Journal of Developmental Origins of Health and Disease

cambridge.org/doh

In vivo and *in vitro* bisphenol A exposure effects on adiposity

M. Desai[1,2], M. G. Ferrini[3], J. K. Jellyman[1], G. Han[1] and M. G. Ross[1,2,4]

[1]Perinatal Research Laboratory, Department of Obstetrics and Gynecology, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, CA, USA, [2]Department of Obstetrics and Gynecology, David Geffen School of Medicine, University of California, Los Angeles, CA, USA, [3]Department of Health and Life Sciences Department of Internal Medicine, Charles R. Drew University, Los Angeles, CA, USA and [4]Department of Obstetrics and Gynaecology, Charles R. Drew University, Los Angeles, CA, USA

Original Article

Cite this article: Desai M, Ferrini MG, Jellyman JK, Han G, Ross MG. (2018) *In vivo* and *in vitro* bisphenol A exposure effects on adiposity. *Journal of Developmental Origins of Health and Disease* **9**: 678–687. doi: 10.1017/S2040174418000600

Received: 15 November 2017
Revised: 19 June 2018
Accepted: 21 June 2018
First published online: 29 August 2018

Key words:
adipogenesis, bisphenol A; lipogenesis; PPARγ; proliferation and differentiation

Address for correspondence:
M. Desai, Perinatal Research Laboratory, Department of Obstetrics and Gynecology, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, 1124 West Carson Street Box 446, Liu Research Bldg., Torrance, CA 90502, USA.
E-mail: mdesai@labiomed.org

Abstract

In utero exposure to the ubiquitous plasticizer, bisphenol A (BPA) is associated with offspring obesity. As adipogenesis is a critical factor contributing to obesity, we determined the effects of *in vivo* maternal BPA and *in vitro* BPA exposure on newborn adipose tissue at the stem-cell level. For *in vivo* studies, female rats received BPA before and during pregnancy and lactation via drinking water, and offspring were studied for measures of adiposity signals. For *in vitro* BPA exposure, primary pre-adipocyte cell cultures from healthy newborns were utilized. We studied pre-adipocyte proliferative and differentiation effects of BPA and explored putative signal factors which partly explain adipose responses and underlying epigenetic mechanisms mediated by BPA. Maternal BPA-induced offspring adiposity, hypertrophic adipocytes and increased adipose tissue protein expression of pro-adipogenic and lipogenic factors. Consistent with *in vivo* data, *in vitro* BPA exposure induced a dose-dependent increase in pre-adipocyte proliferation and increased adipocyte lipid content. *In vivo* and *in vitro* BPA exposure promotes the proliferation and differentiation of adipocytes, contributing to an enhanced capacity for lipid storage. These findings reinforce the marked effects of BPA on adipogenesis and highlight the susceptibility of stem-cell populations during early life with long-term consequence on metabolic homeostasis.

Introduction

The dramatic worldwide increase in obesity and its associated metabolic diseases has been widely attributed to an obesogenic environment created by our increasingly sedentary lifestyles and easy access to highly palatable, energy-dense foods. Although there is little doubt that poor diet and lack of exercise contribute to the obesity epidemic, recent studies have identified an estrogen endocrine disrupter chemical bisphenol A (BPA) that may act as an environmental obesogen and either directly or indirectly influence fat accrual.[1] BPA, a monomer plasticizer, is used in the manufacture of common household goods including polycarbonate plastics (e.g. food and drink containers), paints and adhesives[3–7]. In rodents, maternal BPA exposure increases postnatal body weights (BWs) and growth rates with differing sex-specific effects. Some studies show greater susceptibility to BPA-increased adiposity in females, some in males and other studies show effects on both sexes.[2–6] Critically, fetal exposures to BPA at levels equivalent to or below the established daily human safe dose (50 μg BPA/kg BW/day) not only increase BW and postnatal growth rate but also alter body composition in later life.[2,3,7–9] Energy (calorie) intake, energy expenditure and energy storage are the three key determinants of energy balance.[10] Increased adipose mass in offspring exposed to BPA during pregnancy may result from increased numbers of adipocyte cells (hyperplasia) secondary to enhanced differentiation of pre-adipocytes into mature adipocyte cells, as well as increased adipocyte cell size (hypertrophy) secondary to triglyceride storage.[11,12] Adipogenesis involves pre-adipocyte proliferation, differentiation and lipogenesis.[13,14] This process requires the coordinated interaction of pre-adipocyte proliferative factors (PREF1, SOX9) and several adipogenic differentiation transcription factors, including members of the CCAAT/enhancer-binding family of proteins (C/EBPα, C/EBPβ and C/EBPδ),[15,16] which activate the peroxisome proliferator-activated receptor (PPARγ$_2$).[17] The BPA is associated with increased expression of pro-adipogenic genes *in vivo*[4,18] and with accelerated terminal differentiation of 3T3-L1 cells *in vitro*.[19,20] However, no study to date has determined the effect of prenatal BPA on offspring primary pre-adipocyte proliferation and differentiation and the underlying mechanism.

We sought to confirm the effects of maternal BPA exposure during pregnancy and lactation on offspring BW, while examining effects on measures of adiposity. To more fully explore the mechanisms of BPA-mediated effects, we further utilized established models of newborn rat primary pre-adipocyte stem cells, exploring both proliferative (i.e. trophic) and

CAMBRIDGE UNIVERSITY PRESS

differentiation effects of BPA.[21] We further explored putative signal factors which explain, in part, adipose responses and underlying epigenetic mechanisms mediated by BPA. The data reinforce the marked effects of BPA on adipogenesis and highlight the susceptibility of stem-cell populations during early life with long-term consequence on metabolic homeostasis.

Methods

BPA model

In vivo maternal BPA exposure

Studies were approved by the Animal Care Committee at the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center and were in accordance with the American Accreditation Association of Laboratory Care. All animals were treated humanely and with regard for alleviation of suffering. Virgin Sprague–Dawley female rats (Charles River Laboratories, Hollister, CA, USA) were housed in an animal facility with controlled 12/12 h light/dark cycles, constant temperature and humidity conditions and *ad libitum* access to chow diet (Lab Diet 5001; Brentwood). The chow diet contains soy meal and as this was fed to both Controls and BPA-exposed animals, any comparative differences between the groups are likely due to the BPA exposure rather than the estrogenic activity of the phytoestrogens in the diet. To avoid potential BPA contamination, polypropylene cages and purified water in glass bottles were utilized. Female rats were randomly assigned to Control ($n=5$) or BPA ($n=5$) group. To reflect the most likely route of human exposure,[22–25] dams were exposed to BPA via their drinking water. Control rats had access to purified drinking water, whereas the BPA group received purified drinking water containing BPA (5 mg/l; BPA Sigma-Aldrich, purity ≥99%, CAS no. 80-05-7) for 2 weeks before mating and throughout pregnancy and lactation (Table 1). Studies that administered BPA to pregnant rodents via drinking water, a concentration of 10 mg/l water (consumption of ~ 1.2 mg/kg BW/day)[26] yielded BPA tissue concentrations of 10–25 ng/g tissue[27,28] consistent with that of human samples.[29] A dose five-fold higher (6 mg/kg BW/day) administered via gavage achieved significantly higher maternal plasma BPA levels,[30] whereas a water concentration of only 1 mg/l resulted in low maternal plasma-free BPA levels (0.84 ng/ml).[31] Our dose was selected based upon our confirmation (pilot study) of maternal and newborn serum levels within the lower range of demonstrated human levels with normal BPA exposure.

Maternal BW and water intake were monitored daily, and dual-energy X-ray absorptiometry (DEXA) was undertaken at the end of lactation after which adipose tissue was collected for cell size analysis. Before mating, maternal blood was obtained via tail bleed and at the end of lactation via cardiac puncture in BPA-free tubes for BPA analysis. To avoid inducing maternal stress and fetal resorption,[32] blood samples were not collected during pregnancy, especially as maternal stress has been demonstrated to be an independent risk factor for offspring obesity.[33,34] Free (unconjugated) BPA levels were measured using GC/MS (NMS Labs, PA, USA) with an assay sensitivity of 0.25 ng/ml. Insufficient plasma volume from maternal tail bleeds (before BPA administration) and newborns necessitated pooling of samples and hence only mean values are reported. Following BPA administration at the end of lactation, samples were analyzed individually for BPA levels.

Table 1. *In vivo* maternal BPA exposure: drinking water

	Control ($n=5$ Litters)	BPA ($n=5$ Litters)	Parameters measured
Female non-pregnant Age: 9–12 weeks	BPA-free	BPA	Body weights, water intake
Mating age: 12 weeks	BPA-free	BPA	Body weights, water intake, tail bleed
Pregnancy GA: 0–21 days	BPA-free	BPA	Body weights, water intake
Newborns age: 1 day	BPA-free	BPA	Body weights, blood, adipose tissue
Lactation nursing: 0–21 days	BPA-free	BPA	Body weights, water intake, blood, DEXA
Offspring age: 3 weeks	BPA-free	BPA-free	Body weights, DEXA, adipose tissue
Offspring age: 6 weeks	BPA-free	BPA-free	Body weight, blood pressure
Offspring age: 24 weeks	BPA-free	BPA-free	Body weight, DEXA

BPA, bisphenol A; DEXA, dual-energy X-ray absorptiometry.
In vivo maternal BPA exposure: Non-pregnant female rats at 9 weeks of age were allowed drinking water that was BPA-free (Control group) or contained BPA (BPA group). At 12 weeks of age, tail blood was obtained for BPA analysis and all females were mated and continued on same drinking water regimen throughout pregnancy and lactation. Parameters measured at various time points are indicated. At each offspring ages, $n=5$ males were studied from five separate litters.

Dams gave birth spontaneously and five litters per group (Control and BPA) were utilized for offspring studies. Litter size was standardized to eight per litter (four males and four females) to normalize rearing and all offspring were nursed by their respective mothers till 3 weeks of age. Following weaning, all offspring were given purified water and housed in polypropylene cages.

Offspring studies

DEXA scan

At 3 and 24 weeks of age, one male and one female offspring from one litter ($n=5$) underwent a non-invasive DEXA scanning using DXA system with a software program for small animal (QDR 4500A; Hologic, Bedford, MA, USA). An *in vivo* scan of whole body composition was obtained, including fat tissue mass, total mass and percent body fat.

Adipose tissue retrieval

Excess pups (beyond the four males and four females) were sacrificed at day 1 of life and subcutaneous (inguinal) adipose tissue from two pups of each gender from each litter was pooled according to sex (representing $n=1$) for analysis of tissue protein expression. A total of $n=5$ litters were studied. At 3 weeks of age, one male and one female from one litter ($n=5$) were euthanized and visceral (retroperitoneal) adipose tissue was collected for analysis of protein expression.

Blood pressure

At 6 weeks of age, measurements were undertaken in conscious animals using non-invasive tail-cuff sphygmomanometry (ML125 NIPB System; AD Instruments) method. Several cuff sizes are used depending on the weight of the animal. To circumvent the

https://doi.org/10.1017/S2040174418000600 Published online by Cambridge University Press

potential problem of restraint-induced stress, the animals were acclimatized for at least 1 week with placement in the restraint. One male and one female offspring from one litter ($n = 5$) were studied.

In vitro BPA exposure

An additional four Control litters ($n = 4$) were studied for *in vitro* effects of exogenous BPA on adipocyte cultures. From each litter, inguinal adipose tissue was dissected from each of four, 1-day-old Control males (four pooled samples representing $n = 1$) for cell culture studies. Pre-adipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) medium in absence or presence of differentiation cocktail (see below) and treated with dimethyl sulfoxide (DMSO) (control) or BPA (1, 10, 20 μM) for 5 days. The total number studied was n = 4 from four litters for adipocyte cultures.

Primary cultures

Primary adipocyte cell cultures were established as previously described.[21] Briefly, pooled adipose tissue from 1-day-old newborn males was minced and digested with collagenase type II (5000 U/g) in Krebs-Ringer solution. The pre-adipocyte cultures were resuspended in high glucose (450 mg/dl) DMEM (Invitrogen) with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotics (Invitrogen) and incubated at 37°C with 5% CO_2. Cells were either seeded in 24-well plate (~1 × 10^4 cells/ml) for proliferation studies or flasks (~1 × 10^6 cells/ml) for protein expression and differentiation studies. After 24 h, undifferentiated pre-adipocytes were either treated with DMSO or BPA for 5 days. For differentiation studies, 24-h cultured pre-adipocytes were treated with dexamethasone (1 μM), methylisobutylxanthine (0.1 mM), insulin (10 μg/ml) in presence of DMSO or BPA for 5 days. At the end of exposure, all cells were harvested and protein extracted for analysis as described under Western Blot.

Analysis

Pre-adipocyte proliferation assay

Pre-adipocyte (complete medium) proliferation was determined as previously reported[21] using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay.[35]

Adipocyte cell size

The cross-sectional area of adipocytes was determined as previously described.[36] Briefly, formalin-fixed and paraffin-embedded adipose tissues was sectioned (5 μm), stained with hematoxylin–eosin, photomicrographs captured at 20× magnification and area of adipocytes (three images per section, and three sections per animal) determined using Image PRO software.

Lipid staining

Differentiated adipocytes were fixed with 4% paraformaldehyde, stained with 0.5% oil red O, mounted onto slides with Vectashield mounting medium with 4′,6-diamidino-2 phenylindole (Vector) and images (40× magnification) captured (Zeiss Axioskop 40 microscope with Axiocam HRc camera). For quantification, stained adipocytes were dried for 1 h at 37°C, incubated with a fixed volume of isopropanol for 20 min to elute the oil red O, and absorbance measured at 520 nm.

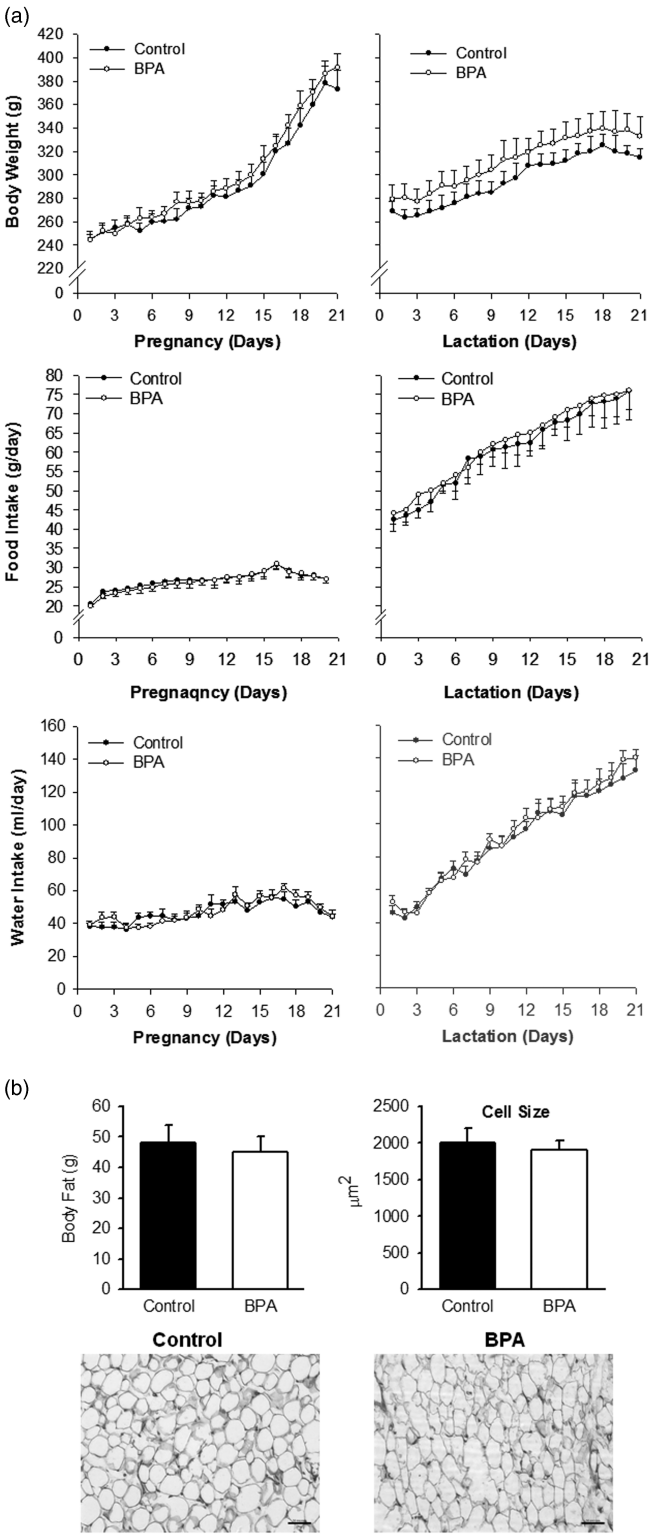

Fig. 1. (*a*) **Effects of maternal bisphenol A (BPA) exposure on maternal body weights, food intake and water intake:** Maternal daily body weights and water intake during pregnancy and lactation of BPA (○) and Control (●) dams. Values are means ± SE of $n = 5$ litters. (*b*) **Effects of maternal BPA exposure on maternal body fat**: Maternal body fat and retroperitoneal adipocyte cell size and images (scale bar = 100 μm) at the end of lactation of BPA (□) and Control (■) dams. Values are means ± SE of $n = 5$ litters.

Western blot

Cell and tissue protein was extracted and Western Blotting performed as described previously.[36] For adipose tissue, protein

https://doi.org/10.1017/S2040174418000600 Published online by Cambridge University Press

expression analysis included pre-adipocyte marker (PREF1, 55 kDa; Millipore), suppressor of pre-adipocyte differentiation (SOX9; 67 kDa; Millipore), adipogenic markers (PPARγ, 57 kDa, Cayman; C/EBPα, 42 kDa; Santa Cruz), lipogenic factor (SREBP1, 125 kDa; Santa Cruz) and epigenetic regulators DNA methyltransferase 3a (DNMT3a, 120 kDa; Santa Cruz) and lysine (K)-specific demethylase 1A (LSD1, 110 kDa; Cell Signaling).

Statistical analyses

In vivo responses between BPA and control offspring were compared by unpaired t-test. *In vitro* responses between BPA-exposed and untreated (DMSO) Control cells were compared by unpaired *t*-test or analysis of variance with Dunnett's *post-hoc* test, as appropriate. *P* values $\leqslant 0.05$ were considered significant.

Results

Maternal BPA effects on maternal phenotype

Of note, the higher BPA levels (shown below) did not impact maternal weight gain and body fat. The maternal BW and water intake during pregnancy and lactation were comparable between BPA and Control dams (Fig. 1a). Further at the end of lactation, body fat and adipocyte cell size were similar in both groups (Fig. 1b).

Maternal BPA effects on plasma BPA levels

The pooled maternal plasma BPA level before BPA administration was 0.46 ng/ml. During the course of pregnancy, the amount of BPA consumed by dams via drinking water was 500–900 µg/kg/day and during lactation, it was higher (approximately 1500 µg/kg/day) due to increased water intake. The maternal BPA plasma levels at the end of lactation were higher in BPA as compared with Control dams (8.2 ± 3.8 *v.* 0.42 ± 0.04ng/ml). Similarly, newborns of BPA dams had higher plasma BPA levels (0.62 ng/ml) as compared with undetectable levels in newborns of Control dams.

Maternal BPA effects on offspring phenotype

Maternal BPA did not alter birth weights of either male or female newborns. However, by the end of the nursing period, 3-week-old BPA males exhibited significantly increased BW that persisted in 24-week-old adults (Fig. 2). Consistent with this, the percentage of body fat was increased in 3- and 24-week-old BPA males as compared with Controls (Fig. 3). Additionally, the systolic blood pressure was significantly higher in 6-week-old BPA than Control males (Fig. 4). In contrast to males, BWs, percentage body fat and systolic blood pressure of BPA females were comparable to Control females (Figs 2–4).

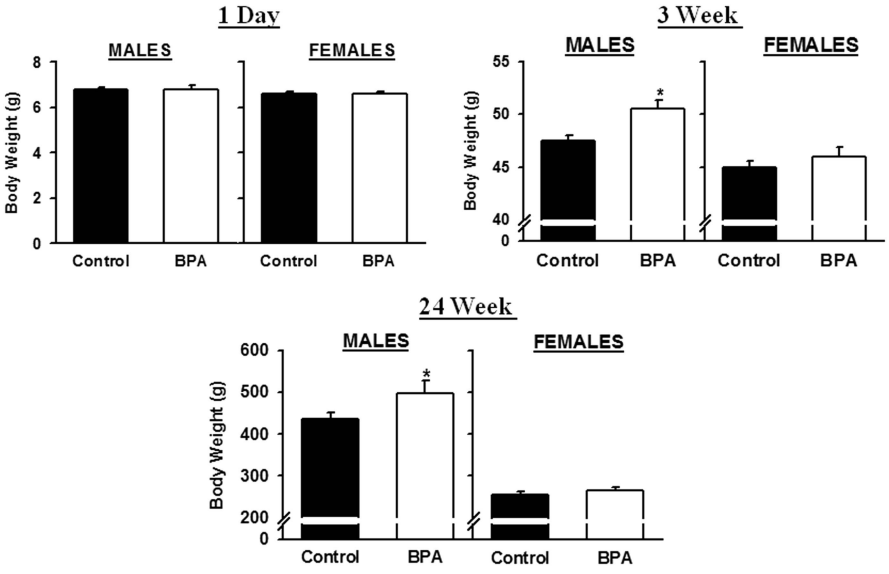

Fig. 2. Maternal bisphenol A (BPA) effects on offspring body weights: Body weights of 1-day-old newborns, and body weights of 3 and 24-week-old male and female offspring. Values are means ± SE of $n = 5$ from 5 litters per group; *$P < 0.05$ BPA (□) *vs.* Control (■).

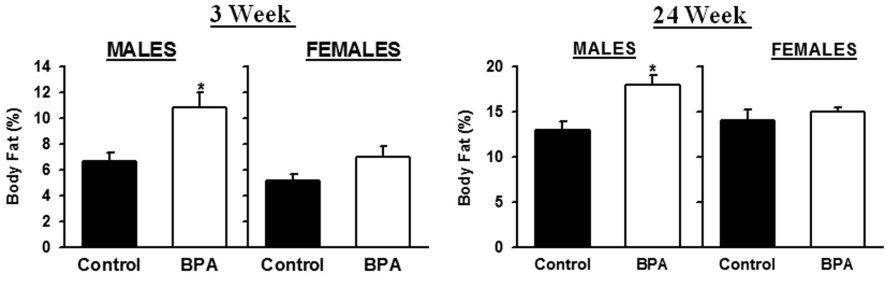

Fig. 3. Maternal bisphenol A (BPA) effects on offspring adiposity: Percentage body fat of 3- and 24-week-old male and female offspring. Values are means ± SE of $n = 5$ from five litters per group; *$P < 0.05$ BPA (□) *vs.* Control (■).

https://doi.org/10.1017/S2040174418000600 Published online by Cambridge University Press

Maternal BPA effects on adipose tissue

As adiposity was not altered in BPA female, we only studied male offspring. At 1 day of age, BPA males had significantly increased protein expression of the adipogenic transcription factor PPARγ though not C/EBPα or the lipogenic factor SREBP1 (Fig. 5a). At 3 weeks of age, BPA males showed increased protein expression of C/EBPα though not PPARγ while the lipogenic factor SREBP1 was increased (Fig. 5b) in conjunction with evidence of hypertrophic adipocytes (Fig. 5c).

Consistent with increased adipose tissue mass and hypertrophic adipocytes in BPA males, protein expression of CD68 (macrophage marker) and TNFα (pro-inflammatory cytokine) was significantly increased at 3 weeks of age (Fig. 6).

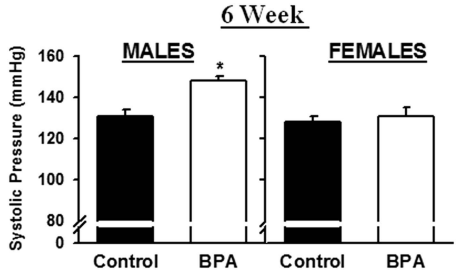

Fig. 4. Maternal bisphenol A (BPA) effects on offspring blood pressure: Systolic blood pressure of 6–week-old male and female offspring. Values are means ± SE of $n = 5$ from 5 litters per group; *$P < 0.05$ BPA (□) *vs.* Control (■).

In vitro BPA effects

Pre-adipocytes from Control offspring cultured in standard media with incremental doses of BPA showed a dose-dependent increase in proliferation (Fig. 7a and 7b), consistent with increased expression of the pre-adipocyte marker (PREF1), though expression of the anti-adipocyte differentiation transcription factor (SOX9) expression was decreased at 10 μM BPA (Fig. 7c). In differentiated adipocytes (grown in DMEM medium), BPA increased the number of adipocytes (1.8-fold), consistent with the increased protein expression of adipogenic transcription factors (PPARγ, C/EBPα), and expression of the pro-inflammatory cytokine TNFα (Fig. 8a). In addition, BPA-treated adipocytes showed increased lipid accumulation consistent with increased expression of SREBP1 (Fig. 8b).

Epigenetic factors

BPA-treated adipocytes demonstrated a dose-dependent increase in DNMT3 though not LSD1 (Fig. 9).

Discussion

The present study determined the effects of BPA exposure *in vivo* and *in vitro* on adipogenesis, and the protein expression of regulatory transcription factors in early life. The data suggest that enhanced pre-adipocyte proliferation and differentiation in early

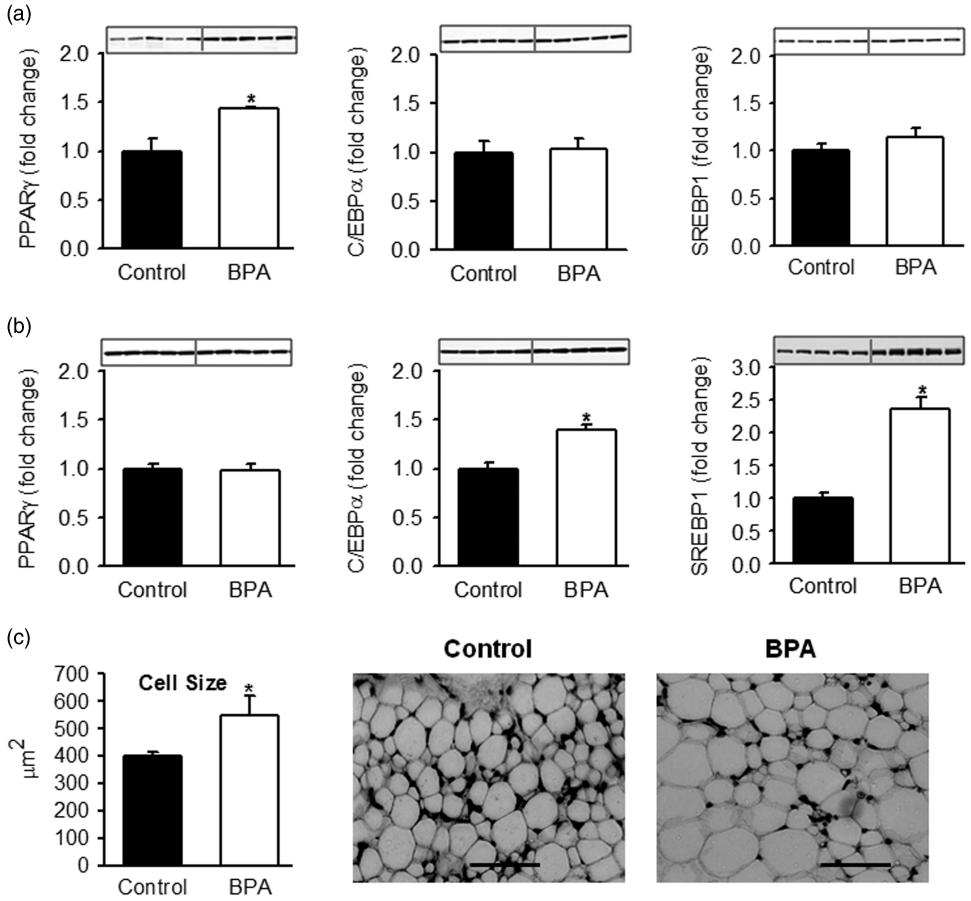

Fig. 5. Maternal bisphenol A (BPA) effects on offspring adipogenic and lipogenic factors: (*a*) inguinal adipose tissue protein expression of peroxisome proliferator-activated receptor (PPARγ) and C/EBPα in 1-day-old male newborns, (*b*) retroperitoneal adipose protein expression of PPARγ, C/EBPα and SREBP1 and (*c*) retroperitoneal adipocyte cell size and images (scale bar = 100 μm) in 3-week-old male offspring. Values are means ± SE of $n = 5$ of pooled adipose from each of five litters per group; *$P < 0.05$ BPA (□) *vs.* Control (■).

https://doi.org/10.1017/S2040174418000600 Published online by Cambridge University Press

life may contribute to the underlying mechanism of BPA-induced obesity.

A wide range of detectable BPA levels are reported in adults and children serum (0.2–20 ng/ml),[37] including breast milk (1.1 ng/ml), and maternal (0.2 to >10 ng/ml) and fetal/newborn serum (0.2–9.2 ng/ml). More significantly, the higher levels of measurable BPA in amniotic fluid (8.3–8.7 ng/ml) and placental tissues (1.0–104.9 ng/ml)[38,39] imply a continued fetal exposure to BPA throughout development. Of note, higher BPA levels are seen in infants and children than in adults[40] and this has been associated with increased adiposity.[41,42] The findings from animal studies corroborate the association of BPA exposure with adiposity. Specifically, studies show that it is lower (≤500 µg/kg/day) rather than higher dose (>5000 µg/kg/day) of maternal BPA that is effective in promoting offspring weight gain.[4,5] Sex-specific effects are seen with some studies demonstrating increased postnatal growth in both males and females at maternal doses between 2.4 and 500 µg/kg/day,[2,43–45] only in males at maternal dose of 500 µg/kg/day[5] and only in females at maternal dose of 100 µg/kg/day.[4] The reported effective maternal BPA dose of 500 µg/kg/day is compatible with our *in vivo* studies where effects are seen only in the male offspring and consistent with study by Bartolomei *et al.*[6] Further regardless of differing metabolism of BPA in rodents and humans,[46] pharmacokinetic studies indicate that BPA exposure of approximately 400–500 µg/kg/day yield blood concentrations of the unconjugated, bioactive form of BPA that is similar to that reported in human blood.[47]

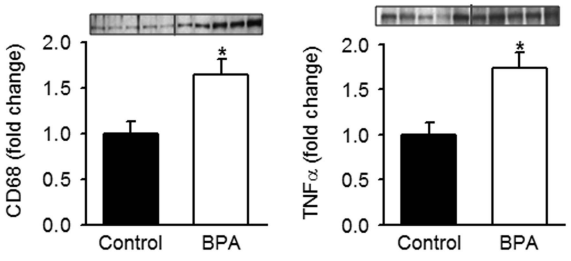

Fig. 6. Maternal bisphenol A (BPA) effects on adipose tissue inflammation in 3-week-old male offspring: Retroperitoneal adipose protein expression of CD68 and TNFα in 3-week-old male offspring. Values are means ± SE of $n = 5$ of pooled adipose from each of five litters per group; *$P < 0.05$ BPA (□) *vs.* Control (■).

Previous studies on mice and rats indicate that endocrine disruptors[48,49] and estrogen[49] have species and strain-dependent as well as organ-specific effects.[49] However, the overall sensitivity of various biological endpoints did not have pronounced differences between strains of rats.[49] With regard to adipogenesis, studies show that the two commonly used strains, Sprague–Dawley and Wistar rats, demonstrate increased adiposity in response to BPA exposure. [50–53] Specifically, pregnant Sprague–Dawley exposed to BPA cause increased adipogenesis[50], metabolic pertubations[54] and induced epigenetic transgenerational inheritance of obesity.[55]

Interestingly unlike in the offspring, increased plasma BPA levels did not adversely impact maternal BW and body fat. This may be a result of continued exposure of developing fetus to BPA as stated above. There is also an implication that α-fetoprotein, which is secreted in high levels during development, may be involved. Normally, α-fetoprotein binds to estrogen and protects the developing fetus from estrogen effects. However, binding properties of some endocrine disruptors to α-fetoprotein may reduce this protection and expose the fetus to estrogen effects.[56–59]

The present study demonstrates that despite the normal birth weight, male newborns exposed to maternal BPA have a markedly increased postnatal growth rate and fat mass accumulation and hypertension. This elevated adipogenic potential is consistent with increased adipogenic (PPARγ, C/EBPα) and lipogenic (SREBP1) factors, which likely contribute to the hypertrophic

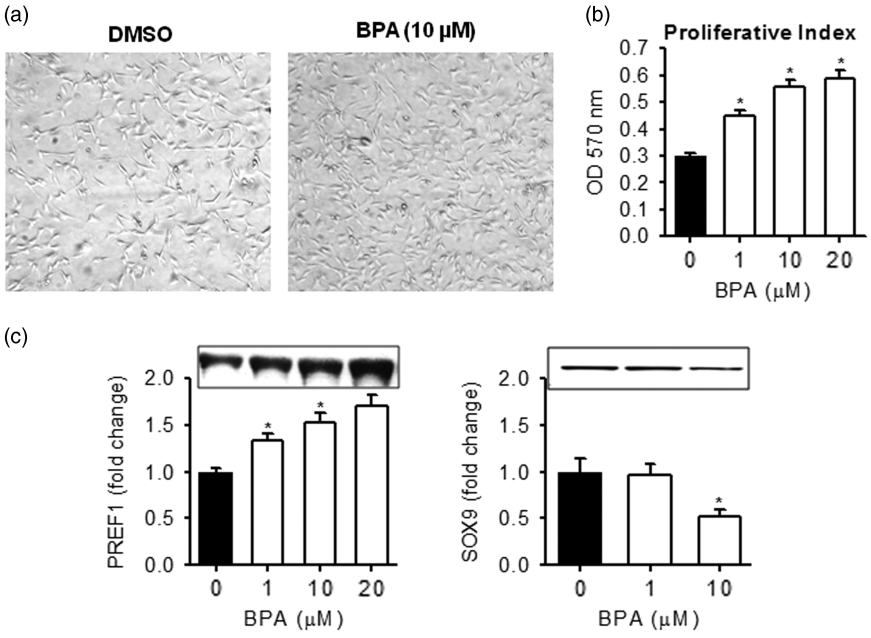

Fig. 7. *In vitro* bisphenol A (BPA) effects on pre-adipocytes: Inguinal adipose tissue from 1-day-old Control newborns were cultured in Dulbecco's modified Eagle's medium (DMEM) (undifferentiated) media and treated with dimethyl sulfoxide (DMSO) (Control) or BPA (0, 1, 10, 20 µM) for 5 days. (*a*) Pre-adipocyte image, (*b*) proliferative index measured at 520 OD and (*c*) protein expression of PREF1 and Sox9 (results are shown for two doses due to loss of 20 µM BPA protein extract) with representative blot shown. Values are fold change (mean ± SE) of $n = 4$ of pooled adipose from each of 4 litters; *$P < 0.05$ BPA (□) *vs.* Control (■).

https://doi.org/10.1017/S2040174418000600 Published online by Cambridge University Press

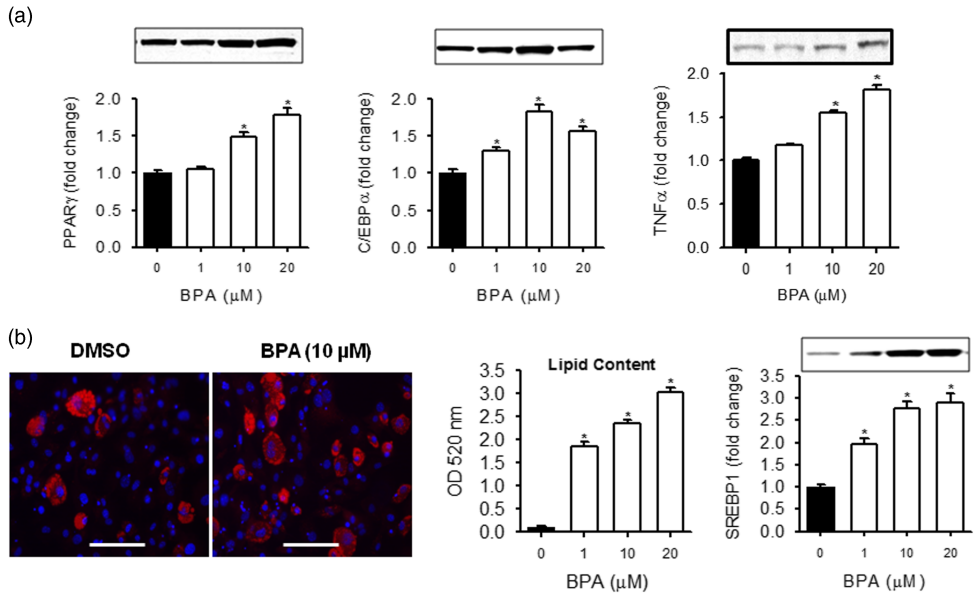

Fig. 8. ***In vitro*** **bisphenol A (BPA) effects on differentiated adipocytes:** Inguinal adipose tissue from 1-day-old Control newborns were cultured in Dulbecco's modified Eagle's medium (DMEM) media and pre-adipocytes were allowed to differentiate in presence of BPA for 5 days. (*a*) Protein expression of peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα with representative blot shown. (*b*) Adipocytes were fixed with 4% paraformaldehyde and stained for lipid (Oil Red O; red) and nucleus (4′,6-diamidino-2 phenylindole (DAPI); blue). Scale bar = 100 μm. Protein expression of SREBP1 with representative blot shown and lipid content. Values are fold change (mean ± SE) of $n = 4$ of pooled adipose from each of four litters;*$P < 0.05$ BPA (□) *vs.* Control (■).

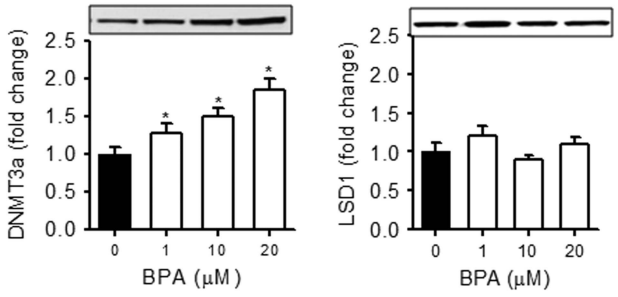

Fig. 9. ***In vitro*** **bisphenol A (BPA) effects on epigenetic factors:** Inguinal pre-adipocytes from 1-day-old Control newborns were cultured in Dulbecco's modified Eagle's medium (DMEM) media with BPA (1, 10, 20 μM) for 5 days. Protein expression of DNA methyltransferase 3a (DNMT3a) and lysine (K)-specific demethylase 1A (LSD1) with representative blot shown. Values are fold change (mean ± SE) of pooled cells $n = 4$ from each of 4 litters; *$P < 0.05$ BPA (□) *vs.* Control (■).

Table 2. Summary data of *in vivo* exposure to maternal BPA

	Ages			
Parameters	1 Day	3 Weeks	6 Weeks	24 Weeks
Body weight	↔	↑	↑	↑
Body fat	–	↑	–	↑
Blood pressure	–	–	↑	–
Adipose tissue				
Size	–	↑	–	–
PPARγ	↑	↔	–	–
C/EBPα	↔	↑	–	–
SREBP1	↔	↑	–	–
CD68	–	↑	–	–
TNFα	–	↑	–	–

↑, increased; ↔, no change; –, not studied; PPARγ, peroxisome proliferator-activated receptor γ.
Changes at various ages are summarized for male offspring exposed to maternal bisphenol A.

adipocytes seen in BPA offspring. The underlying mechanism may be attributed to BPA-induced increased pre-adipocyte proliferation as demonstrated by *in vitro* BPA exposure studies. Specifically, pre-adipocytes exposed to *in vitro* BPA exhibit increased cell proliferation and increased PREF1, suggesting an increased pool of committed pre-adipocyte stem cells for potential adipocyte differentiation.[60] PREF1 is known to suppress adipocyte differentiation by induction of SOX9.[60–62] In our studies of *in vitro* BPA exposure, the failure of PREF1 to induce SOX9, as evident by the suppression of SOX9, indicates dysregulation of PREF1/SOX9 that may facilitate adipocyte differentiation under appropriate stimuli (e.g. increased insulin/glucocorticoid),[63] high carbohydrate or high fat diet.[64–66] Indeed, when pre-adipocytes are allowed to differentiate in presence of BPA, there is increased expression of adipogenic and lipogenic transcription factors together with more lipid-filled adipocytes. Collectively, this results in an inflammatory response as evident by increased expression of macrophage marker (CD68) and pro-inflammatory cytokine (TNFα). These changes are similar to the effects seen with *in vivo* maternal BPA exposure and consistent with previously reported *ex vivo* studies of 3T3-L1 pre-adipocyte cell lines,[19,20,67–69] as well as murine and human[70,71] primary stem cells. Furthermore in the present study, the early increased PPARγ and later increased C/EBPα and SREBP1 suggest the specific influence of BPA during perinatal and postnatal exposures. It is known that although PPARγ and C/EBPα positively regulate each other's expression and cooperate to promote adipogenesis,[72] C/EBPα is required for lipogenesis.[73]

https://doi.org/10.1017/S2040174418000600 Published online by Cambridge University Press

The mechanism underlying BPA-induced enhanced adipocyte proliferation and differentiation may involve epigenetic modifications via DNA and/or histone methylation,[74,75] particularly of genes such as PPARγ[77] and C/EBPα. Methylase and demethylase enzymes that have been implicated in determining stem-cell proliferation (self-renewal) and differentiation include DNMT (DNA methyltransferase) and LSD1 (lysine (K)-specific histone demethylase).[76] For example, treatment of 3T3-L1 pre-adipocytes with an inhibitor of DNA methylation (5′-aza-cytideine) or knockdown of LSD1 decreased proliferation and adipocyte differentiation, resulting in downregulation of PPARγ.[77–79] This is consistent with our findings on *in vitro* BPA exposure, that is, increased pre-adipocyte proliferation with increased protein expression of DNMT3a. The lack of dose–response seen in the case of C/EBPα is presently unknown. However, it may involve BPA- or direct transcription factor-mediated chromatin interaction and accessibility.[74,80] Although the current data suggest BPA alters transcriptional regulation of genes involved in proliferation, the role of DNA methylation in pre-adipocytes remains to be established and further studies of site-specific epigenetic modification of specific genes are required to fully elucidate BPA-induced changes in adipogenesis.

Although the sex-specific effects of BPA are well documented including the differential susceptibility of males and females to different doses of BPA[47,81–85], the underlying mechanism remains unclear.[82] The plausible explanation may involve sex hormones, genomic and non-genomic pathway involving nuclear estrogen receptors, differing developmental pattern and/or epigenetic influence.[47,83,86]

Conclusion

Our data (Table 2) confirm that primary adipose progenitor cells are vulnerable to endocrine disruption by BPA resulting in altered proliferation and differentiation in early life independent of systemic influences. Enhanced adipose proliferation and differentiation indicate the potential for maternal/fetal BPA exposure to program an increased risk of offspring obesity and consequent metabolic abnormalities.[4,18,87,88]

Acknowledgments. The authors thank Stacy Behare for animal assistance.

Financial Support. This work was supported by the National Institute of Environmental Health Sciences (R21ES023112-01; M.D., M.G.R.), National Center for Advancing Translational Sciences UCLA- CTSI (Grant U11TR000124; M.D.), National Institute on Minority Health and Health Disparities (5U54MD007598-06 (M.G.F.) and Flora Foundation (M.D., M.G.R.).

Conflicts of Interest. None.

Ethical Standards. As stated in the Methods, all studies were approved by the Animal Research Committee of the Los Angles Biomedical Research Institute at Harbor-UCLA Medical Center and were conducted in strict accordance with guidelines provided by the American Accreditation Association of Laboratory Care and the Public Health Service Policy on Humane Care and Use of Laboratory Animals and conform to the principles and regulations as described in the Editorial by Grundy.[89] All animals were treated humanely and with regard for alleviation of suffering. Virgin Sprague–Dawley female rats (Charles River Laboratories, Hollister, CA) were housed in an animal facility with controlled 12/12-hour light/dark cycles, constant temperature and humidity conditions and *ad libitum* access to chow diet (Lab Diet 5001; Brentwood, Missouri) and water.

References

1. Janesick A, Blumberg B. Obesogens, stem cells and the developmental programming of obesity. *Int J Androl*. 2012; 35, 437–448.
2. Richter CA, Birnbaum LS, Farabollini F, *et al*. In vivo effects of bisphenol A in laboratory rodent studies. *Reprod Toxicol*. 2007; 24, 199–224.
3. Rubin BS, Soto AM. Bisphenol A: perinatal exposure and body weight. *Mol Cell Endocrinol*. 2009; 304, 55–62.
4. Somm E, Schwitzgebel VM, Toulotte A, *et al*. Perinatal exposure to bisphenol a alters early adipogenesis in the rat. *Environ Health Perspect*. 2009; 117, 1549–1555.
5. Angle BM, Do RP, Ponzi D, *et al*. Metabolic disruption in male mice due to fetal exposure to low but not high doses of bisphenol A (BPA): evidence for effects on body weight, food intake, adipocytes, leptin, adiponectin, insulin and glucose regulation. *Reprod Toxicol*. 2013; 42, 256–268.
6. Susiarjo M, Xin F, Bansal A, *et al*. Bisphenol A exposure disrupts metabolic health across multiple generations in the mouse. *Endocrinology*. 2015; 156, 2049–2058.
7. Alonso-Magdalena P, Morimoto S, Ripoll C, Fuentes E, Nadal A. The estrogenic effect of bisphenol A disrupts pancreatic beta-cell function in vivo and induces insulin resistance. *Environ Health Perspect*. 2006; 114, 106–112.
8. Alonso-Magdalena P, Vieira E, Soriano S, *et al*. Bisphenol A exposure during pregnancy disrupts glucose homeostasis in mothers and adult male offspring. *Environ Health Perspect*. 2010; 118, 1243–1250.
9. vom Saal FS, Nagel SC, Coe BL, Angle BM, Taylor JA. The estrogenic endocrine disrupting chemical bisphenol A (BPA) and obesity. *Mol Cell Endocrinol*. 2012; 354, 74–84.
10. Hill JO, Wyatt HR, Peters JC. Energy balance and obesity. *Circulation*. 2012; 126, 126–132.
11. Ailhaud G, Grimaldi P, Negrel R. Cellular and molecular aspects of adipose tissue development. *Annu Rev Nutr*. 1992; 12, 207–233.
12. Hausman DB, DiGirolamo M, Bartness TJ, Hausman GJ, Martin RJ. The biology of white adipocyte proliferation. *Obes Rev*. 2001; 2, 239–254.
13. Morrison RF, Farmer SR. Insights into the transcriptional control of adipocyte differentiation. *J Cell Biochem*. 1999; 32–33(Suppl.), 59–67.
14. Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM. Transcriptional regulation of adipogenesis. *Genes Dev*. 2000; 14, 1293–1307.
15. Darlington GJ, Ross SE, MacDougald OA. The role of C/EBP genes in adipocyte differentiation. *J Biol Chem*. 1998; 273, 30057–30060.
16. Lane MD, Lin FT, MacDougald OA, Vasseur-Cognet M. Control of adipocyte differentiation by CCAAT/enhancer binding protein alpha (C/EBP alpha). *Int J Obes Relat Metab Disord*. 1996; 20(Suppl. 3), S91–S96.
17. Rosen ED, Spiegelman BM. PPARgamma: a nuclear regulator of metabolism, differentiation, and cell growth. *J Biol Chem*. 2001; 276, 37731–37734.
18. Miyawaki J, Sakayama K, Kato H, Yamamoto H, Masuno H. Perinatal and postnatal exposure to bisphenol A increases adipose tissue mass and serum cholesterol level in mice. *J Atheroscler Thromb*. 2007; 14, 245–252.
19. Chamorro-Garcia R, Kirchner S, Li X, *et al*. Bisphenol A diglycidyl ether induces adipogenic differentiation of multipotent stromal stem cells through a peroxisome proliferator-activated receptor gamma-independent mechanism. *Environ Health Perspect*. 2012; 120, 984–989.
20. Masuno H, Iwanami J, Kidani T, Sakayama K, Honda K. Bisphenol a accelerates terminal differentiation of 3T3-L1 cells into adipocytes through the phosphatidylinositol 3-kinase pathway. *Toxicol Sci*. 2005 ; 84, 319–327.
21. Yee JK, Lee WN, Ross MG, *et al*. Peroxisome proliferator-activated receptor gamma modulation and lipogenic response in adipocytes of small-for-gestational age offspring. *Nutr Metab (Lond)*. 2012; 9, 62.
22. Carwile JL, Luu HT, Bassett LS, *et al*. Polycarbonate bottle use and urinary bisphenol A concentrations. *Environ Health Perspect*. 2009; 117, 1368–1372.
23. Muhamad MS, Salim MR, Lau WJ, Yusop Z. A review on bisphenol A occurrences, health effects and treatment process via membrane technology for drinking water. *Environ Sci Pollut Res Int*. 2016; 23, 11549–11567.
24. Makris KC, Andra SS, Jia A, *et al*. Association between water consumption from polycarbonate containers and bisphenol A intake during harsh environmental conditions in summer. *Environ Sci Technol*. 2013; 47, 3333–3343.

https://doi.org/10.1017/S2040174418000600 Published online by Cambridge University Press

25. Le HH, Carlson EM, Chua JP, Belcher SM. Bisphenol A is released from polycarbonate drinking bottles and mimics the neurotoxic actions of estrogen in developing cerebellar neurons. *Toxicol Lett*. 2008; 176, 149–156.
26. Mendoza-Rodriguez CA, Garcia-Guzman M, Baranda-Avila N, *et al*. Administration of bisphenol A to dams during perinatal period modifies molecular and morphological reproductive parameters of the offspring. *Reprod Toxicol*. 2011; 31, 177–183.
27. Kabuto H, Amakawa M, Shishibori T. Exposure to bisphenol A during embryonic/fetal life and infancy increases oxidative injury and causes underdevelopment of the brain and testis in mice. *Life Sci*. 2004; 74, 2931–2940.
28. Nakajima Y, Goldblum RM, Midoro-Horiuti T. Fetal exposure to bisphenol A as a risk factor for the development of childhood asthma: an animal model study. *Environ Health*. 2012; 11, 8.
29. Schonfelder G, Wittfoht W, Hopp H, *et al*. Parent bisphenol A accumulation in the human maternal-fetal-placental unit. *Environ Health Perspect*. 2002; 110, A703–A707.
30. Yoshida M, Shimomoto T, Katashima S, *et al*. Maternal exposure to low doses of bisphenol a has no effects on development of female reproductive tract and uterine carcinogenesis in Donryu rats. *J Reprod Dev*. 2004; 50, 349–360.
31. Patisaul HB, Sullivan AW, Radford ME, *et al*. Anxiogenic effects of developmental bisphenol A exposure are associated with gene expression changes in the juvenile rat amygdala and mitigated by soy. *PLoS One*. 2012; 7, e43890.
32. Weinstock M. Prenatal stressors in rodents: effects on behavior. *Neurobiol Stress*. 2017; 6, 3–13.
33. Mueller BR, Bale TL. Impact of prenatal stress on long term body weight is dependent on timing and maternal sensitivity. *Physiol Behav*. 2006; 88, 605–614.
34. Hohwu L, Li J, Olsen J, Sorensen TI, Obel C. Severe maternal stress exposure due to bereavement before, during and after pregnancy and risk of overweight and obesity in young adult men: a Danish National Cohort Study. *PLoS One*. 2014; 9, e97490.
35. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983; 65, 55–63.
36. Desai M, Guang H, Ferelli M, Kallichanda N, Lane RH. Programmed upregulation of adipogenic transcription factors in intrauterine growth-restricted offspring. *Reprod Sci*. 2008; 15, 785–796.
37. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. Human exposure to bisphenol A (BPA). *Reprod Toxicol*. 2007; 24, 139–177.
38. Kosarac I, Kubwabo C, Lalonde K, Foster W. A novel method for the quantitative determination of free and conjugated bisphenol A in human maternal and umbilical cord blood serum using a two-step solid phase extraction and gas chromatography/tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2012; 898, 90–94.
39. Padmanabhan V, Siefert K, Ransom S, *et al*. Maternal bisphenol-A levels at delivery: a looming problem? *J Perinatol*. 2008; 28, 258–263.
40. Welshons WV, Nagel SC, vom Saal FS. Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. *Endocrinology*. 2006; 147(6 Suppl.), S56–S69.
41. Harley KG, Aguilar SR, Chevrier J, *et al*. Prenatal and postnatal bisphenol A exposure and body mass index in childhood in the CHAMACOS cohort. *Environ Health Perspect*. 2013; 121, 514–520.
42. Rochester JR. Bisphenol A and human health: a review of the literature. *Reprod Toxicol*. 2013; 42, 132–155.
43. van Esterik JC, Dolle ME, Lamoree MH, *et al*. Programming of metabolic effects in C57BL/6JxFVB mice by exposure to bisphenol A during gestation and lactation. *Toxicology*. 2014; 321, 40–52.
44. Wei J, Lin Y, Li Y, *et al*. Perinatal exposure to bisphenol A at reference dose predisposes offspring to metabolic syndrome in adult rats on a high-fat diet. *Endocrinology*. 2011; 152, 3049–3061.
45. Bansal A, Rashid C, Xin F, *et al*. Sex- and dose-specific effects of maternal bisphenol A exposure on pancreatic islets of first- and second-generation adult mice offspring. *Environ Health Perspect*. 2017; 125, 097022.
46. Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM. Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. *Endocr Rev*. 2009; 30, 75–95.
47. Vandenberg LN, Colborn T, Hayes TB, *et al*. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocr Rev*. 2012; 33, 378–455.
48. Melnick R, Lucier G, Wolfe M, *et al*. Summary of the National Toxicology Program's report of the endocrine disruptors low-dose peer review. *Environ Health Perspect*. 2002; 110, 427–431.
49. Diel P, Schmidt S, Vollmer G, *et al*. Comparative responses of three rat strains (DA/Han, Sprague-Dawley and Wistar) to treatment with environmental estrogens. *Arch Toxicol*. 2004; 78, 183–193.
50. Somm E, Schwitzgebel VM, Toulotte A, *et al*. Perinatal exposure to bisphenol a alters early adipogenesis in the rat. *Environ Health Perspect*. 2009; 117, 1549–1555.
51. Gao L, Wang HN, Zhang L, *et al*. Effect of perinatal bisphenol A exposure on serum lipids and lipid enzymes in offspring rats of different sex. *Biomed Environ Sci*. 2016; 29, 686–689.
52. Schneyer A. Getting big on BPA: role for BPA in obesity? *Endocrinology*. 2011; 152, 3301–3303.
53. Wei J, Lin Y, Li Y, *et al*. Perinatal exposure to bisphenol A at reference dose predisposes offspring to metabolic syndrome in adult rats on a high-fat diet. *Endocrinology*. 2011; 152, 3049–3061.
54. Tremblay-Franco M, Cabaton NJ, Canlet C, *et al*. Dynamic metabolic disruption in rats perinatally exposed to low doses of bisphenol-A. *PLoS One*. 2015; 10, e0141698.
55. Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK. Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. *PLoS One*. 2013; 8, e55387.
56. Nagel SC, vom Saal FS, Welshons WV. Developmental effects of estrogenic chemicals are predicted by an in vitro assay incorporating modification of cell uptake by serum. *J Steroid Biochem Mol Biol*. 1999; 69, 343–357.
57. Montano MM, Welshons WV, vom Saal FS. Free estradiol in serum and brain uptake of estradiol during fetal and neonatal sexual differentiation in female rats. *Biol Reprod*. 1995; 53, 1198–1207.
58. Nunez EA, Benassayag C, Savu L, Vallette G, Delorme J. Oestrogen binding function of alpha 1-fetoprotein. *J Steroid Biochem*. 1979; 11, 237–243.
59. Milligan SR, Khan O, Nash M. Competitive binding of xenobiotic oestrogens to rat alpha-fetoprotein and to sex steroid binding proteins in human and rainbow trout (Oncorhynchus mykiss) plasma. *Gen Comp Endocrinol*. 1998; 112, 89–95.
60. Hudak CS, Sul HS. Pref-1, a gatekeeper of adipogenesis. *Front Endocrinol (Lausanne)*. 2013; 4, 79.
61. Wang Y, Hudak C, Sul HS. Role of preadipocyte factor 1 in adipocyte differentiation. *Clin Lipidol*. 2010; 5, 109–115.
62. Wang Y, Sul HS. Pref-1 regulates mesenchymal cell commitment and differentiation through Sox9. *Cell Metab*. 2009; 9, 287–302.
63. Ailhaud G, Amri E, Bardon S, *et al*. The adipocyte: relationships between proliferation and adipose cell differentiation. *Am Rev RespirDis*. 1990; 142 (6 Pt 2), S57–S59.
64. Faust IM, Miller WH Jr, Sclafani A, *et al*. Diet-dependent hyperplastic growth of adipose tissue in hypothalamic obese rats. *Am J Physiol*. 1984; 247(6 Pt 2), R1038–R1046.
65. Miller WH Jr., Faust IM, Hirsch J. Demonstration of de novo production of adipocytes in adult rats by biochemical and radioautographic techniques. *J Lipid Res*. 1984; 25, 336–347.
66. Wabitsch M. The acquisition of obesity: insights from cellular and genetic research. *Proc Nutr Soc*. 2000; 59, 325–330.
67. Masuno H, Kidani T, Sekiya K, *et al*. Bisphenol A in combination with insulin can accelerate the conversion of 3T3-L1 fibroblasts to adipocytes. *J Lipid Res*. 2002; 43, 676–684.
68. Phrakonkham P, Viengchareun S, Belloir C, *et al*. Dietary xenoestrogens differentially impair 3T3-L1 preadipocyte differentiation and persistently affect leptin synthesis. *J Steroid Biochem Mol Biol*. 2008; 110, 95–103.
69. Sargis RM, Johnson DN, Choudhury RA, Brady MJ. Environmental endocrine disruptors promote adipogenesis in the 3T3-L1 cell line

through glucocorticoid receptor activation. *Obesity (SilverSpring)*. 2010; 18, 1283–1288.
70. Boucher JG, Boudreau A, Atlas E. Bisphenol A induces differentiation of human preadipocytes in the absence of glucocorticoid and is inhibited by an estrogen-receptor antagonist. *Nutr Diabetes*. 2014; 4, e102.
71. Valentino R, D'Esposito V, Passaretti F, *et al*. Bisphenol-A impairs insulin action and up-regulates inflammatory pathways in human subcutaneous adipocytes and 3T3-L1 cells. *PLoS One*. 2013; 8, e82099.
72. Lee JE, Ge K. Transcriptional and epigenetic regulation of PPARgamma expression during adipogenesis. *Cell Biosci*. 2014; 4, 29.
73. Bauer RC, Sasaki M, Cohen DM, *et al*. Tribbles-1 regulates hepatic lipogenesis through posttranscriptional regulation of C/EBPalpha. *J Clin Invest*. 2015; 125, 3809–3818.
74. Bastos SL, Kamstra JH, Cenijn PH, *et al*. Effects of endocrine disrupting chemicals on in vitro global DNA methylation and adipocyte differentiation. *Toxicol In Vitro*. 2013; 27, 1634–1643.
75. Kundakovic M, Champagne FA. Epigenetic perspective on the developmental effects of bisphenol A. *Brain Behav Immun*. 2011; 25, 1084–1093.
76. Adamo A, Sese B, Boue S, *et al*. LSD1 regulates the balance between self-renewal and differentiation in human embryonic stem cells. *Nat Cell Biol*. 2011; 13, 652–659.
77. Fujiki K, Kano F, Shiota K, Murata M. Expression of the peroxisome proliferator activated receptor gamma gene is repressed by DNA methylation in visceral adipose tissue of mouse models of diabetes. *BMC Biol*. 2009; 7, 38.
78. Musri MM, Carmona MC, Hanzu FA, *et al*. Histone demethylase LSD1 regulates adipogenesis. *J Biol Chem*. 2010; 285, 30034–30041.
79. Zych J, Stimamiglio MA, Senegaglia AC, *et al*. The epigenetic modifiers 5-aza-2'-deoxycytidine and trichostatin A influence adipocyte differentiation in human mesenchymal stem cells. *Braz J Med Biol Res*. 2013; 46, 405–416.
80. Hervouet E, Vallette FM, Cartron PF. Dnmt3/transcription factor interactions as crucial players in targeted DNA methylation. *Epigenetics*. 2009; 4, 487–499.
81. Strakovsky RS, Wang H, Engeseth NJ, *et al*. Developmental bisphenol A (BPA) exposure leads to sex-specific modification of hepatic gene expression and epigenome at birth that may exacerbate high-fat diet-induced hepatic steatosis. *Toxicol Appl Pharmacol*. 2015; 284, 101–112.
82. Lejonklou MH, Dunder L, Bladin E, *et al*. Effects of low-dose developmental bisphenol A exposure on metabolic parameters and gene expression in male and female fischer 344 rat offspring. *Environ Health Perspect*. 2017; 125, 067018.
83. Kundakovic M, Gudsnuk K, Franks B, *et al*. Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. *Proc Natl Acad Sci USA*. 2013; 110, 9956–9961.
84. Yang TC, Peterson KE, Meeker JD, *et al*. Bisphenol A and phthalates in utero and in childhood: association with child BMI z-score and adiposity. *Environ Res*. 2017; 156, 326–333.
85. Rubin BS, Paranjpe M, DaFonte T, *et al*. Perinatal BPA exposure alters body weight and composition in a dose specific and sex specific manner: the addition of peripubertal exposure exacerbates adverse effects in female mice. *Reprod Toxicol*. 2017; 68, 130–144.
86. Susiarjo M, Sasson I, Mesaros C, Bartolomei MS. Bisphenol a exposure disrupts genomic imprinting in the mouse. *PLoS Genet*. 2013; 9, e1003401.
87. Ding S, Fan Y, Zhao N, *et al*. High-fat diet aggravates glucose homeostasis disorder caused by chronic exposure to bisphenol A. *J Endocrinol*. 2014; 221, 167–179.
88. Perreault L, McCurdy C, Kerege AA, *et al*. Bisphenol A impairs hepatic glucose sensing in C57BL/6 male mice. *PLoS One*. 2013; 8, e69991.
89. Grundy D. Principles and standards for reporting animal experiments in The. *Journal of Physiology and Experimental Physiology*. *Exp Physiol*. 2015; 100, 755–758.

https://doi.org/10.1017/S2040174418000600 Published online by Cambridge University Press