Perinatal Exposure to Bisphenol A Alters Early Adipogenesis in the Rat

Emmanuel Somm, Valérie M. Schwitzgebel, Audrey Toulotte, Christopher R. Cederroth, Christophe Combescure, Serge Nef, Michel L. Aubert, Petra S. Hüppi

doi: 10.1289/ehp.11342 (available at http://dx.doi.org/)
Online 29 June 2009
Perinatal Exposure to Bisphenol A Alters Early Adipogenesis in the Rat

Emmanuel Somm1, Valérie M. Schwitzgebel2, Audrey Toulotte1, Christopher R. Cederroth3, Christophe Combescure4, Serge Nef3, Michel L. Aubert1, Petra S. Hüppi2

1Faculty of Medicine, University of Geneva, Geneva, Switzerland and Department of Pediatrics, Geneva University Hospitals, 1211 Geneva 4, Switzerland

2Department of Pediatrics, Geneva University Hospitals, 1211 Geneva 4, Switzerland

3Department of Genetic Medicine and Development and National Center for Competence in Research - Frontiers in Genetics, Faculty of Medicine, University of Geneva, Geneva, Switzerland

4Department of Clinical Epidemiology, Geneva University Hospitals, 1211 Geneva 4, Switzerland
Address for correspondence:

Dr. E. Somm, Department of Pediatrics, Geneva University Hospitals, 1211 Geneva 4, Switzerland

Phone: (41 22) 382.45.69

Fax: (41 22) 347.59.79

E-mail: emmanuel.somm@medecine.unige.ch

Acknowledgements

This work was supported by the Swiss National Research Program 50 "Endocrine disruptors: relevance to humans, animals and ecosystems" Grants to M.L Aubert and P.S Hüppi.

Competing financial interest statement: All authors declare they have no potential competing financial interest.

Running title: Perinatal Bisphenol A and Early Adipogenesis

Key words: Adipocyte, Adipose tissue, Bisphenol A, Food intake, Obesity

Abbreviations

BAT: Brown adipose tissue
BPA: Bisphenol A
C/EBP-α: CAAT enhancer binding protein alpha
eWAT: epididymal white adipose tissue
FAS: Fatty acid synthase
GLUT4: Glucose transporter 4
HFD: High fat diet
LPL: lipoprotein lipase
PPAR-γ: Peroxisome proliferator-activated receptor gamma
Pref-1: Preadipocyte factor 1

pWAT: Parametrial white adipose tissue

SCD-1: Stearoyl-CoA desaturase 1

SREBP-1C: Sterol regulatory element binding protein-1C

Outline of section headers

ABSTRACT
BACKGROUND.
OBJECTIVES
METHODS
RESULTS
CONCLUSIONS

INTRODUCTION

MATERIALS AND METHODS
Animal care, diets, and BPA exposure before weaning
Animal care, diets, and BPA exposure after weaning
Histological examination of adipose tissue
RNA preparation and mRNA quantification
Plasma measurements
Glucose tolerance test
Statistics

RESULTS
BPA exposure did not alter maternal physiology during gestation
Prenatal BPA exposure altered body weight of offspring at birth but did not affect sex ratio or litter size
Perinatal BPA exposure altered body weight and fat pad weights at weaning specifically in females
Perinatal BPA exposure increased the expression of genes involved in adipogenesis and lipogenesis in adipose tissue at weaning
Perinatal BPA exposure increased expression of gene involved in lipogenesis in liver at weaning
Perinatal BPA exposure altered post-weaning body weight in a gender-specific manner
Perinatal BPA exposure altered neither post-weaning food intake nor glucose tolerance at adulthood

DISCUSSION

REFERENCES
Figure legends
Figure 1
Figure 2
Figure 3
Figure 4
ABSTRACT

BACKGROUND: The causes of the current obesity pandemic have not been fully elucidated. Implication of environmental endocrine disruptors such as Bisphenol A (BPA) on adipose tissue development has been poorly investigated.

OBJECTIVES: The aim of the present study is to evaluate the effects of perinatal exposure to BPA on early adipose storage at weaning.

METHODS: Pregnant Sprague-Dawley rats had access to drinking water containing 1 mg/L of BPA starting on day 6 of gestation until the end of lactation at postnatal day 21 (PND21). At weaning (PND21), perigonadal adipose tissue of pups was studied (weight/histology/gene expression). Thereafter, body weight (bw) and food intake, on chow or a high fat diet, were measured during their growth.

RESULTS: Gestational exposure to BPA did not alter offspring’s sex ratio or litter size at birth. At PND1, the weight of male and female BPA-exposed pups was increased. At PND21, bw was increased only in females, which parametrial white adipose tissue (pWAT) weight was increased about 3-fold. This excess of pWAT was associated with adipocyte hypertrophy and overexpression of lipogenic genes such as C/EBP-α, PPAR-γ, SREBP-1C, LPL, FAS, SCD-1. In addition, SREBP-1C, FAS and ACC gene expression was also increased in the liver from BPA-exposed females at PND21, without a change in circulating lipids and glucose. After weaning, perinatal BPA exposure predisposed to overweight in a sex- and diet-dependent manner. No change in food intake due to perinatal BPA exposure was observed during growth either on chow or a high fat diet.

CONCLUSIONS: Perinatal exposure to a low dose of BPA increased adipogenesis in females at weaning. Adult bw may be programmed during early life, leading to changes dependant on the gender and the nutritional status. Whereas further studies are required to understand the mechanisms of BPA-action during early life, these results are particularly important with
regard to the increasing prevalence of childhood obesity and the context-dependant action of endocrine disruptors.
INTRODUCTION

Bisphenol A (BPA) is a chemical compound found in plastic products. It is increasingly being identified as a pervasive industrial pollutant as well. In fact, accumulating evidences indicate that since the human population is widely exposed to BPA through polycarbonate plastics, resins, and sealants (Brotons et al. 1995; Olea et al. 1996), environmental BPA is part of the endocrine-disrupting chemicals that can potentially affect human health (Kang et al. 2006; vom Saal and Hughes 2005; vom Saal et al. 2005). Importantly, previous studies have shown that BPA has been detected in serum of pregnant women as well as in cord serum taken at birth (Ikezuki et al. 2002; Takahashi and Oishi 2000) and that BPA accumulates early in fetuses (Schonfelder et al. 2002b). Moreover, BPA content was found to be higher in amniotic fluid and placenta compared with maternal serum (Ikezuki et al. 2002; Takahashi and Oishi 2000). In utero exposure to BPA has been shown to cause adverse effects such as:

- Accelerated puberty and increased body weight (bw) in female mice when gestating dams were injected with BPA (2 and 20 µg/kg) (Honma et al. 2002),
- Alterations in rodent mammary gland when dams were implanted with osmotic pumps delivering 25 to 250 µg/kg/day (Markey et al. 2001),
- Abnormal female genital tract development when dams were exposed to BPA through gavage (Schonfelder et al. 2002a), or osmotic pumps (Markey et al. 2005),
- Altered structure and function of the ventral prostate when dams were exposed to BPA through osmotic pump dose levels between 25 and 250 µg/kg/day (Ramos et al. 2001).

Mechanistically, BPA is a well-known estrogenic compound that binds to the estrogen receptor \( \text{ER}_\alpha \) and \( \text{ER}_\beta \) (Hiroi et al. 1999; Kurosawa et al. 2002). BPA can also act as an antagonist of the thyroid hormone receptor (Moriyama et al. 2002; Zoeller et al. 2005), and targets the protein disulfide isomerase (PDI), a multifunctional protein critically involved in
the folding, assembly, and shedding of many cellular proteins (Hiroi et al. 2006; Okada et al. 2005).

The first evidence that perinatal BPA-exposure could lead to altered metabolic features was provided by Rubin and colleagues, who found that perinatal exposure to BPA in Sprague-Dawley rats resulted in an increase in bw apparent soon after birth and continued into adulthood (Rubin et al. 2001). In addition, numerous in vitro studies have shown metabolic actions of BPA on adipocyte cell lines. First, BPA interferes with glucose homeostasis since BPA treatment enhanced basal and insulin-stimulated glucose uptake in 3T3-F442A adipocytes due to an increased amount of GLUT4 protein (Sakurai et al. 2004). Second, BPA triggers adipocyte differentiation: the confluent cultures of 3T3-L1 fibroblasts treated with BPA presented an increase in triglyceride content, lipoprotein lipase activity, and glycerol phosphate dehydrogenase activity, suggesting that BPA by itself can promote 3T3-L1 fibroblasts to differentiate into adipocytes (Masuno et al. 2002). 3T3-L1 cells treated with BPA also show increased levels of lipoprotein lipase and adipocyte-specific fatty acid binding protein (aP2) mRNAs, confirming that BPA is able to accelerate the terminal differentiation of 3T3-L1 cells into adipocytes (Masuno et al. 2005). In order to determine whether perinatal exposure to BPA could have an impact on adipogenesis in vivo, thus contributing to the partially unexplained increased prevalence of the metabolic syndrome in industrialized countries, we studied adipose tissue deposition and its profile of gene expression at weaning (PND21). We also monitored food intake and body weight during growth in perinatally BPA-exposed animals fed with a standard chow or a high fat diet (HFD) after weaning.
MATERIALS AND METHODS

Animal care, diets, and BPA exposure before weaning

All animals used in this study were treated humanely and with regard for alleviation of suffering. All experimental protocols were approved by the “State of Geneva Veterinary Office” (Geneva, Switzerland). Virgin female and male genitor Sprague-Dawley rats were purchased from Taconic Europe (Laven, Denmark) and provided with a rodent experimental diet (KLIBA NAFAG 3250, PROVIMI KLIBA, Kaiseraugst, Switzerland) low in phytoestrogens, with genistein content below the detection limit, as stated by the provider. Animals were given this diet 10 days before mating until the end of gestation. Day 0 of gestation was declared the day sperm-positive smears were obtained. Pregnant rats were housed individually under standard conditions (22°C, 12 hr light–dark cycle), with free access to food and water. Gestating females were exposed to BPA at concentrations of 1 mg/L beginning on day 6 of pregnancy until the end of lactation (PND21 of offspring), as previously described (Rubin et al. 2001), to mimic the most likely route of human exposure. Control females were provided with water containing 1% of ethanol used as a vehicle for the BPA solution. Based on the measurements of the volume reduction in the bottle, not accounting for possible leakage, evaporation, or spillage, we estimated the mean levels of BPA consumed daily by pregnant females at the end of gestation to be approximately 70 μg of BPA/kg/day. Assuming that all water lost from the bottle was consumed, the estimates of the level of BPA exposure may be somewhat higher than actual exposure levels. Water bottles and cages made of polypropylene utilized for all these studies were devoid of BPA and analog compounds to avoid potential contamination from sources other than administered drinking water. Tap water locally distributed in our animal facilities is not tested for BPA content, but independent organization controlling the quality of water in Lake Geneva (from which water
originates before physicochemical treatment) evaluated its concentration as approximately 7 ng/L (2005 report from the "commission internationale pour la protection des eaux du lac Léman"). After the complex process of filtration, we consider that the residual BPA content in tap water is too low to interfere with the dose studied. Before weaning, maternal food intake and volume drunk as well as bw gain were measured daily. Body weight of offspring were measured at PND1 (at the same time as determination of sex ratio and number of pups per litter) and at PND21.

**Animal care and diets after weaning**

At weaning (PND21), and 2 hours after maternal separation, one cohort of both perinatally BPA-exposed and control animals (n=10 animals of each sex in each group, belonging to three different litters of each group) was anesthetized with isoflurane. After measurement of anogenital distance and bw, animals were sacrificed by decapitation. Truncal blood was collected, centrifuged, and plasma was immediately frozen for later dosage. Epididymal white adipose tissue (eWAT) was removed from areas surrounding the epididymis and testis, while parametrial white adipose tissue (pWAT) was collected from homologous areas surrounding the uterus. Brown adipose tissue was collected from the interscapular region and separated from the attached white adipose tissue. These fat depots, in addition to the liver, were weighed before being frozen in liquid nitrogen and stored at −80°C for later molecular analysis. Adipose depots were selected and dissected using previously described protocols (Ailhaud 2001). A second cohort of both perinatally BPA-exposed and control animals was studied during the post-weaning period. These animals were switched to normal drinking water and were fed *ad libitum* either with a standard chow diet (7% of the calories from fat, 76% from carbohydrates, and 17% from proteins) or with a high fat diet (40% of the calories from fat, 43% from carbohydrates, and 17% from proteins). Metabolizable energy of standard chow
and high fat diet were 2.6 and 4.3 kcal/g, respectively, as described by the manufacturer (PROVIMI KLIBA, Kaiseraugst, Switzerland). Body weight was measured weekly from weeks 4 to 14. Food intake was estimated by the weight of solid pellets placed on grids on top of the cages which was measured weekly from weeks 8 to 14. Values were estimated accurate since visually no pieces of pellet or powder were detected in the bottom of the cages.

**Histological examination of adipose tissue**

Parametrial white adipose tissue (pWAT) of BPA-exposed and control 21 days old rats was fixed in a paraformaldehyde solution for 24 hours and embedded in paraffin. Sections (5 μm) were cut using a microtome (Leica Microsystems, Wetzlar, Germany) before staining with hematoxylin/eosin (H&E). Photographs were taken using an Axiocam camera (Carl Zeiss, Gottingen, Germany).

**RNA preparation and mRNA quantification**

As previously described, adipose tissue depots and liver were carefully dissected and weighed before being frozen in liquid nitrogen and stored at −80°C. Total RNA was subsequently extracted using the Trizol reagent (Invitrogen, Basel, Switzerland), according to the manufacturer’s instructions. RNA quality was assessed using Agilent RNA 6000 Nanokit with an Agilent 2100 Bioanalyzer. Samples with inadequate coefficients of purity were excluded from the subsequent analyses (reverse transcription and real-time PCR). Five micrograms of total RNA were reverse-transcribed using 800 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen, Basel, Switzerland), in the presence of 0.3 units/μL RNAsin (Promega Corp, Madison, WI, USA), 7.5 μM of random primers (oligo(dN)6), 1.2 mM dNTP, and 12 μM of DTT. The relative expression of transcripts coding for rat C/EBP-α, PPAR-γ, SREBP-1C, GATA-2, Pref-1, LPL, ACC, FAS, PPAR-α, PGC-1α, SCD-1, GLUT4 were
determined by quantitative real-time PCR using an ABI 7000 Sequence Detection System (Applied Biosystems, Applera Europe, Rotkreuz, Switzerland), and were normalized using the ribosomal housekeeping gene 36B4. PCR products were quantified using the SYBR Green Core Reagent kit (Applied Biosystems, Applera Europe, Rotkreuz, Switzerland). Primers were designed using the Primer Express software (Applied Biosystems, Applera Europe, Rotkreuz, Switzerland) and tested for efficiency prior use. The sequences of the primers used are listed below:

C/EBP-α: F: AGTTGACCAGTGACAATGACCG, R: TCAGGCAGCTGGCGGAAGAT
PPAR-γ: F: CTGACCCCAATGGTTGCTGATTAC, R: GGACGCAGGCTCTACTTTGATC
SREBP-1C: F: CATCGACTACATCCGCTTTTCTTACA, R:
GTCTTTCACTGATTTGCTTTTGTGA
GATA-2: F: AATCGGCGCCTCATCAAG, R: TCGTCTGACAATTGACACAACA
Pref-1: F: CTGCAGTACCCCATTTTCT, R: TTCCCGGCTTTGTCACA
LPL: F: ACAGTCTTTGGAGCCCATGCT, R: AGCCAGTAATTCTATCGACCTTCTTG
ACC: F: TCCCGGAGCTACTCTTTAAAATG, R: CCCCACGCCACCATG
FAS: F: CTCTGGAAGTGCATGCTGTAAGA; R: GGTAGATGTACATCGAAAGGT
SCD-1: F: CAACACCATGGCCATTTCCA, R: GCGTGTGTCTCAGAAGACTTGT
GLUT4: F: ACTTCTTCGGAGGTTCCCT, R: CTCCCACATAACATAGGCAACCA
PPAR-α: F: TGGAGTCCACGATGTGAAG, R: CCGCAGTCTTTAGCCGAATAG
PGC-1α: F: CTGCATTGTATAGACCGAGAA, R: AGGAGCTCTTTTGTGGCTTTT
36B4: F: TTCCCACTGCGAAAAGGT, R: CGCAGCCGCAAATGC
**Plasma measurements**

Plasma glucose was measured with the glucose oxidase method (Glu, Roche Diagnostics GmbH, Rotkreuz, Switzerland). Plasma non-esterified fatty acid and triglyceride levels were determined using Wako Chemicals GmbH (Neuss, Germany) and Biomérieux (Marcy l’Etoile, France) kits, respectively. Plasma cholesterol levels were measured as previously described (James and Pometta 1990).

**Glucose tolerance test**

For the glucose tolerance tests, 14-week-old male rats, perinatally exposed to BPA, and controls, both fed with a high fat diet since weaning, were fasted from the previous day and injected intraperitoneally (i.p.) with 1.5 mg glucose per gram of body weight. Blood samples were collected by tail puncture before and at selected time points (0, 15, 30, 60, and 120 min) after glucose administration, in order to measure glycemia using a glucose meter (Glucotrend Premium, Roche Diagnostics, Rotkreuz, Switzerland).

**Statistics**

Results are expressed as mean ± SEM. The unpaired Student’s t-test, performed with SYSTAT 10.01 (SPSS, Chicago, IL, USA), was used when appropriate for comparison between groups of rats. A p value of < 0.05 was considered statistically significant. The statistical analysis were performed using litter as the fundamental unit of comparison when appropriate and animals originated from at least three different litters in each group (eight litters for each group for data at birth). To specifically assess the significance of the difference in birth and weaning weight between the control and BPA groups, we also used a regression model taking the correlation between the pups from the same litter into account (Generalized Estimating Equation, GEE, with an exchangeable structure for the working correlation matrix). The factors introduced in the model were the group, the sex, the litter size and the interaction between the sex and group.
RESULTS

BPA exposure did not alter maternal physiology during gestation

To evaluate whether BPA exposure during gestation affected maternal physiology, we measured weight gain, food and water intake of exposed dams during pregnancy. The weight gain throughout the entire pregnancy did not change in the BPA-exposed dams (135 ± 7 g) when compared to control dams (140 ± 6 g; p=0.56). In the same way, total food intake, was similar in BPA-exposed dams (431 ± 15 g) and control dams (428 ± 16 g; p=0.87) between the start of BPA exposure (day 6 of gestation) and parturition. The daily volume drunk was unaltered by absence or presence of BPA in the water, whatever the stage of gestation considered. In fact, at gestational day 7 (G7), control dams drank 24 ± 1 mL and BPA dams drank 25 ± 1 mL (p=0.76). At G14, both control and BPA dams drank 26 ± 1 mL (p=0.83) and at G21, control dams drank 29 ± 2 mL and BPA dams drank 30 ± 2 mL (p=0.88). Based on these measurements, we estimated the mean levels of BPA consumed daily by pregnant females from the drinking water to be at approximately 30 μg of BPA/dams at the end of gestation. Assuming that gestating dams weighed approximately 428 ± 11 g before parturition, we estimated the total BPA exposure around 70 μg/kg/day at the end of gestation. It should be taken into consideration that bottle leakage, evaporation, or spillage could result in actual BPA exposure being lower than our calculated levels which represent a maximal estimation.

Prenatal BPA exposure altered body weight of offspring at birth but did not affect sex ratio or litter size

On day 1, body weight (bw) of male pups born to BPA-exposed dams was significantly increased (7.33 ± 0.12 g, n=45 from 8 litters) when compared to those of male pups born to control dams (6.91 ± 0.15 g, n=55 from 8 litters, p < 0.05). In the same way, the day 1 body
weight of female pups born to BPA-exposed dams was higher (7.03 ± 0.11 g, n=50 from 8 litters) than those born to control dams (6.47 ± 0.12 g, n=47 from 8 litters, p < 0.001) (Figure 1A and 1B). Despite the fact that the number of pups per litter is not statistically different in the BPA group (12.7 ± 1.0, n=8 litters) compared to the control group (14.1 ± 0.9; n=8 litters, p=0.16), the elevation of body weight in pups prenatally exposed to BPA could be an indirect effect of BPA via a slight reduction of litter size. However, some arguments refute this hypothesis. First, when only size-matched litters are used for analysis (n=13.5 and 14 animals per litter in control and BPA group, respectively), body weight of day 1 male and female pups born to BPA-exposed dams remained heavier than those of controls (7.01 ± 0.08 g for BPA, n=54 animals from 4 litters vs 6.44 ± 0.08 g for controls, n=56 animals from 4 litters; p<0.001) (data not shown). Moreover, when a regression model, adjusted on the litter size, is used to analyse these data (Generalized estimating equation, GEE), the day 1 bw of BPA-exposed animals remains higher, both for male (p<0.001) and female (p<0.001). Taken together, these observations argue in favour of a direct effect of BPA on new born body weight, independently of litter size. Finally, we observed that the sex ratio of born pups was not altered by prenatal BPA exposure: the proportion of males per litter was similar in control (53 ± 4%) and in BPA-exposed animals (50 ± 4%; p=0.31).

Perinatal BPA exposure altered body weight and fat pad weights at weaning specifically in females

Since offspring remained exposed to BPA through lactation, we repeated the body weight measurements of these animals at the end of lactating period. At weaning (PND21), the body weight of BPA-exposed male rats (53.36 ± 1.02 g, n=32) was not different to controls (52.54 ± 1.11 g, n=40; p=0.59) (Figure 1C). On the contrary, the body weight of BPA-exposed female rats remained higher (53.73 ± 0.65 g, n=32) than that of controls (47.79 ± 1.44 g, n=26; p<0.001) (Figure 1D). When the GEE regression model was used to analyse these data,
the interaction between sex and group was significant (p=0.02) and for females, the BPA-induced elevation of bw was statistically significant after adjustment on the litter size (p=0.001).

To study the potential impact of BPA exposure on body composition, we sacrificed a fraction of these animals at weaning (PND21) and weighed two different fat pads: interscapular brown adipose tissue (BAT) and perigonadal white adipose tissue (WAT), also named epididymal WAT in male (eWAT) and parametrial WAT (pWAT) in female. Interestingly, we found an increased trend of eWAT mass in male pups exposed to BPA (56 ± 6 mg) compared to controls (39 ± 6 mg; p=0.06), whereas this reached significance in BPA-exposed female pWAT (95 ± 9 mg) in comparison to controls (33 ± 6 mg; p<0.001) (Figure 2A and 2B). BAT weight was unchanged in males (Figure 2A) but was significantly heavier in females exposed to BPA (178 ± 13 mg) when compared to controls (116 ± 11 mg; p=0.002) (Figure 2B). Moreover, in females, BAT weight correlated with pWAT weight (r²=0.74, p<0.001) (Figure 2C). These observations suggest that the increase in bw in BPA-exposed females is, in part, related to an increase in adipose depot weight. In contrast, no change was observed in weight (relative to bw) of others organs such as heart, liver, or kidney (data not shown). Morphologically, no difference was observed in male anogenital distance (AGD) (14.8 ± 0.5 mm for controls vs 16.1 ± 0.7 mm for BPA animals; p=0.18) but a slight increase was observed in female AGD (8.45 ± 0.4 mm for controls vs 10 ± 0.3 mm for BPA animals; p<0.01) (data not shown). Overall, these results suggest that females tend to be more sensitive than males to the BPA-induced fat gain at the dose tested.

**Perinatal BPA exposure increased the expression of genes involved in adipogenesis and lipogenesis in adipose tissue at weaning**

To better characterize the early increase in white adipose tissue deposition observed in perinatally BPA-exposed females pups, we performed histologic sections of their pWAT. As shown in representative photographs of these tissues (Figure 2D), adipocytes of rats perinatally exposed to BPA appeared to be hypertrophied in comparison to those of control animals. These histologic observations correlated with changes in the expression of genes involved in metabolism. Messenger RNA levels of proadipogenic transcription factors such as
CAAT/enhancer binding protein alpha (C/EBP-α), peroxisome proliferator-activated receptor gamma (PPAR-γ) or sterol regulatory element binding protein-1C (SREBP-1C) were significantly increased (respectively by 1.87 ± 0.18 fold; p<0.001, 2.02 ± 0.17 fold; p<0.001, and 1.97 ± 0.23 fold; p<0.002) in pWAT of BPA-exposed females at weaning (Figure 2E). In contrast, mRNA levels of inhibitor of adipogenesis such as GATA-2 or preadipocyte factor 1 (Pref-1) remained unchanged (Figure 2E). Gene expression levels of lipogenic enzymes such as lipoprotein lipase (LPL), fatty acid synthase (FAS), and stearoyl-CoA desaturase 1 (SCD-1) were upregulated (respectively by 1.28 ± 0.09 fold; p<0.05, 2.55 ± 0.63 fold; p<0.05, and 3.10 ± 0.73 fold; p<0.05) in pWAT of BPA-exposed females (Figure 2F). mRNA levels of the glucose transporter 4 (GLUT4) were also increased by 1.59 ± 0.25 fold (p<0.05; data not shown).

Taken together, these findings strongly support that perinatal exposure to BPA enhances adipogenesis and lipogenesis \textit{in vivo} through an increase of the transcription of genes implicated in adipocyte metabolism.

**Perinatal BPA exposure increased expression of gene involved in lipogenesis in liver at weaning**

Liver is also a key organ involved in metabolism since it controls synthesis of many nutrients including lipids and carbohydrates. We measured hepatic change in expression of several metabolic genes. Messenger RNA levels of SREBP-1C were also significantly increased by 2.07 ± 0.47 fold (p<0.05) in the liver of BPA-exposed females at weaning (Figure 3A). In contrast, no change was observed in gene expression of both transcription factors peroxisome proliferator activated receptor-alpha (PPAR-α) and PPAR-gamma-coactivator 1 alpha (PGC1-α), respectively involved in fatty acid oxidation and gluconeogenesis in this organ. mRNA levels of prolipogenic enzymes such as acetyl-CoA carboxylase (ACC) and fatty acid
synthase (FAS) were also upregulated respectively by 2.17 ± 0.43 fold (p<0.02) and 4.94 ± 1.33 fold (p<0.01) in the liver of BPA-exposed females (Figure 3A). To assess whether these early alterations in gene expression were related to changes in circulating levels of nutrients, we measured the levels of triglycerides (TG), non-esterified fatty acid (NEFA), glucose and total cholesterol in female pups at weaning. No statistical difference was detected in circulating TG (control, 123.2 ± 12.1 mg/dL; BPA 118.8 ± 9.6 mg/dL, p=0.84) (Figure 3B), NEFA (control, 0.69 ± 0.04 mmol/L; BPA 0.80 ± 0.06 mmol/L, p=0.18) (Figure 3C), cholesterol (control, 1.45 ± 0.05 g/L; BPA 1.36 ± 0.02 g/L, p=0.13) (Figure 3D), and glucose levels (control, 135.1 ± 3.3 mg/dL; BPA 140.6 ± 4.5 mg/dL, p=0.29) (Figure 3E).

**Perinatal BPA exposure altered post-weaning body weight in a gender-specific manner**

After weaning, we measured body weight of perinatally BPA-exposed animals from 4 weeks to 14 weeks of age. Concerning males, no statistical difference in body weight curve was observed between perinatally BPA-exposed animals and control animals on standard chow diet (Figure 4A). However, perinatally BPA-exposed male were heavier than control males when fed a high fat diet and this difference reached 7 ± 2% of body weight at 14 weeks of age (p<0.05) (Figure 4B), suggesting that a long-term effect of BPA exists in males only when combined with a higher caloric intake. In contrast, differences in body weight curves were observed for females between perinatally BPA-exposed animals and control animals on both standard chow and high fat diets (Figure 4C and Figure 4D). Overall, these results suggest that perinatal exposure to BPA increases the susceptibility to gain weight in a gender-specific manner, even after weaning.
Perinatal BPA exposure altered neither post-weaning food intake nor glucose tolerance at adulthood

To evaluate whether differences in bw observed after perinatal BPA exposure are related to altered appetite regulation, we measured food intake of perinatally BPA-exposed and control rats on the standard chow or the high fat diet from weaning onwards. During the 7 consecutive weeks of measurement, despite the known caloric hyperphagia induced by the high fat diet in all groups, no impact of perinatal BPA exposure on daily food intake and cumulative energy intake was detected in males and females, either eating the chow diet or the high fat diet. Perinatally BPA-exposed males on chow diet ingested $3331 \pm 116$ kcal (versus $3308 \pm 54$ kcal for controls, $p=0.85$) whereas perinatally BPA-exposed males on high fat diet, absorbed $3931 \pm 52$ kcal (versus $3854 \pm 111$ kcal for controls, $p=0.57$) (data not shown). Likewise, perinatally BPA-exposed females on chow diet ingested $2358 \pm 58$ kcal (versus $2251 \pm 7$ kcal for controls, $p=0.15$) whereas perinatally BPA-exposed females on high fat diet absorbed $2655 \pm 81$ kcal (versus $2510 \pm 31$ kcal for controls, $p=0.20$) (data not shown). In conclusion, perinatal exposure to BPA did not affect post-weaning food intake during growth, either in standard conditions or on a high caloric diet.

In order to determine whether the difference in body weight in males fed a high fat diet due to perinatal exposure to BPA displayed an impact on glucose homeostasis, we performed glucose tolerance tests in these two groups of animals. Basal glucose levels after 16 hours of fasting were similar in control rats ($4.63 \pm 0.10$ mmol/L) and BPA-exposed rats ($4.65 \pm 0.08$ mmol/L, $p=0.88$). After i.p. administration of glucose, the circulating glucose clearance was similar in BPA and control rats (data not shown), reflecting no disturbance in glucose utilization. This was confirmed by measuring the area under the curve (AUC) which is equivalent in both BPA rats ($1312 \pm 89$ mmol/L*min) and control animals ($1319 \pm 97$ mmol/L*min).
mmol/L*min, p=0.96) (data not shown). These results suggest that perinatal exposure to BPA has no long-term effects on glucose metabolism in the male rat placed on a high fat diet.
DISCUSSION

Numerous studies have investigated the long-term impact of early BPA exposure during stages of tissue organization on the male reproductive tract (Prins et al. 2006; Watanabe et al. 2003), the female reproductive tract (Suzuki et al. 2002; Vandenberg et al. 2007), as well as on behavior (Aloisi et al. 2002; Della Seta et al. 2005; Dessi-Fulgheri et al. 2002; Farabollini et al. 2002; Fujimoto et al. 2006; Porrini et al. 2005). However, until recently, little data on the metabolic effects of perinatal BPA exposure were available. The doses we tested (1 mg/L in drinking bottle, corresponding to a maximal 70 μg of BPA/kg/day exposure at the end of gestation based on water consumed) as well as the oral route of exposure chosen (similar to exposure through plastic and resin in contact with food) render this model relevant for humans. Despite the fact that this dose cannot be considered as truly environmentally relevant, it can be considered as low (Chapin et al. 2008; Melnick et al. 2002).

First, maternal physiology (assessed by bw gain, food intake, and volume drunk) of the exposed gestating dams was unchanged, as were the sex ratio of pups born and number of pups per litter, as previously reported (Honma et al. 2002; Rubin et al. 2001).

In the present study we also confirmed that the body birth weight of both male and female pups prenatally exposed to BPA were significantly increased. At weaning, after postnatal BPA exposure via milk during lactation, the body weight of BPA-exposed females, but not males, remained higher than controls. These observations are in accordance some previous publications demonstrating increased postnatal growth in different rodent species exposed to maternal BPA doses between 2.4 and 500 μg/kg/day (Howdeshell et al. 1999; Nikaido et al. 2004; Rubin et al. 2001; Takai et al. 2001). However, our present data are in contradiction with others works reporting no alteration of body weight in pups exposed to a very wide range of maternal BPA doses, between 0.001 and 5 mg/kg/day (Tyl et al. 2002; Tyl et al. 2008). Conversely, in these toxicity studies, higher doses of BPA administrated to gestating dams
(between 50 and 600 mg/kg/day) reduced body weight in growing Sprague-Dawley rats and CD-1 mice pups (Tyl et al. 2002; Tyl et al. 2008).

In our study, we observed that the increase in offspring bw due to BPA exposure through placenta and milk was associated with an increase in the early adipose storage at weaning in a gender-dependent manner. Epididymal WAT demonstrated a trend to increase in males and parametrial WAT was increased by nearly 3-fold in females due to BPA exposure. It is interesting to note that our present observations in Sprague-Dawley rats are in agreement with a recent report in which gonadal fat pads were heavier in ICR female mice exposed continuously until 30 days of life to the same dose of BPA as we used (Miyawaki et al. 2007). In the same study (Miyawaki et al. 2007), it should be noted that a 10-fold higher dose was inefficient in inducing overstorage of fat in females but was efficient in increasing fat storage in males. This leads the authors to conclude that BPA caused a nonmonotonic and inverted-U-shape dose response increase in adipose tissue weight in females. Gender-dependent susceptibility to xenoestrogens are highly specific for a given window of exposure and the kind of xenoestrogens utilized. For example, direct oral exposure to genistein (an isoflavone with estrogenic activities found mostly in soy) in 4-week-old mice increases male but not female fat pad weights (Penza et al. 2006), whereas in utero exposure to genistein protects A(vy/a) mice from obesity in association with methylation changes of the epigenome (Dolinoy et al. 2006). In the same A(vy/a) mouse model, in utero exposure to BPA, at 50 mg/kg, also demonstrates epigenetic effects since it decreases CpG methylation in an intracisternal A particle retrotransposon upstream of the Agouti gene (Dolinoy et al. 2007).

We also illustrate for the first time that parametrial WAT enlargement at weaning in females exposed perinatally to BPA is associated to adipocyte hypertrophy, an expanding tissue process more easily reversible than hyperplasia, which corresponds to an increase in preadipocytes recruitment. We show that change in size of adipocytes in BPA-exposed female
is associated with an overexpression of proadipogenic transcription factors (C/EBP-α, PPAR-γ, SREBP-1C). These \textit{in vivo} observations are in accordance with previous \textit{in vitro} observations since BPA by itself can accelerate the differentiation of 3T3-L1 fibroblasts into adipocytes (Masuno et al. 2002). The gene expression of lipogenic enzymes (LPL, FAS, SCD-1) was also upregulated in the WAT of BPA-exposed females, corroborating with the \textit{in vitro} increased levels of lipoprotein lipase mRNAs of 3T3-L1 cell culture treated with BPA (Masuno et al. 2005). Finally, we observed an increase in mRNA levels of the glucose transporter 4 (GLUT4) in WAT of BPA-exposed females, in agreement with the \textit{in vitro} enhanced glucose uptake observed in 3T3-F442A adipocytes treated with BPA due to an increased amount of GLUT4 protein (Sakurai et al. 2004). The molecular pathways implicated in these proadipogenic effects of BPA are unknown, but it has been suggested that the PI3K/Akt pathway could be involved (Masuno et al. 2005) since (i) BPA increased the level of phosphorylated Akt kinase and (ii) LY294002, a chemical inhibitor of PI3K, completely abolished the enhancing effect of BPA on triglyceride accumulation and expression of prolipogenic mRNAs. We finally show that perinatal BPA exposure is associated with an overexpression of lipogenic transcription factor and enzymes (SREBP-1C, ACC, FAS) in the liver. Again, these \textit{in vivo} observations are in accordance with previous \textit{in vitro} observations in which BPA increases lipogenesis through glycerol accumulation in the HuH-7 hepatocellular cell line (Wada et al. 2007). At PND21, we found no difference in circulating levels of triglyceride, non-esterified fatty acid and glucose. We also failed to detect change in cholesterol levels in perinatally BPA-exposed pups contrary to what was observed in ICR mice (Miyawaki et al. 2007). However, in this previous study, measurements were done at PND30, after consumption of a high fat diet and direct exposure of pups to BPA via drinking bottle.
Taken together, these findings provide the first clear direct evidence of early gene expression alterations in adipose tissue and liver after perinatal BPA exposure, an interesting observation since prevalence of childhood obesity is increasing worldwide (Laron 2004; Micic 2001; Reilly 2005) and some countries, such as Canada, prohibit the use of BPA in babies’ bottles. In our study, animals were only indirectly exposed through placenta and milk and BPA exposure was stopped at weaning. In order to uncover a possible programming effect of BPA that persisted beyond the end of exposure, we investigated possible metabolic disturbances after weaning. First, we observed heavier body weight during growth in females perinatally exposed to BPA, independent of the diet provided (chow or high fat diet), in line with those previously reported by Rubin and al. (Rubin et al. 2001). More surprisingly, BPA males presented increased body weight during growth only when placed on a high fat diet, suggesting that BPA predisposition to overweight is potent only when food is hypercaloric.

A possible explanation of enhanced weight gain in BPA-exposed animals is an increase in food intake, since it is known that estrogens can affect neuronal circuits that control appetite by acting on the hypothalamus (Nunez et al. 1980; Wade and Schneider 1992) and BPA is known to cross the brain–blood barrier based on its detection in the brain (Sun et al. 2002). In this way, we investigated post-weaning food intake in perinatally exposed animals fed chow or a high fat diet. No changes in food intake were observed in male or female animals perinatally exposed to BPA compared to their respective controls. As body weight and energy storage result from a balance between energy intake and energy expenditure, this later parameter could more probably explain the difference of bw observed in response to BPA. In this way, it should be noted that besides its estrogenic activities, it has been shown that BPA binds and antagonizes the thyroid hormone receptor and therefore inhibits transcriptional activity stimulated by triiodothyronine (Moriyama et al. 2002; Zoeller et al. 2005a; Zoeller 2005b). It is conceivable that perinatal BPA exposure disrupts the thyroid hormone axis,
which itself plays a predominant role in thermogenesis and energy expenditure. In this way, it is interesting to note the preferential accumulation of BPA in brown adipose tissue (Nunez et al. 2001), a major mediator of thyroid hormone on thermogenesis. Additional studies are required to better understand the precise role of BPA in energy expenditure and thermogenesis. Finally, growth hormone could also be involved in increased bw gain in relation to perinatal BPA exposure since it has recently been shown that BPA induced growth hormone release in vitro in GH3 pituitary cell line (Okada et al. 2007).

In conclusion, the present study demonstrates that direct exposure to BPA through placenta and milk increases early adipose storage and adipogenesis in a gender-specific manner, confirming previous observations in ICR mice (Miyawaki et al. 2007) and in vitro in 3T3-L1 and 3T3-F442A adipocyte lines (Masuno et al. 2005; Masuno et al. 2002; Sakurai et al. 2004). Increased adipose storage caused by perinatal BPA exposure could be a major public health concern in relation to the “epidemic” of childhood obesity (Laron 2004; Micic 2001; Reilly 2005). Moreover, since exposure to BPA is ubiquitous and does not cease during human life (Brotons et al. 1995), further studies are urgently needed to better understand the long-term consequences of permanent BPA exposure lifelong on body weight homeostasis.
REFERENCES


Takahashi O, Oishi S. 2000. Disposition of orally administered 2,2-Bis(4-hydroxyphenyl)propane (Bisphenol A) in pregnant rats and the placental transfer to fetuses. Environ Health Perspect 108(10): 931-935.


Zoeller RT. 2005b. Environmental chemicals as thyroid hormone analogues: new studies indicate that thyroid hormone receptors are targets of industrial chemicals? Mol Cell Endocrinol 242(1-2): 10-15.
Figure legends:

Figure 1
Increased body weight at PND1 and PND21 in 1 mg/L BPA-exposed pups.
The day 1 body weight of male pups (A) and female pups (B) born to 1 mg/L BPA-exposed
dams (■) and controls dams (□). Results are means ± SEM. *P<0.05 for BPA-exposed males
(n=45 from 8 litters) vs control males (n=55 from 8 litters) and *P<0.001 for BPA-exposed
females (n=50 from 8 litters) vs control females (n=47 from 8 litters). The day 21 body weight
of male pups (C) and female pups (D) born to 1 mg/L BPA-exposed dams (■) and controls
dams (□). P=0.59 for BPA-exposed males (n=32 from 6 litters) vs control males (n=40 from 6
litters) and *P<0.001 for BPA-exposed females (n=32 from 6 litters) vs control females (n=26
from 6 litters).

Figure 2
Increased adipogenesis at weaning (PND21) in females perinatally exposed to BPA.
Epididymal white adipose tissue (eWAT), parametrial white adipose tissue (pWAT), and
interscapular brown adipose tissue (BAT) weight in male (A) and female (B) rats exposed
perinatally to BPA. Results are means ± SEM. *P<0.002 for BAT from BPA-exposed females
(n=10 animals from 3 litters) vs BAT from control females (n=10 animals from 3 litters).
*P<0.001 for pWAT from BPA-exposed females (n=10 animals from 3 litters) vs pWAT from
control females (n=10 animals from 3 litters). Correlation between pWAT and BAT weight in
female of both control (○) and BPA (●) groups (n=20 animals, \( r^2=0.74 \), *P<0.001) (C).
Representative histological sections of pWAT from control (upper panel) and BPA-exposed
females (lower panel) (D). Relative mRNA levels, in arbitrary units (A.U), in pWAT of BPA-
exposed (■) and control (□) female rats at weaning (n=9–10 animals in each group from 3
litters) (E and F). Results are means ± SEM. *P<0.001 for C/EBP-α and PPAR-γ, *P<0.002
for SREBP-1C, *P<0.05 for LPL, FAS, and SCD-1 in pWAT from BPA-exposed females vs
pWAT from control females.
Figure 3
Altered gene expression in liver but not circulating metabolites at weaning (PND21) in females perinatally exposed to BPA.
Relative mRNA levels, in arbitrary units (A.U), in liver of BPA-exposed (■) and control (□) female rats at weaning (n=10 animals in each group originate from 3 litters) (A). *P<0.05 for SREBP-1C, *P<0.02 for ACC and *P<0.01 for FAS in liver from BPA-exposed females vs liver from control females. Results are means ± SEM. Circulating levels of triglycerides (TG) (B), non-esterified fatty acid (NEFA) (C), total cholesterol (D), and glucose (E) in plasma of BPA-exposed (■) and control (□) female rats at weaning (n=10 animals in each group from 3 litters).

Figure 4
Body weight curves of animals perinatally exposed to BPA and later fed with control chow or a high fat diet.
Body weight curves of control (○) and perinatally BPA-exposed animals (●). Male rats fed with chow diet (CD) starting weaning (A). Male rats fed with a high fat diet (HFD) starting weaning (B). Female rats fed with chow diet (CD) starting weaning (C). Female rats fed with a high fat diet (HFD) starting weaning (D). *P<0.05 compared to their respective controls, n=8–12 animals per group. Note the smallest curve of body weight for left panels A and C (chow diet) compared to right panels B and D (high fat diet).
FIGURE 1

A  Control  BPA

Male body weight (PND1)

B

Female body weight (PND1)

C

Male body weight (PND21)

D

Female body weight (PND21)
FIGURE 2

A

Female adipose tissue (mg)

Male adipose tissue (mg)

B

Female adipose tissue (mg)

Male adipose tissue (mg)

C

pWAT weight (mg)

BAT weight (mg)

D

Control

BPA

E

Relative mRNA expression (A.U)

F

Relative mRNA expression (A.U)

* * **
FIGURE 3

A

![Graph showing relative mRNA expression (A.U) for SREBP-1C, PPAR-\(\alpha\), PGC-1\(\alpha\), ACC, and FAS in Control and BPA groups.]

B

![Graph showing TG (mg/dl) levels in Control and BPA groups.]

C

![Graph showing NEFA (mmol/l) levels in Control and BPA groups.]

D

![Graph showing Cholesterol (g/L) levels in Control and BPA groups.]

E

![Graph showing Glucose (mg/dl) levels in Control and BPA groups.]

* Indicates significant difference compared to Control group.