tissue (18, 19, 21), and was associated with modulation of the ER stress response during adipogenic differentiation (Fig. 8). Importantly, given the growing evidence of paternal effects on metabolic programming (60, 61), in this work, we have limited the paternal contribution by using a single male parent for all pregnancies. We have also demonstrated that gestational BPS exposure affects steroid receptor expression in adipose tissue and preadipocytes and results in impaired terminal adipogenic differentiation in male fetuses. This finding highlights critical differences between these two bisphenolic compounds on fetal adipose tissue development.

Selection of sheep as the animal model was based on our prior work supporting a metabolic compromise upon gestational BPA exposures (22). Sheep are considered an excellent model to study obesity (34, 35) because of their shared similarities during fetal development with humans (eg, as most adipose tissue deposition is completed before birth) (35, 62, 63). Sheep also respond with increased adipose tissue deposition in sedentary conditions or high caloric diets (22, 64).

Effect of gestational BPA exposure on adipose tissue metabolism

Several animal studies have demonstrated how pre- or perinatal exposure to BPA can lead to increased adipose tissue deposition in adulthood, often accompanied by insulin resistance (19, 22, 65). In this study, we did not evaluate if gestational BPA or BPS exposure resulted in perirenal adipose tissue mass changes (only by gross observation). Although perigestational BPA exposure does not always result in increased adipose mass, in males in particular (19, 21, 66), reported increases in adipose tissue mass upon BPA exposure have only been reported during postnatal life (18–21). Particularly in sheep, prenatal BPA exposure increased adipocyte size during adulthood, but not increased visceral adipose tissue mass (22). Thus, increased adipose tissue mass observed in BPA-exposed animals (19) may occur later in life because of postnatal adipose tissue hypertrophy (67). In addition, lower *ADIPOQ* expression in fetal adipose tissue from BPA fetuses (this study) reflects a similar change to that observed in visceral adipose tissue from adult sheep gestationally exposed to BPA that were insulin resistance (22). *ADIPOQ* has been identified as a predictor of cardiovascular risk and type 2 diabetes in adults (68–70). But, whether differences in *ADIPOQ* during fetal life may be an early indicator of poor

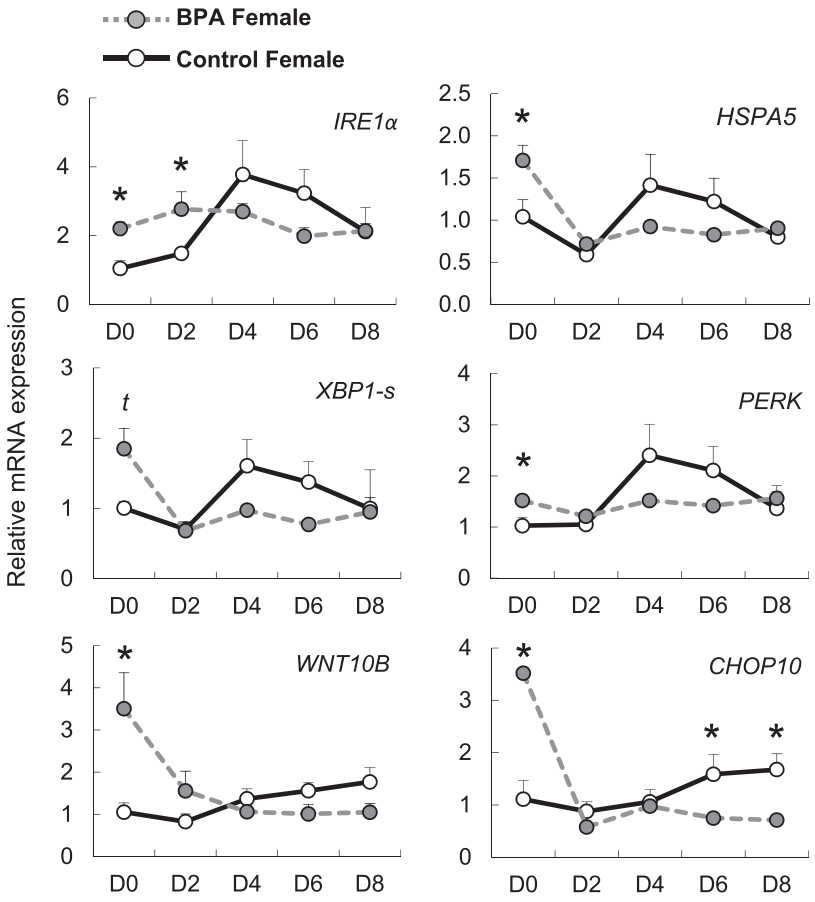

Figure 7. Effect of gestational BPA exposure on ER stress during adipogenic differentiation. mRNA (mean ± SE) expression of ER stress pathway genes *IRE1α*, *XBP1-s*, *WNT10B*, *HSPA5*, *PERK*, and *CHOP10* at differentiation days (D) 0, 2, 4, 6, and 8 in control (solid black line) and BPA (dotted gray line) gestationally exposed female primary preadipocytes (n = 3 per group). *Significance at $P < 0.05$; *t*, statistical trend at $0.05 < P < 0.06$.

cardiometabolic function later in life remains to be demonstrated.

Effect of sex on adipogenic differentiation ability

Our results demonstrate a marked sex-specific effect in adipogenic differentiation ability with fetal male preadipocytes exhibiting very poor adipogenic differentiation ability compared with females. This report is among the first to demonstrate sex-specific adipogenic ability between females and males during fetal life. Whether these sex-specific differences relate to inherent differences in adipose tissue development between female and male fetuses and/or sex-specific critical windows of susceptibility to EDCs in the adipose tissue remains to be investigated. Because the critical role of ESR1 (71) and GR in adipogenesis (72), we hypothesize that the increased expression of *ESR1* and *GR* in control female preadipocytes enables a greater response to adipogenic differentiation stimuli *in vitro* (adipogenic cocktail) than that of male preadipocytes. Although scarce information is available regarding sex-specific steroid receptor expression in preadipocytes and adipocytes, our results demonstrating a sex-specific effect on estrogen receptor expression (higher *ESR1*) in female preadipocytes are in line with human data reporting sex-specific differences in differentiated adipocytes (increased *ESR2*) expression in adult female adipocytes compared with that of adult males (73). To note is that our study did not evaluated protein expression and given the low abundance of *ESR2* (73), *ESR2* gene expression may not be a true reflection of transcript levels. It also remains unknown if a sex-specific pattern of steroid receptor expression also occurs in human preadipocytes. It remains to be tested if our sex-specific adipogenic differentiation results can be extrapolated to other species.

Gestational BPA enhances adipogenic differentiation

In vitro studies using 3T3-L1 cells, a preadipocyte cell line, have demonstrated that BPA interferes with adipogenic differentiation by increasing preadipocyte growth, altering master regulatory genes of adipogenesis, and accelerating terminal differentiation (15, 16, 74); however, the mechanism by which BPA induces increased adipose tissue mass *in vivo* continues to be elusive. We have demonstrated that *in vivo* exposure to BPA increases adipose tissue mass likely by reprogramming the ability of fetal preadipocytes to differentiate into mature adipocytes. The study was designed to discontinue BPA exposure 20 days before tissue harvest for cell isolation. This elicited a washout period to avoid confounding effects from continuous exposure to the EDC at the time of harvest and highlights the persistent nature of the observed effects. Our work is in line with evidence that *in vitro* BPA accelerates terminal differentiation in 3T3-L1 cells (16), and enhances human mesenchymal stem cells (MSCs) differentiation ability *in vitro* (74, 75). Reprogramming of MSC in favor of the adipogenic lineage occurs on exposures to other obesogenic EDC such as tributyltin (76). Although we did not investigate whether the observed effect was due to reprogramming of MSCs before adipogenic lineage commitment, it is possible that MSC were also a target in the current study. If so, it is interesting to speculate that if MSC were the direct target, other MSC-derived cell lineages (osteogenic and myogenic) may also be affected. This has not been investigated in the context of prenatal

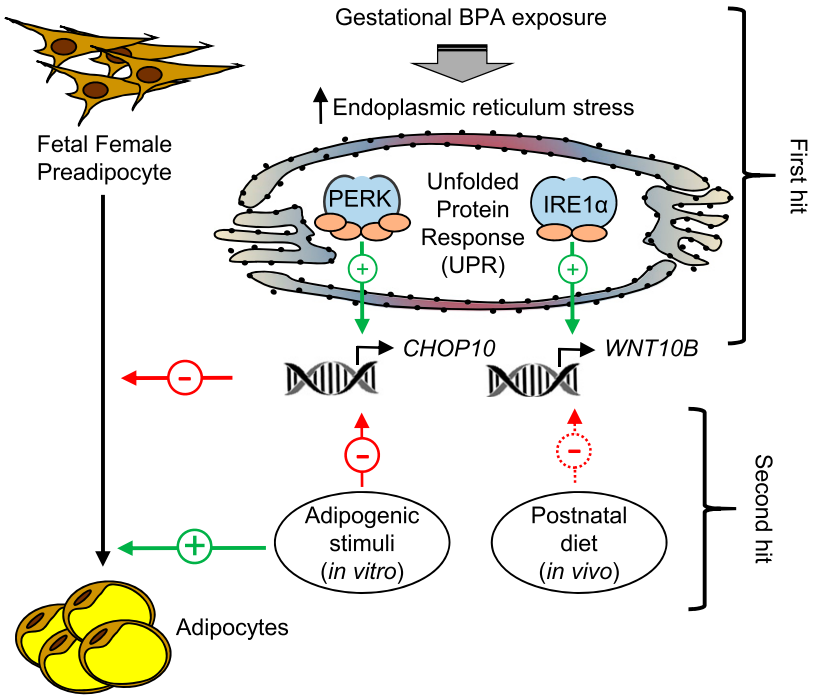

Figure 8. Working model of the effects of gestational BPA exposure on female fetal preadipocyte adipogenesis. We hypothesize that gestational BPA stimulates ER stress, triggering the UPR in female preadipocytes. Activated UPR pathways (PERK and IRE1α) stimulate *CHOP10* and *WNT10B* mRNA expression, in turn inhibiting adipogenic differentiation of fetal preadipocytes. The adipogenic cocktail (0.5 mM 3-isobutyl-1-methylxanthine, dexamethasone, insulin, and PPARγ agonist) counteracts BPA-induced upregulation of *CHOP10* and *WNT10B*. This results in an acceleration and enhancement of adipogenesis in gestationally BPA-exposed female preadipocytes. Further work is required to verify this hypothesis. This second hit (adipogenic cocktail) is necessary to overcome activation of the UPR in BPA-exposed preadipocytes. We hypothesize (dotted lines) that *in vivo*, a second hit, such as high-fat or saturated-fat diets, may exert similar effects to those induced by the adipogenic cocktail *in vitro*, leading to an obesogenic phenotype.

exposures to BPA, but is a much warranted field of study (77).

The need of a "second hit" upon environmental, nutritional, or stress exposures is a common finding in the developmental origins of health and disease field (78). The differentiation stimuli *in vitro* (adipogenic cocktail) could be viewed as a trigger (and second hit) of the pathological phenotype. Because some BPA studies report that a high-fat diet or overfeeding exacerbates BPA's metabolic disruptions (22, 79, 80), we speculate that responses during postnatal life upon prenatal BPA exposure may be enhanced if the individual is faced with a particular adipogenic stimuli (eg, high-fat diet, saturated fat diets, high caloric intake; Fig. 8). This hypothesis would support the discrepancy in obesogenic phenotypes reported after prenatal BPA exposures (13, 14, 19, 21) and other reports where a high-fat diet or overfeeding exacerbates BPA's metabolic disruptions (22, 79, 80). A caveat of this hypothesis is that some studies have reported that BPA, independent of a second hit, promote an adipogenic phenotype, which has contributed to the controversy about the obesogenic ability of BPA (13, 14, 17–22). It is possible that interactions with other factors, such as genetic background or species, may explain the discrepancies in the metabolic effects upon gestational BPA exposures.

Sex-specific effects of gestational BPA on adipogenic differentiation

The sex-specific effect induced by gestational BPA exposure in female (increased adipogenesis) vs male (reduced adipogenesis) preadipocytes is in support of previous sex-specific differences observed upon pre- or perinatal exposures toward increased adipose tissue mass (19) and adipose tissue infiltration (81). That BPA can interfere with ESR1 and ESR2 and modulate GR action (82, 83), coupled with our findings that female preadipocytes had higher *ESR1* and *GR* expression compared with male preadipocytes, supports the hypothesis that female preadipocytes may be more susceptible to BPA's effect than males. In support of sex-specific responsiveness to estrogenic compounds, estradiol-induced preadipocyte proliferation is higher in adult human (84) and rat (85) female preadipocytes compared with that of male preadipocytes. Unfortunately, similar studies assessing sex-specific responsiveness to adipogenic differentiation are lacking in humans. To our knowledge, increased adipogenic ability reported in *in vitro* BPA-exposed human adipose stem cells has only been reported in female primary cells (75, 86).

ESR2 was expressed in ovine fetal preadipocytes, similar to that reported in the 3T3-L1 cell line (87). Although studies have not been able to confirm *ESR2* expression in human preadipocytes (73, 88), available RNA sequencing databases of human preadipocytes prove otherwise (89). When comparing steroid receptor expression between BPA-exposed preadipocytes, *ESR2* expression was higher in BPA female vs control, but not *ESR1* or *GR*. Higher *ESR2* expression is associated to BPA exposure in humans in other systems (90). Because the role of ESR2 in adipogenesis can be more prominent in the absence of ESR1 (91), it is likely that steroid differences (higher *ESR2*) do not contribute to the increased differentiation ability of BPA-exposed female preadipocytes.

ER stress modulation of adipogenesis

ER stress occurs when the ER homeostasis is disrupted. To restore ER homeostasis, UPR is triggered

through three pathways initiated with the transmembrane receptors PERK, ATF6, and IRE1α. The UPR response is highly conserved across species (24) and its activation can modulate adipogenesis (26, 27). The activation of PERK and IRE1 pathways enhance lipogenesis upregulating *C/EBPα*, *PPARγ*, and *SREBP-1c*, but inhibit lipogenesis under severe or prolonged ER stress (26). Our results demonstrate that both PERK and IRE1α pathways are upregulated in fetal female preadipocytes gestationally exposed to BPA. In support of previous work in other model systems, our findings demonstrate that BPA can interfere with ER homeostasis (31, 32). Independent activation of downstream effectors of both PERK and IRE1α, such as CHOP10 and WNT10B, respectively, can suppress adipogenesis (26). In this study, adipogenic induction of BPA-exposed female preadipocytes resulted in a profound downregulation of both PERK (*CHOP10*) and IRE1α (*XBP1-s* and *WNT10B*) pathways to levels close to that of the control preadipocytes by day 2 of differentiation. The last two effectors in these pathways (CHOP10 and WNT10B) control adipogenic differentiation by direct suppression of C/EBPβ and C/EBPα, respectively (26). Thus, we hypothesize that the downregulation of downstream effectors of both PERK and IRE1α pathways may contribute to the enhanced adipogenic differentiation observed in female preadipocytes gestationally exposed to BPA. BPA may have selectively reprogrammed female preadipocytes (higher *ESR1*, *ESR2*, and *GR* expression compared with males), resulting in an increased UPR response. These findings support that the UPR pathway regulates adipogenesis in ovine cells similar to that in mice and humans (26, 28). However, further research including knockdown of specific UPR pathway targets is required to demonstrate a direct role of ER stress in mediating BPA's adipogenic effect.

Even though UPR pathway upregulation can result in cell death (92), BPA-exposed female preadipocytes did not display apoptosis gene upregulation before or during differentiation. This supports that the UPR pathway upregulation (CASP8, MAPK8) may be an adaptive event of the preadipogenic lineage upon BPA. Adaptive responses upon chronic ER stress result in cell survival in several organs (93), but such adaptive responses in adipose tissue are less understood (93). Further studies are needed to investigate how gestational BPA exposure induces UPR signaling, the mechanism that maintains chronic UPR upregulation before differentiation induction, and the mechanism controlling UPR downregulation upon adipogenic stimulation. During prenatal exposures, BPA can alter the epigenetic signature of cells and tissues leading to a disease phenotype (94); however, information on BPA-induced epigenetic modifications on adipose tissue is limited to one study using the 3T3-L1 cell line demonstrating global hypermethylation (95).

Sex-specific effects of BPS on terminal adipogenic differentiation

In our study, BPS altered gene expression and terminal differentiation in gestationally BPS-exposed male, but not female, preadipocytes. During the late adipogenic phase, *FABP4* and *ADIPOQ* were upregulated in male BPS-exposed preadipocytes. Our findings do not support the higher obesogenicity reported for BPS in 3T3-L1 preadipocytes (96), but rather support the contention that gestational BPS exposure may result in a dysfunctional adipocytes as observed with other obesogenic EDCs such as TBT (97). Whether the differential expression of steroid receptors observed in BPS male preadipocytes (upregulation of *ESR1*, *ESR2*, and *GR*) contributes to this dysfunctional adipocyte phenotype requires further investigation.

Conclusion

These findings demonstrate how gestational exposure to EDCs can modify the fate of adipocyte precursors supporting the concept of environmentally mediated metabolic disruption. These results have important implications for the understanding the contributory role of EDCs to the obesity epidemic. Additional research is needed to further understand the underlying mechanisms by which BPA and BPS can modify preadipocyte adipogenic fate and result in dysfunctional adipocytes.

Acknowledgments

We thank Dr. Ehrhardt and the Michigan State University Sheep Teaching and Research Farm for animal procurement and husbandry; Lindsay Hannah, Gabriela Saldana, Igor Suguiura, and Dr. Steve Suhr for help during animal experimentation; and Janina Kavetsky for help with tissue imaging.

Financial Support: Research reported in this publication was supported by National Institute of Environmental Health Sciences, National Institutes of Health Grant 1K22ES026208 (to A.V-L.), Michigan State University (MSU) General Funds, MSU AgBioResearch, and the US Department of Agriculture National Institute of Food and Agriculture Hatch Project Grant MICL02383. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Disclosure Summary: The authors have nothing to disclose.

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