Data Article

Data describing effects of perinatal exposure to bisphenol S on a peripubertal estrogen challenge in intact female CD-1 mice

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ABSTRACT

Bisphenol S (BPS) is an analogue of bisphenol A (BPA), used in consumer products including food packaging and thermal paper. Like BPA, BPS is an estrogen receptor agonist and exposures during perinatal development have been shown to alter growth and morphology of the mouse female mammary gland prior to puberty and in adulthood. Reported here are data describing the effect of exposure to low doses of BPS (2, 200 or 2000 μg/kg/day) during perinatal development on morphology and gene expression in the mammary gland of female CD-1 mice, with or without an additional estrogen exposure (1 μg/kg/day ethinyl estradiol) during the peripubertal period. Additional data document other estrogen-sensitive outcomes including timing of vaginal opening and uterine weight. The data suggest that low doses of BPS induce modest changes in the mammary gland at puberty, but do not appear to sensitize the female to an estrogenic challenge administered during the peripubertal period.

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1. Data

The mammary gland whole mounts and histological sections displayed in Fig. 1A are representative images from female CD-1 mice exposed to vehicle, 2, 200 or 2000 μg BPS/kg/day from gestational day 9 through postnatal day 2 and then challenged with 1 μg ethinyl estradiol/kg/day, from postnatal day 21 through postnatal day 30 (e.g. the peripubertal period); oral route of exposure directly to pup. Whole mount mammary glands collected on postnatal day 31. These samples were also compared to mammary glands collected from females perinatally exposed to BPS and not challenged with ethinyl estradiol (exposed to an oil vehicle) during the postnatal period. Quantification of mammary gland using morphometric tools revealed modest but significant decreases in ductal area in females exposed to 2 μg BPS/kg/day (Fig. 1B). There were no effects of peripubertal ethinyl estradiol treatment. Neither perinatal BPS treatment nor peripubertal ethinyl estradiol exposure affected the number or total area of terminal end buds (TEBs), the highly proliferative structures found in the pubertal mammary gland (Fig. 1C and D).

To further investigate the effects of perinatal BPS exposure on the mammary gland, we evaluated expression of Esr1, the gene encoding estrogen receptor α, and PgR, the gene encoding progesterone receptor. Expression of Esr1 was increased in mammary glands collected from females exposed to 200 μg BPS/kg/day, but expression of this gene was unaffected by a peripubertal ethinyl estradiol challenge.
Expression of PgR was not affected by perinatal BPS treatment or peripubertal ethinyl estradiol exposure (Fig. 2B).

In control females, a peripubertal ethinyl estradiol challenge decreased the time to vaginal opening (from PND 27.6 ± 1.1 to PND 25.2 ± 1.0), although this difference was not statistically significant. No changes in the timing of vaginal opening were observed in females perinatally exposed to BPS (Fig. 3A).

2-way ANOVA also revealed no effect of either perinatal BPS exposure or the peripubertal ethinyl estradiol challenge on anogenital index (AGI, calculated as anogenital distance divided by body weight) in female offspring (Fig. 3B). Uterine weight, normalized for body weight, showed a trend for an effect of perinatal BPS treatment (2-way ANOVA, perinatal treatment, p = 0.06) but no effect of the peripubertal ethinyl estradiol challenge (Fig. 3C).

### 2. Experimental design, materials and methods

#### 2.1. Animal husbandry and necropsy

Pregnant female CD-1 mice (Charles River Laboratories, Raleigh, NC) were housed as described previously [2]. All experimental procedures were approved by the University of Massachusetts Institutional Animal Care and Use Committee.

On pregnancy day 8, dams were randomly assigned to treatment groups. From pregnancy day eight until lactational day 2, dams were orally dosed with BPS or vehicle (Tocopherol Stripped Corn Oil) by gently placing a pipet in the mouth and allowing the mouse to drink the solution. 1 μg oil was administered for every 1 g of body weight. The diluted solutions were designed to deliver 2, 200, or 2000 μg BPS/kg/day. The low dose (2 μg BPS/kg/day) was selected to approximate human exposures; it

Fig. 1. Perinatal exposure to BPS alters mammary gland morphology at postnatal day 31, but does not sensitize the gland pto a peripubertal estrogen challenge. A) Representative whole mount mammary glands collected from females exposed to vehicle, 2, 200 or 2000 μg BPS/kg/day from gestational day 9 through postnatal day 2, and then challenged with ethinyl estradiol (1 μg/kg/day) from postnatal day 21 through postnatal day 30. Mammary whole mounts were also collected from females that were not challenged with ethinyl estradiol (not shown). Mammary glands were collected on postnatal day 31, fixed and stained with carmine alum. Zeiss ZEN software was used to quantify growth and TEB parameters. Scale bar = 2mm. B,C,D) Quantification of data collected from whole mounts including ductal area (B), number of TEBs (C) and total TEB area (D). In panel B, different letters indicate significant differences between groups, p < 0.05, 2-way ANOVA followed by Fisher’s LSD posthoc tests.
is approximately 1–10x higher than typical human intake [3]. The mid dose (200 μg BPS/kg/day) was selected based on prior studies showing that this dose disrupted maternal behaviors, the lactating mother, and development of the mammary gland [2,4,5]. The highest dose (2000 μg BPS/kg/day) was selected based on results from a recent study at the National Toxicology Program which revealed significant effects of BPS on the female mammary gland at a similar dose (5000 μg/kg/day) [6]. Doses were adjusted daily for body weight.

At postnatal day (PND) 21, two females from each litter were selected at random. One pup was assigned to receive vehicle, and one was assigned to receive an estrogen challenge. Each pup was orally dosed via a pipet with either vehicle (Tocopherol Stripped Corn Oil) or 1 μg EE2/kg/day. Dose administration continued for 10 days and was adjusted daily for body weight.

On PND31, pups were euthanized via CO2 inhalation. Anogenital distance was measured using calipers, and the uterus was weighed using an analytical balance. The right fourth inguinal mammary gland was dissected from the skin, spread on a glass slide (Fisher Scientific, Pittsburgh, PA) and fixed in neutral buffered formalin (10%) (Fisher Scientific) overnight (standard whole mount preparation). The third pectoral mammary glands were frozen at −80°C for RNA extraction.

2.2. Whole mount mammary gland preparation and analysis

After fixing in neutral buffered formalin, whole-mounted mammary glands were processed through an alcohol series, defatted with toluene, stained with Carmine-alum, dehydrated in an alcohol and xylene series, and preserved in k-pax heat sealed bags (Fisher Scientific) with methyl salicylate (Acros Organics, Morris Plains, NJ) [7]. Digital images of whole-mount mammary glands were obtained using a Zeiss AxioImager dissection microscope (Carl Zeiss Microscopy, Jena, Germany) and a Zeiss high-resolution color camera. Whole mounts mammary glands from female offspring were imaged using a Zeiss Axio Imager dissection microscope. Using Zen Pro software, the whole mounts are
quantitatively analyzed using methods developed previously [5]. Specific measurements included the area subtended by the ducts (ductal area), the total number of terminal end buds (TEBs, defined as bulb-shaped structures $\geq 0.03 \text{ mm}^2$), and area of TEBs.

2.3. qPCR

Total RNA was extracted from mammary glands of individual mice using Trizol reagent (Ambion, Carlsbad, CA) and a BeadBug microtube homogenizer (Sigma Aldrich, St. Louis, MO). Total RNA was quantified by UV spectrophotometry (Nanodrop 1000; Thermo Scientific). One microgram of RNA from each sample was reverse transcribed to cDNA using reverse transcriptase (Applied Biosystems, Inc). The FastStart Universal SYBR Green Master kit (Roche Diagnostics Corporation, Indianapolis, IN) was used for qPCR along with 1 $\mu$L of cDNA and 300 nM forward and 300 nM reverse primers for each target gene. $\beta$-Actin was used as a housekeeping gene. Every sample was run in triplicate for each gene target. The thermal profile was as follows: 10 minutes at 95 °C; 40 cycles of 15 seconds at 95 °C, 30 seconds at
60 °C, and 15 seconds at 72 °C; a melting-curve analysis was conducted to identify nonspecific products. Relative quantification was determined using the \( \Delta \Delta C_t \) method to correct for differences in \( \beta\)-actin [8].

2.4. Statistical analysis

All analyses were conducted by observers blind to the treatment groups. Data were analyzed using SPSS Version 25 using 2-way ANOVA Univariate analyses with BPS treatment (perinatal) and ethinyl estradiol treatment (peripubertal) as the independent variables, followed by Fisher's LSD post hoc tests. Data were considered statistically significant at \( p < 0.05 \). Graphs illustrate means ± standard error.

Sample sizes for groups that were not given a peripubertal estrogen treatment (peripubertal oil groups) were: control, \( n = 9 \); 2 \( \mu \)g BPS/kg/day, \( n = 9 \); 200 \( \mu \)g BPS/kg/day, \( n = 10 \); 2000 \( \mu \)g BPS/kg/day, \( n = 7 \).

Sample sizes for groups that were given a peripubertal estrogen treatment (peripubertal ethinyl estradiol groups) were: control, \( n = 9 \); 2 \( \mu \)g BPS/kg/day, \( n = 9 \); 200 \( \mu \)g BPS/kg/day, \( n = 10 \); 2000 \( \mu \)g BPS/kg/day, \( n = 7 \).

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