

Sex- and Strain-dependent Effects of Bisphenol: A Consumption in Juvenile Mice

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Abstract

Children are directly exposed to bisphenol A (BPA) and other putative environmental obesogens through both diet and household products. The purpose of this study was to investigate sex- and genetic-dependent effects of BPA consumption on traits relevant to obesity and Type 2 diabetes in juveniles, using mice as models. Two strains of mice (C57BL/6J and DBA/2J) were chosen to represent distinct genetic backgrounds that differ in susceptibility to obesity and insulin resistance. Male and female mice of each strain consumed BPA at three doses in drinking water from four to eleven weeks of age. Male but not female mice of each strain consuming BPA were significantly fatter than controls. BPA induced corresponding changes in adipose tissue gene expression and metabolite abundance that indicate effects on adipogenesis and energy utilization. BPA consumption also altered the synthesis of adipokines, circulating factors that modulate insulin sensitivity and atherogenesis, in C57BL/6J but not DBA/2J males. Conversely, effects of BPA on plasma insulin were only observed in female mice, and with opposite effects in the two strains. These results suggest that dietary BPA may exacerbate childhood obesity and its consequences, and that sex and genetic background are important determinants of the physiological impact of BPA.

Keywords: Adipose; Bisphenol; Glucose; Obesity

Introduction

In recent years, the search for non-genetic contributors to the global obesity epidemic has broadened to include environmental chemicals. Data from a variety of compounds have been sufficiently compelling to prompt the term “environmental obesogen”, used to refer to compounds such as endocrine disruptors that can promote an increase in fatness under certain exposure conditions [1-3]. Bisphenol A is an abundant environmental endocrine disruptor and a candidate environmental obesogen [4]. Human exposure to BPA results from its widespread use to harden consumer plastics, including thermal papers, pipes, consumer plastics and many components of the commercial food and beverage stream [5-7]. As a result, approximately 93% of participants in the National Health and Nutrition Examination Survey (NHANES) who were screened for BPA exhibited detectable levels in urine [6,8]. Epidemiological studies based on NHANES as well as other populations globally link BPA exposure to risk of obesity and its consequences. Urinary level of BPA, the primary means for assessing exposure in humans, is positively associated with incidence of obesity [9-11] and with risk for insulin resistance [12-14] and cardiovascular disease [15-17] in both the U.S. and other countries.

Bisphenol A exposure is a particular concern in children, an age group with heightened sensitivity to environmental chemicals [18]. As in adults, BPA is present at measurable levels in urine from the majority of children sampled worldwide [18-21]. Urinary levels of BPA are higher in children than in adults, and in younger children compared to adolescents [18,19,22]. Also like adults, several studies have reported significant associations between urinary BPA and indices of obesity in juveniles. Analyses of cross-sectional data from children included in NHANES from 2003-2010 support positive relationships between urinary BPA and body mass index (BMI), waist circumference, and risk for obesity, even after adjusting for socioeconomic and lifestyle confounds [23-25]. Similar relationships were found in an independent sampling of predominantly low income Mexican-American children, even though their BPA levels were approximately half those of children in NHANES [26]. The primary source of BPA exposure in children

is thought to be diet [27]. Liao et al. [28] measured BPA content in a variety of foodstuffs and then estimated that daily BPA consumption in infants, toddlers and children is more than twice that of adolescents and adults. Consumption of sodas, school lunches and meals prepared outside the home have been statistically associated with elevated urinary BPA levels [29].

Most experimental BPA studies have focused on the effects of exposure in utero or perinatally by administering BPA in the feed or drinking water of the pregnant or lactating mother. BPA was originally suggested as an obesogen by studies showing that rodents exposed to BPA in utero were heavier at birth and into adulthood [30-32]. Some, but not all, follow-on studies have linked early developmental exposure to increased fatness and to impaired glucose tolerance as adults. In utero exposure studies in rodents may be complicated by the fact that litter size, sex composition and positioning within the uterus impact the sex steroid exposure and sensitivity of each individual pup during development, which may in turn alter individual sensitivity to BPA due to its interaction with estrogen receptors [33]. Although children are predicted to consume the highest levels of BPA, and urinary levels in children have been associated with risk of obesity, relatively few experimental studies with animal models have focused on the specific effects of BPA consumption during a period comparable to childhood. Animal studies are important for filling this gap in knowledge because they allow assessment of defined consumption levels with control over external sources of exposure.

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Risks for both obesity and type 2 diabetes are strongly influenced by genetic background in both humans and experimental models. It is therefore likely that the effects of BPA on endpoints related to these two diseases are also sensitive to genetic variation, rendering some individuals in a population more sensitive to the effects of BPA. The purpose of this study was therefore two-fold: 1.) to test the hypothesis that consumption of BPA in juveniles promotes fat accumulation and alters glucose tolerance, and 2.) to determine if these potential effects of BPA are influenced by genetic susceptibility to obesity and insulin resistance. Bisphenol A was delivered at three doses (50 nM, 500 nM, and 5 μ M) in the drinking water of C57BL/6J and DBA/2J mice beginning at four weeks of age, a time of active adipose accumulation but prior to sexual maturation. These two inbred strains were chosen for their differences in genetic susceptibility to obesity and insulin resistance. C57BL/6J mice are prone to diet-induced obesity and glucose intolerance, while DBA/2J are fatter at baseline but more resistant to obesogenic diets and more glucose tolerant than C57BL/6J [33,34]. These two strains were also chosen because they are the parental strains for a set of recombinant inbred lines [35] that could be useful for mapping genetic modifiers of BPA sensitivity, if implicated in our study. Males and females of each strain were included to evaluate sex-specific effects of BPA consumption, which have been reported in both humans and animal models. We report that consumption of BPA during a four-week window in growing mice altered both adiposity and glucose homeostasis in a strain- and sex-dependent manner.

Experimental (Materials and Methods)

Animals

All protocols were approved by the Institutional Animal Care and Use Committee at the University of Tennessee, Knoxville. Male and female C57BL/6J and DBA/2J mice to be used as breeders were obtained from The Jackson Laboratory (Bar Harbor, ME). Ten females and three males of each strain were used to produce the 64 (32 males and 32 females) pups of each strain, selected from nine litters per strain, used in this study. Pups were weaned at 23 days of age and randomized to a treatment group to avoid litter effects. Mice were housed in BPA-free polypropylene cages, and water was provided in glass bottles to limit exposure to BPA or other estrogenic compounds from the environment. Beginning at four weeks of age and continuing until 11 weeks of age, BPA was administered to the mice via drinking water, which was available ad libitum, at concentrations of 50 nM, 500 nM, or 5 μ M. Bisphenol A (Sigma-Aldrich, St. Louis, MO) was dissolved in a glass vial using ethanol distilled in glass tubing to minimize exogenous BPA from commercial plastics. Controls received vehicle (distilled ethanol) at a dose equal to that used for the highest concentration of BPA. Mice were weighed weekly. Glucose tolerance tests were performed at nine weeks of age using a standard protocol for mice (jaxservices.jax.org). At 11 weeks of age, mice were fasted for five hours and sacrificed using CO₂ asphyxiation and cervical dislocation. Perigonadal fat pads were dissected and weighed, and portions allocated for RNA extraction, histology, tissue metabolomics and adipokine measurements. Blood was collected to for measurement of serum insulin, glucose and adiponectin levels.

RNA isolation

Adipose tissue RNA was extracted using the RNeasy™ Lipid Tissue Mini Kit (Qiagen, Inc., Valencia, CA). RNA quality and concentration were assessed using an Experion RNA StdSens Chips on an Experion™ automated electrophoresis system (Bio-Rad, Hercules, CA).

Gene expression profiling

Genome scale gene expression was performed on adipose tissue from male control and 500 nM BPA mice in each strain (n=6/strain*treatment group). Total RNA (500 ng) was amplified, synthesized to cDNA, and hybridized to the MouseWG-6 v2 Expression BeadChip (Illumina, San Diego, CA). Expression profiling was conducted by the McGill University and G enome Qu ebec Innovation Centre (Quebec, CA). Expression of leptin and the housekeeping gene hypoxanthine phosphoribosyl transferase (Hprt) was measured by quantitative real-time PCR (QPCR) using predesigned and validated primer sets and SYBRgreen chemistry (QuantiTect primers and SYBR Green PCR kit, Qiagen, Inc., Valencia, CA) on a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Relative gene expression was determined using the $\Delta\Delta$ Ct method [36].

Adipokine levels in adipose tissue

Concentrations of leptin, resistin, interleukin 6 (IL-6), tumor necrosis factor alpha (TNF α), monocyte chemoattractant protein 1 (MCP-1), and plasminogen activator inhibitor 1 (PAI-1) in adipose tissue homogenates were analyzed in duplicate by multiplexed flow cytometric assay using a mouse adipokine Milliplex® map kit (MADPK-71K, Millipore, Billerica, MA) on a Luminex® 200 system (Luminex Inc., Austin, TX). Adipokine protein levels were measured in pg/ml and normalized to tissue protein, determined using a bicinchoninic acid assay (Thermo Scientific, Waltham, MA).

Serum insulin, glucose and adiponectin levels

Fasting serum insulin levels were measured using a commercially available ultra-sensitive mouse insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL). Glucose was determined using a commercial colorimetric assay (Cayman Chemical, Ann Arbor, MI). Serum adiponectin levels were measured using a commercially available mouse adiponectin enzyme immunoassay kit (SPI-bio Bertin Pharma, Montigny le Bretonneux, France).

Histological analysis of adipocyte size

Adipocyte size was determined by measuring the area of adipocytes in H&E-stained sections of paraformaldehyde-fixed abdominal adipose tissue using ImageJ (NIH; rsbweb.nih.gov/ij/), as we have previously described [37]. Approximately 200 adipocytes per mouse were measured.

Metabolomics

Metabolite abundance was determined in snap-frozen samples (n=4/strain*treatment group) of abdominal adipose tissue, as previously described [37,38]. Only tissue from males (of both strains) was used for metabolomics. Briefly, samples were homogenized in cold methanol (500 μ L at -78°C) and internal standards, tris (5 μ L of 4.7 mM in H₂O) and benzoic acid (5 μ L of 1.7 mM in H₂O) added to each tube. Samples were extracted on ice, transferred to an autosampler vial, and analyzed by LC-MS/MS using selected reaction monitoring (SRM) in each of positive and negative ionization modes by known methods (9,46). Raw files for each sample were first processed using mzRock (<http://code.google.com/p/mzrock/>), and Xcalibur Quan Browser (Thermo Scientific, Waltham, MA) was used to visualize and manually integrate metabolite peaks over a set time window.

Statistical analysis

Statistical analyses were performed in the language R. Effects

of treatment, strain and treatment *strain interactions in males and females on physiological parameters and QPCR data were determined using mixed models using 95% confidence intervals, with significant F-tests followed by least squares means for post-hoc testing. Microarray data were analyzed in R using the Lumi package (Bioconductor.org), controlling false discovery rate using the q-value method of Storey [39]. Metabolomics data were corrected for normality using a logarithmic transformation and analyzed for main effects of treatment using ANOVA in R, controlling for effects of strain.

Results

Effect of BPA on body weight and adiposity

Bisphenol A was delivered at three fixed concentrations (50 nM, 500 nM and 5 μ M) in drinking water to model repeated consumption due to daily food and beverage intake in children. These concentrations of BPA correspond to daily consumption levels of approximately 2.8, 28 and 280 μ g BPA/kg body weight, based on expected daily water intake of 0.2 ml /g body weight/day in mice (informatics.jax.org). This range of doses was chosen to span the EPA tolerable daily intake (TDI) level of <50 μ g/kg bw/day [40]. Strain and dose interacted to exert modest but statistically significant effects on final body weights, measured at 11 weeks of age, in males ($p=0.025$; Figure 1A). The significant interaction was primarily due to differences in weights between the three levels of BPA treatment. For example, DBA/2J males in the 500 nM group were significantly heavier than those in the 50 nM and 5 μ M groups ($p<0.05$). Only the 50 nM dose differed significantly from controls, and only in C57BL/6J. There was no significant effect of BPA on bodyweight in females of either strain (Figure 1B). Weight gain (11 week – 4 week body weight) was calculated to more specifically reflect the effects of BPA consumption in growth during the treatment period (Figures 1C

and 1D). BPA did not alter weight gain in either sex ($p>0.05$). Food intake did not differ between groups (data not shown).

The weight of perigonadal fat pads was used to assess the specific effects of BPA on adipose accumulation during the treatment period. BPA consumption promoted fatness in male but not female mice. Both absolute fat pad weights (Figure 2A) and adiposity (fat pad weight/body weight; Figures 2B and 2C) were significantly increased by BPA in males ($p=0.0095$ and $p=0.0084$, respectively). This response was independent of strain ($p\text{-value}=0.83$, treatment*strain interaction), suggesting that obesogenic effects of BPA are robust to genetic background. The effect of BPA on adiposity was dose-dependent, but not linear, consistent with nonmonotonic effects of BPA that have been described for other phenotypes. In post-hoc analyses, only the 500 nM BPA dose significantly increased fat pad weight ($p=0.022$; Figure 2A) and adiposity ($p=0.036$; Figure 2C). On average, male mice consuming the 500 nM dose of BPA were 16.4% fatter than controls. Males in the 500 nM treatment group were also significantly fatter than those consuming both the lowest ($p=0.0008$) and highest BPA doses ($p=0.022$; Figure 2C). In contrast, BPA did not alter absolute fat pad weight or adiposity in females (Figures 2B and 2D). Adipocyte size was measured in the male control and 500 nM treatment groups to determine if BPA increased fatness by adipocyte hypertrophy (Figure 3). Adipocytes from DBA/2J males were larger than those from C57BL/6J males ($p=0.034$), as expected due to increased adiposity in DBA/2J vs. C57BL/6J males ($p=1.4 \times 10^{-5}$). BPA consumption significantly increased adipocyte size in both C57BL/6J and DBA/2J mice relative to controls ($p=0.0047$), with no significant interactive effects between strain and treatment ($p>0.05$).

Serum insulin, glucose, and glucose tolerance tests

Circulating glucose and insulin levels and glucose tolerance

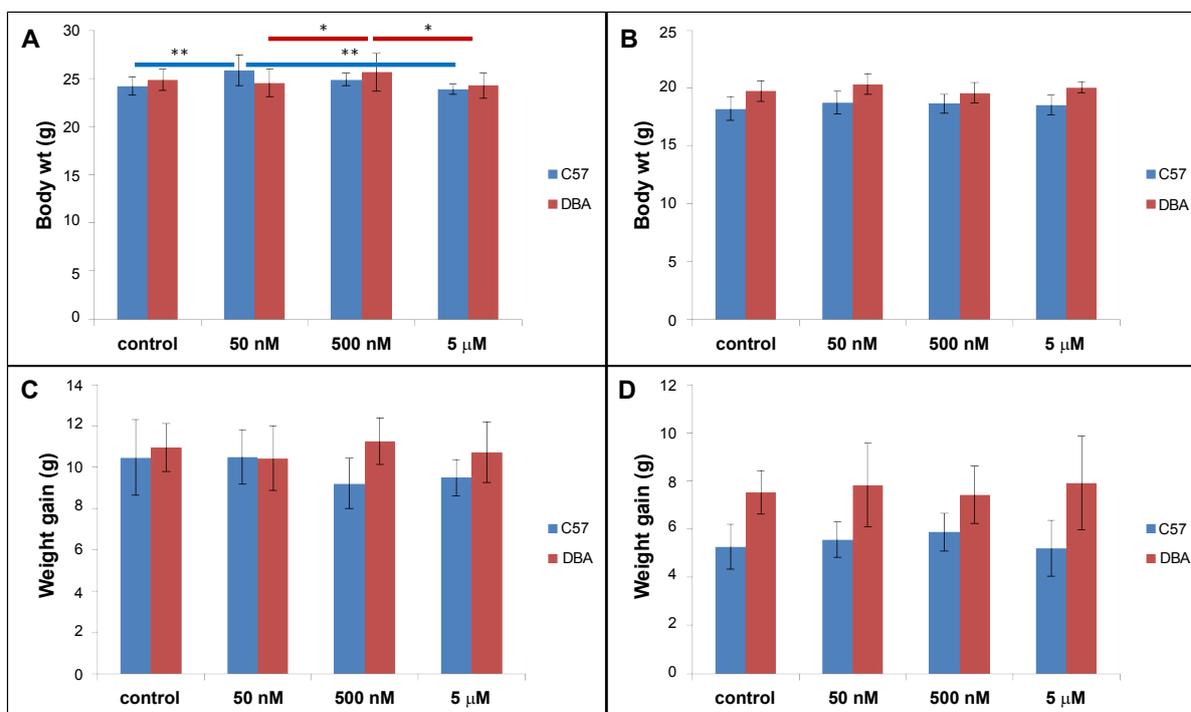


Figure 1: Effects of BPA on bodyweight (A,B) and weight gain (C,D). (A,C) males; (B,D) females; means +/- std. dev; n=8/sex*strain*treatment group; (A, B) body wt. at 11 weeks; (C,D) Weight gain = body wt at 11 weeks – body wt. at 4 weeks; (A). Red (DBA/2J) and blue (C57BL/6J) bars designate post-hoc tests for significant treatment effects within strain; (*) $p<0.05$, (**) $p<0.01$.

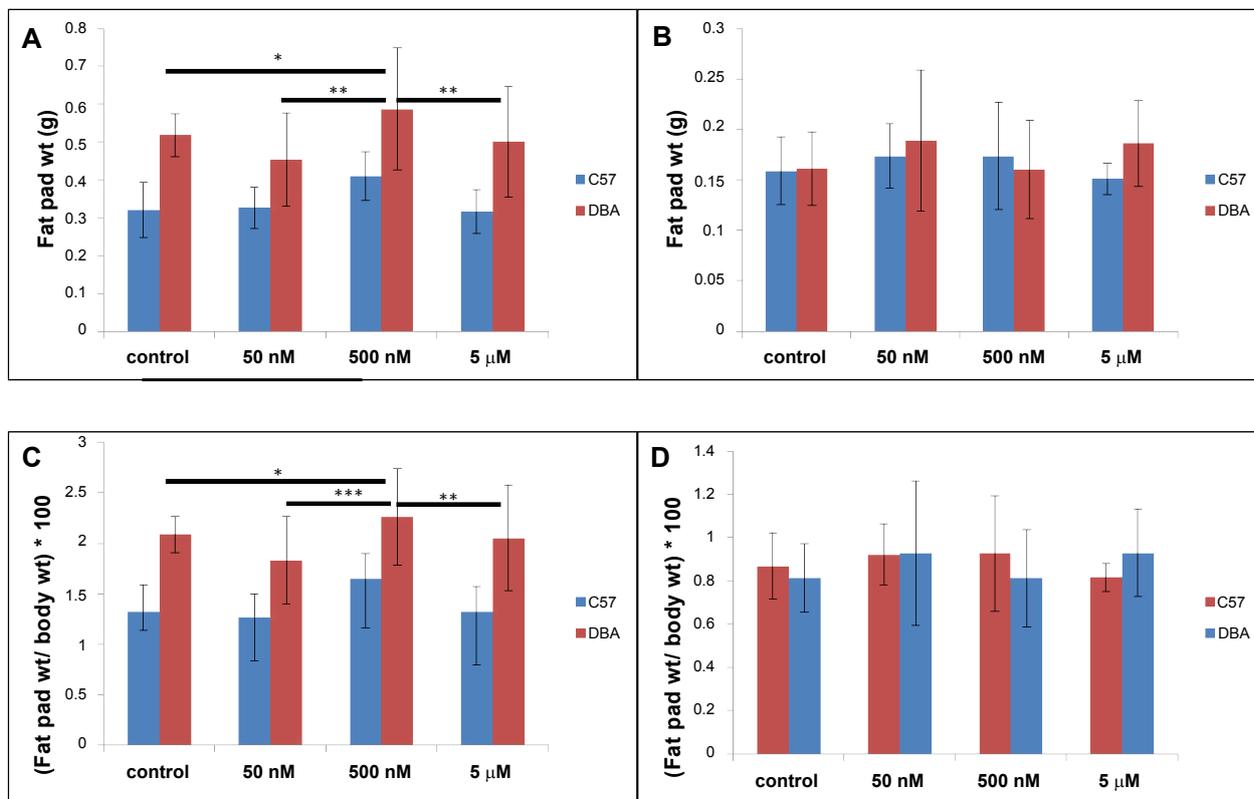


Figure 2: Effects of BPA on fat pad weight (A,B) and adiposity (C,D). (A,C) males; (B,D) females; means +/- std. dev., n=8/sex*strain*treatment group; (A,C). Black bars (A, C) designate post-hoc comparisons between pairs of treatments, (* p<0.05, (** p<0.01, (***) p<0.005. (*) p<0.05.

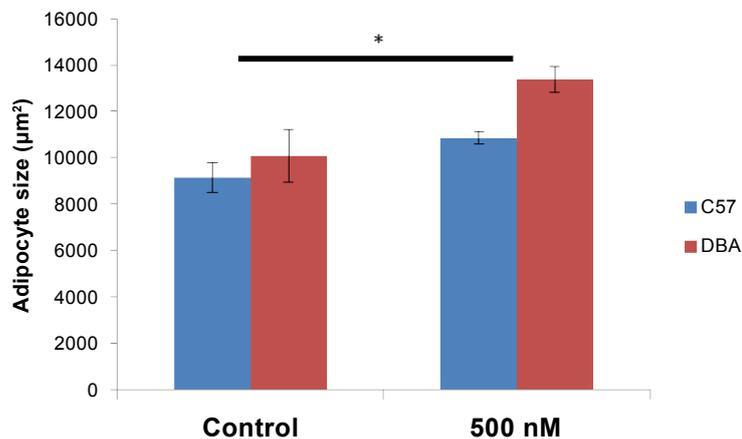


Figure 3: Effects of BPA on adipocyte area in male mice treated with 500 nM BPA; means +/- std. dev.; n=4/ strain*treatment group. (*) p<0.05, effect of 500 nM BPA vs. control.

tests were used to determine if BPA consumption altered glucose metabolism, based on associations between urinary BPA levels risk factors for diabetes in various human studies [10,13,17,40-42]. Fasting serum glucose level was significantly lower in DBA/2J than C57BL/6J mice for both males (p=0.0020; Figure 4A) and females (p=0.0004; Figure 4B), but BPA did not alter glycemia in mice of either sex. However, BPA exerted complex strain and sex-specific effects on serum insulin levels. While there was no effect of BPA on insulin in males (Figure 4C), BPA and strain interacted to control insulin level in

females (p=8.75* E-06; Figure 4D). BPA increased insulin in C57BL/6J females, with significant differences between controls and the 50 nM and 5 μM treatment groups (p=0.05 and p=0.00016, respectively). By contrast, each level of BPA treatment significantly decreased plasma insulin levels (p<0.001) in DBA/2J females relative to controls (Figure 4D). Glucose tolerance tests were performed to determine if BPA consumption in juvenile mice altered glucose disposal. Area under the curve (AUC) was analyzed for effects of strain, BPA, and strain*BPA interactions in males and females. As expected, glucose tolerance

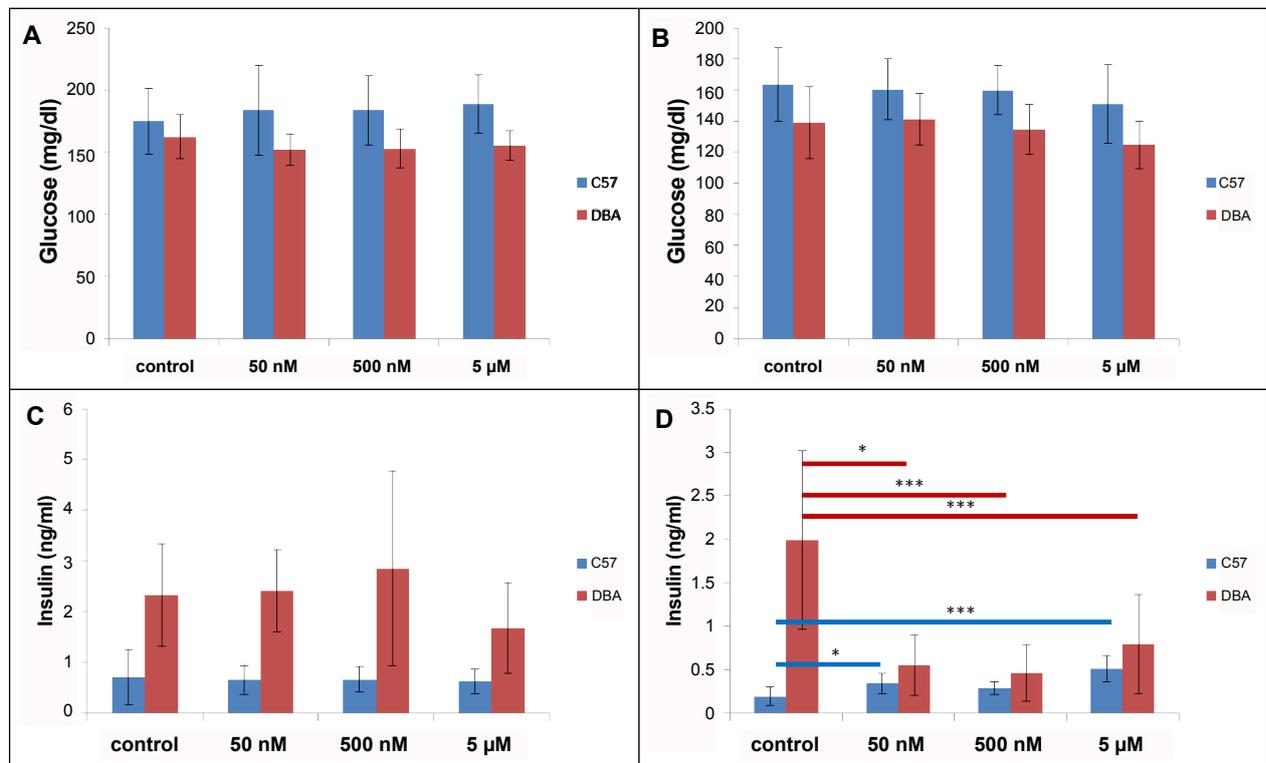


Figure 4: Effects of BPA on fasting serum glucose (A,B) and insulin (C, D) levels. (A,C) males; (B,D) females. Mice were fasted for five hours prior to blood collection; means \pm std. dev., $n=6/\text{sex} \times \text{strain} \times \text{treatment group}$; (D) red (DBA/2J) and blue (C57BL/6J) bars designate post-hoc comparisons between pairs of treatments within strain (* $p < 0.05$, *** $p < 0.005$). Statistical differences between strains are not indicated on the graphs.

differed between strains in both males ($p=2.1 \times 10^{-5}$) and females ($p=6.4 \times 10^{-8}$) due to relatively high serum insulin levels and low blood glucose levels in DBA/2J mice [43]. However, BPA did not significantly affect glucose tolerance in males or females, or in either strain of mice. The results of glucose tolerance tests for C57BL/6J mice are shown in Figures 5A and 5B, males; 5C and 5D, females. Serum adiponectin, an adipokine that promotes insulin sensitivity, was measured in a limited set of samples (control and 500 nM BPA), and only in males, based on reports suggesting that BPA suppresses adiponectin levels. Strain and BPA interacted to control circulating adiponectin ($p=0.045$; Figure 6). BPA significantly suppressed adiponectin level in C57BL/6J males ($p=0.00018$) but not DBA/2J males. Baseline adiponectin levels also differed between the two strains, with lower levels in DBA/2J males ($p=0.0004$).

Adipose adipokine profiles of BPA treated mice

Adipose tissue levels of the adipokines PAI-1, MCP-1, resistin and leptin differed significantly between C57BL/6J and DBA/2J males (Table 1). In females, both MCP-1 and leptin were affected by strain, but none of the adipokines measured were affected significantly by BPA. In males, BPA consumption increased tissue leptin content, although the effect ($p=0.059$) was not significant at the $p < 0.05$ level. Tissue PAI-1 levels in males were affected by BPA in a strain-dependent manner ($p=0.040$, strain \times treatment interaction). PAI-1 content was increased in C57BL/6J males consuming both the 50 nM and 500 nM doses relative to controls ($p=0.024$ and $p=0.042$, respectively). There were no effects of BPA on PAI-I levels between any levels of BPA vs. control in DBA/2J males.

Gene expression profiling and QPCR

To identify changes in gene expression associated with the increased adiposity, global gene expression profiling was performed in adipose tissue from male C57BL/6J and DBA/2J mice in the control and 500 nM BPA treatment groups. Data were analyzed using an ANOVA model that accounted for main effects of strain and BPA; and that included a strain \times BPA interaction term to identify strain-specific responses. After controlling for false discovery (5%) using q-value [39], 2366 genes differed between the two strains regardless of treatment ($FDR < 0.05$). A total of 10 transcripts, two of which are transcript isoforms of the same gene (Neuroepithelial transforming gene 1 (Net1)), were significantly altered by BPA across strains, based on a main effect of treatment (Table 2). Of these, six genes were upregulated and four downregulated by BPA consumption. No genes were significantly influenced by interactions between strain and BPA exposure. Consistent with the effects of BPA on adipocyte size, leptin was upregulated by BPA treatment (~ 1.44 -fold), although the difference did not reach statistical significance. Leptin expression was also determined by QPCR, which indicated a statistically significant effect of 500 nM BPA (1.4 fold-change, BPA vs. controls, $p=0.015$).

Metabolomics

Adipose tissue metabolomics data were analyzed for a main effect of BPA in males because adiposity was only affected in males. No metabolites showed evidence of strain \times treatment interactions ($p > 0.05$). BPA significantly altered the adipose tissue content of six metabolites across the two strains ($p < 0.05$), with suggestive effects ($p < 0.10$) on three additional compounds (Table 3). Included among this group

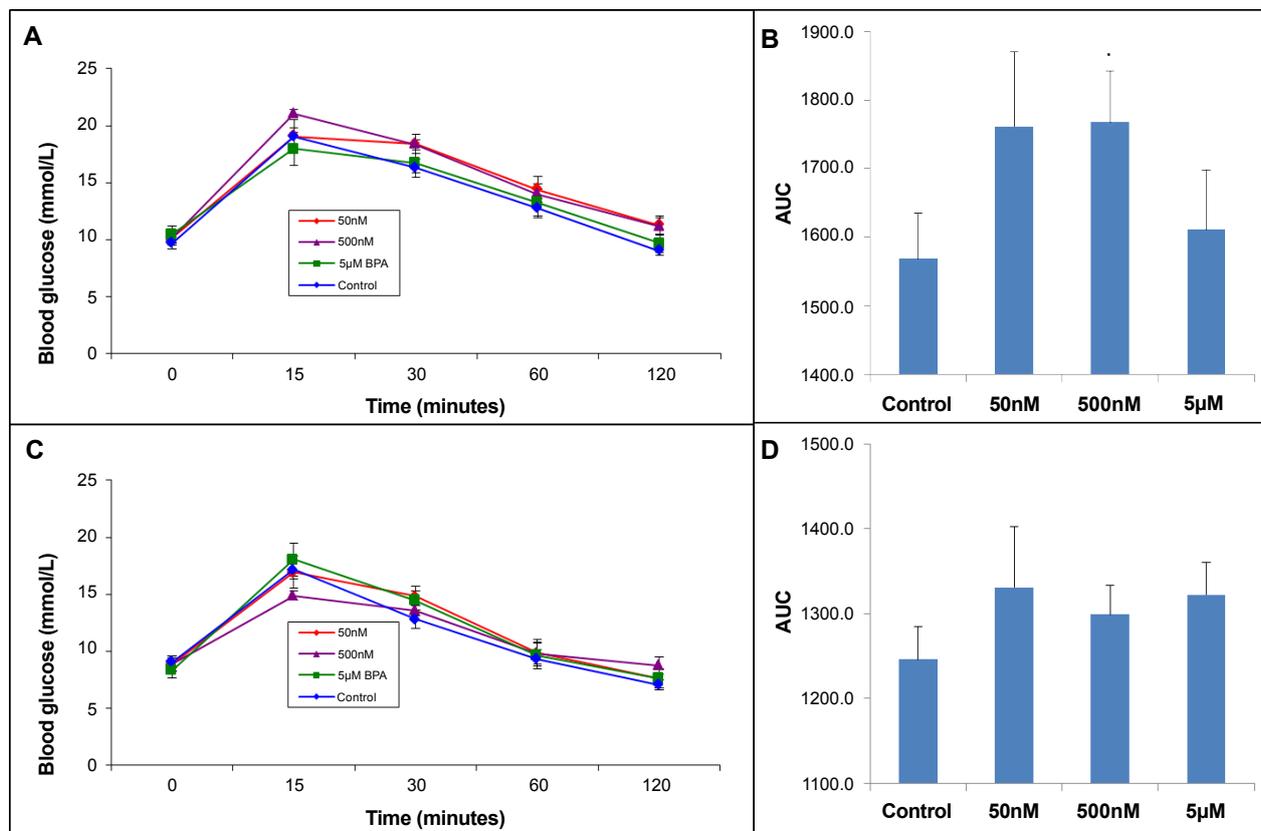


Figure 5: Effects of BPA on glucose tolerance in C57BL/6 mice (A,B), males; (C,D) females; (A,C), time course of glucose disposal; (B,D), area under curve (AUC) based on (A,C); means \pm std. dev.; n=6/ sex*treatment group.

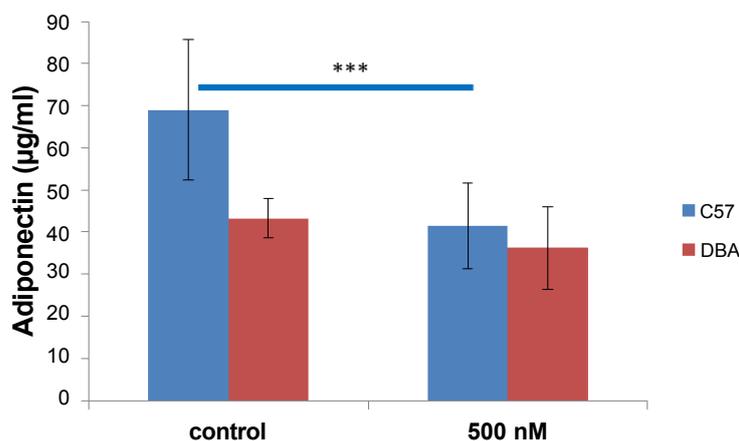


Figure 6: Effects of BPA on serum adiponectin level in male mice treated with 500 nM BPA; means \pm std. dev.; n=4/ strain*treatment group. (***) $p < 0.005$, main effect of 500 nM BPA vs. control.

are metabolites (citrate, isocitrate, fructose-6-phosphate, glucose-6-phosphate and phosphoenolpyruvate) that suggest effects of BPA on intermediary glucose metabolism (Figure 7). Only N-acetylglutamate, phosphoenolpyruvate and thymine levels showed monotonic responses to BPA. The dose of BPA (500 nM) that increased adiposity relative to controls (Figure 2) also significantly increased phosphoenolpyruvate levels (1.51-fold) while decreasing isocitrate (0.75-fold) and citrate (0.50-fold) levels, suggesting effects of BPA on TCA cycle metabolism.

Tissue levels of AMP, ornithine, N-acetylglutamate and thymine were also significantly affected by BPA consumption (Table 3).

Discussion

Most studies into BPA's potential as an obesogen have focused on effects of BPA transmitted in utero or perinatally through maternal circulation or lactation. We modeled BPA consumption in children BPA by adding the compound to drinking water of juvenile mice.

		Leptin		Resistin		MCP1		IL6		Tnfa		PAI1	
Males		Avg. ^a	SD ^b	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD
		$p_{TRT}^c = 0.059$ $p_{STR}^d = 0.021$		$p_{TRT} = 0.687$ $p_{STR} = 0.0086$		$p_{TRT} = 0.0.126$ $p_{STR} = 0.00047$		$p_{TRT} = 0.994$ $p_{STR} = 0.289$		$p_{TRT} = 0.73$ $p_{STR} =$		$p_{TRT} = 0.0261$ $p_{STR} = 0.00296$	
Strain	Treatment												
C57	Cont.	2.16	0.92	18.52	5.57	0.033	0.010	0.070	0.029	0.025	0.008	0.110	0.030
	50 nM	2.17	0.71	17.16	1.90	0.036	0.009	0.077	0.021	0.028	0.004	0.202	0.093
	500 nM	2.75	1.13	15.97	1.20	0.042	0.012	0.064	0.023	0.024	0.006	0.182	0.065
	5 μM	1.82	0.35	17.86	2.77	0.036	0.016	0.066	0.013	0.024	0.005	0.132	0.062
DBA	Cont.	2.89	0.73	17.11	1.63	0.052	0.025	0.069	0.034	0.025	0.009	0.109	0.037
	50 nM	2.76	0.63	18.59	3.51	0.074	0.040	0.064	0.011	0.025	0.007	0.126	0.032
	500 nM	3.31	0.81	17.11	2.27	0.072	0.038	0.065	0.011	0.024	0.006	0.121	0.030
	5 μM	2.96	1.19	18.39	2.36	0.053	0.017	0.071	0.014	0.026	0.006	0.129	0.039
Females													
		$p_{TRT} = 0.207$ $p_{STR} = 0.035$		$p_{TRT} = 0.940$ $p_{STR} = 0.718$		$p_{TRT} = 0.54$ $p_{STR} = 0.00012$		$p_{TRT} = 0.621$ $p_{STR} = 0.203$		$p_{TRT} = 0.457$ $p_{STR} = 0.541$		$p_{TRT} = 0.265$ $p_{STR} = 0.539$	
C57	Cont.	1.16	0.23	18.10	2.34	0.025	0.003	0.143	0.105	0.035	0.005	0.126	0.053
	50 nM	1.10	0.29	17.81	1.48	0.032	0.007	0.182	0.078	0.039	0.005	0.097	0.024
	500 nM	0.96	0.22	19.63	3.71	0.030	0.006	0.186	0.073	0.040	0.016	0.109	0.030
	5 μM	1.08	0.25	17.96	2.43	0.029	0.007	0.153	0.059	0.037	0.009	0.091	0.034
DBA	Cont.	1.37	0.73	18.99	3.01	0.058	0.038	0.110	0.042	0.034	0.012	0.109	0.054
	50 nM	1.26	0.28	16.92	2.93	0.053	0.013	0.121	0.042	0.035	0.009	0.104	0.033
	500 nM	1.09	0.31	18.40	2.81	0.041	0.010	0.164	0.193	0.035	0.007	0.077	0.017
	5 μM	1.57	0.76	18.19	2.01	0.050	0.028	0.143	0.087	0.037	0.016	0.092	0.021

^a Data expressed as pg/mg protein

^b SD, standard deviation

^c p_{TRT} -- p-value for main effect of treatment

^d p_{STR} -- p-value for main effect of treatment

Table 1: Effect of BPA on adipose tissue adipokine content.

Gene name	Gene symbol	Fold change ^a	p-value ^b
Calcium/calmodulin-dependent protein kinase	<i>Camk1</i>	1.22	0.00007
(Heparan sulfate proteoglycan 2	<i>Hspg2</i>	1.27	0.00012
Neuroepithelial cell transforming 1	<i>Net1 (NM_001047159.1)</i>	0.63	0.00013
RUN and SH3 domain containing 2	<i>Rusc2</i>	1.20	0.00014
Reticulon 2	<i>Rtn2</i>	1.31	0.00021
Neuroepithelial cell transforming 1	<i>Net1 (NM_019671.2)</i>	0.68	0.00026
Family with sequence similarity 207, member A	<i>Fam207a</i>	1.16	0.00031
	<i>4631423B10Rik</i>	0.82	0.00031
Cytochrome P450, family 2, subfamily f, polypeptide 2	<i>Cyp2f2</i>	0.39	0.00034
GATS protein-like 3	<i>Gatsl3</i>	1.21	0.00034

^a500 nM BPA vs. control

^b raw p-values corresponding to q-value<0.05

Table 2: Effects of BPA on gene expression.

Using this model, we found that consumption that approximates the EPA tolerable daily intake (TDI) level of <50 μg/kg bw/day [44] significantly increases adiposity. We controlled exogenous sources of BPA by housing mice in cages made of BPA-free plastic, using glass water bottles, distilling ethanol used to solubilize BPA in glass tubing, and feeding a purified diet that lacks plant-based phytoestrogens; the primary source of BPA was drinking water. In contrast, children are exposed through a variety of routes associated with both the foodstream and consumer plastics. Therefore a level of BPA exposure sufficient to promote fatness in children could be reached through cumulative effects of the environment, even if contribution from any single source is less than what we provided in drinking water.

Epidemiological data link BPA to risk of obesity and Type 2 diabetes in humans, both of which are strongly influenced by interactions between genetic variation, gender and the environment

[12-14]. A growing body of literature in humans and rodents supports obesogenic and diabetogenic effects of BPA, but to our knowledge the question of genetic susceptibility to BPA exposure has not been addressed previously. The two inbred mouse strains we used represent many individuals in the U.S. population in that they are neither overtly obese and diabetic nor resistant to these diseases [34,45]. Based on this model, our data suggest broad sensitivity to the obesogenic actions of BPA, at least in males. In contrast, effects of BPA on insulinemia diverged between the two strains of mice, and were limited to females. Unlike genetic susceptibility, sex-specific effects of BPA have been described for a variety of traits, which has been attributed to its interactions with estrogen receptors. BPA may elicit different effects in males and females due to gender-specific expression patterns of estrogen receptors with which it can interact [33]. BPA itself can alter those expression patterns, further creating a basis for sex specific effects

Metabolite	Treatment	Fold-change ^a	P-value (ANOVA)
AMP	50 nm	0.27	0.021
	500 nm	0.99	
	5 μM	2.00	
Citrate	50 nm	0.47	0.054
	500 nm	0.50	
	5 μM	1.12	
Fructose-6-phosphate	50 nm	0.43	0.091
	500 nm	0.45	
	5 μM	1.11	
Glucose-6-phosphate	50 nm	0.99	0.089
	500 nm	1.01	
	5 μM	0.97	
Isocitrate	50 nm	0.86	0.039
	500 nm	0.75	
	5 μM	1.01	
N-acetyl-glutamate	50 nm	1.67	0.017
	500 nm	0.95	
	5 μM	0.88	
Ornithine	50 nm	0.87	0.061
	500 nm	1.38	
	5 μM	1.34	
Phosphoenolpyruvate	50 nm	1.31	0.017
	500 nm	1.51	
	5 μM	1.88	
Thymine	50 nm	0.90	0.009
	500 nm	0.77	
	5 μM	0.49	

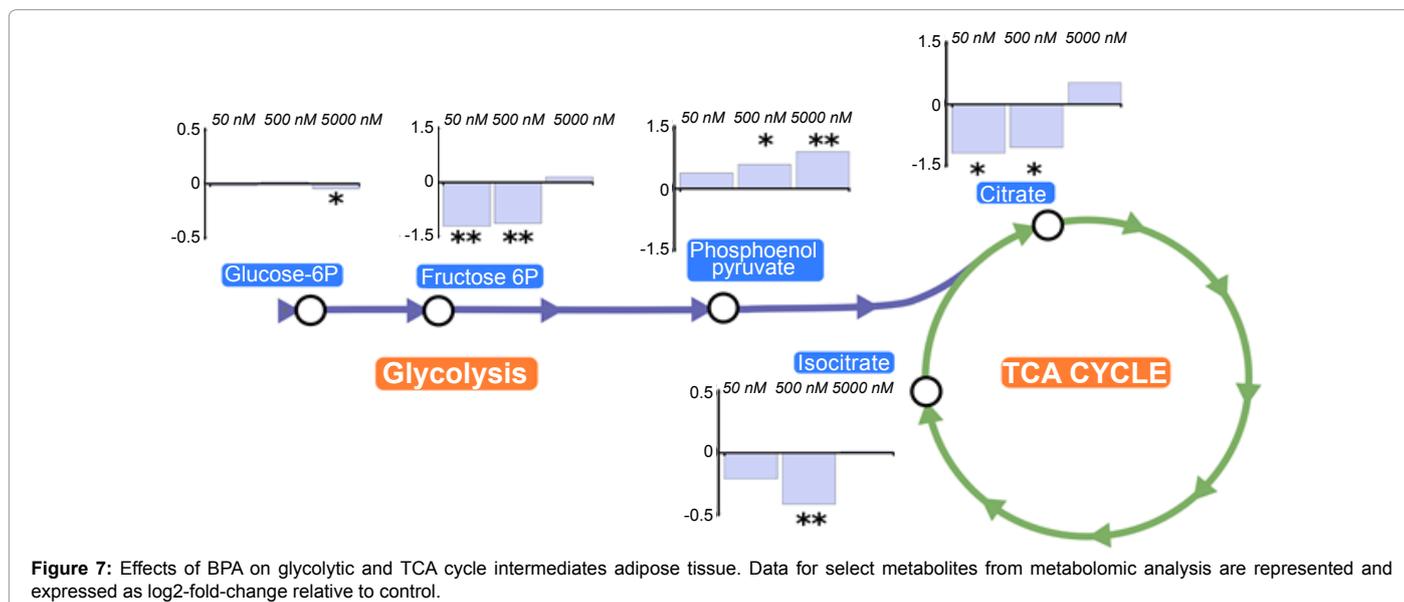
^aVS.control

Table 3: Effects of BPA on adipose tissue metabolites.

of BPA exposure [46]. Less is known about genetic susceptibility to BPA's role in obesity and diabetes, although myriad pathways involved in these disorders are influenced by genetic variation in humans and in animal models. For example, pancreatic β-cells are a direct target of BPA actions [47-49], and numerous aspects of β-cell function differ between C57BL/6J and DBA/2J mice [34,47]. It should be noted that there were no significant consequences for glucose tolerance within the window of BPA exposure used in this study, and whether the divergent effects on insulinemia would eventually impact glucose control requires further study. Nonetheless, our collective data suggest that susceptibility to obesogenic and diabetogenic effects of BPA is not hard-wired. Rather, within a population, individuals may be sensitive to some actions of BPA but not others, depending upon both genetic background and gender.

In addition to obesity and Type 2 diabetes, BPA is clinically associated with risk for coronary artery disease. This relationship appears in both cross-sectional and longitudinal studies, independent of traditional risk factors and with low levels of estimated BPA exposure [17,41,42]. Our data suggest that PAI-1, which was upregulated in males consuming BPA, may play a role in this relationship. PAI-1 is an adipokine that inhibits fibrinolysis [50,51] and is elevated in obesity [52-54]. Elevated PAI-1 levels predispose individuals to atherosclerosis and myocardial infarction [55,56]. Whether increased adipose PAI-1 content in our model was a direct response to BPA or an indirect consequence of fattening cannot be determined from our study. Given the associations between BPA exposure and atherosclerosis, the effect of BPA on PAI-1 warrants further study.

Adipose tissue may serve as an in vivo reservoir of BPA [57] which has direct effects on adipocytes [58]. Although the 500 nM dose of BPA significantly increased adiposity and adipocyte size, the effects on adipose gene expression were relatively modest. This may be because the obesogenic response to BPA (~16% increase in adiposity) was considerably less than for genetic mutations and high fat diet that can increase fatness by more than 3-fold [59,60]. Nonetheless, the set of genes with expression altered by BPA consumption includes candidates that may inform mechanistic studies of BPA as an obesogen. BPA increased expression of heparan sulfate proteoglycan 2 (Hspg2) and reticulon 2 (Rtn2). Hspg2 encodes a heparin sulfate proteoglycan



contained within the extracellular matrix (ECM) [61]. Enriching the ECM in *Hspg2* has been shown to create a tissue microenvironment conducive to adipogenesis [62]. *Rtn2* encodes a member of the reticulon family of proteins that are located primarily on the endoplasmic reticulum membrane where they regulate its structure and function [63]. *Rtn2* plays an important role in translocation of GLUT4 to the cell membrane [64]. Increased expression with BPA exposure may therefore facilitate glucose uptake into adipocytes. As in mice made fatter by BPA consumption, *Rtn2* expression is upregulated in adipose tissue of obese vs. lean humans and of diet-induced obese mice vs. controls [65]. BPA also upregulated expression of CaMKII, a calmodulin kinase family member CaMKII is upregulated by BPA in heart [66], brain [67], Sertoli cells [68], and endothelial cells [69], where it mediates the effects of BPA on oxidative stress. BPA promotes oxidative stress and impairs mitochondrial function in a number of tissues [70-72]. We did not assess mitochondrial function in our model, but it is possible that BPA consumption increased fatness by impairing mitochondrial metabolism. Consistent with this concept, Garcia-Arevalo et al. [73] recently reported that BPA exposure in utero increased adiposity and adipocyte size in adult mice (28 weeks of age) by down-regulating mitochondrial fatty acid oxidation. Changes in the adipose metabolome support an effect of BPA on metabolism. Significant effects on content of phosphoenolpyruvate, a substrate for glyceroneogenesis, as well as citrate, isocitrate and fructose-6-phosphate suggest that BPA may increase adipocyte triglyceride storage by shifting substrate metabolism to enhance the availability of glycerol for fatty acid (re)esterification and triacylglycerol synthesis. In combination, our molecular data suggest that BPA induces fatness through novel mechanisms relative to those characterized in established models of obesity.

Extrapolating to humans, our collective results suggest that BPA may contribute to the increasing incidence of obesity observed in children, both in the U.S. and globally. An expert panel recently estimated that prenatal BPA exposure would be likely to cause ~ 42,000 new cases of childhood obesity in the European Union, with estimated lifetime costs of € 1.54 billion due to associated health consequences [74]. Our results suggest that BPA consumption outside the pre-/perinatal window may further increase this risk. They also indicate that the obesogenic and diabetogenic effects of BPA can be uncoupled, and can manifest differently depending upon gender and genetic background. Given the prevalence and impact of childhood obesity on long term health, continued studies into the effects of BPA consumption in children are warranted.

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