Changes in gene expression and pathway effects of tetrabromobisphenol A (TBBPA) in female Wistar Han rats

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Introduction

Tetrabromobisphenol A (TBBPA; CAS No. 79-94-7) is a brominated flame retardant (BFR) with a global market volume of approximately 150,000 tons/year, representing about 60% of global demand for BFRs.[1] Its primary application is as a reactive product in epoxy resins and laminates in printed circuit boards. TBBPA is also used in Acrylonitrile-Butadiene-Styrene (ABS) plastic casings, as a plasticizer, and as an intermediate for the syntheses of other flame retardants like TBBPA-bis(2,3-dibromopropyl ether), TBBPA-allyl ether, and TBBPA carbonate oligomers.[2] TBBPA production and use is expected to increase as an additive product due to the phase-out of BFRs such as polybrominated diphenyl ethers and hexabromocyclododecanes.[2]

TBBPA has been detected at low levels in environmental samples, and human exposure to TBBPA may be of concern. Repeat-dose subacute and one-generational reproductive studies found that TBBPA exposures resulted in decreased thyroxine levels and other endocrine effects.[3] TBBPA single-exposure bioavailability in rats and humans is low, but chronic exposure effects are not well understood.[4,5] TBBPA chronic exposure studies have found an enhanced susceptibility of female Wistar Han rats to TBBPA toxicity.[6] An NTP 2-year bioassay found that TBBPA exposure led to the development of uterine tumors in Wistar Han rats.[7] Studies were thus conducted to characterize the alterations in genes known to regulate thyroid homeostasis, oxidative stress, and perturbations of lipid and endogenous estrogen metabolism using targeted RT-PCR of suspected genes,[8] microarray, and RNA-seq. The present work investigated modes-of-action of TBBPA-mediated toxicity in female Wistar Han rats with microarray analysis and droplet digital PCR.

Materials & Methods

MODEL ORGANISM Female Wistar Han rats (12 weeks, 181-239 g) from Charles River, Raleigh, NC were used in these studies. Animals were maintained in an AAALAC-approved animal care facility. Animals were housed two per cage, and food and water were provided *ad libitum*. Prior to study initiation, estrus stage was synchronized by exploiting the Whitten effect.[9] All procedures were approved by the NIEHS Institutional Care and Use committee.

DOSING Animals were administered 5 consecutive daily doses of TBBPA or vehicle by gavage (250 mg/kg, 4 mL/kg; N=10/treatment group). Animals were euthanized 24 h after the final dose by CO_2 asphyxiation followed by excision of liver and uterus tissues.

SAMPLE COLLECTIONS Tissues (liver and proximal and distal uterus) were collected at necropsy and stored at -80ºC until analysis. Blood samples were collected via cardiac puncture. Samples were placed in labeled pre-weighed vials after all collections and maintained at -80ºC until analyses.

MICROARRAY RNA was isolated from liver and from the proximal (nearest the cervix) and the distal (nearest the ovaries) sections of the uterine horn. The purity and quality of the RNA were verified and the RNA was reverse transcribed. Gene expression analysis was done using Affymetrix Rat Genome 230 2.0 GeneChip® arrays. Microarray data were processed with Partek Flow to create a list of differentially expressed genes. Using Ingenuity Pathway Analysis, microarray data were evaluated to explore pathways perturbed by TBBPA treatment.

DROPLET DIGITAL PCR To validate the hypothesis generated through microarray analysis that TBBPA treatment may cause immunosuppression in the distal and proximal uterus, genes involved in the immune system were analyzed through droplet digital PCR in a Bio-Rad QX200 system. Probe-based assays were optimized for annealing temperature and cDNA concentration.

STATISTICAL ANALYSES Statistical analyses were performed using the unpaired t-test and Mann-Whitney rank sum test in GraphPad Prism 7.01. Graphs depict the mean ± SD of 9-10 rats/group and 3-6 replicates/rat. The mean was considered significantly different at *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Results & Discussion

Changes in expression of some genes following 5 daily administrations of TBBPA (250 mg/kg/d) were organ-dependent (liver or uterus) or specific to tissue location in the uterine horn.[8] The biological changes observed in that study may correlate to specific toxicities, including the carcinogenic response observed in TBBPA-treated female rats.[6] As the microarray analysis demonstrates, the top pathways in the uterus affected by TBBPA involved the immune system (Table 1).

Table 1. Top ten canonical pathways in TBBPA-treated distal uterus (determined from microarray data using Ingenuity Pathway Analysis)

Ingenuity Canonical Pathways
Graft-versus-Host Disease Signaling
Communication between Innate and Adaptive Immune Cells
Dendritic Cell Maturation
Allograft Rejection Signaling
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis
OX40 Signaling Pathway
Autoimmune Thyroid Disease Signaling
Antigen Presentation Pathway
Phagosome Formation
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses

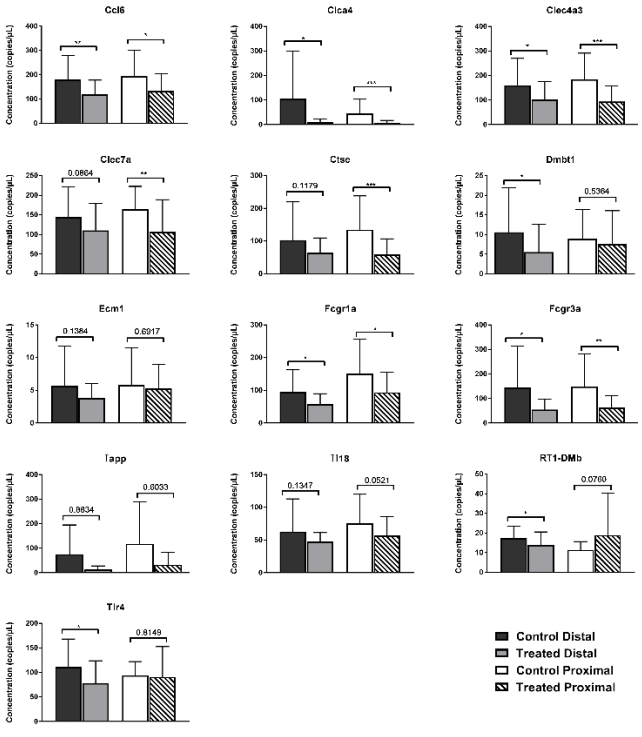
Genes involved in these pathways were selected for further validation through droplet digital PCR (Table 2). Several genes were significantly downregulated ($p<0.05$), and some were significantly downregulated in only one of the uterine tissues (Figure 1).

TBBPA has been linked to immunosuppression by inhibiting CD25 expression in murine splenocytes.[10] The downregulation of *Ctse* in female TBBPA-treated Wistar Han rats observed in this study may help explain the development of uterine tumors. Goto *et al*. observed that female cathepsin E-deficient mice developed swollen uteruses and tumors in the uterine horn.[11] Cathepsin E plays a role in host defense against tumor cells, possibly through tumor-associated macrophage-dependent cytotoxicity.[12] Cytokines regulate various immunological responses; thus, alterations in cytokine activity as implicated through decreased expression of *Ccl6* (a chemoattractant for helper T cells, B cells, and macrophages[13]) may be evidence of an immunotoxic insult caused by TBBPA. CLCA4 has been shown to be downregulated in breast tumors, and CLCA4 expression inhibits breast cancer cell proliferation.[14]

Table 2. Assayed genes for droplet digital PCR

Gene	Gene name and associated function
Ccl6	Chemokine (C-C motif) ligand 6 / cytokine activity, apoptosis
Clca4	Chloride channel accessory 4 / chloride transport
Clec4a3	C-type lectin domain family 4, member A3 / may play a role in immune response, phagocytosis
Clec7a	C-type lectin domain family 7, member A / pathogen recognition, regulated by Esr1
Ctse	Cathepsin E / antigen processing, regulates Il18
Dmbt1	Deleted in malignant brain tumors 1 / associated with endometrioid cancer, role in interaction of tumor cells and immune system
Ecm1	Extracellular matrix protein 1 / angiogenesis, inflammatory response
Fcgr1a	Fc fragment of IgG, high affinity Ia, receptor (CD64) / immune response, antigen presentation
Fcgr3a	Fc fragment of IgG, low affinity IIIa, receptor (CD16) / immune response regulation
Iapp	Islet amyloid polypeptide / apoptosis
Il18	Interleukin 18 / cytokine activity, T cell activation
RT1-DMb	RT1 class II, locus DMb / may play a role in antigen presentation
Tlr4	Toll-like receptor 4 / innate immunity activation, pathogen recognition

Figure 1. TBBPA-dependent changes in assayed gene expression in proximal or distal uterus



C-type lectin receptors (such as *Clec4a3* and *Clec7a*) are involved in antigen presentation; their interaction with tumor glycoproteins indicates a potential for immune surveillance (in which cells with aberrant glycosylation are detected and removed).[15] Decreased *Clec4a3* expression has been found to occur in a rat seminiferous tubule culture model after exposure to the endocrine-disrupting compound bisphenol A (BPA).[16] CLEC7A, or Dectin-1, recognizes N-glycan structures found on the surface of some tumor cells and directs natural killer cells to aid in killing the tumor cell; Dectin-1-deficient mice were found to have increased rates of tumor growth.[17]

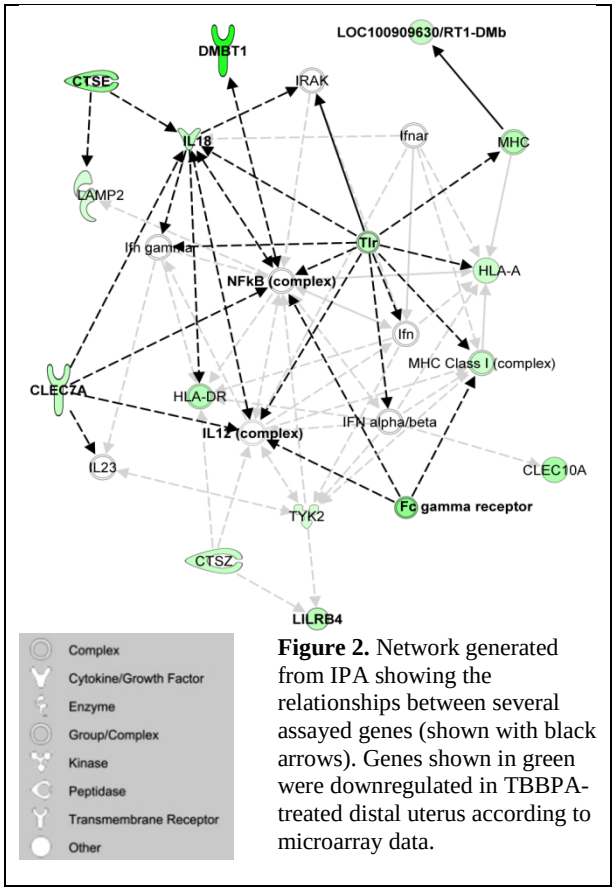


Figure 2. Network generated from IPA showing the relationships between several assayed genes (shown with black arrows). Genes shown in green were downregulated in TBBPA-treated distal uterus according to microarray data.

Sanders *et al.*[8] noted that expression of genes involved in regulation of cell growth and estrogen biosynthesis and metabolism was affected in the rats used for this present study. The work from Sanders *et al*. supported a hypothesis that disruption of estrogen homeostasis is a major mechanism for the increased incidence of uterine tumors observed in the NTP chronic bioassay of TBBPA.[6] Evidence presented here indicates that TBBPA treatment may also downregulate pathways involved in the immune system (Figure 2). It is further hypothesized that this effect may be due to estrogen-mediated immunosuppression in these rats although a direct effect of TBBPA is possible.

Dunnick *et al*. (2016)[18] have found that 13 weeks of repeat TBBPA exposure (1000 mg/kg) in female Wistar Han rats activated the interferon pathway in the liver, adding evidence to the hypothesis that long-term exposure to TBBPA could result in immunomodulatory changes that promote cancer. Furthermore, TBBPA has recently been classified as a Group 2A carcinogen by IARC, indicating TBBPA is probably carcinogenic to humans.[19] One of the findings from the IARC Monograph Working Group influencing this classification was the immunosuppressive effects of TBBPA.[19] The data presented here may aid in further characterization of the mechanism(s) by which chronic exposure to TBBPA may cause uterine adenocarcinomas in female Wistar Han rats.

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