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Involvement of reactive oxygen species in brominated diphenyl ether-47-induced inflammatory cytokine release from human extravillous trophoblasts *in vitro*

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ABSTRACT

Polybrominated diphenyl ethers (PBDEs) are widely used flame retardant compounds. Brominated diphenyl ether (BDE)-47 is one of the most prevalent PBDE congeners found in human breast milk, serum and placenta. Despite the presence of PBDEs in human placenta, effects of PBDEs on placental cell function are poorly understood. The present study investigated BDE-47-induced reactive oxygen species (ROS) formation and its role in BDE-47-stimulated proinflammatory cytokine release in a first trimester human extravillous trophoblast cell line, HTR-8/SVneo. Exposure of HTR-8/SVneo cells for 4 h to 20 μM BDE-47 increased ROS generation 1.7 fold as measured by the dichlorofluorescein (DCF) assay. Likewise, superoxide anion production increased approximately 5 fold at 10 and 15 μM and 9 fold at 20 μM BDE-47 with a 1-h exposure, as measured by cytochrome c reduction. BDE-47 (10, 15 and 20 μM) decreased the mitochondrial membrane potential by 47–64.5% at 4, 8 and 24 h as assessed with the fluorescent probe Rh123. Treatment with 15 and 20 μM BDE-47 stimulated cellular release and mRNA expression of IL-6 and IL-8 after 12 and 24-h exposures: the greatest increases were a 35-fold increased mRNA expression at 12 h and a 12-fold increased protein concentration at 24 h for IL-6. Antioxidant treatments (deferoxamine mesylate, (±)α-tocopherol, or tempol) suppressed BDE-47-stimulated IL-6 release by 54.1%, 56.3% and 37.7%, respectively, implicating a role for ROS in the regulation of inflammatory pathways in HTR-8/SVneo cells. Solvent (DMSO) controls exhibited statistically significantly decreased responses compared with non-treated controls for IL-6 release and IL-8 mRNA expression, but these responses were not consistent across experiments and times. Nonetheless, it is possible that DMSO (used to dissolve BDE-47) may have attenuated the stimulatory actions of BDE-47 on cytokine responses. Because abnormal activation of proinflammatory responses can disrupt trophoblast functions necessary for placental development and successful pregnancy, further investigation is warranted of the impact of ROS and BDE-47 on trophoblast cytokine responses.

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Introduction

Polybrominated diphenyl ethers (PBDEs) are synthetic flame-retardants widely used in polyurethane foam, textiles, plastics, building materials and insulation (Hites, 2004). Among the 209 PBDE congeners, BDE-47 (2,2′,4,4′-tetra-BDE) is one of the most prevalent congers found in human tissues and environmental samples (Hites, 2004). Because of PBDEs' environmental persistence and toxicity, the US EPA has identified PBDEs as a priority human health concern (U.S. Environmental Protection Agency, 2006). Limited studies reported possible reproductive toxicity of PBDEs during pregnancy. Rabbits orally exposed to PBDEs show decreased gestation length (Breslin et al., 1989). Elevated levels of PBDEs in human umbilical cord blood have been correlated with preterm birth, low birth weight or stillbirth (Wu et al., 2010). Although these studies suggest the association between PBDE exposure and adverse birth outcomes, and PBDEs distribute to human placenta (Frederiksen et al., 2009), extraplacental membranes (Miller et al., 2009), amniotic fluid (Miller et al., 2012), and umbilical cord blood (Frederiksen et al., 2009), studies of mechanisms by which PBDEs act on gestational tissues during pregnancy are limited. It is suggested that cytokine dysregulation alters extravillous trophoblast (EVT) processes, leading to placental dysfunction that may compromise pregnancy (Anton et al., 2012). For example, increased levels of inflammatory mediators such as cytokines and C-reactive protein are associated with the pathophysiology of preeclampsia and intrauterine growth restriction (IUGR), possibly contributing to abnormal placental function (Tjoa et al., 2003; Vince et al., 1995). Also, women who delivered preterm had higher rates of placental ischemia and abnormal placentation than controls (Germain et al., 1999; Kim et al., 2003), with high levels of interleukin (IL)-8 and IL-6 in cervical fluid, amniotic fluid and maternal serum (Goldenberg et al., 2005). Although these studies suggest that inflammation occurring at the maternal–fetal interface during pregnancy could contribute to abnormal placental function associated with adverse

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obstetrical outcomes, a recent report on PBDE-stimulated cytokine release in placenta, using second trimester human placental explant cultures (Peltier et al., 2012), showed that pre-exposure of placental explants to a PBDE mixture of congers 47, 99 and 100 enhanced placental proinflammatory response to heat-killed *E. coli*. However, to our knowledge, there are no previous reports of BDE-47 directly altering inflammatory pathways in human placental cells.

Oxidative stress is defined as the imbalance between pro-oxidants and antioxidants resulting in increase of reactive oxygen species (ROS). Oxidative stress in placenta has been associated with pathologies of pregnancy, including preterm labor, preeclampsia, and IUGR (Agarwal et al., 2012). A growing body of literature indicates that oxidative stress can activate a variety of transcription factors, including nuclear factor kappa B (NF-κB), activator protein 1(AP-1), and nuclear factor like 2 (Nrf2), leading to altered expression of genes for inflammatory cytokines, chemokines, and anti-inflammatory molecules (Reuter et al., 2010). Moreover, N-acetylcysteine, which can act as an antioxidant by increasing cellular concentrations of glutathione, prevents lipopolysaccharides (LPS)-stimulated parturition, fetal death in mice, and LPS-induced release of pro-inflammatory cytokines from human extraplacental membranes *in vitro* (Buhimschi et al., 2003; Cindrova-Davies et al., 2007). Together, these findings implicate interplay between cytokines and oxidative stress in the etiology of adverse pregnancy outcomes.

A few studies suggest that PBDEs induce generation of ROS in mammalian cells. He et al. (2008) showed that PBDEs induce lipid peroxidation and DNA damage in primary cultured rat hippocampal neurons. Reistad and Mariussen (2005) reported that pentabrominated diphenyl ether (DE-71) and BDE-47 enhanced the production of ROS, potentially through NADPH oxidase activation in human granulocytes. It is also reported that BDE-47 induced apoptosis in Jurkat cells, possibly through ROS overproduction and mitochondrial dysfunction (Yan et al., 2011). Shao et al (2008) reported that BDE-47 induced ROS overproduction, loss of mitochondrial membrane potential and apoptosis in human fetal liver hematopoietic stem cells. These data suggest a close relationship between ROS formation and toxicity induced by PBDEs. However, there is no previous report on PBDE-stimulated ROS formation in human placental cells and tissues.

Although inappropriate activation of the innate immune response can lead to placental dysfunction and certain environmental contaminants can activate innate immune responses (Campbell, 2004; Lin et al., 2010), there is a paucity of reports on PBDE-stimulated inflammation in first trimester placenta. Moreover, increased oxidative stress in placenta has been observed in pathological pregnancies, and ROS have been implicated in the activation of inflammatory responses in gestational compartments (Buhimschi et al., 2003; Cindrova-Davies et al., 2007). The present study examines the hypothesis that BDE-47 stimulates pro-inflammatory cytokine production via a ROS-mediated mechanism in the first trimester EVT human placental cell line HTR-8/SVneo.

Materials and methods

Chemicals and assay kits. BDE-47 was purchased from AccuStardard (New Haven, CT, USA). DMSO, deferoxamine mesylate (DFO), *tert*-butyl hydroperoxide (TBHP), cytochrome c from bovine heart, superoxide dismutase (SOD) from bovine erythrocytes, N-ethylmaleimide and rhodamine (Rh) 123, 4-hydroxy-TEMPO (tempol), and (±)-α-tocopherol were purchased from Sigma Aldrich (St. Louis, MO, USA). The 6-carboxy-dichlorodihydrofluorescein diacetate (carboxy-H2DCF-DA), CellMask™ Deep Red plasma membrane stain C10046, RPMI medium 1640, fetal bovine serum (FBS), OptiMem 1 reduced-serum medium, Hank's balanced salt solution (HBSS), 0.25% trypsin/EDTA solution and penicillin/streptomycin (P/S) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Recombinant IL-1β and sandwich enzyme-linked immunosorbent assay (ELISA) kits for human IL-6, IL-8 were purchased from R & D systems (Minneapolis, MN, USA). The MultiTox-Glo Multiplex cytotoxicity assay kit was purchased from Promega (Madison, WI, USA). QIAshredder, RNeasy mini plus kit, RT^2 First Strand kit for reverse transcriptase reaction, RT^2 qPCR SYBR Green/ROX Master Mix and primers for human β-microglobulin, IL-6, and IL-8 were purchased from Qiagen (Valencia, CA, USA). BDE-47 was prepared in dimethyl sulfoxide (DMSO) as a 50 mM stock solution. (±)-α-tocopherol was prepared in DMSO as a 100 mM stock solution. Rh123 was prepared in DMSO as a 2 μg/ml stock solution. Carboxy-H2DCF-DA was prepared in DMSO as 50 mg/ml stock solution. Other chemicals were applied directly into media.

Cell Culture and treatment. The human first trimester extravillous trophoblast cell line HTR-8/SVneo was kindly provided by Dr. Charles S. Graham (Queen's University, Kingston, ON, Canada). Cells between passages 71 and 84 were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. Cells were grown to a confluence of 70–90% before treatment. Cells were washed with OptiMem 1 containing 1% FBS and 1% P/S twice and acclimated with the medium for 1 h at 37 °C. From solutions of 5, 10, 15 and 20 mM BDE-47 in DMSO, exposure media containing 5, 10, 15 and 20 μM BDE-47 were made in OptiMem 1 containing 1% FBS and 1% P/S immediately prior to initiating the experiment. The final concentration of DMSO in medium was 0.7% (v/v).

Viability and cytotoxicity assays. Cells were seeded in a white 96-well plate at a density of 1×10^4 cells per well and incubated for 24 h at 37 °C. Cells were exposed to DMSO (solvent control) or BDE-47 (5, 10, 15 or 20 μM) and incubated for 24 h. After the 24-h incubation with BDE-47, cell viability and cytotoxicity were measured by the MultiTox-Glo Multiplex cytotoxicity assay kit. Briefly, this assay is based on two protease activities: one is a live-cell protease, and the other is a dead-cell protease, which is released from cells. Fluorescence is proportional to live cells while luminescence is proportional to dead cells. The assay was performed according to the manufacturer's instructions. Digitonin (300 μg/ml) was used as a positive control.

Dichlorofluorescein assay. Stimulation of ROS generation was assessed using the dichlorofluorescein (DCF) assay. Because artifactual results can occur in the DCF assay due to interactions with toxicants (Tetz et al., 2013), we confirmed that there was no increased DCF fluorescence by BDE-47 in cell free medium (data not shown). The HTR-8/SVneo cells were seeded at a density of 2.4×10^5cells per well in a 6-well plate and cultured for 24 h at 37 °C. Cells were pre-incubated in the presence or absence of 1 mM DFO for 1 h. Cells were washed once with Optimem1 medium containing 10% FBS and 1% P/S, and then exposed to 5, 10, 15 or 20 μM BDE-47 for 4 h. Treatment with 100 μM *tert*-butyl hydroperoxide (TBHP) was included as a positive control. After removal of the treatment and rinsing with HBSS, cultures were incubated for an additional 1 h with 100 μM carboxy-H2DCF-DA in HBSS. After removal of the dye solution and rinsing with HBSS, cells were counterstained with 5 μg/ml CellMask™ Deep Red plasma membrane stain for 5 min. After washing with HBSS and adding fresh HBSS back to the cultures, intracellular DCF fluorescence was visualized at 470 nm excitation and 525 nm emission, and Deep Red stain was visualized at 530 nm excitation and 593 nm emission using an EVOS digital inverted fluorescence microscope. Five images per treatment were taken: one image in each of the four quadrants and one in the center of the well. Equivalent adjustments for brightness and contrast were applied to each image in ImageJ software (National Institutes of Health). Additionally, fluorescence intensity was quantified using the method of He et al. (2008) with a few modifications. Cells exposed to BDE-47 were collected by treatment with 0.25% trypsin/EDTA solution for 2 min and washed twice with HBSS by centrifugation at 1200 rpm for 3 min, then re-suspended in HBSS. After 1-h incubation with 100 μM carboxy-H2DCF-DA in HBSS, the fluorescence intensity of 200,000 cells in a 96-well, black, clear-bottomed plate was measured using the

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