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Influence of Tetrabromobisphenol A, with or without Concurrent Triclosan, upon Bisphenol A and Estradiol Concentrations in Mice

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Background: Humans are commonly exposed to multiple environmental chemicals, including tetrabromobisphenol A (TBBPA; a flame retardant), triclosan (an antimicrobial agent), and bisphenol A (BPA; polycarbonate plastics). These chemicals are readily absorbed and may interact with each other.

Objectives: We sought to determine whether TBBPA, given alone or in combination with triclosan, can modulate the concentrations of BPA and 17β-estradiol (E2).

Methods: Female and male CF-1 mice were each given a subcutaneous injection of 0–27 mg TBBPA, with or without concurrent 0.33 mg triclosan, followed by dietary administration of 50 μg/kg body weight ^{14}C-BPA. Radioactivity was measured in blood serum and tissues through liquid scintillation counting. In subsequent experiments, female and male CF-1 mice were each given a subcutaneous injection of 0 or 1 mg TBBPA and E2 was measured in urine 2–12 h after injection.

Results: Doses as low as 1 mg TBBPA significantly elevated ^{14}C-BPA concentrations in the uterus and ovaries of females; in the testes, epididymides, vesicular-coagulating glands, and preputial glands of males; and in blood serum, heart, lungs, and kidneys of both sexes; urinary E2 concentrations were also elevated. Lower doses of TBBPA or triclosan that had no effects on their own elevated ^{14}C-BPA concentrations when the two substances were given concurrently.

Conclusion: These data indicate that TBBPA, triclosan, and BPA interact *in vivo*, consistent with evidence that TBBPA and triclosan inhibit enzymes that are critical for BPA and E2 metabolism. https://doi.org/10.1289/EHP1329

Introduction

Tetrabromobisphenol A (TBBPA; CAS 79-94-7) is the most-produced flame retardant, with global use over 170,000 metric tons/y (Environment Canada and Health Canada 2013). Approximately 80% of TBBPA is used in reactive applications, where it is covalently bound to the polymer of epoxy resins for printed circuit boards in electronics equipment (Colnot et al. 2014; Shaw et al. 2014). The remaining 20% of TBBPA is used in additive applications, where it is physically blended with rather than chemically bound to the polymer, as in plastic housing for electronics equipment (Colnot et al. 2014; Shaw et al. 2014). Both reactive- and additive-treated products release TBBPA into the environment (Malkoske et al. 2016; Shaw et al. 2014). TBBPA has been detected in soil and sediment (Lee et al. 2015; Wang J et al. 2015; Zhu et al. 2014), surface and waste water (Kim et al. 2016; Xiong et al. 2015), and air and indoor dust (La Guardia and Hale 2015; Ni and Zeng 2013; Wang W et al. 2015; Wu et al. 2016b). Nonoccupational TBBPA exposure in humans occurs via inhalation and ingestion of dust, as well as through dermal contact with dust and free (unreacted) TBBPA in consumer products (Abdallah 2016; Knudsen et al. 2015). TBBPA is bioavailable in humans, as shown by its detection in human serum (Cariou et al. 2008; Fujii et al. 2014), plasma (Ho et al. 2017), breast milk (Abdallah and Harrad 2011; Fujii et al. 2014; Nakao et al. 2015), and adipose tissue (Cariou et al. 2008; Johnson-Restrepo et al. 2008).

The potential for TBBPA to act as an endocrine-disrupting chemical is not well understood. Mechanisms of endocrine disruption by TBBPA could include actions on estrogen, androgen, glucocorticoid, or thyroid hormone receptors, or combinations of any or all of these receptors (Beck et al. 2016; Hamers et al. 2006; Huang et al. 2013). Considering evidence of estrogenic actions, some studies found that TBBPA binds to estrogen receptor (ER) α in *in vitro* assays (Li et al. 2010; Olsen et al. 2003; Suzuki et al. 2013), whereas other studies found that TBBPA failed to bind ERα in *in vitro* assays (Dorosh et al. 2011; Hamers et al. 2006; Lee et al. 2012; Meerts et al. 2001; Miller et al. 2001; Molina-Molina et al. 2013; Riu et al. 2011a, 2011b) and in molecular modeling studies (Zhuang et al. 2014). More recent work has examined indirect mechanisms of estrogenicity whereby TBBPA disrupts estrogen homeostasis (Honkisz and Wójtowicz 2015; Lai et al. 2015; Sanders et al. 2016; Wikoff et al. 2016). One proposed mechanism is that TBBPA inhibits the metabolism of 17β-estradiol (E2), thus increasing its bioavailability, via interactions with conjugating enzymes (Lai et al. 2015; Sanders et al. 2016; Wikoff et al. 2016). These enzymes include estrogen sulfotransferase (SULT), UDP-glucuronosyltransferase (UGT), cytochrome p450 (CYP), and 17β-hydroxysteroid dehydrogenase (17β-HSD) (Dumas and Diorio 2011; Wikoff et al. 2016). Another proposed mechanism is that TBBPA enhances E2 secretion via actions on aromatase (CYP19) expression (Honkisz and Wójtowicz 2015).

We previously demonstrated *in vivo* interactions among bisphenol A (BPA), E2, and triclosan (CAS 3,380-34-5), an antimicrobial agent found in soaps and cosmetics. Compared with vehicle-treated animals, male and female mice given a single dose of 0.6–18 mg triclosan showed greater concentrations of ^{14}C-BPA in blood serum and in reproductive and other tissues (Pollock et al. 2014). Similarly, female mice given a single dose of 1–2 mg triclosan showed greater concentrations of exogenous ^{3}H-E2 in the uterus and natural E2 in urine than did vehicle-treated animals (Pollock et al. 2016). Blastocyst implantation in inseminated female mice can be disrupted by high doses of BPA (Berger et al. 2007, 2008, 2010; Borman et al. 2015) and

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triclosan (Crawford and deCatanzaro 2012); lower doses of each substance that were insufficient on their own disrupted implantation when combined (Crawford and deCatanzaro 2012). Whereas triclosan alone was ineffective in a uterotrophic assay of weanling rats, elevated uterine weight occurred following concurrent triclosan and ethinyl estradiol exposure (Stoker et al. 2010). These findings are consistent with evidence that triclosan is conjugated by SULT, UGT, and CYP (Wu et al. 2016a) and that it can inhibit the activity of SULT and UGT toward other substances, including BPA and E2 (James et al. 2010, 2015; Wang et al. 2004).

Because humans are routinely exposed to multiple potential endocrine-disrupting chemicals, it is important to investigate these chemicals' capacity to interact with each other and with endogenous steroids *in vivo*. Here, we undertook to measure the interactions of TBBPA, triclosan, and BPA. Whereas evidence of direct ER activation by TBBPA and triclosan is weak, BPA is a more established environmental estrogen (Rochester 2013; Seachrist et al. 2016; Ziv-Gal and Flaws 2016). Based on the proposed disruption of estrogen homeostasis via inhibitory actions of TBBPA on conjugating enzyme activity (Lai et al. 2015; Sanders et al. 2016; Wikoff et al. 2016), we hypothesized that TBBPA would elevate BPA concentrations in female and male mice and that this effect would be greatest in serum and in estrogen-binding reproductive tissues. We hypothesized that the actions of TBBPA would be additive with those of triclosan, consistent with evidence that triclosan also inhibits activity of conjugating enzymes (James et al. 2010; Wang et al. 2004). We tested these hypotheses by comparing the impact of TBBPA injection, either alone or in combination with triclosan, on concentrations of ^{14}C-BPA in serum and tissues. We also hypothesized that TBBPA could elevate endogenous levels of E2, the most potent natural estrogen (Kuiper et al. 1997), and tested this hypothesis by measuring the impact of TBBPA injection on urinary E2.

Methods

Animals and Housing

Female (30.5 ± 2.5 g) and male (40.2 ± 3.6 g) CF-1 mice aged 3–4 mo were obtained from Charles River. To standardize timing within the estrous cycle at an easily detected point where estrogen levels are moderate and relatively stable (Miller and Takahashi 2014), we selected diestrous females for use in experiments. These females were identified from a colony of mice with regular estrous cycles by vaginal cytology using published procedures (Byers et al. 2012). Animals were housed in polypropylene cages measuring 28 cm × 16 cm × 11 cm (l × w × h) with *ad libitum* access to food (Teklad 8640 Certified Rodent Chow; Harlan Teklad) and water, except where otherwise stated. The colony was maintained at 21°C with a reversed 14 h light:10 h darkness cycle. All animals were treated humanely and with regard for alleviation of suffering. All procedures adhered to the standards of the Canadian Council on Animal Care and were approved by the Animal Research Ethics Board of McMaster University (Protocol 14-02-03).

Chemicals and Materials

Triclosan [5-chloro-2-(2,4-dichlorophenoxy)phenol, ≥97% purity], 3,3′,5,5′-TBBPA [4,4′-isopropylidenebis(2,6-dibromophenol), ≥97% purity], 17β-E2 (≥98% purity), and creatinine standards were obtained from Sigma-Aldrich. ^{14}C-BPA {[ring-[^{14}C](U)]-BPA, in ethanol, 0.1 mCi/mL, 106 mCi/mmol} was obtained from Moravek Biochemicals. SOLVABLE solubilization cocktail, Ultima Gold scintillation cocktail, and 8-mL midivial scintillation vials were obtained from PerkinElmer. E2 antibodies and horseradish peroxidase (HRP) conjugates were obtained from the Department of Population Health and Reproduction at the University of California, Davis, CA.

Experimental Design and Dosing

This research followed procedures previously published by this laboratory (Pollock et al. 2014, 2016). In brief, mice were weighed, individually housed, and each given a dietary supplement of 1 g peanut butter. Approximately 14–16 h later at the onset of darkness on the following day, animals were randomly assigned to treatment conditions involving a single subcutaneous (sc) injection of TBBPA and/or triclosan dissolved in 0.05 mL peanut oil. In experiment 1, males and diestrous females received vehicle or 1, 3, 9, or 27 mg TBBPA ($n = 7$ per dose). In experiment 2, males ($n = 6$ per dose) and diestrous females ($n = 7$ per dose) received a single sc injection of vehicle, 0.33 mg TBBPA, 0.33 mg triclosan, or 0.33 mg TBBPA + 0.33 mg triclosan. Table 1 provides TBBPA and triclosan doses in milligrams/kilogram for each treatment condition. At 30 min after injection, each animal was given a dietary supplement of 50 μg/kg ^{14}C-BPA in 0.2 g peanut butter. Food, water, and bedding were removed to prevent contamination of the ^{14}C-BPA treatment. At 1 h after ^{14}C-BPA administration, each animal was anesthetized with isoflurane, and blood was collected via cardiac puncture. Each animal was perfused with 20 mL phosphate-buffered saline (PBS), and tissues were collected in preweighed scintillation vials. Tissue samples taken included the heart, lung, superficial adductor muscle from the hind leg, abdominal adipose, liver, and a cross-section of the kidney encompassing both the medulla and the cortex. Male reproductive tissues taken included one testis, one epididymis, one vesicular-coagulating (VC) gland, and one preputial gland. Female reproductive tissues taken included the whole uterus and both ovaries. Vials were reweighed following tissue collection to determine the sample wet mass; no significant changes in tissue weights were observed (data not shown).

In experiment 3, mice were weighed and were individually placed in a Plexiglas apparatus measuring 21 cm × 15 cm × 13 cm (l × w × h) with a wire-mesh grid floor raised approximately 1 cm above a Teflon-coated stainless-steel surface covered with wax paper. Animals acclimated to the novel cages for 3 d before the start of the experiment. At the onset of darkness on the fourth day, males and diestrous females received a sc injection of vehicle or 1 mg TBBPA (corresponding to 33.8 ± 3.7 mg TBBPA/kg for females and 23.4 ± 2.4 mg TBBPA/kg for males) dissolved in 0.05 mL peanut oil ($n = 15$ per dose). Urine was collected noninvasively at 2, 4, 6, 8, 10, and 12 h postinjection. All urine samples were placed into labeled vials and frozen at −20°C at the time of collection.

We administered triclosan and TBBPA via sc injection to mimic dermal absorption of triclosan from personal care products (Fang et al. 2016; Queckenberg et al. 2010) and free (unreacted) TBBPA from dust and consumer products (Abdallah 2016; Knudsen et al. 2015). However, percutaneous penetration is incomplete compared with sc injection; ≤70% of dermally applied TBBPA is absorbed through rat skin (Knudsen et al. 2015), and ≤85% of dermally applied triclosan is absorbed through mouse skin (Fang et al. 2016). We administered ^{14}C-BPA in a dietary supplement to mimic ingestion of BPA from dust, food, and beverages, which accounts for approximately 85–95% of total exposure in adults (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids 2015). Dietary BPA exposure leads to less-efficient first-pass hepatic metabolism and to higher serum BPA concentrations than oral bolus (Sieli et al. 2011). The 30-min latency between TBBPA and ^{14}C-BPA administration and the 1-h latency between ^{14}C-BPA treatment and tissue collection

Table 1. Mean (±SD) TBBPA and triclosan doses in milligrams/kilogram for each treatment condition.

	n	BPA dose (μg/kg)	TBBPA dose (mg)	TBBPA dose (mg/kg)	Triclosan dose (mg)	Triclosan dose (mg/kg)
Experiment 1						
Females	7	50	0	0.0 ± 0.0		
	7	50	1	32.7 ± 2.2		
	7	50	3	95.5 ± 4.8		
	7	50	9	291.3 ± 22.2		
	7	50	27	860.8 ± 67.2		
Males	7	50	0	0.0 ± 0.0		
	7	50	1	24.6 ± 1.4		
	7	50	3	76.7 ± 1.9		
	7	50	9	221.5 ± 9.6		
	7	50	27	686.4 ± 26.5		
Experiment 2						
Females	7	50	0	0.0 ± 0.0	0	0.0 ± 0.0
	7	50	0	0.0 ± 0.0	0.33	11.4 ± 0.6
	7	50	0.33	10.9 ± 0.6	0	0.0 ± 0.0
	7	50	0.33	10.8 ± 0.8	0.33	10.8 ± 0.8
Males	6	50	0	0.0 ± 0.0	0	0.0 ± 0.0
	6	50	0	0.0 ± 0.0	0.33	8.4 ± 0.6
	6	50	0.33	9.6 ± 0.5	0	0.0 ± 0.0
	6	50	0.33	8.0 ± 0.5	0.33	8.0 ± 0.5

Note: BPA, bisphenol A; *n*, number of animals; SD, standard deviation; TBBPA, tetrabromobisphenol A.

were chosen based on an effective paradigm used in previous studies (Pollock et al. 2014, 2016). We selected 1–27 mg doses of TBBPA in experiment 1 to establish the impact of a wide range of TBBPA exposures on tissue concentrations of ^{14}C-BPA. To investigate potential additive effects of TBBPA and triclosan in experiment 2, we selected doses of 0.33 mg so that the quantity of either substance was below the lowest effective dose of triclosan (0.6 mg) required to elevate ^{14}C-BPA concentrations (Pollock et al. 2014). When both substances were given concurrently, the combined quantity (0.66 mg) was greater than the lowest effective dose of triclosan used previously (Pollock et al. 2014). We selected 1 mg TBBPA for experiment 3 because this dose was sufficient to modulate ^{14}C-BPA concentrations in experiment 1. We measured urinary E2 because there are very low concentrations of estrogen conjugates in mouse urine (Muir et al. 2001), whereas unconjugated E2 is abundant in urine and reflects systemic trends (deCatanzaro et al. 2003, 2004; Muir et al. 2001; Thorpe et al. 2014).

Blood and Tissue Processing for Liquid Scintillation Counting

Blood and tissue samples were processed for liquid scintillation counting following previously published procedures (deCatanzaro and Pollock 2016; Pollock et al. 2014, 2016; Pollock and deCatanzaro 2014). Blood samples were centrifuged at 1,500 × *g* for 10 min, and 10 μL serum was added to a scintillation vial containing 5 mL Ultima Gold scintillation cocktail. Tissue samples were solubilized by adding 1 mL SOLVABLE tissue solubilizer to each vial and placing the vials in a 50°C water bath for 4–5 h until completely dissolved. Following the addition of 5 mL Ultima Gold, the vials were agitated to promote mixing of the sample with the scintillation cocktail. Each vial was stored in the darkness chamber of a Tri-Carb 2910TR Liquid Scintillation Analyzer (PerkinElmer) for 5 min to eliminate noise in the form of heat and luminescence. Radioactivity was then measured for 5 min per vial. The amount of radioactivity per sample, in disintegrations per minute (dpm), was automatically calculated via QuantaSmart software by subtracting background radiation, which is continually monitored by the scintillation counter. Frequent cleaning and monitoring of all work surfaces and equipment ensured that contamination of samples did not occur. The final dpm measures were then normalized to the weight of the sample wet mass and were reported as equivalent nanograms BPA/gram tissue or nanograms BPA/milliliter serum.

Measurement of Urinary E2

Full procedures and validations for enzyme immunoassays for mouse urine have been reported previously (Muir et al. 2001). Cross-reactivities for anti-E2 are as follows: E2, 100%; estrone, 3.3%; progesterone, 0.8%; testosterone, 1.0%; androstenedione, 1.0%, and all other measured steroids, <0.1%. Urinary E2 levels were considered with and without adjustment for urinary creatinine, which corrects for differential hydration and urinary concentration among animals, and were reported as nanograms E2/milligram creatinine and nanograms E2/milliliter urine, respectively.

Statistical Analyses

All statistical analyses were performed using the R software environment (R Core Team). A comparison-wise error rate of $\alpha < 0.05$ was employed for all tests. Differences between treatments in experiment 1 were analyzed using univariate analysis of variance (ANOVA) for each tissue, with Holm-Bonferroni adjustments to correct for the number of tissues (Holm 1979). Observation of significant effects in ANOVA was followed by pairwise Newman-Keuls multiple comparisons. Differences between treatments in reproductive tissues and in serum of animals in experiment 2 were analyzed using Student's *t*-test. Differences between urinary E2 concentrations of animals in experiment 3 were analyzed by factorial ANOVA comparing the effects of treatment and collection time point (repeated measures). Significant main effects or interactions in ANOVA were followed by pairwise Newman-Keuls multiple comparisons of treatment at each collection time point. Data from each experiment for individual animals are provided in Tables S1–S6.

Results

Experiment 1: Measurement of ^{14}C-BPA in Mice Given TBBPA

This experiment was designed to determine the impact of TBBPA on the distribution of BPA. Radioactivity was measured in tissues and serum of diestrous females (Figure 1; see also Table S1) and of males (Figure 2; see also Table S2) that received a sc injection of TBBPA followed by a dietary supplement of ^{14}C-BPA. Concentrations of ^{14}C-BPA in the liver and

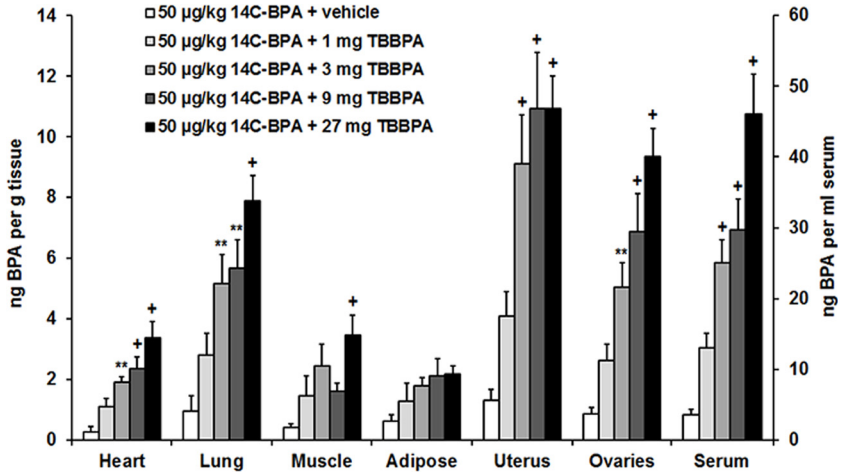

Figure 1. Mean (+SE) concentration of ^{14}C-bisphenol A (BPA) in the heart, lung, muscle, adipose, uterus, ovaries, and serum of diestrous females following subcutaneous (sc) injection of vehicle, 1, 3, 9, or 27 mg tetrabromobisphenol A (TBBPA) and subsequent dietary administration of 50 µg/kg ^{14}C-BPA ($n = 7$ per dose). Difference from vehicle treatment in the same tissue: $^{**}p < 0.01$; $+p < 0.001$. See Table S1 for individual animal data.

kidney are reported in Table 2. Pretreatment with TBBPA induced a dose-dependent increase in concentrations of ^{14}C-BPA in serum and in most tissues of both sexes.

Comparisons were made among the five treatments for each of nine tissues in females. ANOVA followed by Holm-Bonferroni correction produced significant effects of treatment for the heart, $F(4,30) = 12.47$, $p < 0.001$; lung, $F(4,30) = 11.14$, $p < 0.001$; muscle, $F(4,30) = 4.23$, $p = 0.024$; uterus, $F(4,30) = 11.92$, $p < 0.001$; ovary, $F(4,30) = 16.97$, $p < 0.001$; kidney, $F(4,30) = 8.19$, $p < 0.001$; and serum, $F(4,30) = 20.41$, $p < 0.001$. Multiple comparisons revealed that the vehicle-treated group differed from the 3-, 9-, and 27-mg groups for the heart, lung, uterus, ovaries, kidney, and serum. The vehicle-treated group also differed from the 27-mg group for muscle.

Comparisons were made among the five treatments for each of eleven tissues in males. ANOVA followed by Holm-Bonferroni correction produced significant effects of treatment for the heart, $F(4,30) = 8.35$, $p < 0.001$; lung, $F(4,30) = 7.31$, $p = 0.002$; testis, $F(4,30) = 11.13$, $p < 0.001$; epididymis, $F(4,30) = 13.76$, $p < 0.001$; VC gland, $F(4,30) = 4.43$, $p = 0.020$; preputial gland, $F(4,30) = 6.98$, $p = 0.002$; liver, $F(4,30) = 4.61$, $p = 0.020$; kidney, $F(4,30) = 8.06$, $p = 0.001$; and serum, $F(4,30) = 18.32$, $p < 0.001$. Multiple comparisons revealed that the vehicle-treated group differed from the 1-, 3-, 9-, and 27-mg groups for the heart, epididymis, VC gland, kidney, and serum. The vehicle-treated group also differed from the 1-mg group for the liver; the 3-, 9-, and 27-mg groups for the testis; the 9- and 27-mg groups for the lung; and the 27-mg group for the preputial gland.

Experiment 2: Measurement of ^{14}C-BPA in Mice Given TBBPA and/or Triclosan

This experiment was designed to determine whether actions of TBBPA or triclosan on the distribution of BPA would be additive

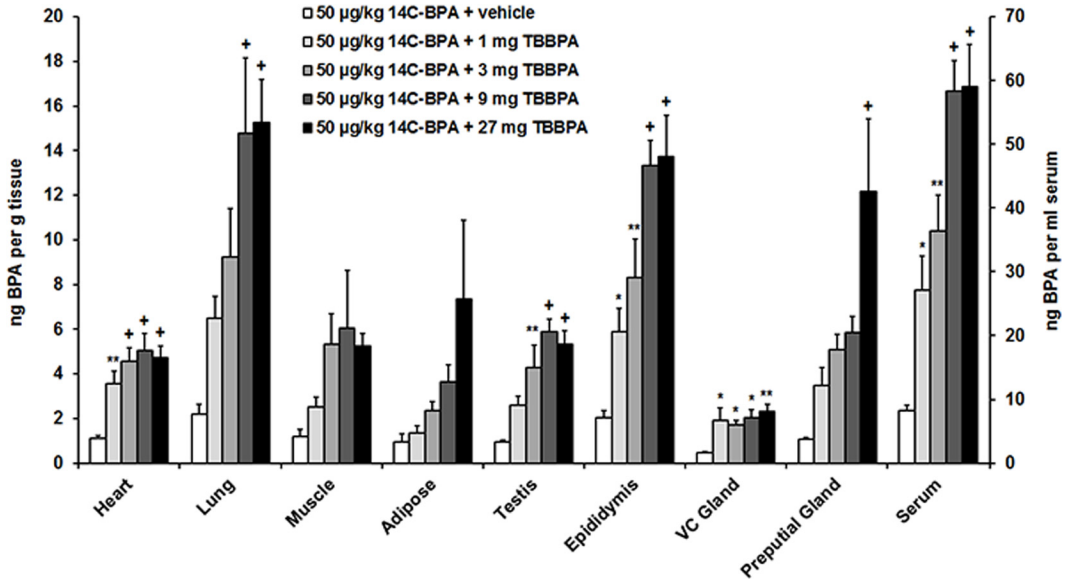

Figure 2. Mean (+SE) concentration of ^{14}C-bisphenol A (BPA) in the heart, lung, muscle, adipose, testis, epididymis, vesicular-coagulating (VC) gland, preputial gland, and serum of males following subcutaneous (sc) injection of vehicle, 1, 3, 9, or 27 mg tetrabromobisphenol A (TBBPA) and subsequent dietary administration of 50 µg/kg ^{14}C-BPA ($n = 7$ per dose). Difference from vehicle treatment in the same tissue: $^{*}p < 0.05$; $^{**}p < 0.01$; $+p < 0.001$. See Table S2 for individual animal data.

Table 2. Mean (±SE) concentration of ^{14}C-BPA in the liver and kidney of diestrous females and males following subcutaneous injection of TBBPA and/or triclosan and subsequent dietary administration of 50 μg/kg ^{14}C-BPA.

	TBBPA dose (mg)	Triclosan dose (mg)	Liver (ng BPA/g)	Kidney (ng BPA/g)
Experiment 1				
Females	Vehicle		23.9 ± 4.0	17.6 ± 4.3
	1		23.4 ± 5.1	38.5 ± 7.4
	3		36.2 ± 5.7	70.4 ± 11.7‡
	9		14.7 ± 2.4	48.2 ± 8.3*
	27		16.7 ± 2.7	77.4 ± 8.9‡
Males	Vehicle		39.8 ± 8.3	76.0 ± 6.9
	1		70.2 ± 8.4*	184.5 ± 25.1†
	3		51.3 ± 8.5	193.5 ± 21.5†
	9		47.7 ± 6.8	265.3 ± 31.1‡
	27		27.2 ± 3.7	164.0 ± 27.5*
Experiment 2				
Females	Vehicle		25.9 ± 2.3	15.3 ± 2.9
	0	0.33	20.8 ± 2.9	14.0 ± 1.8
	0.33	0	24.7 ± 2.4	16.7 ± 3.4
	0.33	0.33	38.2 ± 5.3	19.5 ± 1.4
Males	Vehicle		39.1 ± 4.4	86.2 ± 12.0
	0	0.33	43.7 ± 2.7	114.6 ± 9.0
	0.33	0	38.6 ± 3.8	106.1 ± 24.9
	0.33	0.33	45.2 ± 4.2	119.6 ± 20.1

Note: Significance marks indicate differences from vehicle treatment in the same tissue. BPA, bisphenol A; *n*, number of animals; SD, standard deviation; TBBPA, tetrabromobisphenol A.
* $p < 0.05$.
† $p < 0.01$.
‡ $p < 0.001$.

when the two substances were given concurrently. Radioactivity was measured in the tissues of diestrous females (Figure 3, Table 2; see also Table S3) and of males (Figure 4, Table 2; see also Table S4) that received a sc injection of TBBPA, triclosan, or both followed by a dietary supplement of ^{14}C-BPA. Triclosan and TBBPA showed a greater impact on ^{14}C-BPA concentrations in serum and reproductive tissues when administered concurrently. Tests between treatment conditions in females revealed that the vehicle-treated group differed from the group given 0.33 mg TBBPA + 0.33 mg triclosan for the uterus, $t(12) = 4.13$, $p = 0.001$; ovaries, $t(12) = 2.66$, $p = 0.021$; and serum, $t(12) = 4.02$, $p = 0.002$. Tests between treatment conditions in males revealed that the vehicle-treated group differed from the group given 0.33 mg TBBPA + 0.33 mg triclosan for the testis, $t(10) = 2.63$, $p = 0.025$; epididymis, $t(10) = 2.96$, $p = 0.014$; and serum, $t(10) = 2.89$, $p = 0.016$.

Experiment 3: Measurement of Urinary E2 in Mice Given TBBPA

This experiment was designed to determine the impact of TBBPA on endogenous E2. Urinary E2 concentrations of diestrous females (Figure 5; see also Table S5) and of males (Figure 6; see also Table S6) were measured after a sc injection of vehicle or TBBPA. Concentrations of E2 are reported for uncorrected (nanograms E2/milliliter urine) and corrected (nanograms E2/milligram creatinine) measures. In females, ANOVA on uncorrected measures showed a significant main effect of collection time point, $F(5,100) = 17.26$, $p < 0.001$, and a significant interaction, $F(5,100) = 2.62$, $p = 0.029$. ANOVA on creatinine-corrected measures showed significant main effects of treatment, $F(1,20) = 17.12$, $p < 0.001$, and collection time point, $F(5,100) = 6.98$, $p < 0.001$, and a significant interaction, $F(5,100) = 4.04$, $p = 0.002$. Multiple comparisons revealed that the vehicle-treated females differed from the TBBPA-treated females at 8 h after injection for the corrected measures, as well as at 10 and 12 h after injection for both the uncorrected and corrected measures. In males, ANOVA on uncorrected measures showed significant main effects of treatment, $F(1,12) = 8.18$, $p = 0.014$, and collection time point, $F(5,60) = 2.87$, $p = 0.022$, but no significant interaction. ANOVA on corrected measures showed only a significant main effect of collection time point, $F(5,60) = 4.86$, $p < 0.001$. Multiple comparisons revealed that the vehicle-treated males differed from the TBBPA-treated males at 2 and 4 h after injection for both the uncorrected and corrected measures, as well as at 10 h after injection for the uncorrected measures.

Discussion

These data show that TBBPA greatly magnifies concentrations of BPA in serum and tissues and that it elevates urinary concentrations of E2. When animals received an oral dose of ^{14}C-BPA,

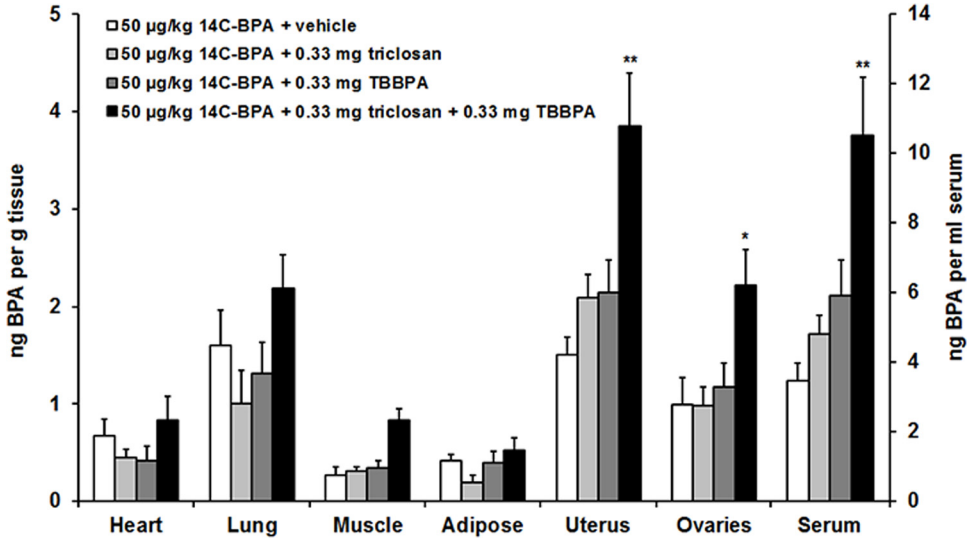

Figure 3. Mean (+SE) concentration of ^{14}C-bisphenol A (BPA) in the heart, lung, muscle, adipose, uterus, ovaries, and serum of diestrous females following subcutaneous (sc) injection of vehicle, 0.33 mg triclosan, 0.33 mg tetrabromobisphenol A (TBBPA), or 0.33 mg triclosan + 0.33 mg TBBPA and subsequent dietary administration of 50 μg/kg ^{14}C-BPA ($n = 7$ per dose). Difference from vehicle treatment in the same tissue: *$p < 0.05$; **$p < 0.01$. See Table S3 for individual animal data.

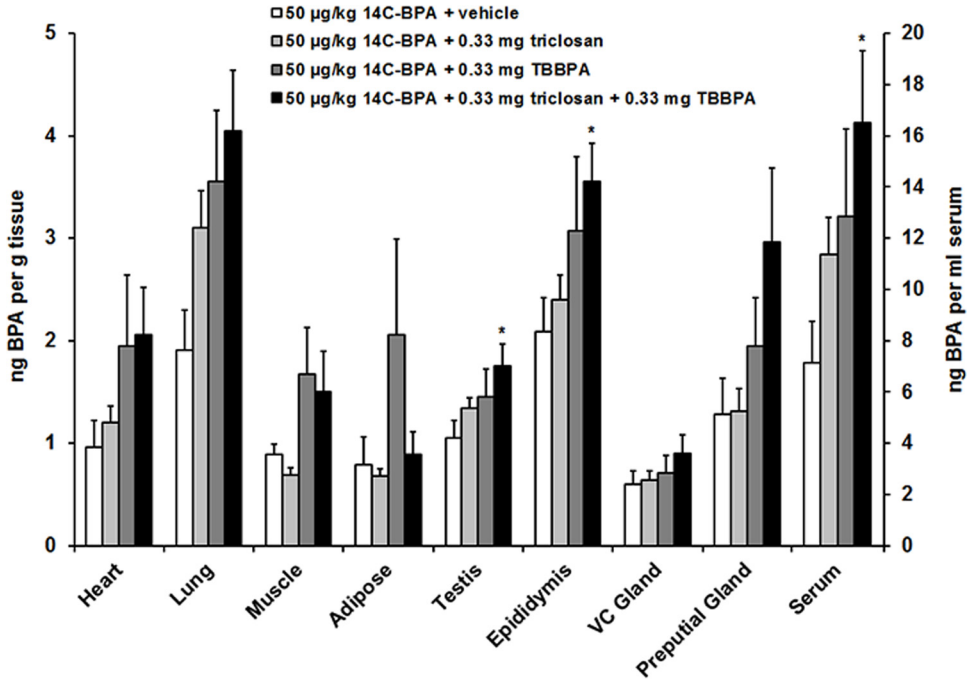

Figure 4. Mean (+SE) concentration of ^{14}C-bisphenol A (BPA) in the heart, lung, muscle, adipose, testis, epididymis, vesicular-coagulating (VC) gland, preputial gland, and serum of males following sc injection of vehicle, 0.33 mg triclosan, 0.33 mg tetrabromobisphenol A (TBBPA), or 0.33 mg triclosan + 0.33 mg TBBPA and subsequent dietary administration of 50 µg/kg ^{14}C-BPA ($n = 6$ per dose). Difference from vehicle treatment in the same tissue: *$p < 0.05$. See Table S4 for individual animal data.

radioactivity was greater in serum, reproductive tissues, and elsewhere in female and male mice that were pretreated with 1–27 mg TBBPA. Radioactivity was also greater in serum and reproductive tissues of mice given 0.33 mg TBBPA + 0.33 mg triclosan. Urinary E2 concentrations were elevated in animals given 1 mg TBBPA. Our novel findings that TBBPA modulates E2 and BPA concentrations underscore a concern (Osimitz et al. 2014) that molecular modeling and *in vitro* studies may not address biological activity *in vivo*.

There are several potential mechanisms through which TBBPA, triclosan, BPA, and E2 could interact. These include direct actions at ER, transport proteins in blood, and enzymes involved in steroid synthesis and metabolism. There is conflicting evidence regarding direct binding to ER of TBBPA (Lee et al. 2012; Li et al. 2010; Molina-Molina et al. 2013; Suzuki et al. 2013) and triclosan (Gee et al. 2008; Henry and Fair 2013; Stoker et al. 2010). Insofar as there is such binding, actions of TBBPA or triclosan would be competitive with binding of ^{14}C-BPA, producing an opposing effect to that observed in experiments 1 and 2. Similarly, competition for transport proteins in blood would presumably reduce ^{14}C-BPA concentrations in serum and tissues. Our findings in experiments 1 and 2 are much more consistent with competition among TBBPA, triclosan, and ^{14}C-BPA for metabolic enzymes. Enzymes of particular interest are those involved in phase II metabolism, including UGT and SULT (Dumas and Diorio 2011; Wikoff et al. 2016). The major metabolite of BPA in rodents is the monoglucuronide conjugate resulting from interaction with hepatic UGT 2B1 and potentially other isoforms (Inoue et al. 2001; Kurebayashi et al. 2010; Yokota et al. 1999; Zalko et al. 2003). Other metabolites of BPA in rodents include the monosulfate conjugate resulting from interaction with SULT 1A1 (Yalcin et al. 2016; Zalko et al. 2003), as well as the diglucuronide (Zalko et al. 2003), disulfate (Yalcin et al. 2016), and glucuronide/sulfate (Inoue et al. 2016) diconjugates.

Sulfate and glucuronide conjugates of TBBPA (Borghoff et al. 2016) and triclosan (Fang et al. 2016) have also been

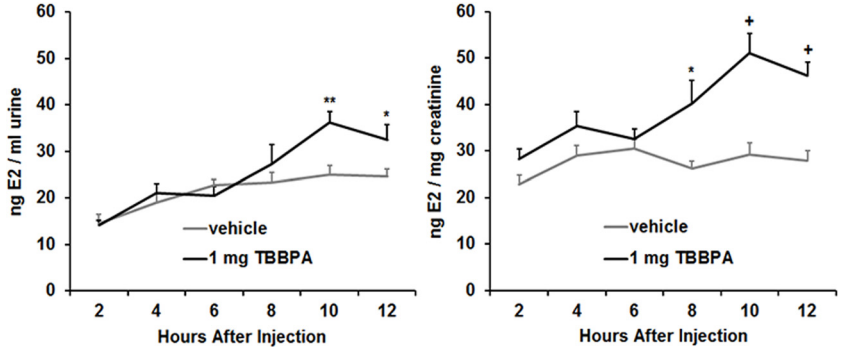

Figure 5. Mean (+SE) concentration of urinary E2, expressed as nanograms 17β-estradiol (E2)/milliliter urine and nanograms E2/milligram creatinine, following subcutaneous (sc) injection of vehicle or 1 mg tetrabromobisphenol A (TBBPA) in diestrous females ($n = 15$ per dose). Significant difference from vehicle treatment at the same time point: *$p < 0.05$; **$p < 0.01$; +$p < 0.001$. See Table S5 for individual animal data.

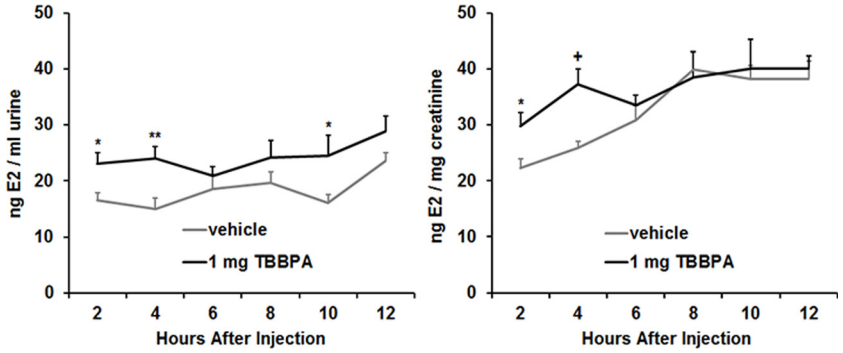

Figure 6. Mean (+SE) concentration of urinary 17β-estradiol (E2), expressed as nanograms E2/milliliter urine and nanograms E2/milligram creatinine, following subcutaneous (sc) injection of vehicle or 1 mg tetrabromobisphenol A (TBBPA) in males ($n = 15$ per dose). Significant difference from vehicle treatment at the same time point: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{+}p < 0.001$. See Table S6 for individual animal data.

observed in rodents. TBBPA can inhibit the activity of SULT 1E1 and SULT 1A1 (Gosavi et al. 2013; Hamers et al. 2006; Harju et al. 2007; Kester et al. 2002) and can reduce the expression of genes in the liver that encode SULT 1E1 and SULT 2A1 (Sanders et al. 2016). Triclosan can inhibit sulfonation and glucuronidation of BPA in human liver fractions (Wang et al. 2004).

Our findings in experiment 3 can be explained by competition between TBBPA and E2 for metabolic enzymes. In addition to phase II metabolism (described above), phase I metabolism involving CYP and 17β-HSD is important for estrogen metabolism (Dumas and Diorio 2011; Wikoff et al. 2016). TBBPA can inhibit CYP 2C9 and CYP 3A4 activity (Ames 2013) and 17β-HSD4 activity (NIH/NCBI) in human liver fractions. TBBPA can reduce expression of genes in the liver that encode 17β-HSD, but it can increase expression of genes that encode certain CYP isoforms (Sanders et al. 2016). Our findings in experiment 3 can also be explained by actions of TBBPA on enzymes involved in steroid synthesis. One study found that TBBPA upregulates aromatase expression and activity in human choriocarcinoma cells for up to 72 h *in vitro* (Honkisz and Wójtowicz 2015). The latency of TBBPA action on E2, up to 8–12 h in females, could be attributed to increased E2 biosynthesis.

We found that TBBPA magnified concentrations of ^{14}C-BPA in the heart, lung, kidney, and blood serum of mice, as well as in the uterus and ovary of females and in the testis, epididymis, VC gland, and preputial gland of males. The impact of 1–27 mg TBBPA on ^{14}C-BPA concentrations in tissues and in serum is consistent with that previously shown for 0.6–18 mg triclosan (Pollock et al. 2014), except TBBPA appears to have larger effects. This is particularly evident in males because pretreatment with triclosan elevated ^{14}C-BPA concentrations in only the epididymis and blood serum (Pollock et al. 2014). The greatest impact of TBBPA on ^{14}C-BPA concentrations was in the lung, reproductive tissues, kidney, and blood serum. The localization of ^{14}C-BPA to the lung and reproductive tissues is consistent with the high expression of ERα and ERβ in these tissues (Couse et al. 1997; Kuiper et al. 1997). Of the tissue samples collected, the highest concentrations of ^{14}C-BPA were in the liver and in the kidney. This observation is consistent with findings from previous studies of the distribution of BPA at doses ranging from 0.5 to 100,000 μg/kg (Kim et al. 2004; Kurebayashi et al. 2005; Pollock and deCatanzaro 2014). These organs are involved in the metabolism and excretion of ingested BPA, and radioactivity in these tissues does not necessarily reflect tissue deposition of ^{14}C-BPA. Concentrations of ^{14}C-BPA were greater in males than in females in most nonreproductive tissues and in blood serum. In vehicle-treated animals in experiment 1, average ^{14}C-BPA concentrations in males were greater than those in females for the heart (394%), lung (230%), muscle (294%), adipose tissue (152%), serum (228%), liver (167%), and kidney (433%). These findings are consistent with the distribution of 100 μg/kg BPA in certain tissues of male and female rats (Kurebayashi et al. 2005) and may be explained by differences in BPA metabolism, as shown by sex- and tissue-specific expression of numerous UGT isoforms (Buckley and Klaassen 2007).

Greater concentrations of urinary E2 following TBBPA administration were most evident in females approximately 8–12 h postinjection but were also observed in males approximately 2–4 h postinjection. This discrepancy in latency between males and females may be influenced by differences in the metabolism of TBBPA, differences in estrogen synthesis, or both. The rate of TBBPA glucuronide production is faster in male rat liver fractions (Zalko et al. 2006), whereas aromatase expression is greater in the ovaries than in the testes (Golovine et al. 2003). Taken together, these processes may hasten the influence of TBBPA on E2 concentrations in males but result in greater effects of TBBPA on E2 concentrations in females. Slight but persistent elevations in E2 can lead to adverse reproductive and health outcomes in mammals. In mice, heightened E2 levels can prevent intrauterine blastocyst implantation and cause pregnancy failure (Thorpe et al. 2013). In humans, elevated E2 from hormone-replacement therapy correlates with increased risk of breast, endometrial, and ovarian cancer (Million Women Study Collaborators 2003, 2005, Beral and Million Women Study Collaborators 2007).

Data from the 2011–2012 U.S. National Health and Nutrition Examination Survey (NHANES) indicated that 72% of human urine samples contain detectable triclosan concentrations ranging from 2.3 to 3,830 μg/L (Han et al. 2016). Although NHANES did not report TBBPA concentrations in urine, TBBPA was detected in 93% of plasma samples and in 89% of urine samples in a study of 140 healthy adults in China (Ho et al. 2017). Some published reports have estimated daily TBBPA exposure levels in the range of 3.2×10^{-7} to 1.95×10^{-4} mg/kg/day (Environment Canada and Health Canada 2013; NTP 2014; Wikoff et al. 2015). However, these exposure estimates are derived from concentrations of TBBPA in environmental media and may not account for all exposure pathways. Furthermore, interactions among chemicals may influence their distribution, metabolism, and excretion, as indicated by our data. One study suggested that disruption of homeostatic control of TBBPA and estrogen conjugation is unlikely in humans because the doses required to produce uterine tumors in rodents are orders of magnitude greater than exposure estimates in humans (Borghoff et al. 2016). However, our data show clear *in vivo* interaction between TBBPA and triclosan, indicating that it is not appropriate to consider only one chemical

in isolation. Inhibition of enzymes involved in estrogen metabolism has been shown for a number of environmental chemicals and their metabolites, including parabens (Ozaki et al. 2016; Prusakiewicz et al. 2007), phthalates (Ozaki et al. 2016), polychlorinated biphenyls (Kester et al. 2000; Wang and James 2007), and polyhalogenated aromatic hydrocarbons (Kester et al. 2002). Given the potential adverse reproductive and carcinogenic outcomes of persistently elevated estrogenic activity, these findings demonstrate the importance of considering multiple toxicants when determining regulatory exposure limits.

Conclusion

These data demonstrate that concurrent exposure to TBBPA elevates concentrations of dietary BPA in reproductive and other tissues. TBBPA and triclosan have additive effects in their capacity to modulate concentrations of BPA. TBBPA also elevates measures of urinary E2 in mice. These effects are consistent with competition among these synthetic chemicals and endogenous steroids for conjugating enzymes. These results indicate that TBBPA and triclosan, both of which have negligible direct effects on ER, can have indirect estrogenic effects.

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