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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 μ M BDE-47 for 24 h increased intracellular glutathione (GSH) levels compared to solvent control. Treatment of HTR-8/SVneo cells with 20 μ M 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- κ B) 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.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Keywords

Polybrominated diphenyl ethers (PBDEs); antioxidant response; HTR-8/SVneo cells; human placental cells; cytokines; nuclear factor E2-related factor 2 (Nrf2)

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 congeners 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 (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.*, 2003a; 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 have been implicated in a variety of experimental models (Khor *et al.*, 2006; Rangasamy *et al.*, 2004; Rangasamy *et al.*, 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- κ B) 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.*, 2003b; 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 AccuStandard (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 Signal reporter assay kit, NF- κ B Signal 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% CO₂ 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 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 10X 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 calculated from CT values (gene of interest CT values – Average of all housekeeping gene CT values) using the CT method. Mean CT values were compared between groups using paired t-tests from the Limma package of Bioconductor (Smyth, 2004). With qRT-PCR, we validated the findings of the array for those genes with significant mRNA expression changes that were approximately two-fold or more with 20 μ M BDE-47 treatment: solute carrier family 7 (anionic amino acid transporter light chain, xc- system), member 11 (SLC7A11), heme oxygenase (decycling) 1 (HMOX1), aldehyde oxidase 1 (AOX1), sulfiredoxin 1 (SRXN1), prostaglandin-endoperoxide synthase 2 (PTGS2), sequestosome 1 (SQSTM1), prion protein (PRNP), glutathione reductase (GSR), ring finger protein 7 (RNF7), thioredoxin reductase 1 (TXNRD1), four and a half LIM domains 2 (FHL2), glutamate-cysteine ligase, modifier subunit (GCLM), glutathione peroxidase 1 (GPX1), ferritin, heavy polypeptide 1 (FTH1), and 24-dehydrocholesterol reductase (DHCR24). Sequences for primer pairs are provided in Supplemental material, Table 1. The qRT-PCR reactions were prepared with SsoAdvanced SYBR Green Supermix and primers, and run on a Bio-Rad CFX96 Real time C1000 thermal cycler following the manufacturer's recommended protocols. The mRNA levels of each gene of interest were normalized to β -2-microglobulin mRNA levels and presented as fold change compared to solvent controls.

Measurement of ARE reporter activity

Nrf2 activity was assessed using a commercially available reporter construct (SABiosciences, Qiagen). The reporter consists of a mixture of inducible firefly luciferase gene downstream of tandem antioxidant response element (ARE) consensus binding site repeats and constitutive Renilla luciferase gene controlled by the cytomegalovirus (CMV) promoter. HTR-8/SVneo cells were seeded at a density of 20,000/well in white, clear bottom 96-well plates containing transfection reagent complexed with negative control, positive control, or ARE reporter constructs in Opti-MEM 1 supplemented with 1% non-essential amino acid (NEAA) and 3% FBS. After transfection for 6 h at 37°C, the transfection complex was replaced with fresh medium and cells were incubated for 18 h at 37°C. Cells were then pretreated with tBHQ for 1 h or with sulforaphane for 24 h prior to treatment with BDE-47 for 24 h. Treatment solutions were prepared in OptiMEM 1 supplemented with 1% NEAA, 1% FBS and 1% P/S. After treatment with BDE-47, medium was aspirated, and cells were passively lysed. Dual luciferase assays were performed according to manufacturer's instructions. Luminescence was measured using a GloMax Multi Plus detection system (Promega) with two injectors. ARE firefly luciferase activity was normalized to luciferase activity of Renilla, included as an internal control accounting for cell number and transfection efficiency. Data are presented as the fold change in luciferase activity normalized to the control.

Measurement of NF- κ B reporter activity

NF- κ B activity was assessed using a commercially available reporter construct (SABiosciences, Qiagen). The reporter consists of a mixture of inducible firefly luciferase gene downstream of tandem NF- κ B consensus binding site repeats and constitutive Renilla

luciferase gene controlled by a CMV promoter. The assay was conducted as described above for ARE reporter activity. NF- κ B firefly luciferase activity was normalized to luciferase activity of Renilla. Data are presented as the fold change in luciferase activity normalized to the control.

Measurement of cytokine release

The HTR-8/SVneo cells were seeded at a density of 5×10^4 cells per well in a 24-well plate and cultured for 24 h at 37 °C. Cells were washed once with OptiMem1 medium containing 1 % FBS and 1% penicillin/streptomycin, and pretreated with tBHQ for 1 h or sulforaphane for 24 h prior to 20 μ M BDE-47 treatment for 24 h. The concentration of IL-6 in the medium was then analyzed by ELISA as described above, expressed as pg/ml.

Statistical analysis

Statistical analysis was performed with Sigma Plot 11.0 software (Systat Software Inc., San Jose, CA, USA). Data were analyzed either by one-way analysis of variance (ANOVA) or repeated measures two-way ANOVA. If significant effects were detected, the ANOVA was followed by Tukey post-hoc comparison of means. A $P < 0.05$ was considered statistically different. Data were expressed as means $\pm$ SEM.

RESULTS

Effect of BDE-47 on cellular GSH

Because our previous work showed that BDE-47 increases generation of reactive oxygen species (Park *et al.*, 2014), intracellular GSH concentration as an indicator of oxidative stress was quantified in HTR-8/SVneo cells after 24-h treatment with BDE-47 using a luminescence based assay. Treatment with 5, 10, 15 and 20 μ M BDE-47 increased GSH production by 22%, 39%, 29%, and 52%, respectively, compared to the solvent control (Figure 4.1A; $P < 0.05$). Treatment with 20 μ M BDE-47 resulted in significantly increased GSH production compared to treatment with 5 μ M BDE-47, also, indicating a concentration-dependent response (Figure 1A; $P < 0.05$). To examine the temporal changes in GSH production in BDE-47-treated cells, GSH levels were measured at 0.5, 4, and 24 h after treatment with 20 μ M BDE-47. BDE-47 stimulated GSH production after 24 h (51% compared to solvent control; $P < 0.05$), as observed in the prior experiment, but there were no statistically significant changes in GSH levels at 0.5 or 4 h (Figure 1B; $P < 0.05$). Treatment with 50 μ M BSO, included as a positive control, almost completely depleted GSH after 24 h.

Effect of BDE-47 treatment on ARE reporter activity

We investigated Nrf2 activation as a possible explanation for the increased cellular GSH concentrations observed with BDE-47 treatment, using an ARE reporter activity assay. After 24 h treatment, 10 and 20 μ M BDE-47 increased ARE activity by 1.7-fold and 2-fold, respectively, compared to solvent control, indicating Nrf2 activation (Figure 2; $P < 0.05$). We did not detect statistically significant changes at 6 h, although slight increases in ARE activity were suggested.

Oxidative stress PCR array

Probing BDE-47 activation of antioxidant responses further, we used a commercial Oxidative Stress PCR Array to investigate changes in expression of redox-sensitive genes. We identified 15 genes with mRNA expression significantly changed two-fold or more by 20 μ M BDE-47 treatment compared to solvent control (for complete mRNA array data, see Supplemental material, Table 2). Changes in expression of the array-identified genes were then examined by qRT-PCR. Consistent with the array results, treatment with 20 μ M BDE-47 for 24 h significantly increased mRNA expression of HMOX1, PTGS2, and PRNP by 4.9 fold, 4.7 fold, and 2.5 fold, respectively, and nearly abolished mRNA expression of DHCR24 to 0.08 fold relative to solvent control (Figure 3A, $P<0.05$). The mRNA expression of genes involved in GSH redox cycling was also induced by 20 μ M BDE-47 treatment, with SLC7A11, SRXN1, GCLM, and GPX1 increased 3 fold, 1.8 fold, 1.5 fold and 1.7 fold, respectively (Figure 3B, $P<0.05$). BDE-47 suppressed mRNA expression of GSR to 0.7 fold relative to solvent control (Figure 3B, $P<0.05$). We did not observe any significant changes in mRNA expression with 15 μ M BDE-47.

Effect of Nrf2 inducers on ARE reporter activity

We validated the efficacy of tBHQ and sulforaphane as Nrf2 inducers in the HTR-8/SVneo cells and investigated effects of the Nrf2 inducers on BDE-47-stimulated Nrf2 activation using an ARE reporter activity assay. Treatment with 20 and 50 μ M tBHQ increased ARE activity by 1.7-fold and 2.4-fold, respectively, compared with controls not exposed to tBHQ (No BDE-47 with 0 μ M tBHQ, Figure 4A; $P<0.05$). Similarly, 10 μ M sulforaphane increased ARE activity by 1.8 fold compared with controls not exposed to sulforaphane (No BDE-47 with 0 μ M sulforaphane, Figure 4B; $P<0.05$). No statistically significant changes were observed with 5 μ M tBHQ, or with 5 and 7.5 μ M sulforaphane. These results identified 20 and 50 μ M tBHQ, and 10 μ M sulforaphane, as effective concentrations for Nrf2 activation. Because subsequent experiments would utilize co-treatments of BDE-47 with the Nrf2 inducers, we also measured ARE activity in the presence of BDE-47. Pretreatment with 0, 5, 20, and 50 μ M tBHQ followed by 20 μ M BDE-47 treatment increased ARE activity 1.9 fold, 2.8 fold, 3 fold, and 3.5 fold, respectively, compared to control (No BDE-47 with 0 μ M tBHQ) (Figure 4A; $P<0.05$). Treatment with 50 μ M tBHQ increased ARE activity significantly higher than 0, 5 and 10 μ M tBHQ within the 20 μ M BDE-47 pretreatment group, showing a concentration-dependent response. BDE-47-treated cells always showed significantly higher ARE activity compared to cells with no BDE-47 and the same tBHQ concentration (Figure 4A; $P<0.05$). Similarly, pretreatment with 0, 5, 7.5, and 10 μ M sulforaphane followed by 20 μ M BDE-47 treatment increased ARE activity 1.6 fold, 1.8 fold, 2 fold, and 3 fold, respectively, in BDE-47-pretreated cells compared to control (No BDE-47 with 20 μ M tBHQ) (Figure 4B; $P<0.05$). Treatment with 10 μ M sulforaphane resulted in significantly increased activity compared to 0, 5 and 7.5 μ M sulforaphane, showing a concentration-dependent increase (Figure 4B; $P<0.05$). BDE-47-treated cells showed significantly higher ARE activity compared to cells with no BDE-47 and the same sulforaphane concentration (Figure 4.4B; $P<0.05$). These data show that pretreatment with Nrf2 inducers stimulates Nrf2 transactivation, leading to increased antioxidant capacity in HTR-8/SVneo cells.

Effect of Nrf2 inducers on BDE-47-stimulated GSH production

To examine the effect of Nrf2 transactivation by tBHQ on cellular antioxidative capacity, changes in intracellular antioxidant GSH concentrations were assayed. In cells without BDE-47-treatment, 20 and 50 μ M tBHQ increased GSH production 20% and 37%, respectively, compared to control (No BDE-47 with 0 μ M tBHQ) (Figure 5; $P<0.05$). Following pretreatment with 0, 10, 20 and 50 μ M tBHQ, 20 μ M BDE-47 increased GSH concentration by 17%, 27%, 40%, and 63%, respectively, compared to control (No BDE-47 with 0 μ M tBHQ) (Figure 5; $P<0.05$). tBHQ induced GSH production in a concentration-dependent manner, such that pretreatment with 50 μ M tBHQ significantly increased GSH compared with pretreatment with 10 and 20 μ M tBHQ in those cells subsequently exposed to 20 μ M BDE-47 (Figure 5; $P<0.05$). BDE-47-treated cells showed significantly higher GSH concentrations compared to cells not exposed to BDE-47 at the same tBHQ concentration (Figure 4A; $P<0.05$) (Figure 5; $P<0.05$). These findings suggest that cells pretreated with tBHQ may have an augmented antioxidant capacity against BDE-47 treatment.

Effect of Nrf2 inducers on expression of HMOX1 and GCLM

To evaluate the role of Nrf2 in BDE-47-induced expression of antioxidant genes, mRNA expression of HMOX1 and GCLM was quantified in HTR-8/SVneo cells after pretreatment with tBHQ without and with subsequent exposure to BDE-47. Treatment with 20 μ M BDE-47 significantly increased mRNA expression of HMOX1 and GCLM compared to control (No BDE-47 with 0 μ M tBHQ) at all tBHQ concentrations (0, 10, 20 and 50 μ M) (Figure 6A and 6B; $P<0.05$). Pretreatment with 50 μ M tBHQ significantly increased HMOX1 mRNA expression by 68% compared to no pretreatment (0 μ M tBHQ) in BDE-47-treated cells (Figure 6A; $P<0.05$). However, mRNA expression of GCLM was not statistically significantly increased (Figure 6B; $P=0.062$). We did not observe any significant changes in solvent controls (No BDE-47) with tBHQ pretreatment. Increased expression of antioxidant enzymes by tBHQ suggests that cells pretreated with tBHQ might have increased protection from BDE-47-stimulated oxidative damage.

Effect of Nrf2 inducers on IL-6 production

Because IL-6 was shown to regulate trophoblast migration and invasion *in vitro* (Jovanovic and Vicovac, 2009), and our previous study showed that BDE-47-stimulated IL-6 production was dependent on ROS formation (Park *et al.*, 2014), we investigated the role of the antioxidant transcription factor Nrf2 in regulation of BDE-47-stimulated IL-6 release. HTR-8/SVneo cells were pretreated with the known Nrf2 inducers tBHQ or sulforaphane prior to exposure to BDE-47. Neither tBHQ nor sulforaphane had a significant effect on IL-6 release in the absence of BDE-47 exposure (Figure 7A and 7B, respectively). Treatment with 20 μ M BDE-47 increased IL-6 release compared to cells not exposed to BDE-47 for all pretreatment conditions (0–50 μ M tBHQ or 0–10 μ M sulforaphane, Figures 7A and 7B, respectively; $P<0.05$). Though still elevated compared with no BDE-47 treatment, pretreatment with the Nrf2 inducers reduced BDE-47-stimulated IL-6 release: 50 μ M tBHQ significantly suppressed BDE-47-stimulated IL-6 release by 55% compared with 0 μ M

tBHQ (Figure 7A; $P<0.05$), and 10 μM sulforaphane decreased BDE-47-induced IL-6 release by 65% compared with 0 μM sulforaphane (Figure 8B; $P<0.05$).

Effect of tBHQ on NF- κ B transactivation

Because prior studies implicated that Nrf2 activation exhibits its anti-inflammatory effect partly via suppression of the pro-inflammatory transcription factor NF- κ B (Jin *et al.*, 2011), we examined the possible involvement of Nrf2 in BDE-47-stimulated NF- κ B reporter activity. Treatment with tBHQ alone had no statistically significant effect on NF- κ B activation compared with solvent control (0 μM tBHQ with no BDE-47; Figure 8). Treatment with 20 μM BDE-47 increased NF- κ B reporter activity 3.4-fold compared to control (0 μM tBHQ with no BDE-47) (Figure 8; $P<0.05$). Though elevated compared with no BDE-47 treatment, pretreatment with 50 μM tBHQ suppressed the BDE-47-stimulated activation of NF- κ B by 32% (Figure 4.8; $P<0.05$).

DISCUSSION

PBDEs are flame retardant chemicals commonly detected in human serum, with BDE-47 among the most abundant of the PBDE congeners detected (Sjodin *et al.*, 2008). Previously, we showed that BDE-47 directly stimulates ROS generation and proinflammatory cytokine responses in the first trimester human EVT cell line, HTR-8/SVneo (Park *et al.*, 2014). In the present study, we show that BDE-47 activated oxidative stress response pathways, stimulating differential expression of redox-sensitive genes and augmentation of intracellular GSH, with concurrent transactivation of Nrf2 and NF- κ B in HTR-8/SVneo cells. Especially, we report novel findings that induction of Nrf2 activity by Nrf2 inducers suppressed BDE-47-stimulated release of the proinflammatory cytokine IL-6 and activation of a NF- κ B reporter in HTR-8/SVneo cells, implicating crosstalk between Nrf2 and NF- κ B pathways.

The concentrations of BDE-47 in this study are several orders of magnitude higher (100-fold or more) than the median concentrations reported previously in human gestational fluids and tissue: 337 – 21842 pg/ml in amniotic fluid (Miller *et al.*, 2012), 0.11–3000 ng/g lipid in placenta (Doucet *et al.*, 2009; Frederiksen *et al.*, 2009), and 0.46 to 504 ng/g lipid in umbilical cord blood (Frederiksen *et al.*, 2009; Guvenius *et al.*, 2003; Wu *et al.*, 2010). However, the concentrations of PBDEs in placenta can be as high as $\sim 8 \mu\text{M}$ (Doucet *et al.*, 2009). Moreover, correcting for adsorption onto plastic, estimated at 73% (Barber *et al.*, 2006; Mundy *et al.*, 2004), the corrected concentrations of BDE-47 in culture medium in this study range from 2.7 nM to 5.4 μM .

Consistent with ROS generation previously described (Park *et al.*, 2014), BDE-47 treatment resulted in differential expression of the redox-sensitive genes HMOX1, PTGS2, PRNP, DHCR24, SLC7A11, SRXN1, GSR, GCLM, and GPX1 in HTR-8/SVneo cells. Several lines of evidence suggest that the antioxidant and anti-inflammatory enzyme heme oxygenase (HO)-1 (Tjoa *et al.*, 2003), encoded by the gene HMOX1, is a key regulator during pregnancy (Vince *et al.*, 1995). For example, HMOX1 polymorphisms have been associated with increased incidence of idiopathic recurrent miscarriages in women (Denschlag *et al.*, 2004), and placentas from human pathologic pregnancies including spontaneous abortion, choriocarcinoma, and hydatidiform mole, express lower levels of

HO-1 compared with normal pregnancies (Zenclussen *et al.*, 2003). PRNP, the gene for prion protein, is known to protect cells from oxidative damage and to prevent apoptosis (Liang *et al.*, 2006; Watt *et al.*, 2005). It was recently reported that PRNP is highly expressed in placentas from preeclamptic pregnancies (Hwang *et al.*, 2010). We suggest that HMOX1 and PRNP may play protective roles against BDE-47-stimulated generation of ROS and pro-inflammatory mediators in placental cells.

Similarly, SLC7A11, SRXN1, GSR, GCLM, and GPX1 are genes involved in GSH redox cycling. The first step of GSH synthesis is rate-limiting and is catalyzed by glutamate-cysteine ligase composed of a glutamate-cysteine ligase catalytic subunit (GCLC) and a glutamate-cysteine ligase modifier subunit (GCLM) (Lu, 2009). Furthermore, SLC7A11 encodes an amino acid antiporter that mediates the exchange of extracellular L-cystine and intracellular L-glutamate across the cellular plasma membrane, a critical step in glutathione production and anti-oxidant protection (Lewerenz *et al.*, 2013), and GSR encodes glutathione reductase, an antioxidant enzyme that catalyzes the reduction of GSH disulfide leading to increased availability of reduced GSH (Harvey *et al.*, 2009). Thus, the upregulation of GCLM, SLC7A11, and GSR is consistent with the augmented GSH concentrations observed in BDE-47-stimulated HTR-8/SVneo cells. Increased expression of SRXN1 and GPX1 may implicate increased oxidation of proteins and lipids by BDE-47-stimulated ROS generation (Park *et al.*, 2014) because SRXN1 codes for sulfiredoxin 1, which plays a role in reduction of oxidative modification on proteins (Findlay *et al.*, 2005), and GPX1 codes for glutathione peroxidase 1, which catalyzes the reduction of hydroperoxides and lipid peroxides (Chance *et al.*, 1979). However, further experiments beyond the scope of the present study are needed to measure oxidation of proteins and lipids. Genes involved in glutathione redox regulation have been linked with adverse pregnancy outcomes. For example, female GCLM^{-/-} mice had compromised fertility due to early preimplantation embryo mortality (Nakamura *et al.*, 2011). In addition, GSR activity was increased in human placentas from preterm delivery (Prokopenko *et al.*, 2002) and GPX1 activity was decreased in human preeclamptic placentas (Mistry *et al.*, 2010).

Among other genes, expression of PTGS2, the gene for COX-2, was highly induced in our study. COX-2 is a rate-limiting enzyme in the synthesis of prostaglandins (Shanmugam *et al.*, 2006). Increased PTGS2 mRNA expression and prostaglandins in gestational compartments have been associated with preterm birth (Cox *et al.*, 1993; Mijovic *et al.*, 1998). In addition, prostaglandin E2 (PGE2) has been reported to regulate trophoblast migration and invasion that are critical for proper placentation (Biondi *et al.*, 2006; Horita *et al.*, 2007a; Nicola *et al.*, 2005a). Given the critical roles for prostaglandins in pregnancy, further study could investigate the effects of BDE-47 on PGE2 production and trophoblast cellular function. In addition, expression of DHCR24, the gene for 3 β -hydroxysterol-D24 reductase, was decreased in the present study. Because DHCR24 catalyzes the last step in cholesterol biosynthesis, reduced DHCR24 expression could potentially interfere with synthesis of steroid hormones, including progesterone, which plays critical roles in maintenance of pregnancy (Luu *et al.*, 2014). Moreover, DHCR24 expression was downregulated in IUGR placentas (Diplas *et al.*, 2009). Whether the BDE-47-stimulated DHCR24 gene responses observed in the present study are relevant to human pregnancy requires additional experiments beyond the scope of the present study. Furthermore, changes

in expression of critical genes should be confirmed with protein analysis in future experiments to ascertain functional relevance.

Expression of the genes identified in the PCR array are either directly or indirectly regulated by Nrf2 (Ma, 2013; Taylor *et al.*, 2008; Wakabayashi *et al.*, 2010), suggesting that Nrf2 may play a critical role in the regulation of BDE-47-mediated cellular defense responses. Nrf2 is a well-known redox-sensitive transcription factor that translocates to the nucleus where it binds to ARE and activates ARE-mediated gene expression (Itoh *et al.*, 1997; Motohashi and Yamamoto, 2004; Osburn *et al.*, 2006). Although we did not measure Nrf2 translocation, we did observe increased ARE reporter activity in BDE-47-exposed HTR-8/SVneo cells, indicating Nrf2 activation in response to BDE-47. BDE-47 stimulates increased ROS generation (Park *et al.*, 2014), and ROS can oxidize cysteine residues on the Nrf2 inhibitor Keap1, leading to conformational changes in Keap1, with subsequent Nrf2 release and translocation of Nrf2 to activate ARE-dependent gene expression (Rushmore *et al.*, 1991), ultimately serving as a defensive mechanism to protect cells from ROS and inflammation. Experiments beyond the scope of this investigation, including knockdown of Nrf2 and Keap1, are needed to provide a more complete understanding of the roles of Nrf2 in the BDE-47-stimulated responses. Nonetheless, although there have been extensive studies on the protective role of Nrf2 against carcinogens and xenobiotics *in vitro* and *in vivo* (Fahey *et al.*, 2002; Kensler *et al.*, 2007), this is the first study to report BDE-47-stimulated activation of Nrf2 pathways in human placental cells.

In limited studies, increased Nrf2 activity was reported in cytotrophoblasts and EVT's from placentae with IUGR or preeclampsia (Kweider *et al.*, 2012; Wruck *et al.*, 2009). In addition, genome-wide transcriptional profiling of preeclamptic and normal pregnancies showed that the Nrf2-mediated oxidative stress response was dysregulated in preeclampsia (Chigusa *et al.*, 2012; Loset *et al.*, 2011). Furthermore, decreased expression of HO-1, a hallmark of Nrf2 activation, was associated with lower cell motility and trophoblast invasion (Bilban *et al.*, 2009). Together, these data imply that Nrf2 may play a critical role in the regulation of trophoblast cellular function and invasion, and that dysregulation of Nrf2 may contribute to the etiology and progression of birth complications.

Migration and invasion of extravillous trophoblast into the spiral arteries are critical events during placentation (Brosens *et al.*, 1967; Pijnenborg *et al.*, 1983). It has been suggested that inflammation within the gestational compartment may lead to impaired trophoblast cellular function, contributing to the placental dysfunction seen in pregnancy-related disorders (Anton *et al.*, 2012) such as IUGR, preeclampsia and preterm birth (Germain *et al.*, 1999; Gomez *et al.*, 2008; Kim *et al.*, 2002; Ness and Sibai, 2006; Riewe *et al.*, 2010). IL-6 has been shown to increase migration and invasion in HTR-8/SVneo cells (Jovanovic *et al.*, 2010; Jovanovic and Vicovac, 2009) and in JEG-3 choriocarcinoma cells (Dubinsky *et al.*, 2010). In addition, LPS reduced invasion of HTR-8/SVneo cells with increased production of IL-6 (Anton *et al.*, 2012). Moreover, high levels of IL-6 in the cervicovaginal fluid, amniotic fluid, and maternal serum were associated with increased risk for preterm birth (Dortbudak *et al.*, 2005; Goepfert *et al.*, 2001; Romero *et al.*, 2002; Wenstrom *et al.*, 1996) with evidence of higher rates of placental ischemia and abnormal placentation than controls (Germain *et al.*, 1999; Kim *et al.*, 2003). These findings implicate a critical role for IL-6 in

regulating trophoblast cellular function during placentation, and suggest that dysregulation of IL-6 production may contribute to adverse birth outcomes associated with abnormal placentation.

Our previous study showed that BDE-47 stimulated production of proinflammatory IL-6 in HTR-8/SVneo cells (Park *et al.*, 2014). Because the role of Nrf2 in the regulation of trophoblast cellular function and invasion is implicated (Bilban *et al.*, 2009) and many studies have provided evidence of an anti-inflammatory effect of Nrf2 in a variety of experimental models (Khor *et al.*, 2006; Rangasamy *et al.*, 2004; Rangasamy *et al.*, 2005; Thimmulappa *et al.*, 2006), the present study examined the hypothesis that induction of Nrf2 would suppress BDE-47-stimulated IL-6 release in HTR-8/SVneo cells. Our results showed that pretreatment with the Nrf2 inducers tBHQ or sulforaphane suppressed BDE-47-stimulated IL-6 production NF- κ B transactivation in HTR-8/SVneo cells. NF- κ B is a transcription factor that plays a crucial role in immune and inflammatory response (Blackwell and Christman, 1997). Although we did not assess a causal relationship between BDE-47-stimulated NF- κ B activity and IL-6 release, NF- κ B is well known to regulate the transcription of IL-6 (Blackwell and Christman, 1997; Reuter *et al.*, 2010). As such, decreased NF- κ B activity partially explains the anti-inflammatory effect of tBHQ in the present study. These findings are consistent with previous studies showing that tBHQ or sulforaphane decrease NF- κ B activation, production of inflammatory cytokines (TNF- α , IL-1 β , and IL-6), COX-2 expression, and PGE2 release *in vivo* and *in vitro* (Heiss *et al.*, 2001; Jin *et al.*, 2010; Khodagholi and Tusi, 2011; Koh *et al.*, 2009). However, to the best of our knowledge, the present study is the first to report the protective role of Nrf2 activation on toxicant-stimulated inflammatory responses in human placental cells.

A wide range of natural and synthetic small molecules including tBHQ and sulforaphane induce Nrf2 activity (Ma, 2013). tBHQ-stimulates ROS formation as a consequence of redox cycling, which subsequently stimulates nuclear translocation of Nrf2 and activates ARE-dependent gene expression (Gharavi *et al.*, 2007; Itoh *et al.*, 1999; Sian *et al.*, 1994). Sulforaphane is an electrophile that can react with protein thiols to form thionoacyl adducts and is believed to modify cysteine residues in Keap 1 protein to a sulfenic acid (–SOH), resulting in a conformational change of Keap1, translocation of Nrf2, and upregulation of antioxidant genes (Imhoff and Hansen, 2010; Keum, 2011). The augmented GSH concentrations and increased antioxidant gene expression observed in the present study agree with prior reports that Nrf2 inducers increase cellular antioxidative capacity (Alfieri *et al.*, 2011; Hara *et al.*, 2003). Consistent with augmented antioxidant responses, pretreatment with tBHQ or sulforaphane suppressed BDE-47-stimulated ROS formation (as visualized by microscopic detection of DCF fluorescence) after 24 h of exposure to BDE-47 (data not shown). Although the precise mechanism regarding the anti-inflammatory activity of Nrf2 inducers remains elusive, it is suggested that the anti-inflammatory properties might result from augmentation of cellular antioxidant systems via up-regulation of the Nrf2 signaling pathway and inhibition of the NF- κ B signaling pathway (Jin *et al.*, 2011; Khodagholi and Tusi, 2011).

The Nrf2 and NF- κ B signaling pathways interact at several points through mechanisms of regulation ranging from direct effects on the transcription factors themselves to protein –

protein interactions and second-messenger effects on target genes (Wakabayashi *et al.*, 2010). It is suggested that Nrf2 may reduce available co-activator levels and promote recruitment of a co-repressor, leading to interruption of NF- κ B binding to DNA. In addition, Nrf2 target genes such as HO-1, NQO1, and thioredoxin (TRX) are able to influence NF- κ B activity (Wakabayashi *et al.*, 2010). Moreover, Nrf2 interferes with NF- κ B inflammatory signaling pathways through the maintenance of cellular redox status because NF- κ B is activated in the oxidizing environment (Kabe *et al.*, 2005). Our results showed suppression of BDE-47-stimulated NF- κ B transactivation by tBHQ pretreatment, implicating cross talk between the Nrf2 and NF- κ B signaling pathways. In addition, tBHQ stimulated mRNA expression of HMOX1, the gene for HO-1, and augmented GSH production concomitant with suppression of NF- κ B reporter activity. Based on our findings and other relevant reports, the following model is suggested: that BDE-47-stimulated ROS may activate Nrf2 to restore cellular redox status to a less oxidizing environment via increased cellular antioxidant capacity, resulting in suppression of NF- κ B activity, and, in turn, decreasing IL-6 production in HTR-8/SVneo cells. In addition, the increased antioxidant capacity may scavenge cellular ROS further leading to suppression of IL-6 secretion that is dependent on BDE-47-mediated ROS formation (Park *et al.*, 2014). However, we should note that the observed anti-inflammatory effects may originate from not a single mechanism, but from multiple mechanisms involving various proteins and signaling molecules (Kabe *et al.*, 2005). Moreover, the direct dependence of the anti-inflammatory effect on Nrf2 should be tested using genetic knockdown approaches such as iRNA.

In the present study, IL-6 was the only cytokine studied. However, overproduction of IL-6 alone may not accurately represent trophoblast inflammatory response to BDE-47, and the impact of BDE-47-stimulated inflammatory responses on trophoblast cellular function and invasion should be investigated further to confirm potential relevance of our findings to placentation and pregnancy. In addition, the present study did not examine direct anti-inflammatory effects of Nrf2 activation, but rather presented the concomitant activation of Nrf2-mediated pathways with suppression of IL-6 release when pretreated with Nrf2 inducers. Moreover, the pharmacological Nrf2 inducers tBHQ and sulforaphane may bind to proteins in a non-specific manner to affect other redox-sensitive transcription factors and protein kinases that impact cellular mechanisms other than Nrf2 signaling (Reuter *et al.*, 2010). Nonetheless, our conclusion that Nrf2 activation may be involved in BDE-47-stimulated inflammatory responses is supported by the use of two Nrf2 inducers, tBHQ and sulforaphane, which activate Nrf2 pathways by different mechanisms yet resulted in a similar effect on IL-release in response to BDE-47.

Another limitation of the present study is that the results of *in vitro* experiments may not accurately reflect responses *in vivo* that include complex interactions between inflammatory mediators and trophoblast invasion involving a number of autocrine and paracrine factors such as growth factors, growth factor-binding proteins, proteoglycans, other cytokines/chemokines, integrins, adhesion and proteolytic molecules (Anton *et al.*, 2012; Chakraborty *et al.*, 2002; Lala and Chakraborty, 2003). Likewise, our experiments used a transformed cell line that may not reflect primary extravillous trophoblast cells. Although HTR-8/SVneo cells have a similar phenotype compared to their primary counterparts (Biondi *et al.*, 2006; Graham *et al.*, 1993; Jovanovi *et al.*, 2010), retaining migratory capability and expressing

specific placental trophoblast markers (Biondi *et al.*, 2006; Khan *et al.*, 2011), it is reported that the cells have a different transcriptomic and epigenetic profile compared to primary extravillous trophoblast cells (Bilban *et al.*, 2010; Novakovic *et al.*, 2011). To address the latter issue, further investigation using primary trophoblasts or placental tissues will be needed to validate the potential relevance of our results to pregnancy.

Despite these limitations, our findings suggest potential adverse impacts of PBDE exposure during pregnancy. Invasion of EVT_s into maternal spiral arteries is a key event during placentation (Brosens *et al.*, 1967; Pijnenborg *et al.*, 1983; Pijnenborg *et al.*, 1980), and impaired EVT invasion has been attributed to pathologies of adverse birth outcomes with the evidence of abnormal placentation. Because IL-6 has been shown to regulate EVT proliferation, migration, and invasion during first trimester of pregnancy (Biondi *et al.*, 2006; Horita *et al.*, 2007b; Jovanovic *et al.*, 2010; Jovanovic and Vicovac, 2009; Nicola *et al.*, 2005b), overproduction of IL-6 in HTR-8/SVneo cells by BDE-47 suggests that BDE-47 exposure may disrupt trophoblast cellular function, leading to improper trophoblast invasion and abnormal placentation, thereby potentially contributing to adverse obstetrical outcomes. Ongoing research in our laboratory on the effects of PBDEs on trophoblast cellular function will lead us toward a better understanding of the mechanisms and relevant risks associated with PBDE exposures during pregnancy.

In summary, BDE-47, a predominant flame retardant chemical found in human tissues, activates Nrf2-dependent antioxidant responses in human first trimester EVT_s as indicated by differential expression of oxidative stress genes, stimulated ARE reporter activity, and augmented production of GSH. Our results provide evidence that Nrf2 activation by chemical inducers suppressed BDE-47-stimulated IL-6 production in human placental cells with stimulated ARE reporter activity, reduced NF- κ B reporter activity, increased GSH production, and stimulated expression of antioxidant genes compared to non-Nrf2 inducer pretreated groups. This is the first study to show that BDE-47 activated Nrf2-dependent oxidative stress pathways in human first trimester EVT_s and to link PBDE-stimulated pro-inflammatory responses with Nrf2 signaling pathways. Because proper trophoblast function is necessary for placental development and successful pregnancy, and dysregulation of inflammatory responses are associated with altered trophoblast invasion and placental dysfunction, further investigation of the impact of BDE-47 on trophoblast function is warranted. In addition, further studies about the role of Nrf2 on BDE-47-stimulated responses will be needed to confirm its protective effects and to consider Nrf2 as a potential therapeutic target to prevent adverse birth outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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	<i>tert</i> -butyl hydroquinone
TRX	thioredoxin
TXNRD1	thioredoxin reductase 1

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Highlights

- BDE-47 stimulated ARE reporter activity and GSH production.
- BDE-47 resulted in differential expression of redox-sensitive genes.
- Nrf2 inducers upregulated Nrf2-mediated oxidative stress responses.
- Nrf2 inducers reduced BDE-47-stimulated IL-6 release and NF- κ B activity.

Figure 1.

BDE-47-stimulated intracellular GSH production in HTR-8/SVneo cells. A) Cells were non-treated control (NT), or were exposed to solvent control (0.07% v/v DMSO) or BDE-47 (0.01–20 μM) for 24 h, and then GSH levels were quantified using a luminescence-based assay. B) Time-course of GSH levels. Bars represent means±SEM (n=3 experiments). Each experiment was performed in triplicate. *P<0.05, significantly increased compared to solvent control within same time point. #P<0.05, significantly different from each other. &P<0.05, different from NT, solvent control, and BDE-47 treated groups within same time point.

Figure 2.

BDE-47-stimulated ARE reporter activity in HTR-8/SVneo cells. Cells were exposed to solvent control (0.7% v/v DMSO) or BDE-47 treatment for 6 or 24 h, and then ARE reporter activity was assessed as an indicator of Nrf2 activation. Data are presented as means $\pm$ SEM fold change over solvent control (dashed line) for each respective time point. To derive fold changes, firefly luciferase relative light unit (RLU) values were first normalized to Renilla luciferase to compensate for cell number and transfection efficiency, then fold changes were calculated relative to solvent control for each time point (n=3 experiments). Each experiment was performed in triplicate.*P<0.05, significant compared to solvent control within same time point.

Figure 3.

BDE-47 effects on HTR-8 cell mRNA expression of genes previously identified with a targeted gene expression array. Cells were exposed to solvent control (0.7% v/v DMSO) or BDE-47 treatment for 24 h. Then, mRNA expression of redox-sensitive genes was quantified by qRT-PCR. A) The mRNA expression of HMOX1, PTGS2, PRNP, and DHCR24. B) The mRNA expression of SLC7A11, SRXN1, GSR, GCLM, and GPX1. Bars represent means $\pm$ SEM (n=3 experiments). Each experiment was performed in triplicate. *P<0.05, significantly different compared to solvent control.

Figure 4.

Effect of Nrf2 inducers on ARE reporter activity in HTR-8/SVneo cells without or with subsequent exposure to BDE-47. A) Cells were pretreated with *tert*-butyl hydroquinone (tBHQ) for 1 h prior to subsequent incubation without or with 20 μM BDE-47 for 24 h. B) Cells were pretreated with sulforaphane for 24 h prior to subsequent incubation without or with 20 μM BDE-47 for 24 h. ARE reporter activity was measured using a firefly luciferase reporter construct and expressed as fold change. To derive fold changes, firefly luciferase relative light unit (RLU) values were first normalized to Renilla luciferase to compensate for cell number and transfection efficiency, and then fold changes were calculated relative to control. Data are presented as means $\pm$ SEM fold change over control, with the group not exposed to Nrf2 inducer (0 μM) and no BDE-47 pretreatment serving as control (dashed line). Each experiment was performed three times with three replicates in each experiment. * $P < 0.05$, compared to control (dashed line). & $P < 0.05$, compared with lower concentrations of the Nrf2 inducer within 20 μM BDE-47 treatment. # $P < 0.05$, significantly different compared to cells in No BDE-47 group receiving the same Nrf2 inducer treatment

Figure 5.

Effect of pretreatment with the Nrf2 inducer *tert*-butyl hydroquinone (tBHQ) on BDE-47-stimulated GSH in HTR-8/SVneo cells. Cells were pretreated with tBHQ (10–50 μM) prior to subsequent incubation without or with 20 μM BDE-47 for 24 h. GSH levels were quantified using a luminescence-based assay (n=3 experiments). Each experiment was performed in triplicate. Data are presented as means±SEM fold change over control, with the group exposed to neither tBHQ (0 μM) nor BDE-47 pretreatment (No BDE-47) serving as control (dashed line). *P<0.05, compared to control (dashed line). +P<0.05, compared to 0 μM tBHQ within 20 μM BDE-47 treatment. &P<0.05, compared to 0, 10, and 20 μM tBHQ within 20 μM BDE-47 treatment. #P<0.05, significantly different compared to cells in No BDE-47 group receiving the same Nrf2 inducer treatment

Figure 6.

Effect of pretreatment with the Nrf2 inducer *tert*-butyl hydroquinone (tBHQ) on mRNA expression of the antioxidant genes HMOX1 and GCLM. HTR-8/SVneo cells were pretreated with tBHQ, and then exposed to solvent control (No BDE-47, 0.7% v/v DMSO) or BDE-47 for 24 h. Bars represent means±SEM fold change over control (dashed line, No BDE-47 with 0 μM tBHQ). The target gene expression from each sample was first normalized to the housekeeping gene B2M, and then fold changes were calculated relative to the normalized control (n=3 experiments). Each experiment was performed in triplicate. A) The mRNA expression of HMOX1. B) The mRNA expression of GCLM. *P<0.05, compared to control (dashed line). #P<0.05, significantly different compared to cells in No BDE-47 group receiving the same Nrf2 inducer treatment. &P<0.05, compared to no pretreatment (0 μM tBHQ) in BDE-47-treated cells.

Figure 7.

Effect of pretreatment with Nrf2 inducers on BDE-47-stimulated IL-6 release from HTR-8/SVneo cells. A) Cells were pretreated with *tert*-butyl hydroquinone (tBHQ) for 1 h prior to subsequent incubation without or with 20 μ M BDE-47 for 24 h. B) Cells were pretreated with sulforaphane for 24 h prior to subsequent incubation without or with 20 μ M BDE-47 for 24 h. IL-6 levels were quantified using ELISA. Bars represent means $\pm$ SEM (n=3 experiments). Each experiment was performed in triplicate. *P<0.05, significantly different compared to control (0 μ M tBHQ or 0 μ M sulforaphane with no BDE-47 treatment). #P<0.05, significantly different from each other. &P<0.05, significantly decreased compared with 0 μ M and 5 μ M sulforaphane pretreatment with BDE-47 exposure.

Figure 8.

Effect of pretreatment with *tert*-butyl hydroquinone (tBHQ) on BDE-47-induced NF-κB reporter activity in HTR-8/SVneo cells. Cells were pretreated with tBHQ for 1 h prior to subsequent treatment without or with 20 μM BDE-47 for 24 h. NF-κB reporter activity was quantified using a firefly luciferase reporter construct and expressed as fold change. To derive fold changes, firefly luciferase relative light unit (RLU) values were first normalized to Renilla luciferase to compensate for cell number and transfection efficiency, then fold changes were calculated relative to control (n=3 experiments). Data are presented as means ±SEM fold change over control (0 μM tBHQ with no BDE-47, dashed line). *P<0.05, significantly different compared to control (dashed line). #P<0.05, significantly different from each other. &P<0.05, significantly different compared to 50 μM tBHQ pretreatment with BDE-47 exposure.