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Protective effect of nuclear factor E2-related factor 2 on inflammatory cytokine response to brominated diphenyl ether-47 in the HTR-8/SVneo human first trimester extravillous trophoblast cell line

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ABSTRACT

Polybrominated diphenyl ethers (PBDEs) are widely used flame retardants, and BDE-47 is a prevalent PBDE congener detected in human tissues. Exposure to PBDEs has been linked to adverse pregnancy outcomes in humans. Although the underlying mechanisms of adverse birth outcomes are poorly understood, critical roles for oxidative stress and inflammation are implicated. The present study investigated antioxidant responses in a human extravillous trophoblast cell line, HTR-8/SVneo, and examined the role of nuclear factor E2-related factor 2 (Nrf2), an antioxidative transcription factor, in BDE-47-induced inflammatory responses in the cells. Treatment of HTR-8/SVneo cells with 5, 10, 15, and 20 μM BDE-47 for 24 h increased intracellular glutathione (GSH) levels compared to solvent control. Treatment of HTR-8/SVneo cells with 20 μM BDE-47 for 24 h induced the antioxidant response element (ARE) activity, indicating Nrf2 transactivation by BDE-47 treatment, and resulted in differential expression of redox-sensitive genes compared to solvent control. Pretreatment with *tert*-butyl hydroquinone (tBHQ) or sulforaphane, known Nrf2 inducers, reduced BDE-47-stimulated IL-6 release with increased ARE reporter activity, reduced nuclear factor kappa B (NF-κB) reporter activity, increased GSH production, and stimulated expression of antioxidant genes compared to non-Nrf2 inducer pretreated groups, suggesting that Nrf2 may play a protective role against BDE-47-mediated inflammatory responses in HTR-8/SVneo cells. These results suggest that Nrf2 activation significantly attenuated BDE-47-induced IL-6 release by augmentation of cellular antioxidative system via upregulation of Nrf2 signaling pathways, and that Nrf2 induction may be a potential therapeutic target to reduce adverse pregnancy outcomes associated with toxicant-induced oxidative stress and inflammation.

Introduction

Polybrominated diphenyl ethers (PBDEs) are synthetic flame retardants widely used in polyurethane foam, textiles, plastics, building materials and insulation (Hites, 2004). BDE-47 (2,2′,4,4′-tetra-BDE) is one of the most prevalent congers found in human tissues and environmental samples (Hites, 2004), detected in nearly all human serum samples from the NHANES 2003–2004 biomonitoring assessment (Sjodin et al., 2008). 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 report associations between PBDE exposure and adverse birth outcomes such as preterm birth, low birth weight or stillbirth (Breslin et al., 1989; Wu et al., 2010). Although PBDEs have been found in gestational tissues such as 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.

Improper regulation of the inflammatory networks has been associated with adverse pregnancy outcomes such as miscarriage, preeclampsia, intrauterine growth restriction (IUGR), and preterm labor (Orsi and Tribe, 2008; Tjoa et al., 2004). Specifically, increased levels of inflammatory mediators such as interleukin (IL)-6 and C-reactive protein were associated with the pathophysiology of preeclampsia and IUGR

Abbreviations: AOX1, aldehyde oxidase 1; ARE, antioxidant response element; B2M, β-2-micoglobulin; BDE-47, 2,2′,4,4′-tetra-BDE; BSO, buthionine sulfoximine; CMV, cytomegalovirus; COX-2, cyclooxygenase-2; DHCR24, 24-dehydrocholesterol reductase; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; EVT, extravillous trophoblast; FBS, fetal bovine serum; FHL2, four and a half LIM domains 2; FTH1, ferritin, heavy polypeptide 1; GCLM, glutamate-cysteine ligase, modifier subunit; GPX1, glutathione peroxidase 1; GSH, glutathione; GSR, glutathione reductase; GST, glutathione S-transferase; HMOX1, heme oxygenase (decycling) 1; HO-1, heme oxygenase-1; IL, interleukin; IUGR, intrauterine growth restriction; Keap1, kelch-like ECH-associated protein 1; NAC, N-acetylcysteine; NF-κB, nuclear factor kappa B; NQO1, NAD(P)H dehydrogenase, quinone 1; Nrf2, nuclear factor E2-related factor 2; PBDEs, polybrominated diphenyl ethers; PGE2, prostaglandin E2; PRNP, prion protein; PTGS2, prostaglandin-endoperoxide synthase 2; RNF7, ring finger protein 7; ROS, reactive oxygen species; SLC7A11, solute carrier family 7 (anionic amino acid transporter light chain, xc-system), member 11; SQSTM1, sequestosome 1; SRXN1, sulfiredoxin 1; tBHQ, *tert*-butyl hydroquinone; TRX, thioredoxin; TXNRD1, thioredoxin reductase 1.

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(Tjoa et al., 2003; Vince et al., 1995), and women who delivered preterm had higher rates of placental ischemia and abnormal placentation than women who delivered at term (Germain et al., 1999; Kim et al., 2003). Moreover, increased levels of IL-8 and IL-6 in cervical fluid, amniotic fluid and maternal serum have been associated with preterm birth (Goldenberg et al., 2005). It is suggested that cytokine dysregulation alters extravillous trophoblast (EVT) functions during placentation, leading to placental alterations that may compromise pregnancy (Anton et al., 2012).

A few studies reported modulation of innate immune responses by BDE-47 treatment in peripheral blood mononuclear cells or placental explants (Ashwood et al., 2009; Peltier et al., 2012). Our previous study showed that BDE-47 treatment of a human first trimester EVT cell line, HTR-8/SVneo, stimulated mRNA and protein expression of the pro-inflammatory cytokines IL-6 and IL-8 (Park et al., 2014). Furthermore, suppression of BDE-47-induced IL-6 production by antioxidant treatments implicates a role for reactive oxygen species (ROS) in the initiation and regulation of BDE-47-stimulated inflammatory responses in the cells (Park et al., 2014). Similarly, the antioxidant N-acetylcysteine (NAC) prevented 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 suggest an interaction between oxidative stress and inflammatory pathways in gestational compartments. In fact, a growing body of literature shows that ROS can function as signaling molecules in mammalian cells (Finkel, 1998; Khan and Wilson, 1995; Remacle et al., 1995) to regulate expression of genes for inflammatory cytokines, chemokines, and anti-inflammatory molecules (Reuter et al., 2010).

Nuclear factor E2-related factor 2 (Nrf2) is the master transcriptional regulator of oxidative and xenobiotic stress responses (Tjoa et al., 2003). In response to oxidative insults, Nrf2 binds to the antioxidant response element (ARE) in a promoter and activates ARE-regulated genes. A wide range of natural and synthetic small molecules such as *tert*-butyl hydroquinone (tBHQ) and sulforaphane induce Nrf2 activity to exert cytoprotective activities (Gharavi et al., 2007; Juge et al., 2007). Especially, the anti-inflammatory effect of Nrf2 activation has been implicated in a variety of experimental models (Khor et al., 2006; Rangasamy et al., 2004, 2005; Thimmulappa et al., 2006). Although the mechanisms for the anti-inflammatory effects of Nrf2 are not fully understood, it is suggested that augmentation of cellular antioxidant responses via up-regulation Nrf2 signaling pathway and inhibition of proinflammatory nuclear factor kappa B (NF-κB) signaling pathway may have roles in these responses (Jin et al., 2011; Khodagholi and Tusi, 2011).

Despite its importance, there are few studies about the roles of Nrf2 in gestational tissues during pregnancy. It has been recently reported that dysregulation of Nrf2 signaling pathways is associated with adverse birth outcomes such as preeclampsia and IUGR (Chigusa et al., 2012; Kweider et al., 2012; Loset et al., 2011; Wruck et al., 2009). To our knowledge, however, there is no report about the role of Nrf2 activation in the regulation of toxicant-stimulated inflammatory responses in human first trimester placental cells. Because ROS have been implicated in the activation of inflammatory responses in gestational compartments (Buhimschi et al., 2003; Cindrova-Davies et al., 2007) and our previous study showed that BDE-47-stimulated cytokine production was dependent on ROS formation (Park et al., 2014), we suggest that the antioxidative transcription factor Nrf2 may play a protective role on BDE-47-induced inflammatory responses in the HTR-8/SVneo cell model. The present study aimed to investigate BDE-47-stimulated antioxidant responses in HTR-8/SVneo cells and examined the roles of Nrf2 activation by Nrf2 inducers on BDE-47-induced inflammatory cytokine responses in the cells.

Materials and methods

Chemicals and assay kits. BDE-47 was purchased from AccuStardard (New Haven, CT, USA). Dimethyl sulfoxide (DMSO), *tert*-butyl hydroquinone (tBHQ), and sulforaphane were purchased from Sigma Aldrich (St. Louis, MO, USA). RPMI medium 1640, fetal bovine serum (FBS), OptiMem 1 reduced-serum medium, 10 mM non-essential amino acids in minimal essential medium, 0.25% trypsin/EDTA solution and penicillin/streptomycin were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Sandwich enzyme-linked immunosorbent assay (ELISA) kit for human IL-6 was purchased from R&D systems (Minneapolis, MN, USA). Antioxidant Response Cignal reporter assay kit, NF-κB Cignal reporter assay kit, Attractene transfection reagent, QIAshredder columns, and RNeasy kits were purchased from Qiagen (Valencia, CA). Dual Luciferase, GSH-Glo™ Glutathione Assays were purchased from Promega (Madison, WI). The iScript cDNA synthesis kits and SsoAdvanced SYBR Green Supermix were purchased from Bio-Rad (Hercules, CA). Primers were synthesized by Integrated DNA Technologies (Coralville, IA).

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% penicillin/streptomycin 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% penicillin/streptomycin immediately prior to initiating the experiment. The final concentration of DMSO in the medium was 0.7 % (v/v).

Measurement of intracellular glutathione concentration. Changes in intracellular glutathione (GSH) levels by BDE-47 treatment on HTR-8/SVneo cells were quantified using the GSH-Glo Glutathione assay kit (Promega). The assay is based on the conversion of a luciferin derivative into luciferin in the presence of GSH, catalyzed by glutathione S-transferase (GST). The luminescence generated in a coupled reaction with firefly luciferase is proportional to the amount of glutathione present in the sample. Cells were seeded at a density of 10,000/well in a white, clear-bottomed 96-well plate and incubated for 24 h at 37 °C. Then, cells were exposed to BDE-47 for 0.5, 4 or 24 h at 37 °C. After exposure, the culture medium was removed and 100 μl of GSH-Glo™ Reagent was added to each well. After a 30 min-incubation, 100 μl of Luciferin Detection Reagent was added to each well, followed by an additional 15-min incubation. The plate was then read in a luminometer. A standard curve was generated by serial 2-fold dilutions of a 10× GSH solution. GSH levels per well were calculated based on luminescence. To examine the effect of Nrf2 induction on GSH production, cells were pretreated with tBHQ for 1 h or sulforaphane for 24 h prior to BDE-47 treatment. After treatment with BDE-47, GSH levels were measured following the manufacturer's protocol as described above.

Oxidative stress gene array and qRT validation. Changes in mRNA expression of 84 target genes by BDE-47 treatment on HTR-8 cells were quantified using the commercial Oxidative Stress Responses PCR Array (SA Biosciences; Valencia, CA). Cells were treated with DMSO (solvent control, 0.7% v/v) or BDE-47 (20 μM) for 4 or 24 h. A concentration of 20 μM BDE-47 was used because our previous studies showed that this was an effective concentration for stimulating increased ROS formation in HTR-8/SVneo cells without being cytotoxic (Park et al., 2014). After incubation, cell lysates were collected and homogenized using QIA shredder (Qiagen). Total RNA was extracted from homogenized lysates using RNeasy mini plus kit (Qiagen), and cDNA was synthesized from 1 μg of total RNA using iScript cDNA synthesis kits (Bio-Rad) following the manufacturer's protocols. For the array, cDNA from the solvent control and BDE-47 treatment groups were analyzed using the Applied Biosystems 7900HT Sequence Detection System following the SABiosciences recommended protocol. Fold changes were