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Disruption of estrogen homeostasis as a mechanism for uterine toxicity in Wistar Han rats treated with tetrabromobisphenol A

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ABSTRACT

Chronic oral treatment of tetrabromobisphenol A (TBBPA) to female Wistar Han rats resulted in increased incidence of cell proliferation at 250 mg/kg and tumor formation in the uterus at higher doses. The present study was designed to test the hypothesis that disruption of estrogen homeostasis was a major mode-of-action for the observed effects. Biological changes were assessed in serum, liver, and the proximal (nearest the cervix) and distal (nearest the ovaries) sections of the uterine horn of Wistar Han rats 24 h following administration of the last of five daily oral doses of 250 mg/kg. Expression of genes associated with receptors, biosynthesis, and metabolism of estrogen was altered in the liver and uterus. TBBPA treatment also resulted in changes in expression of genes associated with cell division and growth. Changes were also observed in the concentration of thyroxine in serum and in expression of genes in the liver and uterus associated with thyroid hormone receptors. Differential expression of some genes was tissue-dependent or specific to tissue location in the uterus. The biological responses observed in the present study support the hypothesis that perturbation of estrogen homeostasis is a major mode-of-action for TBBPA-mediated cell proliferation and tumorigenesis previously observed in the uterus of TBBPA-treated Wistar Han rats.

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1. Introduction

Some high production volume brominated flame retardants (BFRs) such as polybrominated diphenyl ether (PBDEs) mixtures have been, or are being phasedout due to their environmental persistence and potential for bioaccumulation and toxicity (USEPA, 2014). In contrast, production of tetrabromobisphenol A (TBBPA), used primarily as a reactive BFR in epoxy resins for printed circuit boards, continues at a high volume (Canada, 2012; de Wit et al., 2010; ECB, 2006). TBBPA is well absorbed in rats, rapidly conjugated and eliminated from systemic circulation, has little potential to accumulate in tissues, and acute toxicity is in the g/kg range (ECB, 2006; Hakk et al., 2000; Knudsen et al., 2014; Kuester et al., 2007; USEPA, 2008). TBBPA may be degraded by photochemical, thermal, and bacterial decomposition in the environment (Li et al., 2014). Even so, TBBPA is categorized as moderately persistent (USEPA, 2008). TBBPA has minimal potential to leach from products when used as a reactive BFR (ECB, 2006; USEPA, 2008); however, the chemical more easily enters the environment when used in an additive fashion similar to that of PBDEs (Canada, 2012; de Wit et al., 2010).

The safety of TBBPA is of concern based on effects on the endocrine system in animals and a recent study reporting carcinogenic activity in TBBPA-treated rats and mice (Dunnick et al., 2015). Studies conducted *in vitro* indicate that TBBPA competes with thyroxine (T4) for binding to transthyretin (TTR), inhibits binding of triiodothyronine (T3) to thyroid hormone receptors (TR), affects gene or protein expression of TR and peroxisome proliferator-activated receptors (PPARs), and may bind to PPARγ (Hamers et al., 2006; Kitamura et al., 2002; Meerts et al., 2000; Riu et al., 2011). TBBPA was estrogenic in a uterotropic assay in ovariectomized mice and competed with 17β-estradiol (E2) for receptor binding in MtT/E-2 cells (Kitamura et al., 2002, 2005). However, other studies indicate that TBBPA has minimal estrogenic activity. TBBPA had no activity in estrogen receptor (ER)-CALUX® assays and had little affinity for ERα in MCF-7 or HepG2 cells (Dorosh et al., 2010; Hamers et al., 2006; Meerts et al., 2001; Riu et al., 2011). Recently, the National Toxicology Program (NTP) reported a positive 2-year carcinogenicity study for TBBPA in both Wistar Han rats and B6C3F1 mice (NTP, 2014). Results indicated an increased incidence of epithelial atypical hyperplasia in uterine tissue of rats at the lowest dose tested (250 mg/kg) and an increased incidence of uterine tumors (adenomas, adenocarcinomas, and malignant Müllerian cell tumors) at higher doses. This response in rats raises concern for human exposure to TBBPA. TBBPA has been detected in serum, milk, and/or adipose samples from disparate populations (NTP, 2014). Although not correlated with

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any specific chemical exposure, cancers of the uterine corpus are estimated to be the fourth most common of new cancer cases in U.S. women (Siegel et al., 2013).

The mechanism(s) of the carcinogenic response in the uterus of TBBPA-treated rats may involve tissue insult from accumulation of TBBPA or a reactive metabolite. TBBPA can be metabolized to a 2,6-dibromobenzoquinone radical in male Sprague-Dawley rats (Chignell et al., 2008). However, several studies investigating the fate of TBBPA in rats detected no evidence of oxidative cleavage of the molecule (Hakk et al., 2000; Knudsen et al., 2014; Kuester et al., 2007). Furthermore, neither persistence nor accumulation of TBBPA-derived material was observed in uterine tissue following repeated oral administration of ^{14}C-labeled TBBPA to female Wistar Han rats (Knudsen et al., 2014). TBBPA-mediated disruption of estrogen homeostasis may be the best explanation for observed carcinogenic effects in the uterus (Dunnick et al., 2015). Excessive estrogen exposure has been linked to uterine tumors in humans (Lax, 2004). Inhibition, induction, or saturation of enzymes involved in estrogen synthesis, metabolism, and elimination could result in increased concentrations of the hormone in uterine tissue of TBBPA-treated rats.

Conjugation with glucuronic acid and/or sulfate is the major pathways for TBBPA metabolism in female Wistar Han rats (Knudsen et al., 2014). Estrogen is also metabolized through these conjugation pathways, with sulfation being the major mechanism for regulation of serum concentrations (Raftogianis et al., 2000). TBBPA and E2 have similar binding affinities for human estrogen sulfotransferase (Gosavi et al., 2013) and TBBPA is a potent inhibitor of E2 sulfation *in vitro* (Hamers et al., 2006). Decreased availability of the enzyme following TBBPA exposure could lead to prolonged or elevated levels of estrogen in target tissues.

Cell proliferation occurred in the uterus after chronic administration of 250 mg/kg to rats (Dunnick et al., 2015). This dose could be considered to be well above a threshold needed to alter kinetics of TBBPA and/or estrogen metabolism following short-term repeated administration. Therefore, it was postulated that effects on estrogen biosynthesis and/or metabolism would be observed after administration of as few as five daily doses of 250 mg/kg. The results of the present study confirmed the study design and supported the proposed hypothesis that disruption of estrogen homeostasis is a major mode-of-action for the histological changes observed in the uterus of Wistar Han rats chronically exposed to TBBPA.

2. Materials and methods

2.1. Animals and treatments

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the National Institute of Environmental Health Sciences (NIEHS). Female Wistar Han rats (*circa* 9 weeks old) were obtained from Charles River, Raleigh, NC. Upon receipt into the animal facilities, bedding from male rat cages was added to the cages housing the females to stage the estrous cycle prior to dosing with TBBPA (Whitten, 1958). The estrous cycle of each rat was monitored daily using the vaginal cytology assay as described by Hubscher et al. (2005). The randomized rats were initially dosed on a morning when the assay indicated that 18 of 20 rats were in diestrus (one TBBPA-treated rat was in metestrus and one vehicle control rat was in proestrus; see Supplemental material, Table S1). The rats were *circa* 12 weeks old and weighed 197 ± 17 g at dosing. Each rat (n = 10/group) received either five consecutive daily doses of 250 mg/kg of TBBPA or vehicle (1:1:3 ratio of ethanol, Cremophor EL®, and water) by gavage. The TBBPA (3,3′,5,5′-tetrabromobisphenol A) used in this study was obtained from Sigma-Aldrich (St. Louis, MO) and had a chemical purity of 97%. The doses were administered in a volume of 4 ml/kg using a #16 ball-tipped feeding needle attached to a syringe. Rats of the same treatment group were housed two per cage and received water and food (NIH #31) for *ad libitum* consumption. The stage of the estrous cycle was determined and recorded daily. All rats were euthanized by CO_2 asphyxiation 24 h following the final dose. Blood, liver, and uterine tissue were collected rapidly for processing.

2.2. Tissue collection and preparation

Blood was collected by cardiac puncture at time of death, allowed to clot, and centrifuged at 2000*g* for 10 min to produce serum. The liver and uterus were excised, the central portion of the left lobe of the liver was collected and cubed, and the uterus was collected in three sections by locating the approximate midpoint of the branched uterine horns and cutting one to two mm to either side of the midpoint. The uterine collection technique followed a protocol used by the NTP in the chronic study; however, the histopathologic data were reported for the complete uterus, not by section (NTP, 2014). The proximal section (nearest the cervix) and the distal portion (nearest the ovaries) of the uterus were analyzed separately as described below. The small portion of tissue between the proximal and distal sections was not analyzed. The uterine and liver tissues were flash-frozen in liquid nitrogen upon collection and were stored along with the serum at −80 °C. The concentrations of T3, T4, and E2 were determined in serum using RIA kits (Siemens Diagnostics, Los Angeles, CA) and an APEX automatic gamma counter (ICN Micromedic Systems, Inc., Huntsville, AL). The concentration of thyroid stimulating hormone (TSH) was determined in serum using an EIA assay kit (ALPCO, Salem, NH) and a SpectraMAX 340PC plate reader with SOFTmax PRO software (Molecular Devices Corp., Sunnyvale, CA). Frozen tissue samples (50–60 mg) were weighed quickly and minced in Qiagen (Germantown, MD) RLT buffer containing β-mercaptoethanol (1:100). Sample volume was brought to 1.4 ml (liver) or 1 ml (uterus) and samples were processed with an Omni μH handheld homogenizer (Omni International, Kennesaw, GA) using disposable OmniTip homogenizer probes (soft tissue for liver, hard tissue for uterus) to prevent cross-contamination. Homogenates were centrifuged at maximum speed in an Eppendorf 5430R microcentrifuge and 3 × 350-μl aliquots of lysate were transferred into 2-ml tubes (additional buffer was added to bring the triplicate uterine samples to 350 μl each). Total RNA was isolated from the lysate in a QIAcube (Qiagen) following the standard QIAcube Protocol for animal tissues and cells using the RNeasy Mini Kit with DNase treatment; elution volume was 50 μl (liver) or 30 μl (uterus). A second 30 μl elution of uterus samples was performed manually to maximize sample recovery. RNA concentrations were determined for individual eluates using a Nanodrop 2000c (Thermo Scientific, Wilmington, DE). Aliquots with similar concentrations from the same sample were pooled prior to further analysis. RNA integrity was measured on a QIAxcel instrument (Qiagen) using a QIAxcel RNA QC Kit V2.0, with analysis performed with QIAxcel ScreenGel version 1.2.0. All samples were stored at −80 °C.

cDNA was prepared using 2.5 U/μl Moloney Murine Leukemia Virus (MuLV) Reverse Transcriptase (Applied Biosystems, Life Technologies, Grand Island, NY), 5 mM $MgCl_2$, PCR Buffer II (ABI), 1 mM dNTP mix, 2.5 μM Random Hexamers, 0.25 U/μl RNase Inhibitor, and either 100 ng/μl or 20 ng/μl RNA. Reactions were incubated in a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA) for one cycle of 10 min at 25 °C, 60 min at 42 °C, 5 min at 95 °C, then held at 4 °C until removal to storage at −20 °C.

TaqMan® Gene Expression Assays (20×) and TaqMan Universal PCR Master (2×) were purchased from Applied Biosystems. Quantitative PCR was performed in 10 μl assays containing master mix, primer/probe (0.9 μM/0.25 μM), RNase-free water, and the indicated amount of cDNA in a Bio-Rad CFX384 Real-Time PCR System. Cycling parameters included an initial activation of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Assays for *Cyp19a1* and *Hsd3b5* were purchased from Bio-Rad. Quantitative PCR was performed for these