BROMINATED DIPHENYL ETHER-47 ACTIVATES REACTIVE OXYGEN-MEDIATED INFLAMMATORY PATHWAYS IN HUMAN FIRST TRIMESTER EXTRAVILLOUS TROPHOBLASTS *IN VITRO*

by

Hae-Ryung Park

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Doctoral Committee:

Professor Rita Loch-Caruso, Chair
Professor Craig Harris
Associate Professor John D. Meeker
Professor Stephen W. Ragsdale

DEDICATION

To my family and friends

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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. Exposure to BDE-47 has been linked to adverse pregnancy outcomes in humans including preterm birth, low birth weight, and stillbirth. Although underlying mechanisms of adverse birth outcomes are poorly understood, critical roles of impaired trophoblast invasion and placental dysfunction characterized with dysregulated inflammatory pathways have been implicated. The present study examined the hypothesis that BDE-47 stimulates oxidative stress-mediated activation of inflammatory pathways in a human first trimester extravillous trophoblast (EVT) cell line, HTR-8/SVneo, and that the antioxidant transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) plays a protective role against BDE-47-induced inflammatory pathways.

Our results provide evidence of altered mitochondrial membrane potential, enhanced production of reactive oxygen species (ROS), and enhanced production of the pro-inflammatory interleukin (IL)-6, IL-8, and prostaglandin E2 (PGE2) stimulated by BDE-47 in human placental cells. The inhibition of stimulated release of IL-6 and PGE2 by a variety of antioxidant treatments implicates the involvement of ROS in the regulation of BDE-47-stimulated inflammatory pathways in HTR-8/SVneo cells. In addition, treatment with BDE-47 activated Nrf2-mediated oxidative stress responses as indicated by increased Nrf2 transactivation, differential expression of redox-sensitive genes, and augmented glutathione (GSH) production. Pretreatment with the Nrf2 inducers *tert*-butyl hydroquinone (tBHQ) or sulforaphane suppressed BDE-47-stimulated IL-6 production and nuclear factor kappa B (NF-κB) transactivation in HTR-

8/SVneo cells, with stimulated Nrf2 transactivation, intracellular GSH production, and mRNA expression of antioxidant genes compared with non-pretreated controls. The latter findings suggest that Nrf2 may play a protective role against BDE-47-stimulated inflammatory responses.

In conclusion, BDE-47, a predominant flame retardant chemical found in human tissues, activates proinflammatory responses in human first trimester EVTs. The present study provides the first experimental data to support a mechanism by which PBDE exposure could contribute to increased risk for adverse birth outcomes. By demonstrating that a common toxicological effect, oxidative stress, activates inflammatory pathways associated with impaired trophoblast function and placental dysfunction, these data provide support for a plausible biological explanation for environmental contaminant exposure associations with adverse obstetrical outcomes. Furthermore, this research contributes new information for potential interventions to reduce adverse obstetrical outcomes originating from abnormal placental function, with attendant possible economic, societal and public health benefits.

CHAPTER 1. **INTRODUCTION**

The hallmark of placentation - trophoblast invasion

The placenta plays critical roles during pregnancy ranging from anchoring the conceptus and preventing its rejection by the maternal immune system to enabling the transport of nutrients and wastes between the mother and the embryo/fetus (Maltepe *et al.*, 2010). Proper placental development is a prerequisite for a successful pregnancy. Placentation is a unique biologic process which involves the intimate dialogue between fetal and maternal tissues leading to profound remodeling of the uterine vasculature. Invasion of extravillous trophoblast into the spiral arteries is a key event during placentation (Brosens *et al.*, 1967; Pijnenborg *et al.*, 1983; Pijnenborg *et al.*, 1980). The extravillous trophoblasts (EVTs) are a highly proliferative and migratory cell population that invades the decidual and myometrial segments of the spiral arteries, resulting in the reversible remodeling of the arterial wall architecture (Anton *et al.*, 2012; Brosens, et al., 1967; Pijnenborg, et al., 1983; Pijnenborg, et al., 1980) (Figure 1.1). This transformation leads to reduced peripheral vascular resistance in the placental bed, thereby maximizing blood flow to the fetus (Brosens, et al., 1967; Pijnenborg, et al., 1983; Pijnenborg, et al., 1980). Abnormal placentation has contributes to the pathophysiology of adverse obstetrical complications such as preeclampsia (PE) (Brosens, 1977; Gerretsen *et al.*, 1981; Robertson *et al.*, 1967; Sheppard and Bonnar, 1976) , intrauterine growth restriction (IUGR) (Gerretsen, et al., 1981; Hustin *et al.*, 1983; Labarrere and Althabe, 1987; Sheppard and Bonnar, 1981), spontaneous abortion (Hustin *et al.*, 1990; Khong *et al.*, 1987), preterm premature rupture of membranes (Kim *et al.*, 2002), and preterm birth (Kim *et al.*, 2003). Although the mechanisms

responsible for improper placentation are not fully understood, impaired trophoblast invasion has been implicated (Zhou *et al.*, 1997a; Zhou *et al.*, 1997b).

Regulation of trophoblast invasion

Trophoblast invasion is tightly regulated by trophoblast-derived as well as maternal factors in time- and distance-dependent manners (Bischof *et al.*, 2000; Lala and Hamilton, 1996). These regulators include a number of autocrine and paracrine factors, including growth factors, growth factor-binding proteins, and proteoglycans (Chakraborty *et al.*, 2002; Lala and Chakraborty, 2003). Recently, inflammatory mediators such cytokines and prostaglandins have been shown to play a role in the regulation of trophoblast function during first trimester of pregnancy (Biondi *et al.*, 2006; Horita *et al.*, 2007a; Jovanovic *et al.*, 2010; Jovanovic and Vicovac, 2009; Nicola *et al.*, 2005b).

-Cytokines

Cytokines are small (8-30 kDa) hydrophilic signaling peptides and glycoproteins used in autocrine, paracrine and endocrine signaling throughout the body (Miller, 2009). In the placenta and extraplacental membranes, cytokines are produced by trophoblasts and diverse decidual cell types, and are assumed to play a role in establishment of successful pregnancy (Salamonsen *et al.*, 2007)

Interleukin (IL)-6 is a pleiotropic cytokine that belongs to the family of gp130 cytokines (Dimitriadis *et al.*, 2005). IL-6 is produced by many cell types and is involved in various processes such as immune response, acute phase reaction and hematopoiesis (Jovanovic and Vicovac, 2009). It has been shown that IL-6 is released from human first trimester placenta in

tissue culture (Kameda *et al.*, 1990). IL-6 was expressed in both syncytiotrophoblasts and extravillous trophoblasts (Jauniaux *et al.*, 1996; Kauma *et al.*, 1993). Receptor protein for IL-6 (IL-6R) and gp130 have been detected in trophoblast (Nishino *et al.*, 1990; Sawai *et al.*, 1995)

IL8 (CXCL8) is a pro-inflammatory molecule belonging to the CXC chemokine subfamily (Jovanovic, et al., 2010). IL8 is shown to play roles in regulation of neutrophil transendothelial migration (Huber *et al.*, 1991), angiogenesis (Koch *et al.*, 1992), and proliferation myeloid progenitor cells (Sanchez *et al.*, 1998). IL-8 is constitutively expressed in trophoblasts and placental macrophages, and its expression is highest at term (Shimoya *et al.*, 1992). mRNA and protein expression of IL-8 was reported in cytotrophoblast, syncytiotrophoblast, and Hofbauer cells of the placenta (Saito *et al.*, 1994). Expression of IL-8 receptors, CXCR1 and CXCR2, has been reported in human first trimester EVT (Hanna *et al.*, 2006), cytotrophoblast, and the human choriocarcinoma BeWo cell line (Hirota *et al.*, 2009; Tsui *et al.*, 2004).

-Prostaglandins

Prostaglandins are small lipid molecules synthesized from membrane phospholipids in response to various physiological and pathological stimuli (Nicola, et al., 2005b). Prostaglandin E2 (PGE2) is one of the most extensively studied prostaglandins, with critical roles for successful pregnancy, including implantation (Psychoyos *et al.*, 1995; Yee *et al.*, 1993), immunoprotection of the semiallogenic conceptus (Parhar *et al.*, 1988), and parturition (Keelan *et al.*, 2003). Prostaglandin production begins with arachidonic acid liberation from membrane-bound phospholipids by the action of phospholipase A2 (PLA2) (Jakobsson *et al.*, 1999; Kuroda and Yamashita, 2003; Stichtenoth *et al.*, 2001). Then, liberated arachidonic acid is converted to

prostaglandin H2 (PGH2) by two cyclooxygenase (COX) isoforms, COX-1 and COX-2 (Jakobsson, et al., 1999; Kuroda and Yamashita, 2003; Stichtenoth, et al., 2001). Finally, prostaglandins are further isomerized by tissue-specific prostaglandin synthases. In the case of PGE2, PGH2 is converted to PGE2 by PGE2 synthase (PGES) (Horita, et al., 2007a). Prostaglandins can be further catabolized to biologically inactive keto-metabolites by 15-hydroxyprostaglandin dehydrogenase (HPGD) (Tai *et al.*, 2006). It has been reported that COX-2 and microsomal PGES-1 are expressed in human first trimester EVT cells (Meadows *et al.*, 2004).

Inflammation and adverse birth outcomes

There is a growing body of evidence that improper regulation of the inflammatory networks may lead to adverse pregnancy outcomes (Orsi and Tribe, 2008; Tjoa *et al.*, 2004). Specifically, increased levels of cytokines, prostaglandins, adhesion molecules, and C-reactive protein in cervical fluid, amniotic fluid and maternal serum have been linked to the pathophysiology of preterm birth, PE, and IUGR (Cox *et al.*, 1993; Goldenberg *et al.*, 2005; Lyall *et al.*, 1994; Romero *et al.*, 2002; Tjoa *et al.*, 2003; Vince *et al.*, 1995). High levels of IL-6, IL-8 and PGE2 in the cervicovaginal fluid and amniotic fluid of pregnant women have been associated with increased risk for preterm birth (Dortbudak *et al.*, 2005; Goepfert *et al.*, 2001; Romero, et al., 2002; Wenstrom *et al.*, 1996). PTGS2 mRNA levels were approximately seven times higher in chorionlaeve from spontaneous preterm extra-embryonic membranes compared to non-laboring tissues of equivalent gestational age (Mijovic *et al.*, 1998). Through pathologic activation of pro-inflammatory pathways, pregnancies complicated with bacterial vaginosis (Flynn *et al.*, 1999; Leitich *et al.*, 2003) or intrauterine infection (Goldenberg *et al.*, 2008; Romero *et al.*, 2001) have been associated with increased risk of preterm birth. These studies

suggest that inflammation occurring at the maternal–fetal interface during pregnancy contributes to adverse obstetrical outcomes. Although the etiology of inflammation-related adverse birth outcomes is not fully understood, it has been recently 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).

Inflammation and placentation

A possible link between placental dysfunction and inflammation has been implicated in studies showing that 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 IL-6 and IL-8 in cervical fluid, amniotic fluid and maternal serum (Goldenberg, *et al*., 2005). In HTR-8/SVneo cells, LPS reduced invasion activity (in a matrigel-based assay) with increased production of IL-8 and IL-6 (Anton, *et al*., 2012). However, treatment with IL-6 increased gelatinase (matrix metalloproteinase-9) activity when assessed with a gelatin zymography assay (Jovanovic, *et al*., 2010; Jovanovic and Vicovac, 2009). Inhibition of endogenous IL-6 in JEG-3 choriocarcinoma cells inhibited migration and invasion (Dubinsky *et al.*, 2010). Roles for PGE2 in trophoblast cellular function have been suggested, also, although reported findings are not consistent. For example, PGE2 promoted migration of HTR-8 cells with suppressed migration by COX-2 inhibition (Horita *et al.*, 2007b; Nicola *et al.*, 2005a), whereas Biondi *et al*. showed that PGE2 suppressed the proliferation and migration of HTR-8/SVneo cells (Biondi, *et al*., 2006). Despite the inconsistencies, these published reports suggest that inflammatory mediators including IL-6, IL-8 and PGE2 may play critical roles in regulating trophoblast cellular function during placentation.

Reactive oxygen species as a signaling molecule

Oxidative stress is defined as the imbalance between cellular 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, PE, and IUGR (Agarwal *et al.*, 2012). A growing body of literature has reported that ROS can function as signaling molecules in mammalian cells (Finkel, 1998; Khan and Wilson, 1995; Remacle *et al.*, 1995) to regulate signal transduction pathways that control gene expression and posttranslational changes of proteins (Allen and Tresini, 2000; Palmer and Paulson, 1997) involved in various cellular functions (Droge, 2002; Valko *et al.*, 2007). ROS 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 oxidative stress and inflammation in the etiology of adverse pregnancy outcomes.

Nrf2-Keap1 pathway: cellular defensive mechanism

To defend against exogenous toxicants and injury, cells possess a variety of cytoprotective and detoxifying enzymes. The expression of some of these genes is regulated by a common promoter element called the antioxidant response element (ARE). Nrf2 is a redox-

sensitive transcription factor that binds to ARE and activates the ARE-mediated gene expression (Itoh *et al.*, 1997; Motohashi and Yamamoto, 2004; Osburn *et al.*, 2006). Under basal conditions, Nrf2 is sequestered in the cytoplasm by the cytosolic regulatory protein Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein1 (Keap1) (Imhoff and Hansen, 2010). While sequestered, Nrf2 constantly undergoes ubiquitination and subsequent proteosomal degradation. Keap1 contains many cysteine residues which sense changes in the redox status in the intracellular environment and are modified directly through either conjugation or oxidation to a sulfenic acid (−SOH) (Imhoff and Hansen, 2010). Modifications on the cysteine residues causes a conformational change of Keap1, leading to the release of Nrf2. Then, Nrf2 translocates to the nucleus and binds to ARE (Rushmore *et al.*, 1991) resulting in the upregulation of phase II detoxification enzymes and antioxidants such as NAD(P)H dehydrogenase, quinone 1 (NQO1), heme oxygenase-1 (HMOX1), glutamate–cysteine ligase catalytic subunit (GCLC), and glutamate–cysteine ligase modifier subunit (GCLM) (Kensler *et al.*, 2007). 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).

-Anti-inflammatory effects of Nrf2

Recently, many studies provided evidence implicating the 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). In response to traumatic brain injury (TBI), mice lacking Nrf2 exhibited increased intestinal inflammatory response and mucosal injury (Jin *et al.*, 2008; Jin *et al.*, 2009). In addition, oral administration of the synthetic Nrf2 inducer tert-butylhydroquinone (tBHQ) decreased NF-κB activation, inflammatory cytokines production

(TNF-α, IL-1β, and IL-6), and ICAM-1 expression in TBI-induced gut injury in mice (Jin *et al.*, 2010). In rat neuron-like PC12 cells, tBHQ inhibited LPS-induced cyclooxygenase 2 (COX-2), TNF-α, and NF-κB expression in a dose-dependent manner while stabilizing Nrf2 (Khodagholi and Tusi, 2011). Moreover, pretreatment of murine BV-2 microglial cells with tBHQ attenuated the LPS-derived overproduction of pro-inflammatory TNF-α, IL-1β, IL-6, and PGE2 (Koh *et al.*, 2009). Although the precise mechanism regarding the anti-inflammatory ability of tBHQ remains elusive, several lines of evidence indicate that the anti-inflammatory properties of tBHQ might result from the augmentation of the cellular antioxidant response via up-regulation of the Nrf2 signaling pathway and inhibition of NF-κB signaling pathway, along with suppression of MAP kinase (p38, ERK1/2, and JNK) phosphorylation (Jin *et al.*, 2011; Khodagholi and Tusi, 2011).

-Nrf2 in gestational compartments

Despite the importance of Nrf2 in cellular anti-oxidative and anti-inflammatory responses, there have been relatively few studies regarding Nrf2 activation in placenta and pregnancy. In vitro cell culture studies have been a useful tool to show the involvement of Nrf2 in the regulation of placental cell functions. It has been reported that Nrf2 may play a critical role in regulating angiogenesis by human umbilical vein endothelial cells (HUVECs) (Valcarcel-Ares *et al.*, 2012) and human choriocarcinoma BeWo cells (Kweider *et al.*, 2011). In addition, genetic profiling of highly migratory human EVT and villous cytotrophoblasts revealed that reduced expression of HO-1, a hallmark of Nrf2 activation, is associated with decreased cell motility and trophoblast invasion (Bilban *et al.*, 2009). Wruck *et al*. provided the first experimental data showing that Nrf2 is active within cytotrophoblasts of preeclamptic placentae, with PE associated with increased Nrf2 activity compared to normal gestation-matched controls (Wruck

et al., 2009). Later, Kweider *et al*. (Kweider *et al.*, 2012) reported that not only cytotrophoblasts but also extravillous trophoblasts are a source of Nrf2-regulated proteins in human placenta. Moreover, the latter study showed that extravillous trophoblasts showed an increased cytoplasmic expression of Nrf2 and 4-hydroxynonenal (4-HNE), a marker of oxidative stress, in IUGR/PE cases. In addition, the genome-wide transcriptional profiling of preeclamptic and normal pregnancies showed that the Nrf2-mediated oxidative stress response is upregulated in PE (Loset *et al.*, 2011). Another study appears to contradict the prior studies, reporting that Nrf2 activation and HO-1 mRNA were significantly decreased in preeclamptic placentae compared with normal controls (Chigusa *et al.*, 2012). Nonetheless, when assessed together these reports suggest 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. Although studies have linked Nrf2 to adverse pregnancy outcomes, the present study is the first to explore the role of Nrf2 activation in the regulation of inflammatory responses in the gestational compartment, to the best of our knowledge.

Environmental exposures and adverse birth outcomes

There has been increasing global concern over the adverse health impacts attributed to environmental pollutants (Luo *et al.*, 2010). In particular, maternal exposure to various chemicals, such as heavy metals, xenoestrogen and tobacco, during critical periods of pregnancy has been identified as a rising public health concern, because those chemicals are reported to pass across the placental barrier into the fetal blood stream and can be transferred to the fetus (Barr *et al.*, 2007). In addition, there has been growing evidence that environmental exposure can play an important role in the etiology of adverse birth outcomes (Stillerman *et al.*, 2008). For

example, cigarette smoking has been associated with increased risk for PE, preterm birth, low birth weight, IUGR, and pregnancy loss (Cnattingius, 2004; Delpisheh *et al.*, 2006), associated with increased systemic markers of oxidative stress and inflammation (Yanbaeva *et al.*, 2007). Similarly, air pollution (Sram *et al.*, 2005) and organochlorine pesticides including dichlorodiphenyltrichloroethane (DDT) (Pathak *et al.*, 2010) have been associated with adverse birth outcomes with increased markers for ROS. Exposure to lead and phthalates has also been associated with increased risk for IUGR, low birth weight, and preterm birth (Dietrich, 1991; Jelliffe-Pawlowski *et al.*, 2006; Meeker *et al.*, 2009; Torres-Sanchez *et al.*, 1999). A critical knowledge gap that is identified and addressed in this study is the lack of information on biological explanations that link environmental contaminant exposures with adverse birth outcomes. Because pregnant women are constantly exposed to many environmental chemicals, an improved understanding of the impacts of those chemicals on gestational tissues is warranted.

Polybrominated diphenyl ethers – growing public health concerns

Polybrominated diphenyl ethers (PBDEs) are commercially produced synthetic flame-retardants consisting of two phenyl rings linked by an ether bond with variable hydrogen to bromine substitutions (Figure 1.2A). PBDEs have been used in textiles, plastics, building materials and insulation (Miller *et al.*, 2009b). Because PBDEs are not chemically bound to the products, they may migrate from the products into the environment. In addition, they are persistent and bioaccumulative because of their lipophilic chemical properties (Frederiksen *et al.*, 2009a). Due to widespread use and bioaccumulation of PBDEs, human exposure to PBDEs increased exponentially over recent decades (Hites, 2004). Analysis of 2,062 human serum samples from the NHANES 2003-2004 detected PBDEs in nearly all participants, with BDE-47

(Figure 1.2B), one of the most prevalent PBDE congeners, having the highest concentration of the PBDE congeners measured (Sjodin *et al.*, 2008). Moreover, PBDEs have been reported to exhibit neurodevelopmental (Branchi *et al.*, 2003; Viberg *et al.*, 2006), hepatic (Zhou *et al.*, 2001; Zhou *et al.*, 2002), immunological (Fowles *et al.*, 1994; Thuvander and Darnerud, 1999) and thyroid toxicities (Zhou, *et al*., 2002) in animal studies, leading to growing public concerns about their use.

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). Production of penta-BDE (tri- to hexa-BDE mixtures) and octa-BDE (hexa- to nona-BDE mixtures) ceased in the United States and in Europe (European Chemicals Bureau, 2003). Recently, tetra-, penta-, hexa- and hepta-BDEs were listed as persistent organic compounds at the 4th meeting of the Convention of Parties of the Stockholm Convention on Persistent Organic Chemicals and banned in over 160 countries (Stockholm Convention News Release, 2009). Deca-BDE is still produced, although Sweden and some states in the USA (Maine and Washington) have prohibited the use of deca-BDE (Costa *et al.*, 2008; Frederiksen, *et al*., 2009a).

Despite these efforts to limit production and use of PBDEs in recent years, PBDE exposure still persists and remains a potential risk to human health. The globalization of commerce has allowed access the goods from regions with little or no regulation on PBDEs (Miller, 2009). Furthermore, there still exists a wide stock of old products containing penta- and octa-BDEs, allowing continued exposures. Moreover, a growing body of literature suggested that highly brominated deca BDEs could be degraded into lower brominated congeners (Stapleton *et al.*, 2006; Thuresson *et al.*, 2005). Finally, many PBDE congeners have long environmental and

biological half-lives (Hakk and Letcher, 2003), which lead to persistent exposure to PBDEs after disposal of the products. Besides, the potential risk of bioaccumulation through the food chain has been implicated in many animal studies (Debruyn *et al.*, 2009; Letcher *et al.*, 2009; Yu *et al.*, 2009). High levels of PBDEs in human samples from people who work at recycling facilities have also been reported (Bi *et al.*, 2007).

-PBDEs and reproductive toxicity

Limited studies report possible reproductive toxicity of PBDEs during pregnancy. Rabbits orally exposed to PBDEs show decreased gestation length (Breslin *et al.*, 1989). In human studies, Main *et al*. report a significantly higher risk of cryptorchidism for sons born to mothers with elevated PBDE levels in breast milk (Main *et al.*, 2007). In addition, Chao *et al*. found elevated levels of PBDEs in breast milk correlated with decreased infant birth weight, infant birth length, infant chest circumference and infant body mass index (Chao *et al.*, 2007). 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.*, 2009b), extraplacental membranes (Miller *et al.*, 2009a), amniotic fluid (Miller *et al.*, 2012), and umbilical cord blood (Frederiksen, *et al*., 2009b), studies of mechanisms by which PBDEs act on gestational tissues during pregnancy are limited.

-PBDEs and Oxidative Stress

A few studies suggest that PBDEs induce increased 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 reported that pentabrominated diphenyl ether (DE-71) and BDE-47 enhanced the production of ROS, potentially through NADPH oxidase activation in human granulocytes (Reistad and Mariussen, 2005). 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. Together, these reports 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.

-PBDEs and inflammation

Oxidative stress has been implicated in inflammatory disease, and a growing body of literature shows that ROS can play a role in regulation of inflammatory pathways (Finkel, 1998; Reuter, *et al*., 2010). Although BDE-47-stimulated oxidative stress has been extensively studied, little is known about interactions of BDE-47 with the innate immune response. It was recently reported that BDE-47-pretreatment of peripheral blood mononuclear cells from children with autism spectrum disorders exhibit divergent LPS-stimulated cytokine responses compared with age-matched controls (Ashwood *et al.*, 2009). Peltier *et al.* (2012) reported that pre-exposure of human second trimester placental explants to a PBDE mixture of congers 47, 99 and 100 enhanced placental proinflammatory response to heat-killed *E. Coli*, with increased IL-1β and reduced IL-10 production. In the latter study by Peltier *et al*.(2012), however, PBDE treatment alone did not stimulate proinflammatory cytokine production in placental explant cultures. To

our knowledge, there are no previous reports of PBDEs directly altering inflammatory pathways in human placental cells.

Research hypothesis

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 and prostaglandin production via a ROS-mediated mechanism in the first trimester EVT human placental cell line HTR-8/SVneo. Furthermore, the roles of the redox sensitive transcription factor Nrf2 on the regulation of BDE-47-stimulated inflammation were investigated as a potential therapeutic target for adverse birth outcomes.

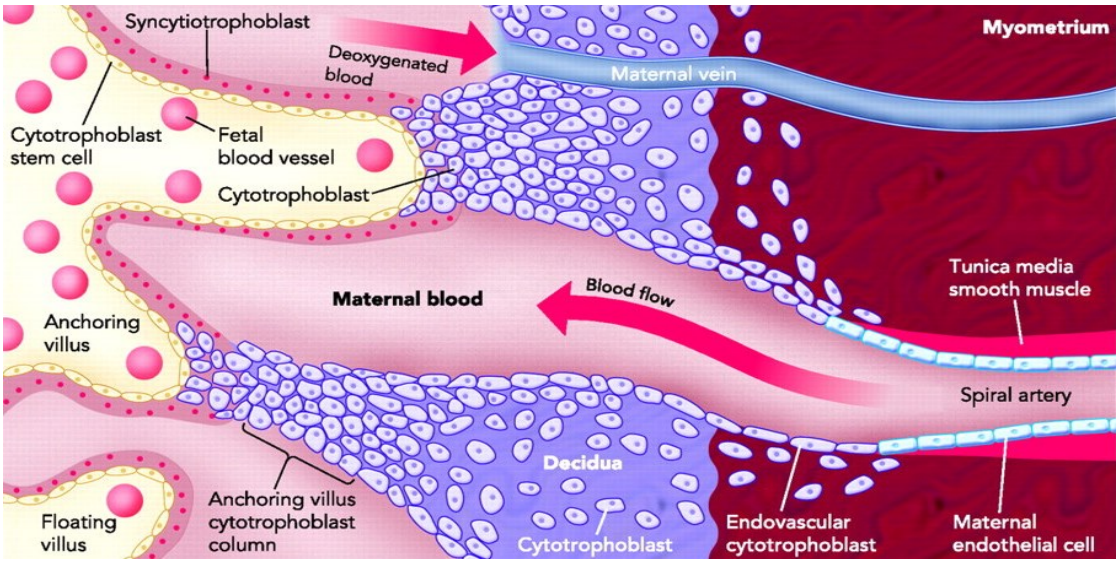

Figure 1.1. Trophoblast invasion during placentation.
In the first trimester of human pregnancy, fetal extravillous trophoblasts (EVTs) invade the decidua and maternal spiral arteries. This physiologic transformation leads to reduced peripheral vascular resistance enabling the transport of nutrients and wastes between the mother and the fetus. Figure adapted from Wang *et al*. (2009).

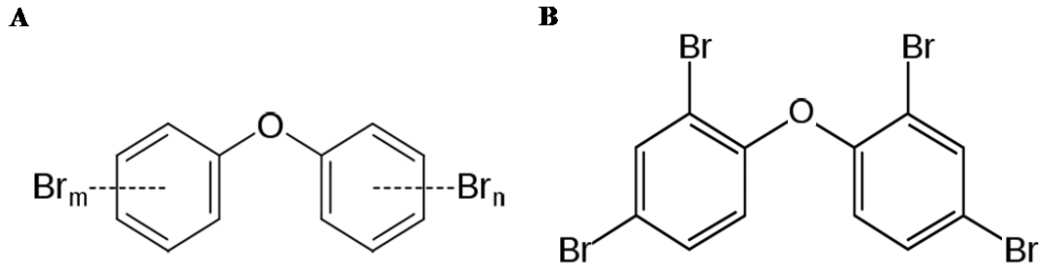

Figure 1.2. Chemical structure of PBDEs
A) General chemical structure of PBDEs (m+m=1-10). B) Chemical structure of BDE-47.

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CHAPTER 2. INVOLVEMENT OF REACTIVE OXYGEN SPECIES IN BROMINATED DIPHENYL ETHER-47-INDUCED INFLAMMATORY CYTOKINE RELEASE FROM HUMAN EXTRAVILLOUS TROPHOBLASTS IN VITRO

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 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.

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 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 reported that pentabrominated diphenyl ether (DE-71) and BDE-47 enhanced the production of ROS, potentially through NADPH oxidase activation in human granulocytes (Reistad and Mariussen, 2005b). 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^{TM} 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 2μg/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^{TM} 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 Molecular Devices SpectraMax Gemini M2e at an excitation wavelength of 492 nm and emission wavelength of 522 nm.

Cytochrome c reduction assay. Superoxide production in HTR-8/SVneo cells exposed to BDE-47 was quantified by the cytochrome C reduction assay based on the method of Boota *et al*. (1996) with a few modifications. Superoxide production in HTR-/SVneo cells exposed to BDE-47 was quantified by the cytochrome C reduction assay. HTR-8/SVneo cells were seeded at a density of 1×10^4 cells per well in a white, clear bottomed, 96-well plate, and incubated for 24 h at 37 °C. Cells were exposed to 5, 10, 15 or 20 μM BDE-47 for 1 h. Pyrogallol (100 μM) was included as a positive control. Reaction buffer was prepared in HBSS with 70 μM ferricytochrome c with or without 80 μg/ml of SOD. After treatment with BDE-47, cells were washed once with HBSS and a 100 μl-aliquot of the reaction buffer solution was added to each

well of the plates. Cytochrome c reduction was measured at 550 nm after 10, 30, 60 and 90 min incubation with reaction buffer: results are shown for the 90-min time point because this was the reaction time that yielded maximal response. Superoxide production was determined based on the difference in cytochrome c reduction with or without SOD. An extinction coefficient of 28.0 $mM^{-1}cm^{-1}$ was used for calculations. Results were expressed as nmoles superoxide released per 1×10^4 cells.

Determination of the mitochondrial membrane potential (MMP). Rh123, which can bind specifically to mitochondria, was used to estimate MMP based on the methods of Yan *et al*. (2011) with few modifications. HTR-8/SVneo cells were seeded at a density of 3×10^4 cells per well in a black, clear bottomed, 96-well plate, and incubated for 24 h at 37 °C. Cells were exposed to 5, 10, 15 or 20 μM BDE-47 for 4, 8 or 24 h. Treated cells were washed once with HBSS and incubated with Rh123 (2.5 μg/ml) in HBSS for 60 min in the dark at 37 °C. After replacing Rh123 with fresh HBSS, the fluorescence was measured with a fluorescence spectrophotometer using 507 nm Ex and 529 nm Em filter settings.

Measurement of cytokine release. 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 washed once with OptiMem1 medium containing 10 % FBS and 1% P/S and exposed to 5, 10, 15 or 20 μM BDE-47 for 4, 8 or 24 h. After incubation with BDE-47, culture medium was collected and centrifuged to remove any residual cell lysates. The concentration of IL-6 and IL-8 in the supernatant was measured by sandwich ELISA following the manufacturer's protocols. To determine oxidative stress-mediated activation of inflammatory pathways by BDE-47, HTR-8/SVneo cells were pretreated with 1 mM DFO for 1 h prior to BDE-47 treatment for 24 h, or co-treated either with 20 μM (±)-α-tocopherol, a peroxyl radical scavenger, or with 1mM tempol, a

membrane-permeable SOD mimetic for 24 h. Concentrations of IL-6 and IL-8 in the medium was analyzed by ELISA as described above. Release of cytokines was expressed as pg/ml.

RNA extraction and Quantitative real-time polymerase chain reaction. After 4, 12 or 24-h incubation with BDE-47, cell lysates were collected and homogenized using QIA shredder. Total RNA was extracted from homogenized lysates using RNeasy mini plus kit and cDNA was synthesized from 1μg of total RNA using RT^2 First Strand Kit. The procedures were performed according to the instructions of the manufacturer. Quantitative real-time polymerase chain reaction (qPCR) was performed in a total volume of 25 μL containing 4 μL of cDNA template, 1 μL of a gene-specific primer (IL-6, IL-8, TNF-α, or IL-10), 12.5 μL of RT^2 SYBR Green qPCR Master Mix and 7.5 μL of nuclease-free H_2O using CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). A housekeeping gene, β-microglobulin, was co-amplified as an internal control. qRT-PCR was performed with an initial denaturation step of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 5 s at 60°C. At the end of each cycle, the fluorescence emitted by the SYBR Green was measured. After completion of the cycling process, samples were subjected to a temperature ramp (from 65°C to 95°C at 0.5°C/s) with continuous fluorescence monitoring for melting curve analysis. Signal intensities of target genes were quantified and normalized to the signal of β-microglobulin using Bio-Rad CFX manager software. The level of mRNA expression was presented as fold change compared to solvent controls.

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 measured two-way ANOVA. If significant effects are detected, the ANOVA will be followed by Tukey post-hoc comparison of means. A $P < 0.05$ was considered

statistically different. Data were expressed as means ± SEM. All experiments were repeated at least three times and all treatments were performed at least in triplicate in each experiment.

Results

Cytotoxicity of BDE-47

To investigate the cytotoxic effect of BDE-47 on HTR-8/SVneo cells, protease-based viability and cytotoxicity assays were performed. Exposure to BDE-47 with concentrations up to 20 μM for 24 h did not result in a significant loss of cell viability in HTR-8/SVneo 8 cells as measured by cellular retention of proteases and indicated by sustained cellular fluorescence (Supplementary Figure 1A). In contrast, the loss of cell viability was clearly evident in cells treated with digitonin, included as a positive control. Similarly, cytotoxic effects were not significant with concentrations of BDE-47 up to 20 μM, as measured by increased luminescence due to protease release from dead cells, although digitonin-mediated cytotoxicity of HTR-8/SVneo cells was apparent (Supplementary Figure 1B; $P<0.05$).

Effect of BDE-47 on ROS production

Treatment of HTR-8/SVneo cells with 20 μM BDE-47 for 4 h increased DCF fluorescence compared with solvent controls as visualized with epifluorescence microscopy, indicating increased carboxy-H_2DCF-DA oxidation to the fluorescent DCF moiety, an indication of cellular reactive species generation (Figure 1A). Pretreatment with the iron-chelating antioxidant DFO decreased BDE-47-stimulated DCF fluorescence (Figure 1A). There were no differences in fluorescence comparing cells from control cultures incubated in HBSS alone, HBSS with 0.7% DMSO, or HBSS with 1 mM DFO (data not shown). Quantification of the fluorescence intensity using a spectrophotometer showed that treatment with 20 μM BDE-47 induced 1.7-fold increase

in the DCF fluorescence in the HTR-8/SVneo cells (Table 1; $P<0.05$), which was inhibited by DFO pretreatment to the equivalent level of the solvent control (Table 2; $P<0.05$). There were no statistically significant differences between non-treated controls and solvent controls (Table 1 and 2).

Effect of BDE-47 on superoxide production

BDE-47 treatment increased production of superoxide in HTR-8/SVneo cell cultures as measured with the cytochrome c reduction assay (Figure 2; $P<0.05$). Specifically, superoxide production was 0.49, 0.5 and 0.93 nmoles/10^4 cells in cells exposed to 10, 15, and 20 μM BDE-47 for 1 h, significantly increased compared to the solvent control (Figure 2; $P<0.05$). Treatment with 100 μM pyrogallol, included as a positive control, increased ferricytochrome c reduction to 2.19 nmoles/10^4 cells, a significant increase compared to the solvent control and BDE-47-treated groups (Figure 2; $P<0.05$). The negative values detected in solvent controls with the cytochrome c reduction assay suggest oxidation of ferricytochrome c under our basal experimental conditions, as observed by others (Arthur *et al.*, 1987).

Changes in mitochondrial membrane potential (MMP) by BDE-47 treatment

Because mitochondria are potential sources of cell-generated ROS, MMP was assessed by measuring fluorescence of Rh123, a dye specifically taken up by mitochondria in the normal polarized state. Treatment with 10, 15 and 20 μM BDE-47 significantly decreased Rh123 fluorescence compared to solvent controls at 4, 8 and 24 h (Figure 3; $P<0.05$), indicating decreased MMP. Reduction in Rh123 fluorescence ranged from 47% to 65%, but was neither concentration-dependent nor time-dependent. The decrease of MMP was significant compared to

non-treated control, also, at 4 h with 20 μM BDE-47 and at 8 h with 10, 15, and 20 μM BDE-47 (Figure 3; $P<0.05$). Treatment with 5 μM BDE-47 did not significantly change Rh123 fluorescence at any time point up to 24 h. There were no statistically significant differences between non-treated controls and solvent controls at any time points.

Effect of BDE-47 on cytokine production

Because cytokines play critical roles in pregnancy (Keelan *et al.*, 2003; Orsi, 2008), we investigated the effect of BDE-47 on IL-6 and IL-8 production in HTR-8/SVneo cells. BDE-47 treatment for 12 and 24 h stimulated concentration-dependent and time-dependent increases in IL-6 (Figure 4A; $P<0.05$) and time-dependent increases in IL-8 release (Figure 4B; $P<0.05$). Treatment with 15 or 20 μM BDE-47 significantly increased IL-6 3.7- fold and 6.3- fold at 12 h, and 3.7-fold and 12-fold at 24 h, respectively, relative to the solvent control (Figure 4A; $P<0.05$). After 24 h, the lower concentration of 10 μM BDE-47 also induced a significant 1.9-fold increase of IL-6 compared to the solvent control (Figure 4A; $P<0.05$). Moreover, 15 and 20 μM BDE-47 treatment increased IL-6 release in a time-dependent manner from 12 h to 24 h (Figure 4A; $P<0.05$). No statistically significant changes in IL-6 concentrations were observed with 4 h treatment at any BDE-47 concentration examined or with the lowest concentration evaluated, 5 μM BDE-47, at any time point. Pro-inflammatory chemokine IL-8 concentrations in the medium was significantly increased after a 12-h treatment with 15 and 20 μM BDE-47 by 2.1-fold and 2.3-fold, respectively, compared with solvent control (Figure 4B; $P<0.05$). In addition, 24-h treatment with 20 μM BDE-47 increased IL-8 release 1.8-fold compared with solvent control, to an average concentration that was significantly increased compared the average IL-8 concentration observed after 12 h of exposure to 20 μM BDE-47 (Figure 4B; $P<0.05$). No statistically significant changes in IL-8 concentrations were observed with 4-h treatment at any

BDE-47 concentration examined or with 5 and 10 μM BDE-47 at any time point. There were no statistically significant differences between non-treated controls and solvent controls at any time point.

Effect of BDE-47 on mRNA expression of cytokines in HTR-8/SVneo cells

Expression of inflammatory cytokine genes in HTR-8/SVneo cells exposed to BDE-47 was quantified using real time qRT-PCR. Treatment with 15 and 20 μM BDE-47 for 12 h increased IL-6 mRNA expression by 14.7 fold and 35.4 fold, respectively, compared to the solvent control (Figure 5A; $P<0.05$). Treatment with 20 μM BDE-47 for 24 h increased IL-6 mRNA 20.1 fold compared to the solvent control (Figure 5A; $P<0.05$). Likewise, IL-8 expression increased with 20 μM BDE-47 after 4, 12 and 24-h exposures compared to solvent control (Figure 5B; $P<0.05$). IL-8 mRNA expression increased with 12-h exposure to 15 and 20 μM BDE-47 by 12.1 fold and 24.9 fold, respectively, compared with solvent control (Figure 5B; $P<0.05$). For both IL-6 and IL-8, concentration-dependent increases in mRNA expression were observed with 15 and 20 μM at 12 h only. Treatment with 5 and 10 BDE-47 did not result in significant changes in IL-6 and IL-8 mRNA expression at any time point. Treatment with DMSO (solvent control) suppressed IL-8 mRNA expression at 12 h compared to non-treated control (Figure 5B, $P<0.05$). There were no other statistically significant differences between non-treated controls and solvent controls for IL-6 and IL-8 mRNA expression.

Effect of Antioxidant treatment on BDE-47-stimulated cytokine production in HTR-8/SVneo cells

To investigate the role of reactive oxygen species on BDE-47-mediated cytokine release in HTR-8/SVneo cells, cells were pretreated with 1 mM DFO for 1 h prior to BDE-47 treatment for 24 h,

or co-treated with 20 μM (±)α-tocopherol for 24 h. As shown in Figure 6A, DFO pretreatment inhibited IL-6 release stimulated by 20 μM BDE-47 in HTR-8/SVneo cells, reducing IL-6 release by 54.1% compared to cultures exposed to BDE-47 without DFO pretreatment (Figure 6A; $P<0.05$). Although IL-6 concentrations in cultures pretreated with DFO prior to exposure to 15 μM BDE-47 were not significantly reduced compared with cultures exposed to 15 μM BDE-47 without DFO pretreatment, they also were not statistically significantly different from the non-treated or solvent controls. Similar to DFO, (±)α-tocopherol and tempol co-treatment for 24 h resulted in 56.3% (relative to No Vehicle Control group, Figure 6B; $P<0.05$) and 37.7% (Figure 6C; $P<0.05$) reduction in BDE-47-mediated IL-6 production in HTR-8/SVneo cells. In the absence of (±)α-tocopherol cotreatment, a significant reduction in IL-6 release was observed for cultures exposed to DMSO (0.7 % v/v) as the solvent control group for BDE-47 treatments compared to non-treated controls (Fig. 6B; $P<0.05$); however, there were no statistically significant differences between (DMSO) solvent and non-treated controls that also received the vehicle (DMSO 0.02% v/v) used with (±)α-tocopherol cotreatment (Fig. 6B). There were no significant changes in IL-8 production from HTR-8/SVneo cells with DFO pretreatment, (±)α-tocopherol co-treatment, or tempol co-treatment (data not shown).

Discussion

Due to widespread use as flame-retardants in household and commercial products, human exposure to PBDEs increased exponentially over recent decades (Hites, 2004). Analysis of 2,062 human serum samples from the NHANES 2003-2004 detected BDE-47 in nearly all participants, with BDE-47 having the highest concentration of the PBDE congeners measured (Sjodin *et al.*, 2008). Despite BDE-47 presence in human placental tissues (Frederiksen, et al., 2009; Miller, et

al., 2009) and the importance of cytokine regulation in placental development during early pregnancy (Anton, et al., 2012), there are no previous reports of BDE-47-stimulated effects on inflammatory pathways in human first trimester placental cells. Moreover, we identified only a single previous report on PBDE-stimulated cytokine release in placenta with results showing that pre-exposure of second trimester human placental explant cultures to a PBDE mixture of congers 47, 99 and 100 enhanced placental proinflammatory response to heat-killed *E. Coli* (Peltier, et al., 2012). The present study is distinct from the latter study in that we showed direct stimulation of proinflammatory cytokines in the absence of pathogen exposure in a first trimester human extravillous trophoblast cell line treated with a single BDE congener.

Oxidative stress has been suggested to play a role in human pregnancy-related disorders, such as preterm labor, preeclampsia, and IUGR (Agarwal, et al., 2012). The present study provides new information that BDE-47 increased ROS generation in the human trophoblast cell line HTR-8/SVneo. Moreover, BDE-47 decreased mitochondrial membrane potential, indicating mitochondrial dysfunction (Brand and Nicholls, 2011). Because mitochondrial defects can lead to enhanced mitochondrial production of ROS and superoxide is a major type of ROS generated by mitochondrial respiration (Sohal *et al.*, 1995), we suggest that the BDE-47-stimulated superoxide production in HTR-8/SVneo cells may have originated from mitochondria. Superoxide also acts as a precursor for formation of other types of ROS (Al-Gubory *et al.*, 2010). As such, the increased DCF fluorescence observed with BDE-47 could be explained by subsequent formation of peroxyl, hydroxyl radical and other ROS from mitochondrial superoxide. However, our findings are correlative only and require further research to confirm the present findings and to elucidate our mechanistic hypothesis.

Similar to our results with HTR-8/SVneo cells, BDE-47-stimulates intracellular ROS formation in rat neuronal cells (He, et al., 2008), Jurkat cells (Yan, et al., 2011), human fetal liver hematopoietic stem cells (Shao, et al., 2008), and human neutrophil granulocytes (Reistad and Mariussen, 2005a). In contrast to previously reported studies that BDE-47 induces apoptosis in Jurkat cells (Yan, et al., 2011) and human fetal liver hematopoietic stem cells (Shao, et al., 2008), we observed no significant loss of cell viability in HTR-8/SVneo cells after a 24-h treatment with BDE-47 (Supplementary Figure 1). A possible explanation for these inconsistencies is that the range of BDE-47 concentrations used in our study (5-20 μM) was much lower than concentration ranges used in studies reporting BDE-47-induced cytotoxicity and/or a mitochondrial membrane potential reduction: 25-100 μM (Yan, et al., 2011) and 50 μM (Shao, et al., 2008). Moreover, different types of cells (human trophoblasts *versus* rat neuronal cells, Jurkat cells, human fetal liver hematopoietic stem cells, or human neutrophil granulocytes) and experimental conditions (media, serum concentration, exposure duration, cell density, etc.) may generate divergent responses to the same chemical. Notably, our study provides the first evidence that BDE-47 decreases mitochondrial membrane potential at lower concentrations of BDE-47 (10, 15 and 20 μM) than reported previously.

Treatment with BDE-47 reduced mitochondrial membrane potential, though the observed reduction was not concentration-dependent for the BDE-47 concentration range examined (10, 15, and 20 μM). The lack of a concentration-dependent reduction in mitochondrial membrane potential could be explained by the narrow range of BDE-47 concentrations in our studies, especially if the sensitivity of Rh123 may not be sufficient to detect the differences in mitochondrial membrane potential within this range of concentrations. Similar to our findings, Yan *et al*. (2011) did not show a concentration-dependent decrease in mitochondrial membrane

potential in BDE-47-treated Jurkat cells with 5, 10 and 25 μM BDE-47 using Rh123, although a decreasing trend was observed as concentrations increased up to 100 μM. Shao *et al*.(2008) also reported a decrease in mitochondrial membrane potential in human fetal liver hematopoietic stem cells at 12.5 and 50 μM using another mitochondria-specific dye, JC-1, but did not show a significant concentration-dependent reduction, either. Further study using an expanded range of BDE-47 concentrations will be needed to clarify reasons for the lack of a concentration-dependent effect on the mitochondrial membrane response.

Little is known about interactions of BDE-47 with the innate immune response. It was recently reported that BDE-47-pretreatment of peripheral blood mononuclear cells from children with autism spectrum disorders exhibit divergent LPS-stimulated innate cytokine responses compared with age-matched controls (Ashwood *et al.*, 2009). Peltier *et al.* (2012) reported 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*, with increased IL-1β and reduced IL-10 production. In the latter study by Peltier *et al*.(2012), however, PBDE treatment alone did not stimulate proinflammatory cytokine production in placental explant cultures. We observed that BDE-47-stimulated cytokine mRNA expression increased for IL-8 as early as 4 h with 20 μM exposure, then peaked 12 h after initiating exposure, the only time point in our study where we observed significantly increased IL-6 and IL-8 mRNA expression at the lower concentration of 15 μM, also. Compared with mRNA expression, cytokine protein release lagged temporally, with initial increases after 12 h and further increases after 24 of exposure to BDE-47. The lowest effective concentration for cytokine response was observed with 10 μM BDE-47-stimulated increase of IL-6 protein after 24 h of exposure. As such, our study is the first to report direct effect of BDE-47 on regulation of cytokine production in human placental cells, showing that

BDE-47 increased proinflammatory IL-8 and IL-6 in HTR-8/SVneo cells at the transcriptional and protein levels. Moreover, the BDE-47-stimulated increased mRNA levels of IL-6 and IL-8, suggesting that activation of transcription contributes to the overall increased cytokine expression. Because the fold change in cytokine protein production was modest compared to the fold change in cytokine mRNA expression, additional post-transcriptional mechanisms may modulate protein expression (Griesinger *et al.*, 2001; Wang *et al.*, 2011; Ye *et al.*, 2011).

Inflammation within the gestational compartment may lead to impaired trophoblast cellular function, contributing to the placental dysfunction seen in pregnancy-related disorders. Histologic examination found evidence of localized inflammation (histologic chorioamnionitis) in 85% of placentae from spontaneous preterm births delivered at 28 weeks gestation (Yoon *et al.*, 2000) with higher rates of placental ischemia and abnormal placentation compared with controls at term (Germain, et al., 1999; Kim, et al., 2003). A recent study reported that LPS increases production of IL-8 and IL-6 and decreases invasion activity in HTR-8/SVneo cells (Anton, et al., 2012). Our study showed that exposure to BDE-47 stimulated pro-inflammatory IL-6 and IL-8 production in HTR-8/SVneo cells, suggesting that BDE-47 could potentially impair normal trophoblast cellular function and invasion. However, we did not measure BDE-47 effects on trophoblast invasion, and further investigation is needed to ascertain the potential relevance of BDE-47 exposure to placental function and pregnancy.

It has been reported that ROS can regulate signal transduction pathways in mammalian cells as second messengers (Khan and Wilson, 1995). Our study clearly showed the novel finding that ROS play a role in activation of BDE-47-mediated inflammatory response in HTR-8/SVneo cells. Three different antioxidant treatments suppressed BDE-47-stimulated IL-6 production in HTR-8/SVneo cells. Although mechanisms of regulation of inflammatory response

by ROS are not fully understood, involvement of the redox-sensitive transcription factor NF-κB has been implicated (Reuter, et al., 2010). NF-κB plays a crucial role in immune and inflammatory response, regulating gene expression of a large number of genes, including cytokines, growth factors, adhesion molecules, immunoreceptors, and acute-phase proteins (Blackwell and Christman, 1997). Several lines of evidence suggest a role for ROS in activation of NF-κB, and antioxidants inhibit NF-κB activation *in vitro* and *in vivo* (Blackwell and Christman, 1997). Moreover, NF-κB plays a crucial role in the transcription of IL-6 and IL-8 (Blackwell and Christman, 1997; Reuter, et al., 2010). Although we did not assess BDE-47-induced NF-κB activation in HTR-8/SVneo cells, we speculate that NF-κB may be involved in BDE-47-stimulated cytokine production in HTR-8/SVneo cells because both cytokines were notably increased with BDE-47 treatment.

The mechanisms of PBDE toxicity have not been fully resolved. Because of the similar chemical structures of PBDEs and their metabolites to thyroid hormones, polychlorinated biphenyls (PCBs), and 2,3,7,8-tetra- chlorodibenzo-p-dioxin (TCDD) (Ren and Guo, 2013), other studies have focused on PBDEs' toxic effects through nuclear hormone receptor (NR) mediated pathways involving thyroid hormone receptor (TR), estrogen receptor (ER), aryl hydrocarbon receptor (AhR), androgen receptor (AR) and progesterone receptor (PR) (Ibhazehiebo *et al.*, 2011; Kojima *et al.*, 2009; Mercado-Feliciano and Bigsby, 2008) (Liu *et al.*, 2011; Ren and Guo, 2013; Stoker *et al.*, 2005). For example, hydroxylated BDE-47 metabolites showed modest binding potency with rat TR (Kitamura *et al.*, 2008) whereas BDE-199, 153, 154, 209 and DE-71 (a commercial PBDE mixture) showed antagonistic activity for TRβ in CV-1 monkey fibroblast-derived cells (Ibhazehiebo, et al., 2011). BDE-47 showed agonistic activities in the ERα and ERβ by the ER-CALUX assay using Chinese hamster ovary cells

(Kojima, et al., 2009). Chen *et al*. (2001) reported that 18 PBDE congeners and 3 commercial mixtures bound to rat hepatic AhR with affinities 10^{-2} to 10^{-5} times that of TCDD. Anti-androgenic activity of PBDEs also has been observed (Kojima, et al., 2009; Liu, et al., 2011). Although these studies suggest potential roles of NRs on endocrine disruption by PBDEs, the contribution of NR activation to BDE-47-stimulated inflammatory responses in the present study is not clear. It was recently suggested that NRs regulate inflammatory pathways by altering the turnover or recruitment of co-repressors and co-activators in a gene-specific manner. These NR-dependent trans-repression pathways may play roles in controlling the initiation, magnitude and duration of pro-inflammatory gene expression (Glass and Saijo, 2010; Huang and Glass, 2010). However, further study on the biological mechanisms of action of PBDEs is warranted to investigate roles of potential receptors and mediators in the activation and regulation of BDE-47 mediated inflammation in human placental cells.

In the present study, we used DMSO at a final concentration of 0.7% to deliver BDE-47 to the cell cultures. Although previous reports used lower DMSO concentrations to deliver similar or higher concentrations of BDE-47 to cell cultures (Shao, et al., 2008; Yan, et al., 2011), we found that BDE-47 precipitated out over time in cultures at final DMSO concentrations below 0.7% in our laboratory. DMSO is widely used as a vehicle for hydrophobic pharmaceutical agents in biomedical research. In preliminary experiments, we evaluated 1,4-dioxane as an alternative vehicle for BDE-47, but decided on using DMSO because BDE-47 had better solubility in DMSO and because DMSO is more commonly used in cell culture studies with PBDEs. However, DMSO's reported antioxidant and anti-inflammatory properties raise cautions when interpreting results from studies using DMSO as a solvent. It has been reported that DMSO (≥1% v/v) is a strong antioxidant that scavenges hydroxyl free radicals (Bektasoglu

et al., 2006; Halliwell *et al.*, 1987; Panganamala *et al.*, 1976), and reduces production of hydroxyl radicals, lipid peroxidation, and protein carbonyl formation (Sanmartin-Suarez *et al.*, 2011). DMSO also exhibits anti-inflammatory properties by inhibiting secretion and/or mRNA expression of pro-inflammatory mediators such as TNF-α, IL-6, and IL-8, by decreasing prostaglandinE_2 production related to COX-2 activity, and by reducing NF-κB activation in in vivo and in vitro (Chang *et al.*, 1999; DeForge *et al.*, 1992; Hollebeeck *et al.*, 2011; Kelly *et al.*, 1994; Nakamuta *et al.*, 2001). Although means of solvent controls and non-treated controls had divergent values in most experiments in the present study, statistically significant differences between these control groups were detected only in experiments measuring IL-8 mRNA expression and IL-6 protein. Preliminary data prior to this study showed that treatment of HTR-8/SVneo cells with higher DMSO concentrations (0.75-1%) suppressed BDE-47-stimulated IL-6 release in a concentration-dependent manner (Supplementary Figure 2). Because DMSO effects were in the opposite direction than the observed BDE-47-stimulated effects, it is likely that any confounding by DMSO on IL-6 release would result in muting the IL-6 response, making it more difficult to observe significant increases. We clearly show that BDE-47 stimulated ROS overproduction and inflammatory responses in HTR-8/SVneo cells, outweighing possible opposing DMSO effects. We suggest that the DMSO effects on cytokine production are due to its radical scavenger or anti-inflammatory properties. However, it is out of the scope of the present work to clarify the mode of actions of DMSO in cytokine production because DMSO was used purely as a vehicle control in this investigation.

A limitation of our study is that overproduction of IL-6 and IL-8 alone may not accurately represent the proinflammatory response and the possible impact of BDE-47 exposure on trophoblast cellular function *in vivo*. Although changes in cytokine levels by an activated

immune response play an important role in regulating trophoblast function, there are complex interactions between cytokines and trophoblast invasion involving other pro- and anti-invasive factors, such as other cytokines/chemokines, integrins, and adhesion and proteolytic molecules (Anton, et al., 2012).

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 utero and placenta: 337 - 21842 pg/ml in amnionic fluid (Miller, et al., 2012), 0.11- 3000 ng/g lipid in placentae (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, PBDE concentrations vary markedly among samples and can differ by three orders of magnitude, possibly due to factors such as proximity to the source of contamination, length of exposure, individual life style, occupation, nutritional status, absorption, metabolism, and excretion (Athanasiadou *et al.*, 2008; Doucet, et al., 2009; Gill *et al.*, 2004; Stapleton *et al.*, 2008). Taking this variability of exposure into consideration and assuming that the lipid content in placentae is 1.31%, the concentrations of PBDEs in placentae can be as high as ~8 μM (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 1.34 to 5.4 μM. In addition, we have not examined the effect of multiple or chronic exposures to PBDEs, and it is unlikely for people to be exposed to a single PBDE congener like BDE-47 (Shao, et al., 2008). Moreover, some PBDE congeners showed the ability to act synergistically when combined (Tagliaferri *et al.*, 2010) or with other toxicants (Fischer *et al.*, 2008; Pellacani *et al.*, 2012). Ongoing research in our laboratory on the effects of other prevalent PBDE congeners, such as BDE-49 and 99, will lead us toward a better understanding

of the mechanisms and relevant risks associated with PBDE exposures in gestational compartments. Moreover, ongoing analysis of gene expression array data is expected to guide us in future experiments to elucidate the mechanisms of BDE-47-stimulated ROS formation and cytokine production.

In summary, BDE-47, a predominant flame retardant chemical found in human tissues, activates proinflammatory responses in human first trimester EVTs. Our results provide evidence of altered mitochondrial membrane potential, enhanced production of ROS, and enhanced production of the proinflammatory cytokines IL-6 and IL-8 stimulated by BDE-47 in human placental cells. The inhibition of stimulated release of IL-6 by a variety of antioxidant treatments implicates the involvement of ROS in regulation of cytokine production in HTR-8/SVneo cells. This is the first study to show that BDE-47 activates proinflammatory pathways in human first trimester EVTs and to link PBDE-stimulated pro-inflammatory responses with ROS. Because proper trophoblast function is necessary for placental development and successful pregnancy, further investigation of the impact of BDE-47 on trophoblast function is warranted.

Table 2.1. Quantification of BDE-47-Stimulated ROS Production in HTR-8 cells[a]

Treatment	DCF fluorescence intensity
NT	174.60 ± 8.17
Solvent control	183.10 ± 12.36
5 μM BDE-47	257.50 ± 23.77
10 μM BDE-47	260.60 ± 25.88
15 μM BDE-47	247.40 ± 27.36
20 μM BDE-47	309.10 ± 16.72*
100 μM TBHP	555.00 ± 109.40*

[a] HTR-8 cells were non-treated (NT; control), or were treated with DMSO (solvent control), BDE-47 or *tert*-butyl hydroperoxide (TBHP, positive control) for 4 h, then loaded with carboxy-H2DCF-DA for 1 h. Values represent the means ± SE of 3 independent experiments containing 4 replicates each. *$P<0.05$, significant compared to solvent control.

Table 2.2. Inhibition of BDE-47-Stimulated ROS Production by Antioxidant Pretreatment in HTR-8 cells[b]

Treatment	DCF Fluorescence Intensity	
	No DFO	1mM DFO
NT	216.30 ± 7.71	218.80 ± 5.71
Solvent control	216.70 ± 7.53	215.60 ± 6.96
20 μM BDE-47	279.60 ± 8.98*	215.40 ± 6.98#

[b] HTR-8 cells were pretreated for 1 h with or without deferoxamine mesylate (DFO) prior to exposure to non-treated control (NT), DMSO (solvent control) or BDE-47 for 4 h, and then loaded with carboxy-H2DCF-DA for 1 h. Values represent the means of 3 independent experiments containing 4 replicates each ± SE. *$P<0.05$, significant compared to solvent control. #$P<0.05$, significantly different compared to non-DFO treated group.

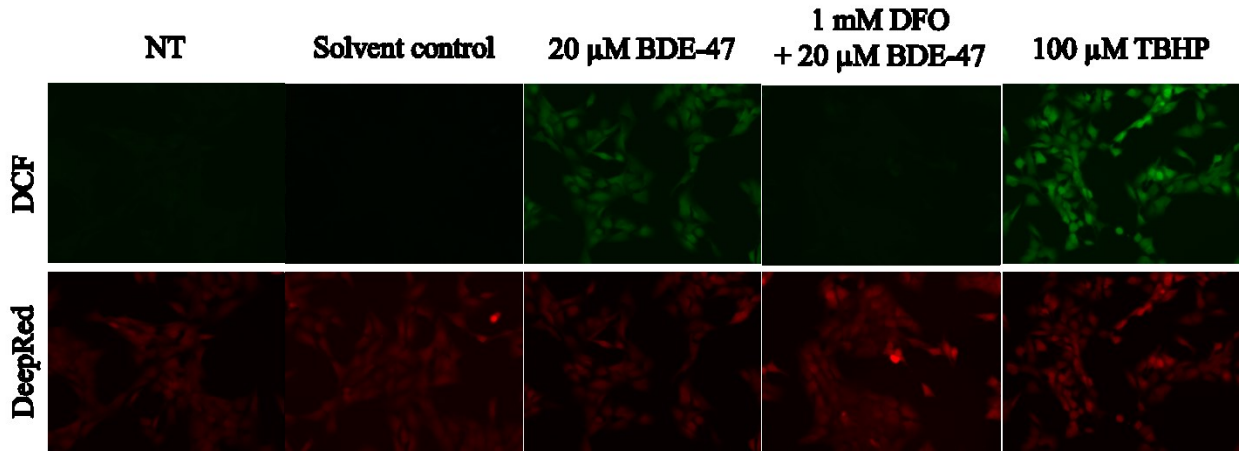


Figure 2.1. Fluorescence microscopy visualization of BDE-47-stimulated dichlorofluorescein (DCF) fluorescence, an index of reactive oxygen species production, in a first trimester human extravillous trophoblast cell line, HTR-8/SVneo.
HTR-8/SVneo cells were pretreated for 1 h with or without 1 mM deferoxamine mesylate (DFO), and then received no further treatment (NT, non-treated control) or were exposed to DMSO (solvent control), 20 µM BDE-47 or 100 µM *tert*-butyl hydroperoxide (TBHP; positive control) for 4 h. Subsequently, the cells were loaded with the non-fluorescent pro-dye carboxy-H2DCF-DA for 1 h, counterstained with CellMask^{TM} Deep Red plasma membrane dye, and photographed using an epifluorescence microscope. The top panel shows representative images of intracellular DCF fluorescence, and the bottom panel show corresponding Deep Red membrane staining. Representative images of 3 independent experiments.

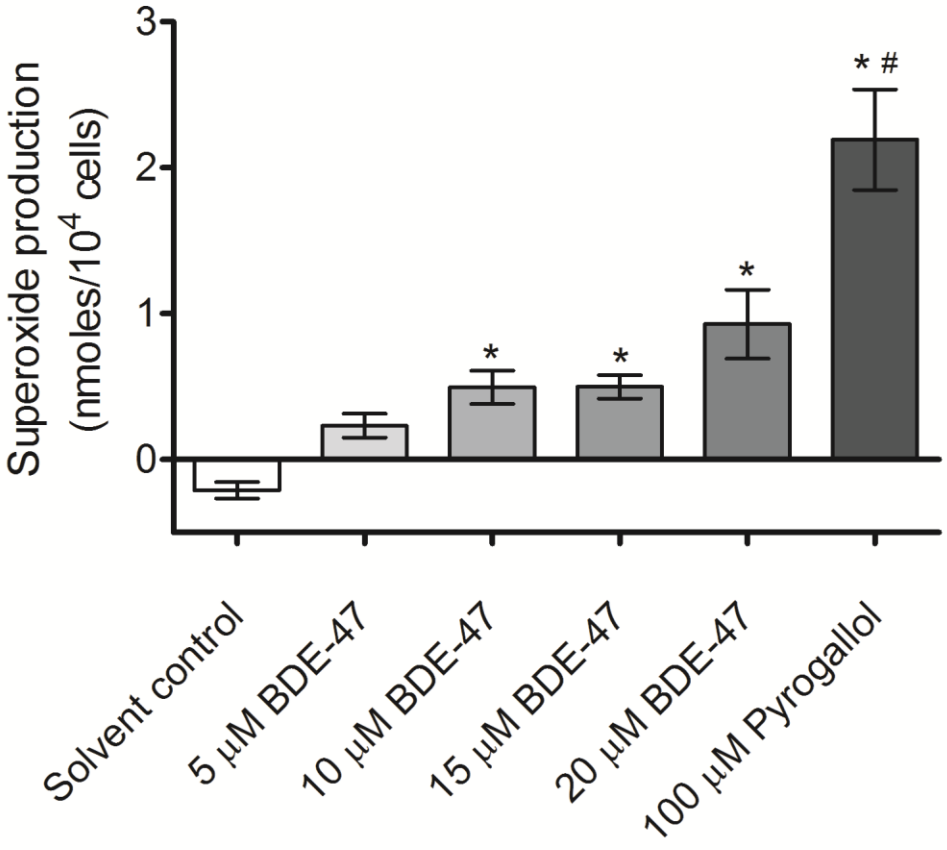


Figure 2.2. BDE-47-induced cytochrome c reduction, an index of superoxide anion production, in HTR-8/SVneo cells.

HTR-8/SVneo cells were treated for 1 h with DMSO (solvent control) or BDE-47, then incubated with cytochrome c reaction buffer with or without SOD for 90 min. Bars represent the means of 3 independent experiments containing 6 replicates each ±SE. *P<0.05, significant compared to solvent control. $^{\#}$P<0.05, significant compared to 5, 10, 15, and 20 μM BDE-47.

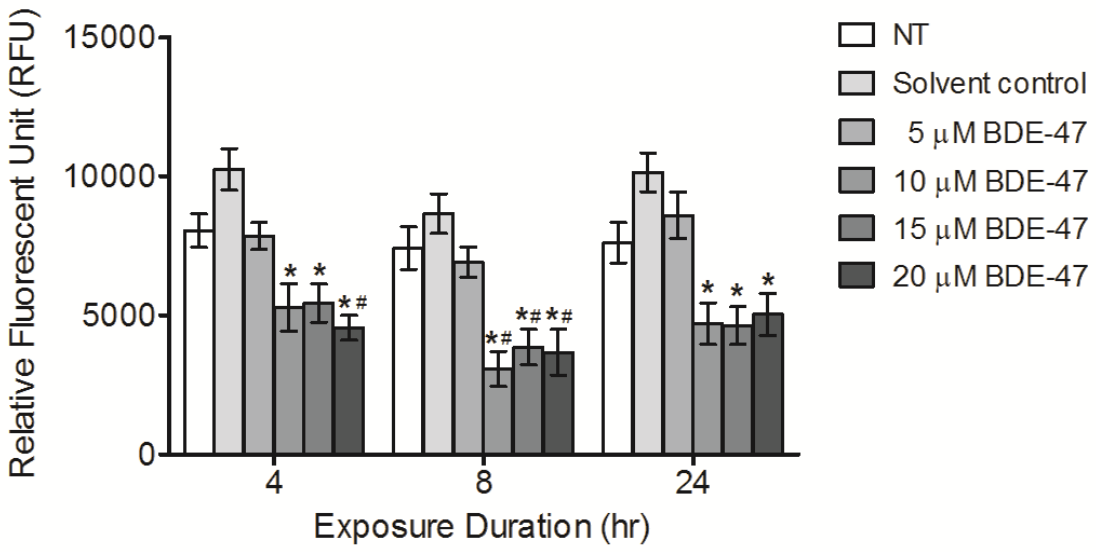


Figure 2.3. BDE-47 effects in HTR-8/SVneo cells on mitochondrial membrane potential (MMP). HTR-8/SVneo cells were treated for 4, 8 or 24 h with non-treated control (NT), DMSO (solvent control) or BDE-47, and then loaded with Rh123 for 1 h. Bars represent the means of 6 independent experiments containing 4 replicates each ±SE. *P<0.05, significant compared to solvent control within same time point.

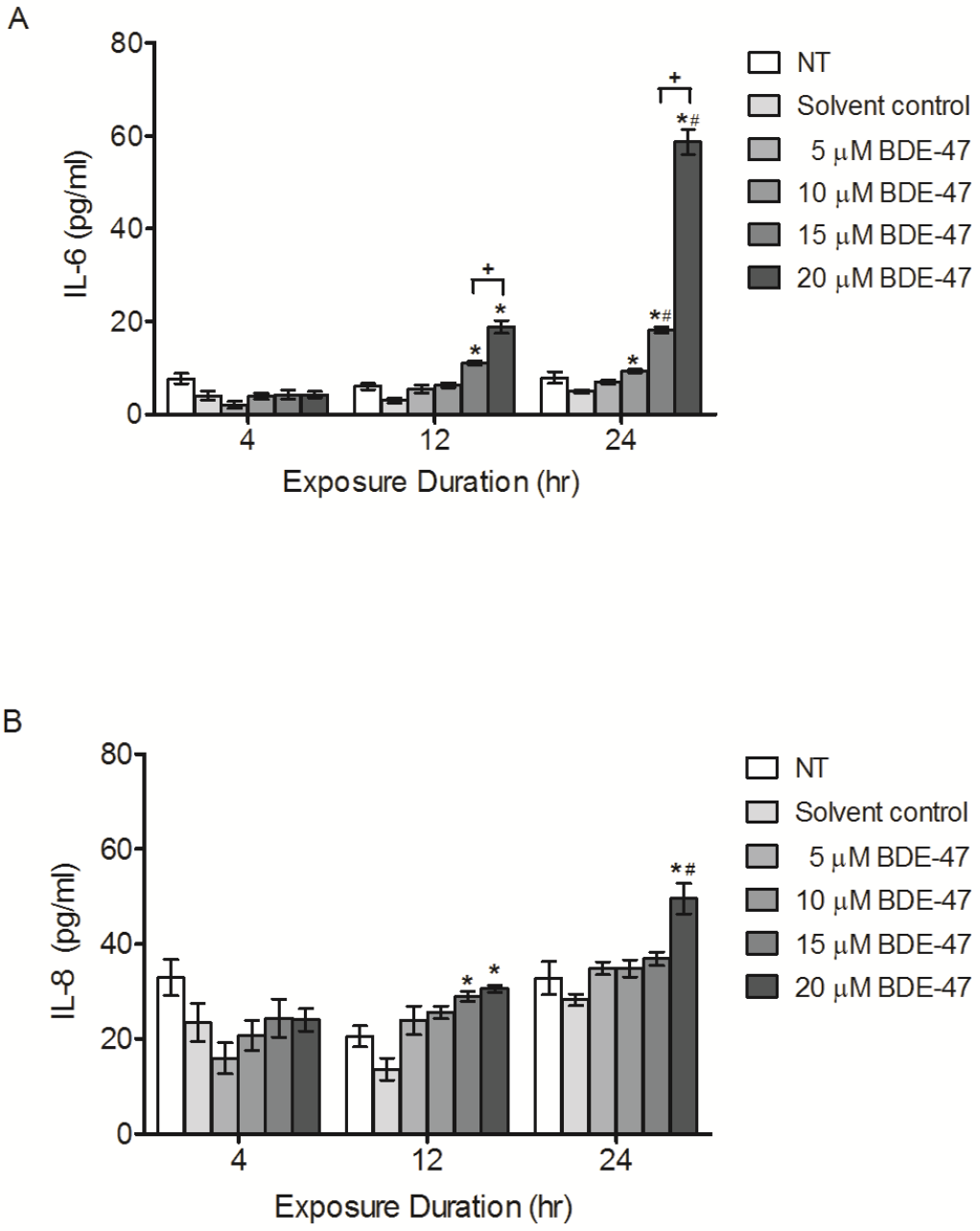


Figure 2.4. BDE-47-induced pro-inflammatory cytokine production in HTR-8/SVneo cells. HTR-8/SVneo cells were untreated (NT, non-treated control), or treated for 4, 8 or 24 h with DMSO (solvent control) or BDE-47, and then concentrations of IL-6 (A) and IL-8 (B) in culture medium were quantified by EIA. Bars represent the means ± SE of 3 independent experiments containing 3 replicates each. *P<0.05, significant compared to solvent control within same time point. $^{\#}$P<0.05, significant compared to same treatment at12 h. $^{+}$P<0.05, significantly different from next lowest concentration within time point.

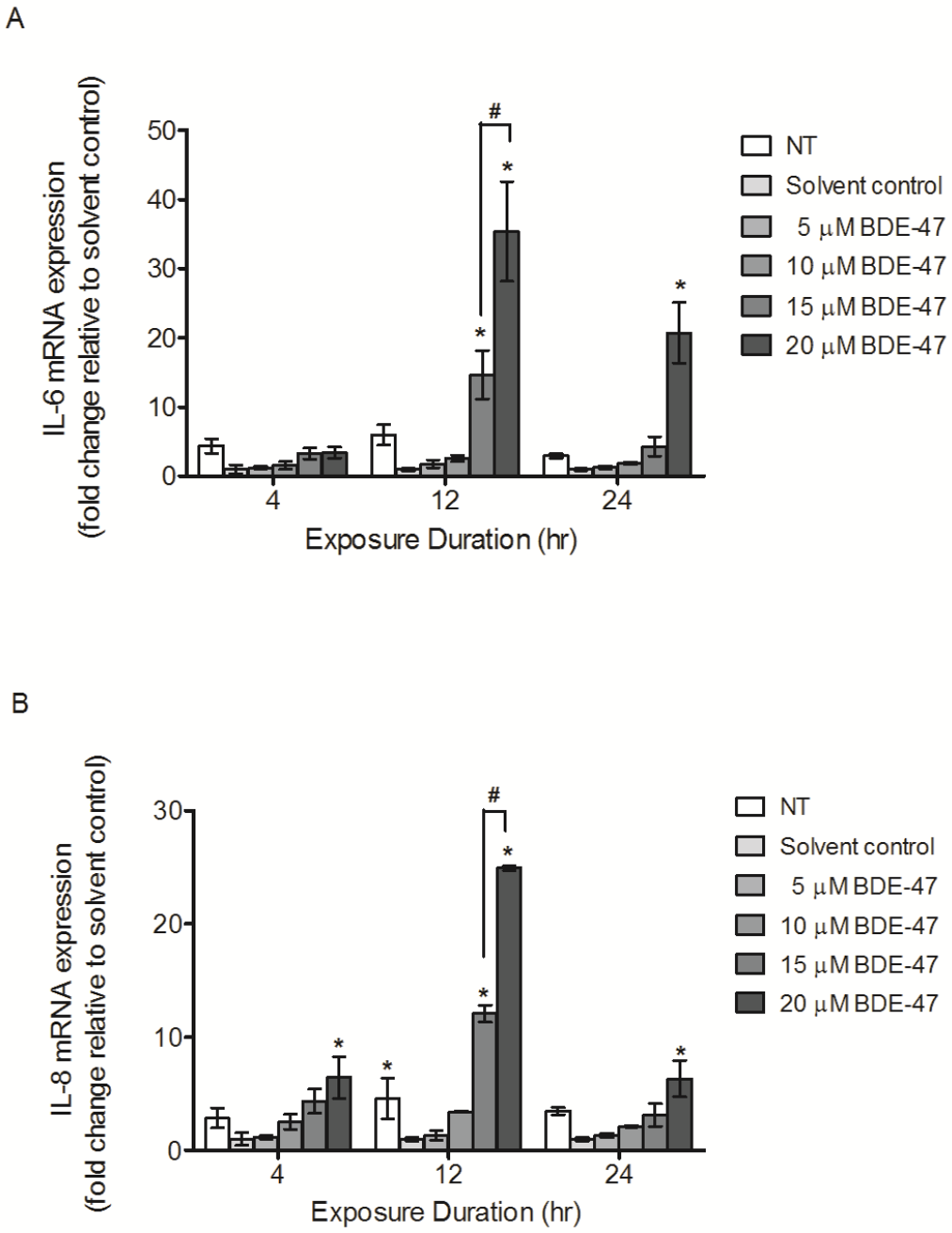


Figure 2.5. BDE-47 effects on mRNA expression of inflammatory cytokine genes in HTR-8/SVneo cells.

HTR-8/SVneo cells were treated for 4, 8 or 24 h with non-treated control (NT), DMSO (solvent control) or BDE-47. The mRNA expression of IL-6 (A) and IL-8 (B) was quantified by qRT-PCR. Bars represent the means ± SE of 3 independent experiments containing 3 replicates each. *P<0.05, significant compared to solvent control within same time point. #P<0.05, significantly different from next lowest concentration within time point.

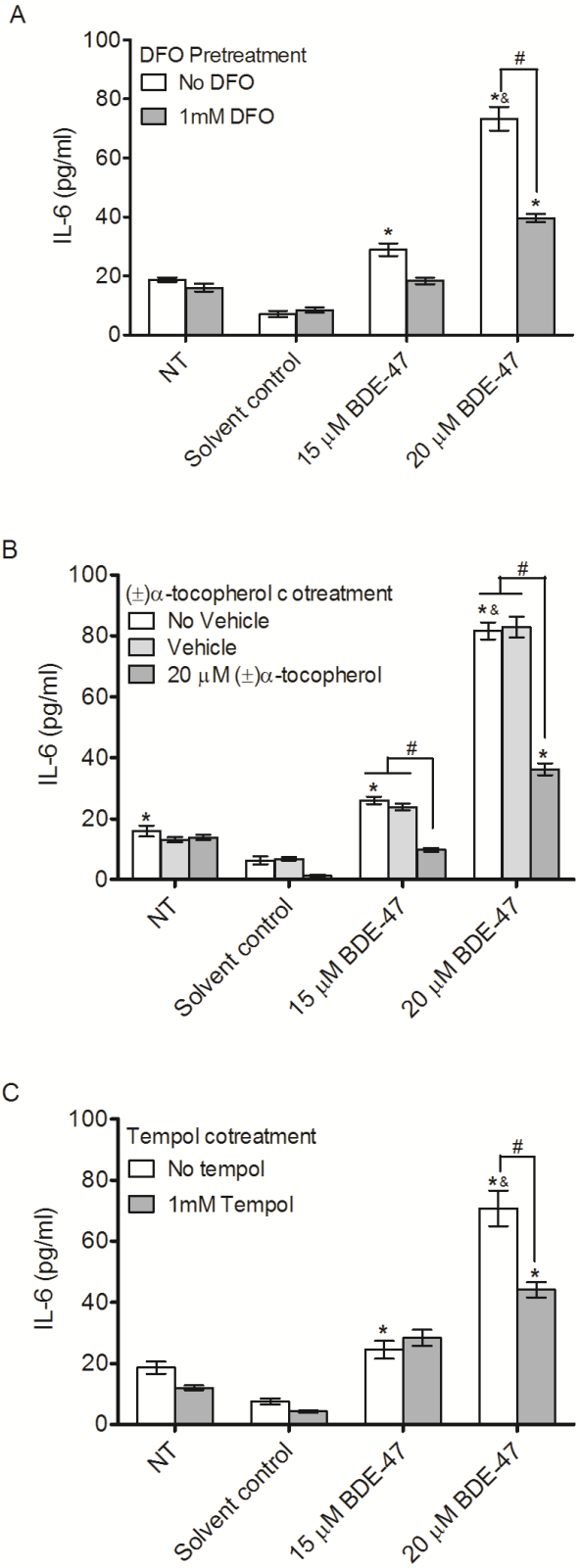
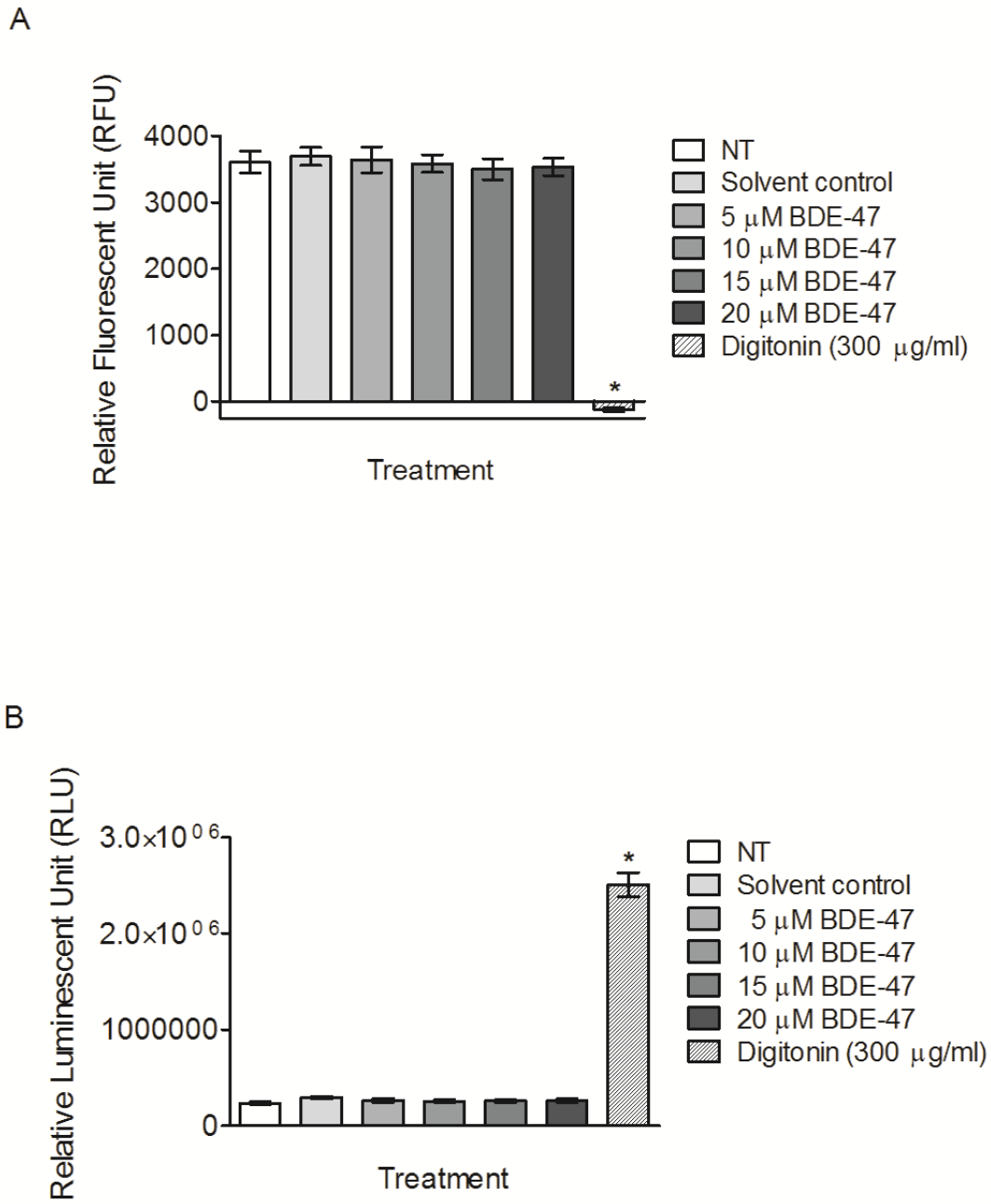
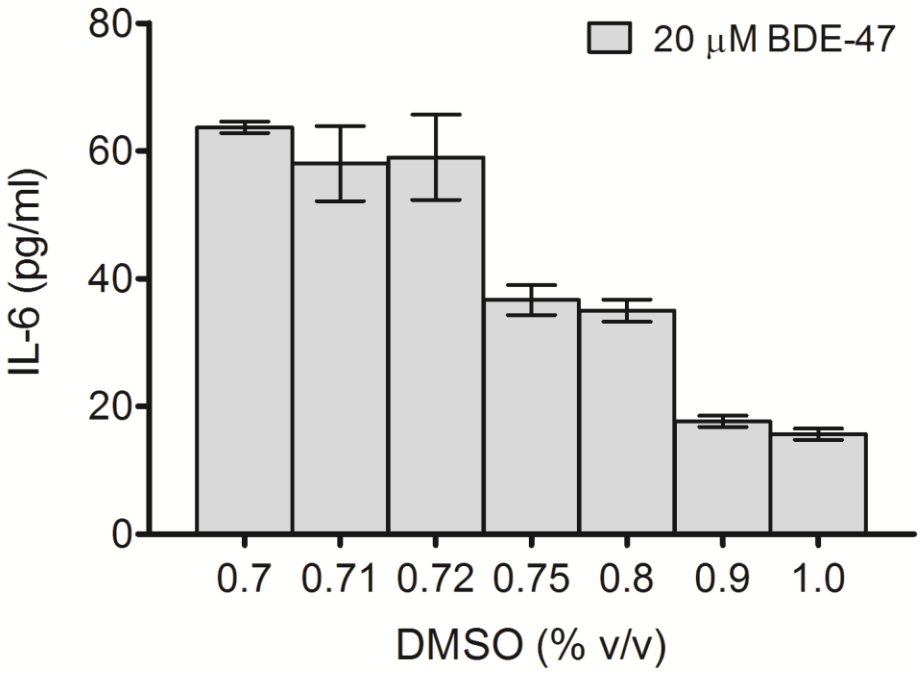

A
DFO Pretreatment
No DFO
1mM DFO
IL-6 (pg/ml)
100
80
60
40
20
0
NT
Solvent control
15 μM BDE-47
20 μM BDE-47
B
(±)α-tocopherol c otreatment
No Vehicle
Vehicle
20 μM (±)α-tocopherol
IL-6 (pg/ml)
NT
Solvent control
15 μM BDE-47
20 μM BDE-47
C
Tempol cotreatment
No tempol
1mM Tempol
IL-6 (pg/ml)
NT
Solvent control
15 μM BDE-47
20 μM BDE-47

Figure 2.6. Effects of antioxidant treatments on BDE-47-stimulated IL-6 release in HTR-8/SVneo cells.

HTR-8/SVneo cells receiving antioxidant treatments were pretreated with DFO for prior to exposure to had no further treatment (non-treated control, NT), or were exposed to DMSO (solvent control), 15 μM BDE-47 or 20 μM BDE-47, or were co-treated with (±)-α-tocopherol or tempol. A) Effects of 1-h pretreatment with DFO on BDE-47-stimulated IL-6 release. B) Effects of (±)-α-tocopherol cotreatment on BDE-47-stimulated IL-6 release; vehicle controls were exposed to DMSO (used to deliver (±)-α-tocopherol, 0.02% v/v) and additional controls received no vehicle. C) Effects of tempol cotreatment on BDE-47-stimulated IL-6 release. Bars represent the means ± SE of 3 independent experiments containing 3 replicates each. *$P<0.05$, significant compared to respective solvent controls. $^{\&}P<0.05$, significant compared to 15 μM BDE-47-treated group. $^{\#}P<0.05$, significantly different from each other.



Appendix 2.1. BDE-47 effects in HTR-8/SVneo cells on A) cytotoxicity and B) cell viability



Appendix 2.2. DMSO effects in HTR-8/SVneo cells on BDE-47-stimulated IL-6 release.

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CHAPTER 3. **PROTECTIVE EFFECT OF (±)α-TOCOPHEROL ON BDE-47-STIMULATED PROSTAGLANDIN PATHWAYS IN HUMAN EXTRAVILLOUS TROPHOBLASTS *IN VITRO***

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. Exposure to BDE-47 has been linked to adverse pregnancy outcomes in humans including preterm birth, low birth weight and stillbirth. Although the underlying mechanisms of adverse birth outcomes are poorly understood, critical roles of prostaglandin pathways are implicated. The present study investigates BDE-47- activation of prostaglandin pathways in a human extravillous trophoblast cell line, HTR-8/SVneo. In addition, the role of the peroxyl radical scavenger (±)-α-tocopherol on the regulation of prostaglandin production was evaluated. HTR-8/SVneo cells were treated with BDE-47 in the presence or absence of (±)-α-tocopherol for 24 h. Then, prostaglandin E2 (PGE2) release was measured using enzyme-linked immunesorbant assay. mRNA expression of prostaglandin-endoperoxide synthase 2 (PTGS2), prostaglandin E synthase (PTGES), and 15-hydroxyprostaglandin dehydrogenase (HPGD) was quantified by qRT-PCR. Protein expression of COX-2 and prostaglandin E synthase (PGES) was measured by western blot. At 24 h, 20 μM BDE-47 induced significant increases in PGE2 concentration in the culture medium, suggesting increased COX activity. The PGE2 increases were accompanied by significant 5.3, 4.4 and 4.7-fold increases in mRNA expression of PTGS2 at 4, 12 and 24 h, respectively. Furthermore, treatment with 15 and 20 μM BDE-47 significantly decreased mRNA expression of PTGES and HPGD. Treatment with 20 μM BDE-47 for 24 h induced a significant 2.0-fold increase in COX-2

expression. (±)-α-Tocopherol cotreatment suppressed BDE-47-stimulated increases of PGE2 without affecting COX-2 mRNA and protein expression, implicating post-translational regulations of COX activity by (±)-α-tocopherol. Because abnormal activation of prostaglandin production can disrupt trophoblast functions that are necessary for placental development and successful pregnancy, further investigation is warranted of the impact of BDE-47 on trophoblast cellular responses.

Introduction

Proper placental development is prerequisite for a successful pregnancy. Abnormal placentation contributes to the pathophysiology of adverse obstetrical complications such as preeclampsia (Brosens, 1977; Gerretsen *et al.*, 1981; Robertson *et al.*, 1967; Sheppard and Bonnar, 1976), intrauterine growth restriction (IUGR) (Gerretsen, *et al*., 1981; Hustin *et al.*, 1983; Labarrere and Althabe, 1987; Sheppard and Bonnar, 1981), spontaneous abortion (Hustin *et al.*, 1990; Khong *et al.*, 1987), preterm premature rupture of membranes (Kim *et al.*, 2002), and preterm birth (Kim *et al.*, 2003). Although the mechanisms responsible for improper placentation have not been fully elucidated, the role of impaired trophoblast invasion has been implicated (Zhou *et al.*, 1997). The extravillous trophoblasts (EVTs) are a highly proliferative and migratory cell population that invades the decidual and myometrial segments of the spiral arteries, resulting in the reversible remodeling of the normal arterial wall architecture (Anton *et al.*, 2012; Brosens *et al.*, 1967; Pijnenborg *et al.*, 1983; Pijnenborg *et al.*, 1980). Trophoblast invasion is tightly regulated by a number of autocrine and paracrine factors including growth factors, growth factor-binding proteins, and proteoglycans (Chakraborty *et al.*, 2002; Lala and Chakraborty, 2003). Recently, inflammatory mediators such cytokines and prostaglandins have been shown to play a role in the regulation of trophoblast function during first trimester of

pregnancy (Biondi *et al.*, 2006; Horita *et al.*, 2007a; Jovanovic *et al.*, 2010; Jovanovic and Vicovac, 2009; Nicola *et al.*, 2005b).

Prostaglandins are small lipid inflammatory molecules synthesized from membrane phospholipids in response to various physiological and pathological stimuli (Nicola, *et al*., 2005b). Of these, prostaglandin E2 (PGE2) is one of the most extensively studied prostaglandins, and has been shown to play critical roles in successful pregnancy, for example in implantation (Psychoyos *et al.*, 1995; Yee *et al.*, 1993), immunoprotection of the semiallogenic conceptus (Parhar *et al.*, 1988), and parturition (Keelan *et al.*, 2003). PGE2 is produced by a series of biochemical reactions invoving conversion of arachidonic acid to PGH2 by cyclooxygenase (COX)-1 and COX-2 (Jakobsson *et al.*, 1999; Kuroda and Yamashita, 2003; Stichtenoth *et al.*, 2001), followed by PGH2 metabolism to PGE2 by PGE2 synthase (PGES) (Horita, *et al*., 2007a). PGE2 can be catabolized further to the biologically inactive keto-metabolites by 15-hydroxyprostaglandin dehydrogenase (HPGD) (Tai *et al.*, 2006). Dysregulation of PGE2 production within the gestational compartment has been linked to adverse birth outcomes such as intrauterine growth restriction, preeclampsia and preterm birth (Germain *et al.*, 1999; Ness and Sibai, 2006). Although it is not fully understood how dysregulated prostaglandin pathways lead to these adverse impacts, it is suggested that PGE2 regulates trophoblast cellular functions that are critical for successful placentation (Biondi, *et al*., 2006; Horita *et al.*, 2007b; Nicola *et al.*, 2005a).

Polybrominated diphenyl ethers (PBDEs) are commercially produced synthetic flame-retardants that have been used in textiles, plastics, building materials and insulation (Miller *et al.*, 2009b). 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 report reproductive toxicity of PBDEs during pregnancy. Rabbits orally exposed to PBDEs showed decreased gestation length (Breslin *et al.*, 1989). In human studies, Main *et al*. reported a significantly higher risk of cryptorchidism for sons born to mothers with elevated PBDE levels in breast milk (Main *et al.*, 2007). In addition, Chao *et al*. found that elevated levels of PBDEs in breast milk correlated with decreased infant birth weight, infant birth length, infant chest circumference and infant body mass index (Chao *et al.*, 2007). 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 report associations between PBDE exposure and adverse birth outcomes, and although PBDEs distribute to human placenta (Frederiksen *et al.*, 2009), extraplacental membranes (Miller *et al.*, 2009a), 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. Specifically, we identified one study reporting that pre-exposure of placental explants to a PBDE mixture of congers 47, 99 and 100 enhanced placental pro-inflammatory response to heat-killed *E. Coli*, with increased PGE2 release and COX-2 expression (Peltier *et al.*, 2012).

Our previous study showed that treatment with BDE-47 stimulated production of reactive oxygen species in in the first trimester EVT human placental cell line HTR-8/SVneo (Park *et al.*, 2014). Increased oxidative stress in placenta, possibly due to increased generation of ROS, 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). Moreover, formation of reactive oxygen species (ROS) has been shown to modulate

pathways in various experimental models (Basu, 1999; Davidge, 1998; Gonzalez *et al.*, 2000; Wentzel *et al.*, 1999). The present study examines the hypothesis that BDE-47 stimulates PGE2 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 AccuStandard (New Haven, CT, USA). Dimethyl sulfoxide (DMSO), *tert*-butyl hydroperoxide (TBHP), indomethacin, NS398, and (±)-α-tocopherol were purchased from Sigma Aldrich (St. Louis, MO, USA). Purchase of 6-carboxy dichlorodihydrofluorescein diacetate (carboxy-H2DCF-DA), Hoechst 33342 dye, 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) was from Invitrogen Life Technologies (Carlsbad, CA, USA). PGE2 ELISA kit and arachidonic acid was purchased from Cayman Chemical (Ann Arbor, MI, 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, PTGS2, PTGES and HPGD were purchased from Qiagen (Valencia, CA, USA). IGEPAL The NP-40 substitute, CA-630 was purchased from United States Biological (Salem, MA). PhosStop protease inhibitor cocktail and complete mini protease inhibitor cocktail tablets were from Roche (Indianapolis, IN). Reducing Laemmli SDS sample buffer was purchased from Boston BioProducts (Ashland, MA). Memcode reversible protein staining kit and bicinchoninic acid (BCA) assay kit were from Thermo Scientific (Waltham, MA). Alkaline phosphatase-linked secondary antibody was purchased from Cell Signaling Technology (Beverly, MA). Enhanced chemifluorecence (ECF) substrate and PVDF membrane Hybond-P were purchased from GE Healthcare Life Sciences (Pittsburgh, PA).

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 70-90% confluence before treatment. Cells were washed twice with OptiMem 1 containing 1% FBS and 1% P/S, and then acclimated with the medium for 1 h at 37 °C. From solutions of 20 mM BDE-47 in DMSO, exposure media containing 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).

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 washed once with OptiMem 1 medium containing 1% FBS and 1% P/S, and then were untreated (NT, non-treated controls), or exposed to solvent control (DMSO 0.7% v/v), 15 μM BDE-47 or 20 μM BDE-47 for 4 h in the absence or presence of 20 μM (±)-α-tocopherol. Treatment with 100 μM *tert*-butyl hydroperoxide (TBHP) was included as a positive control (Vessey *et al.*, 1992). After removal of the exposure media and rinsing with HBSS, cells were collected by treatment with 0.25% trypsin/EDTA solution for 2 min, washed twice by centrifugation and resuspension in HBSS, and 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 Molecular Devices SpectraMax

Gemini M2e plate reader at an excitation wavelength of 492 nm and emission wavelength of 522 nm.

Cyclooxygenase activity assay. 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% P/S, and then exposed to 20 μM BDE-47 in the absence and presence of 10 μM indomethacin, a non-selective COX inhibitor, or 5 μM NS398, a COX-2 specific inhibitor. After 24-h incubation, the culture medium was removed and cells were washed once with HBSS. Then, cells were incubated with 2.5 μM arachidonic acid in HBSS for 4 h at 37 °C. After the 4-h incubation, the concentration of PGE2 was measured by sandwich ELISA following the manufacturer's protocols. To determine ROS-related activation of prostaglandin pathways by BDE-47, HTR-8/SVneo cells were co-treated with 20 μM (±)-α-tocopherol, a peroxyl radical scavenger for 24 h. Concentrations of PGE2 in the medium were analyzed by ELISA as described above. Release of PGE2 was expressed as pg/ml.

RNA extraction and quantitative real-time polymerase chain reaction. After a 24-h incubation with BDE-47, cell lysates were collected and homogenized using QIA shredder. Total RNA was extracted from homogenized lysates using a RNeasy mini plus kit, and cDNA was synthesized from 1 μg of total RNA using a RT^2 First Strand Kit. The procedures were performed according to instructions of the manufacturer. Quantitative real-time polymerase chain reaction (qPCR) was performed in a total volume of 25 μL containing 4 μL of cDNA template, 1 μL of a gene-specific primer (PTGS2, PTGES, HPGD), 12.5 μL of RT^2 SYBR Green qPCR Master Mix, and 7.5 μL of nuclease-free H_2O using CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). A housekeeping gene, β-microglobulin, was co-amplified as an internal control. qRT-PCR was performed with an initial denaturation step of 10 min at 95°C, followed

by 40 cycles of 15 s at 95°C, 5 s at 60°C. At the end of each cycle, the fluorescence emitted by the SYBR Green was measured. After completion of the cycling process, samples were subjected to a temperature ramp (from 65°C to 95°C at 0.5°C/s) with continuous fluorescence monitoring for melting curve analysis. Signal intensities of target genes were quantified and normalized to the signal of β-microglobulin using Bio-Rad CFX manager software. The level of mRNA expression was presented as fold change compared to solvent controls.

Western blot The HTR-8/SVneo cells were seeded at a density of 2.4×10^5 cells per well in a 6-well plate and cultured for 24 h at 37 °C. Cells were washed once with Optimem1 medium containing 1% FBS and 1% P/S, and then exposed to 20 μM BDE-47 in the absence or presence of 20 μM (±)-α-tocopherol. After a 24-h incubation, the culture medium was removed, and cells were washed twice with ice-cold dPBS, incubated with lysis buffer (0.5% IGEPAL, 250 mM NaCl, 50 mM tris-HCl, with a protease inhibitor tablets), and then scraped from the plates to collect cell lysates. After centrifugation of lysates, the supernatant was collected and stored at -80°C until analysis. Total protein was quantified by BCA assay. The protein samples were boiled in sample buffer, and then 75 μg protein was subjected to SDS-polyacrylamide gel electrophoresis followed by electrotransfer to a PVDF membrane. Transfer efficiency was confirmed by reversible membrane staining (Memcode or Ponceau). Membranes were blocked at room temperature for 1 h with 5% milk in Tris-buffered saline supplemented with 0.1% tween (TBST; 20 mM Tris-HCl, 137 mM NaCl, pH 7.6). Membranes were probed with primary antibodies overnight at 4°C with agitation in 5% BSA TBST. Following washing with TBST under agitation for 3 min three times, membranes were incubated with alkaline phosphatase-conjugated secondary antibodies for 1 h at RT in 5% milk TBST. All antibodies were diluted at 1:2000. Bands were imaged after developing the blot with ECF for 5 min, and imaged on a

Fujifilm Fluorescent Image Analyzer FLA-5000. Images shown are representative of 3 individual experiments. Densitometry was used to semi-quantitate data using Multi Gauge software (Fujifilm).

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 measured 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 ± SEM. All experiments were repeated at least three times and all treatments were performed at least in triplicate in each experiment.

Results

Effects of (±)-α-tocopherol on BDE-47-stimulated ROS production

The DCF fluorescence assay was used to assess the effect of (±)-α-tocopherol on BDE-47-stimulated ROS production. Treatment with 20 μM BDE-47 increased DCF fluorescence by 66% in the HTR-8/SVneo cells indicating increased generation of reactive species, and this BDE-stimulated response was blocked by (±)-α-tocopherol cotreatment ($P<0.05$, Table 1.). Treatment with 100 μM TBHP, included as a positive control, increased DCF fluorescence by 176%. There were no statistically significant differences between non-treated controls, solvent controls, and (±)-α-tocopherol-treated groups, nor was the DCF fluorescence observed with 15 μM BDE-47 statistically different from solvent controls.

Effects of BDE-47 on PGE2 release

COX activity in HTR-8/SVneo cells was determined by measuring PGE2 release into culture medium from cells supplemented with exogenous arachidonic acid for 4 h. Treatment with 20 μM BDE-47 induced a significant 1.8 fold-increase in PGE2 release from HTR-8/SVneo cells compared to the solvent control ($P<0.05$, Figure 3.1A). Co-treatment with indomethacin, a nonspecific COX inhibitor, or NS-398, a COX-2 specific inhibitor, resulted in the complete suppression of BDE-47-stimulated PGE2 release to the levels comparable of the solvent control ($P<0.05$, Figure 3.1B), indicating that BDE-47-induced PGE2 release was dependent on COX activity. Notably, NS-398-mediated PGE2 decrease was similar to indomethacin-mediated PGE2 decrease, suggesting that BDE-47-stimulated PGE2 production is mainly dependent on COX-2 activity. There were no statistically significant differences between non-treated controls and solvent controls, nor did treatment with COX inhibitors alone significantly alter PGE2 release.

Effects of BDE-47 on mRNA expression of PTGS2, PTGES and HPGD

Expression of enzymes involved in prostaglandin synthesis and catabolism was measured at the mRNA levels. Treatment with 20 μM BDE-47 significantly increased mRNA expression of PTGS2 in HTR-8/SVneo cells compared to the solvent control at 4, 12 and 24 h by 5.3-fold, 4.5-fold, and 4.7-fold, respectively ($P<0.05$, Figure 3.2A), in agreement with BDE-47-stimulated COX activity shown in Figure 3.1A. On the other hand, mRNA expression of PTGES, the gene for membrane-bound PGES-1 (mPGES-1), was suppressed 66% with 20 μM BDE-47 treatment compared with solvent control ($P<0.05$, Figure 3.2B). The mRNA expression of HPGD, the gene for 15-hydroxyprostaglandin dehydrogenase, was also reduced by 66% and 44% with 15 and 20 μM BDE-47, respectively ($P<0.05$, Figure 3.2C). There were no statistically significant differences between non-treated controls and solvent controls at any time point, nor was the

mRNA expression observed with 5 and 10 μM BDE-47 statistically different from solvent controls.

Effects of (±)-α-tocopherol treatment on BDE-47-stimulated PGE2 release

To investigate the role of reactive oxygen species in BDE-47-induced PGE2 production, HTR-8/SVneo cells were co-treated with 20 μM (±)-α-tocopherol for 24 h. As shown in Fig. 3.3, (±)-α-tocopherol cotreatment inhibited COX activity stimulated by 20 μM BDE-47 in HTR-8/SVneo cells, reducing PGE2 concentrations in culture medium 22.5% compared to cultures exposed to BDE-47 without (±)-α-tocopherol pretreatment (Figure 3.3; $P<0.05$). There were no statistically significant differences between non-treated controls and solvent controls, nor did treatment with (±)-α-tocopherol alone significantly alter PGE2 release.

Effects of (±)-α-tocopherol treatment on COX-2 and PGES expression

To test whether suppression of BDE-47-induced COX activity by (±)-α-tocopherol cotreatment stems from changes in expression of COX-2 and PGES, qRT-PCR or western blot were conducted. Treatment with 20 μM BDE-47 induced a significant 3.2-fold increase in PTGS2 mRNA expression in HTR-8/SVneo cells compared with solvent control ($P<0.05$, Figure 3.4). The mRNA expression of PTGS2 was not significantly changed with (±)-α-tocopherol cotreatment compared to non-(±)-α-tocopherol-treated groups (Figure 3.4), suggesting that ROS do not regulate COX-2 expression at the transcription level. Treatment with 20 μM BDE-47 increased COX-2 protein expression by 2.0-fold compared with control (dashed line, Solvent Control with no (±)-α-tocopherol cotreatment) ($P<0.05$, Figure 3.5B). Cotreatment with (±)-α-tocopherol did not significantly change BDE-47-stimulated COX-2 expression compared to non-(±)-α-tocopherol-treated groups, implicating that ROS do not regulate COX-2 expression at the

translational level, either (Figure 3.5B). Protein expression of PGES was not significantly changed either with BDE-47 treatment or with (±)-α-tocopherol cotreatment (Figure 3.6).

Discussion

PGE2 is a pro-inflammatory mediator of critical trophoblast functions during placentation (Biondi, *et al*., 2006; Horita, *et al*., 2007b; Nicola, *et al*., 2005a; Zhou, *et al*., 1997). The present study demonstrated that BDE-47, a prevalent flame retardant chemical in the environment and in human tissue samples, stimulated COX activity leading to increased PGE2 release from a human first trimester EVT cell line, HTR-8/SVneo. In addition, we showed that treatment with BDE-47 resulted in differential expression of genes relevant to PGE2 pathways such as PTGS2, PGTES, and HPGD. Furthermore, we showed that BDE-47-stimulated COX-2 activity was dependent on ROS formation in HTR-8/SVneo cells. The interaction of PBDEs and prostaglandin pathways in gestational tissues has not been extensively explored previously. Indeed, we found only one related previous study, which showed that pre-exposure of placental explants to a PBDE mixture of congers 47, 99 and 100 enhanced placental pro-inflammatory response to heat-killed *E. Coli*, with increased PGE2 release and COX-2 expression (Peltier, *et al*., 2012).

PGE2 production is mainly regulated by substrate availability (arachidonic acid) and the activity of COX, the rate limiting step in PGE2 production (Beharka *et al.*, 2002; Shanmugam *et al.*, 2006). Because each treatment group was supplemented with exogenous arachidonic acid in the present study, stimulated PGE2 production is not affected by substrate availability but may be a reflection of increased COX activity (Hayek *et al.*, 1994; Hayek *et al.*, 1997). Suppression of PGE2 release by co-treatment with COX inhibitors confirmed that BDE47-induced PGE2 production was dependent on COX activity. Because treatment with NS-398, a COX-2-specific

inhibitor, was sufficient to completely suppress BDE47-stimulated PGE2 release, it is suggested that BDE47-mediated PGE2 production was mainly dependent on COX-2 activity in HTR-8/SVneo cells.

Stimulated PGE2 release could result from changes in the rate of protein synthesis or the rate of mRNA transcription (Beharka, *et al*., 2002). Our results showed that mRNA expression of PTGS2 was highly induced by BDE-47 treatment in HTR-8/SVneo cells whereas mRNA expression of PTGES and HPGD was reduced. Stimulated PTGS2 expression is consistent with increased COX activity we observed, supporting the hypothesis that increased gene transcription may contribute to the increased COX activity. Decreased HPGD expression may also contribute to the increased PGE2 concentrations in medium, due to reduced conversion of PGE2 to inactive metabolites (Tai, *et al*., 2006). Because PGES plays a role in the final step of PGE2 synthesis by converting PGH2 to PGE2, decreased PTGES mRNA expression is inconsistent with our findings of elevated PGE2 concentrations. However, western blot analysis on PGES protein showed that PGES protein abundance was not affected by BDE-47 treatment, suggesting that PGES protein remains at a level with sufficient activity for PGE2 production even in the circumstance of decreased PGES mRNA. An alternative explanation may involve isoforms of PGES, because there are three different PGESs including cytosolic PGES (cPGES) and two membrane-bound PGES (mPGES-1 and mPGES-2) (Samuelsson *et al.*, 2007). Of these isoforms, cPGES and mPGES-2 are constitutively expressed, whereas mPGES-1 (PTGES) is mainly an induced isoform (Samuelsson, *et al*., 2007). Although the present study only measured mRNA expression of inducible mPGES-1 (PTGES), constitutively expressed cPGES and mPGES-2 would convert PGH2 produced by COX to PGE2. Moreover, the rate of PGE2 synthesis is mainly dependent on COX activity (Beharka, *et al*., 2002; Shanmugam, *et al*., 2006).

The present study provides new information that ROS play a role in activation of BDE-47-mediated prostaglandin pathways in HTR-8/SVneo cells. Our finding that cotreatment with the antioxidant (±)-α-tocopherol suppressed BDE-47-stimulated ROS production and PGE2 release suggests that ROS likely play a key role in BDE-47 stimulation of PGE2 release from HTR-8/SVneo cells. Our results are in agreement with previous findings that α-tocopherol diminished ROS-stimulated placental PGF2α and thromboxane B2 (TXB2), as well as lipoperoxide levels (White *et al.*, 2002). Interestingly, (±)-α-tocopherol cotreatment led to reduced COX activity without changing its expression, implicating post-translational regulation of COX activity by (±)-α-tocopherol. Similarly, α-tocopherol inhibited PGE2 production and COX activity, but had no effect on the expression of COX in murine macrophages and in Caco2 cells (Jiang *et al.*, 2000; O'Leary *et al.*, 2004; Wu *et al.*, 1998).

Vitamin E (tocopherols and tocotrienols) is an effective biological antioxidant and lipid peroxide chain-breaking free radical scavenger (Wu, *et al*., 1998). It is reported that COX activity requires the presence of oxidant hydroperoxides (Hemler and Lands, 1980; Kulmacz and Wang, 1995; Smith *et al.*, 1992).Therefore, it has been proposed that vitamin E may attenuate COX activity by scavenging the oxidant hydroperoxides necessary for COX activation (Wu *et al.*, 2001). Increased lipid peroxidation by BDE-47 treatment *in vitro* is consistent with this mechanism (He *et al.*, 2008; Shao *et al.*, 2008); however, we did not measure lipid peroxidation in the present study. Another proposed mechanism involves nitric oxide (NO) and peroxynitrite (ONOO) in regulation of COX activity (Wu, *et al*., 2001). Specifically, NO and ONOO stimulate COX activity without affecting COX expression (Salvemini *et al.*, 1995; Wu, *et al*., 2001), and vitamin E reduces COX activity in murine macrophages by decreasing NO and ONOO production(Wu, *et al*., 2001). Production of NO, ONOO, and NO synthase activity were

reported in human first trimester primary trophoblasts, first trimester trophoblast cell lines, term primary trophoblasts, and term placenta (Al-Hijji *et al.*, 2003; Asagiri *et al.*, 2003; Dash *et al.*, 2003). Because NO can combine with superoxide to form ONOO (Wu, *et al*., 2001), our previous report of increased superoxide production by BDE-47 in HTR-8/SVneo cells (Park, *et al*., 2014) is consistent with the potential production of ONOO in BDE-47-treated HTR-8/SVneo cells. However, further study will be need to measure NO and ONOO levels in HTR-8/SVneo cells stimulated by BDE-47 to test the roles of NO and ONOO on COX activity in human trophoblasts.

Sakamoto *et al*. suggested an alternative explanation to post-translational modulation of COX activity by vitamin E (Sakamoto *et al.*, 1993). They reported that PGE2 production stimulated by phorbol 12-myristate 13-acetate or A-23187 was inhibited by intraperitoneal injection of vitamin E via suppression of PLA2 activity and the subsequent decrease in arachidonic acid release (Sakamoto *et al.*, 1991; Sakamoto, *et al*., 1993). The latter mechanism may be relevant to our findings because we observed augmented PGE2 production with endogenous arachidonic acid in BDE-47-treated HTR-8/SVneo cells compared to controls without exogenous arachidonic acid supplementation (data not shown). However, we used an experimental approach that supplemented the cell culture medium with exogenous arachidonic acid because the observed PGE2 levels were close to the limit of detection in the assay otherwise. Therefore, the effect of vitamin E on PLA2 activity and subsequent arachidonic acid release was not tested in the present study because arachidonic acid was not limited in our experimental setting. Because prostaglandin production involves multiple step-wise reactions, we suggest that multiple mechanisms, not a single mechanism, may contribute to modulation of COX activity mediated by vitamin E. Besides, (±)-α-tocopherol treatment was not able to

suppress BDE-47-stimulated COX activity completely, supporting additional mechanisms. Therefore, further study will be needed to better understand the mechanisms for modulatory effects by vitamin E on COX activity.

Our findings implicate PGE2 as a potential mediator of adverse impacts on trophoblast cellular function by PBDE exposure. A few studies indicate that PGE2 regulates trophoblast cellular functions *in vitro*. For example, PGE2 promoted migration of HTR-8/SVneo cells (Horita, *et al*., 2007b; Nicola, *et al*., 2005a) and the stimulated migration was suppressed by COX-2 inhibition. In contrast, Biondi *et al*. (2006) showed that PGE2 suppressed the proliferation and migration of HTR-8/SVneo cells. These contradictory results may be due to different experimental conditions (media, serum concentration, exposure duration, cell density, etc.) generating divergent responses to the same stimuli. Regardless of these inconsistencies, these data implicate that PGE2 may play a role in regulating trophoblast cellular function and that dysregulation of PGE2 production at the gestational compartment may affect trophoblast invasion and migration that are critical for proper placentation (Pijnenborg, *et al*., 1983; Pijnenborg, *et al*., 1980). Moreover, dysregulation of PGE2 production within the gestational compartment has been linked to adverse birth outcomes such as intrauterine growth restriction, preeclampsia and preterm birth (Germain, *et al*., 1999; Ness and Sibai, 2006). Because improper placentation has been associated with adverse obstetrical complications (Brosens, 1977; Hustin, *et al*., 1983; Kim, *et al*., 2003), further investigation will be needed to ascertain the potential relevance of BDE-47 stimulation of PGE2 on trophoblast invasion and placental function.

In conclusion, this is the first study to show that treatment with BDE-47, a predominant flame retardant chemical found in human tissues, stimulated COX activity and expression of COX2, leading to increased conversion of arachidonic acid to PGE2 in human first trimester placental

cells. In addition, (±)-α-tocopherol cotreatment reduced BDE-47-stimulated COX activity without affecting mRNA and protein expression of COX2, implicating post-translational regulation of COX activity. Because dysregulation of PGE2 has been implicated in improper trophoblast invasion and placental dysfunction, and associated with adverse birth outcomes, further investigation of the impact of BDE-47 on trophoblast function is warranted.

Table 3.1. Quantification of reactive oxygen species production in HTR-8/SVneo cells[a]

Treatment	DCF fluorescence intensity
NT	177.88 ± 5.22
Solvent control	178.79 ± 7.38
15 μM BDE-47	236.63 ± 16.60
20 μM BDE-47	296.81 ± 18.70*
20 μM BDE-47+ 20 μM (±)-α-tocopherol	183.88 ± 7.96
100 μM TBHP	493.82 ± 40.47*
20 μM (±)-α-tocopherol	181.39 ± 6.97[#]

[a] HTR-8 cells were non-treated (NT; control), or were treated with DMSO (solvent control), BDE-47 or *tert*-butyl hydroperoxide (TBHP, positive control) in the absence or presence of (±)-α-tocopherol for 4 h. After 1-h incubation with 100 μM carboxy-H2DCF-DA, the fluorescence intensity was measured using a spectrophotometry. Values represent the means ± SE of 3 independent experiments containing 3 replicates each. *$P<0.05$, significant compared to solvent control. [#]$P<0.05$, significantly different compared to 20 μM BDE-47-treated group.

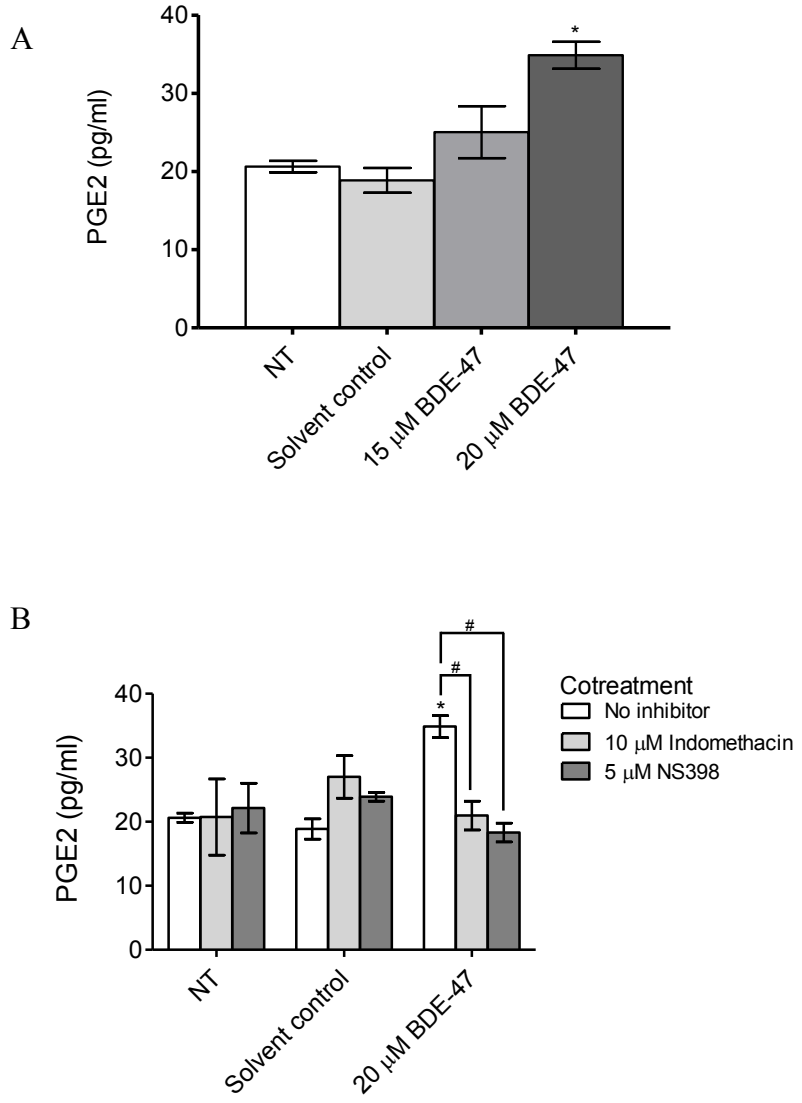


Figure 3.1. BDE-47 effects on COX activity in HTR-8/SVneo cells.
HTR-8/SVneo cells were non-treated (NT, control), or treated with solvent control (DMSO, 0.7% v/v) or BDE-47 for 24 h in the absence or presence of the nonspecific cyclooxygenase (COX) inhibitor indomethacin or the COX-2 specific inhibitor NS 398. COX activity was inferred by quantification of PGE2 in the culture medium from cells stimulated with exogenous arachidonic acid after BDE-47 treatment. A) BDE-47 effects on COX activity in HTR-8/SVneo cells. B) Suppression of BDE47-stimulated PGE2 release by treatment with COX inhibitors. Bars represent the means of 3 independent experiments containing 3 replicates each ±SE. *$P<0.05$, significant compared to solvent control. #$P<0.05$, significantly different from each other. *$P<0.05$, significant compared to solvent control. $^{\#}P<0.05$, significantly different from each other.

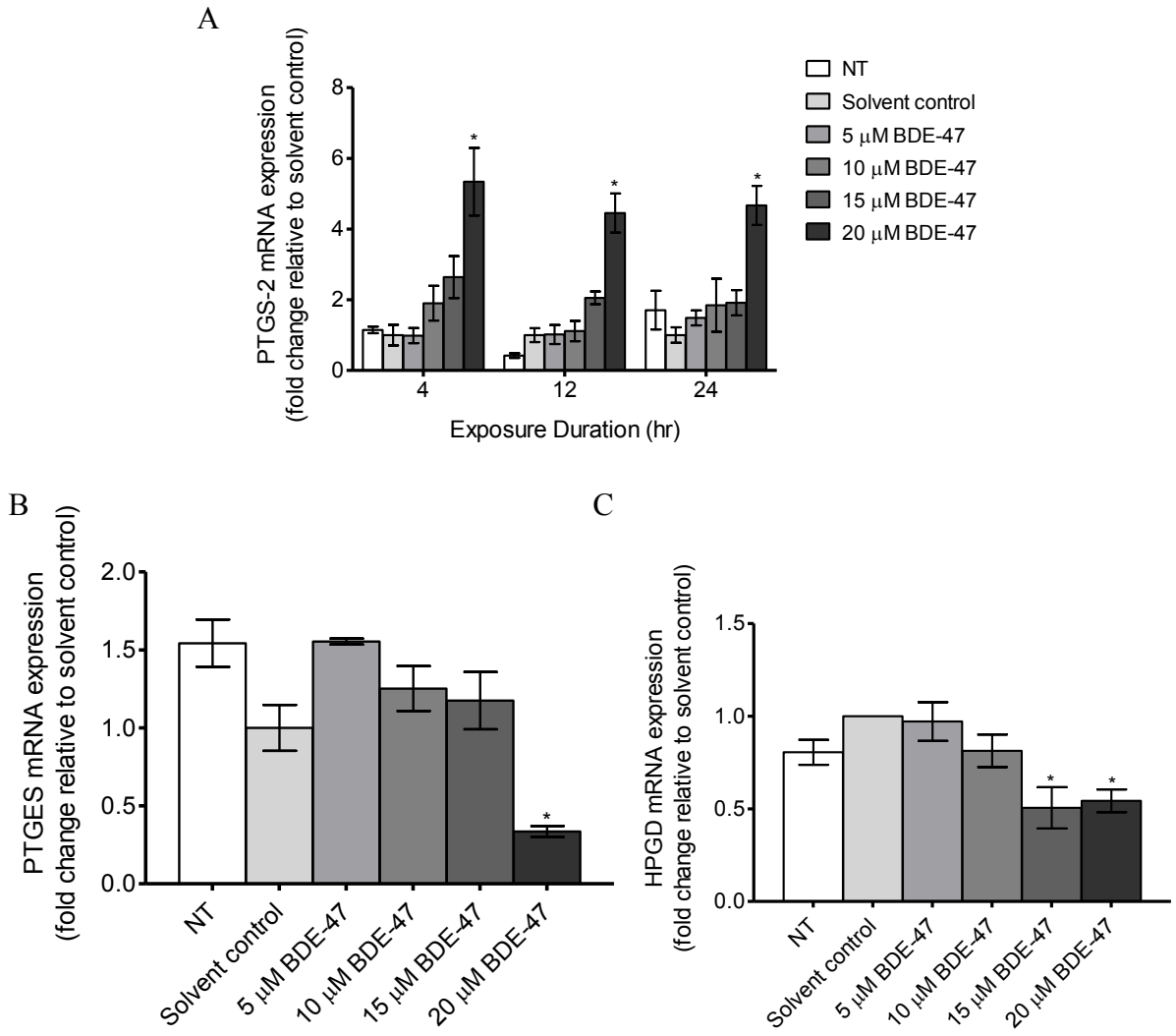


Figure 3.2. BDE-47 effects on mRNA expression of PTGS-2, PTGES, and HPGD. HTR-8/SVneo cells received no treatment (non-treated control, NT), or were treated with solvent control (DMSO, 0.7% v/v) or BDE-47 for 4, 12, or 24 h, and then mRNA expression of target genes was quantified by qRT-PCR. A) Time-course of BDE-47 concentration-dependent effects on PTGS2 mRNA expression. B) BDE-47 concentration-dependent effects on PTGES mRNA expression. C) BDE-47 concentration-dependent effects on HPGD mRNA expression. Bars represent the means of 3 independent experiments containing 3 replicates each ±SE. *P<0.05, significant compared to solvent control within each time point.

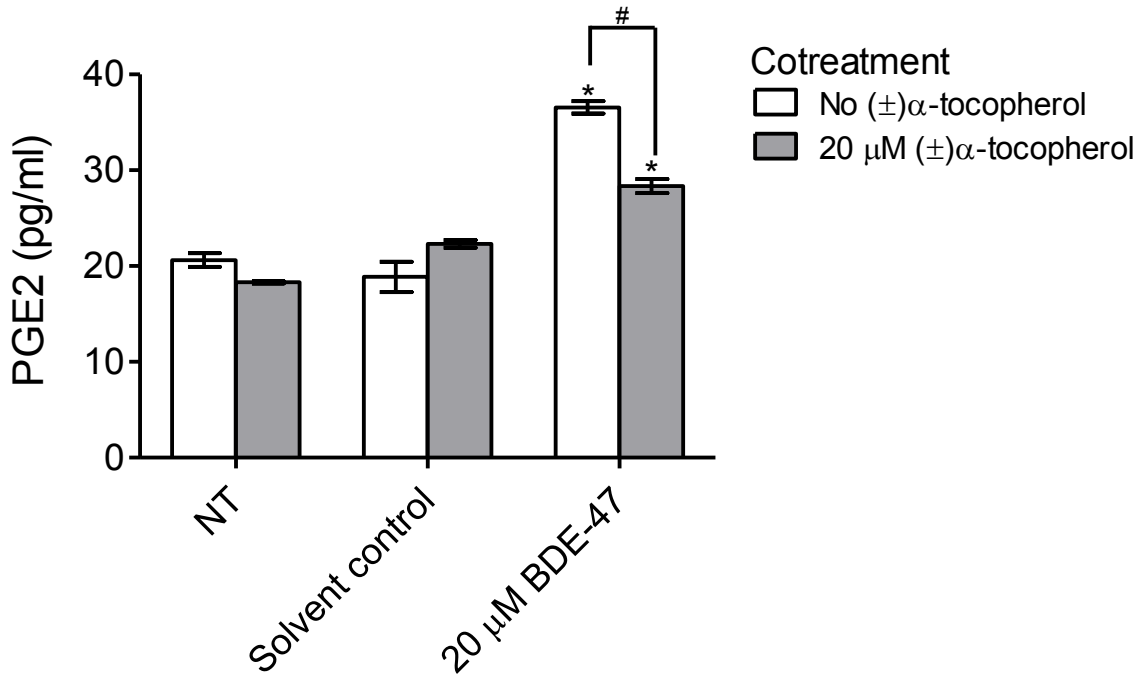


Figure 3.3. (±)-α-Tocopherol effects on BDE-47-stimulated PGE2 release.
HTR-8/SVneo cells received no treatment (non-treated control, NT), or were treated with solvent control (DMSO, 0.7% v/v) or BDE-47 for 24 h in the absence or presence of (±)-α-tocopherol. COX activity was inferred by quantification of PGE2 in the culture medium of cells stimulated with exogenous arachidonic acid after BDE-47 treatment. Bars represent the means of 3 independent experiments containing 3 replicates each ±SE. *P<0.05, significant compared to solvent control. #P<0.05, significantly different from each other.

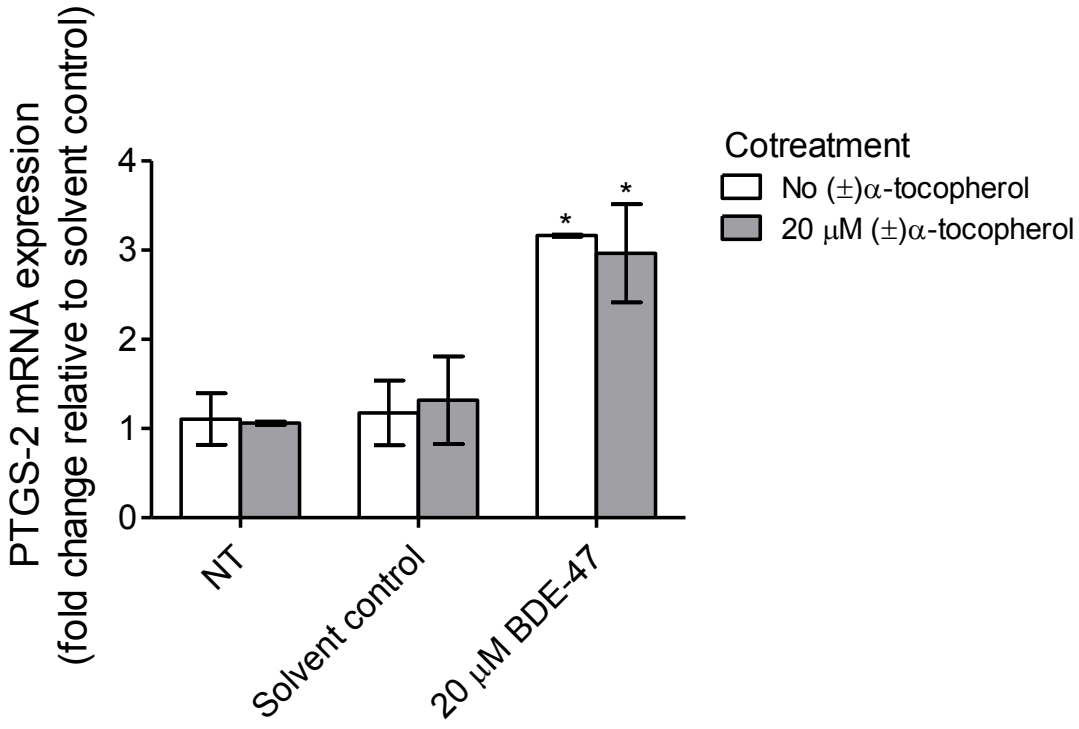
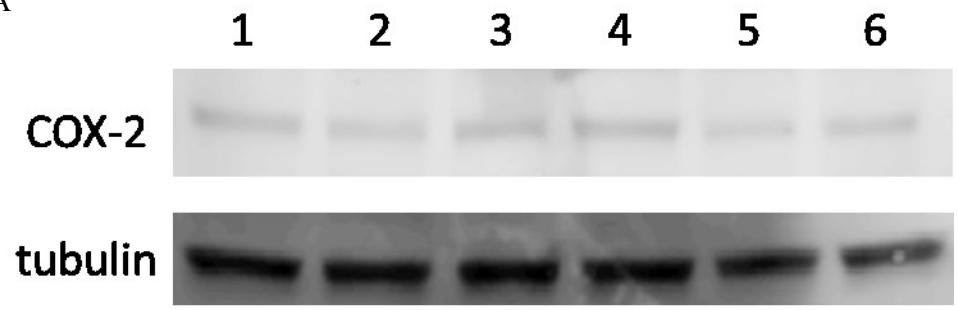


Figure 3.4. (±)-α-Tocopherol effects on BDE-47-stimulated PTGS2 mRNA expression. HTR-8/SVneo cells received no treatment (non-treated control, NT), or were treated with solvent control (DMSO, 0.7% v/v) or BDE-47 for 24 h in the absence or presence of (±)-α-tocopherol. The mRNA expression of PTGS2 was quantified by qRT-PCR. Bars represent the means of 3 independent experiments containing 3 replicates each ±SE. *P<0.05, significant compared to solvent controls.

A



B

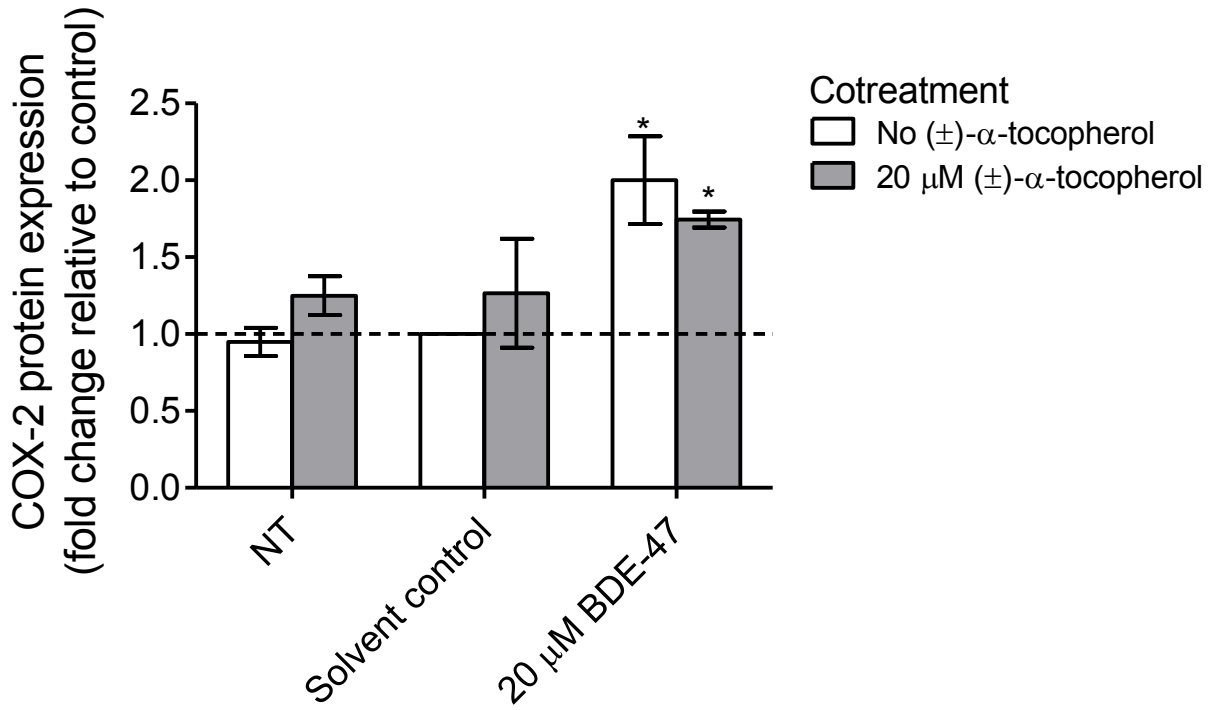


Figure 3.5. (±)-α-Tocopherol effects on BDE-47-stimulated COX-2 protein expression. HTR-8/SVneo cells received no treatment (non-treated control, NT), or were treated with solvent control (DMSO, 0.7% v/v) or BDE-47 for 24 h in the absence or presence of (±)-α-tocopherol. A) Representative image of western blotting for COX-2 and β-tubulin loading control. 1: NT, 2: Solvent control, 3: 20 μM BDE-47, 4: NT+20 μM (±)-α-tocopherol, 5: Solvent control +20 μM (±)-α-tocopherol, 6: 20 μM BDE-47+20 μM (±)-α-tocopherol . B) Fold changes in COX-2 protein expression relative to control (dashed line, Solvent control with no (±)-α-tocopherol cotreatment). Densitometry data for COX-2 were normalized to the β-tubulin loading control. Data represent mean ± SEM for 3 experiments.*$P<0.05$, significant compared to control.

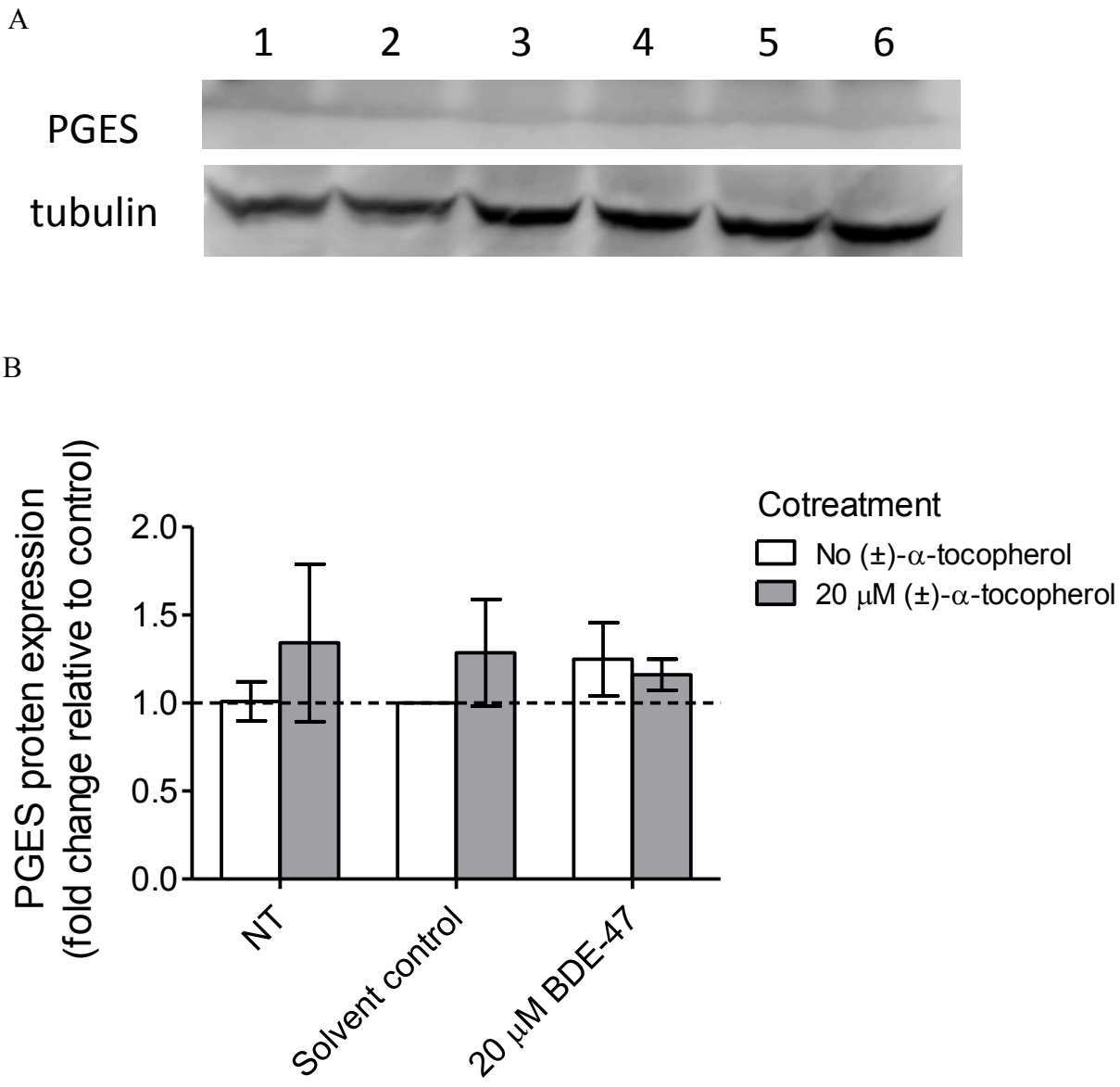


Figure 3.6. (±)-α-Tocopherol effects on PGES protein expression.

HTR-8/SVneo cells received no treatment (non-treated control, NT), or were treated with solvent control (DMSO, 0.7% v/v) or BDE-47 for 24 h in the absence or presence of (±)-α-tocopherol. A) Representative image of western blotting for PGES and β-tubulin loading control. 1: NT, 2: Solvent control, 3: 20 μM BDE-47, 4: NT+20 μM (±)-α-tocopherol, 5: Solvent control +20 μM (±)-α-tocopherol, 6: 20 μM BDE-47+20 μM (±)-α-tocopherol . B) Fold changes in PGES protein expression relative to control (dashed line, Solvent control with no (±)-α-tocopherol cotreatment). Densitometry data for PGES were normalized to the β-tubulin loading control. Data represent mean ± SEM for 3 experiments.*$P<0.05$, significant compared to control.

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CHAPTER 4. PROTECTIVE EFFECT OF NUCLEAR FACTOR E2-RELATED FACTOR 2-DEPENDENT ANTIOXIDANT RESPONSE ELEMENT ACTIVATION ON INFLAMMATORY RESPONSE IN HUMAN FIRST TRIMESTER EXTRAVILLOUS TROPHOBLASTS EXPOSED TO BROMINATED DIPHENYL ETHER-47

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. Exposure to BDE-47 has been linked to adverse pregnancy outcomes in humans including preterm birth, low birth weight and stillbirth. Although the underlying mechanisms of adverse birth outcomes are poorly understood, critical roles for oxidative stress and inflammation are implicated. The present study investgated the role of nuclear factor E2-related factor 2 (Nrf2), a redox-sensitive transcription factor, in oxidative stress responses in a human extravillous trophoblast cell line, HTR-8/SVneo cells, and examined the protective roles of Nrf2 on BDE-47-induced inflammatory re sponses in the cells. After 6 or 24 h exposure of HTR-8 cells to BDE-47, intracellular glutathione (GSH) concentration, Nrf2 transactivation, and expression of 84 redox-regulated genes were assayed. Treatment of HTR-8 cells with 20 μM BDE-47 for 24 h resulted in differential expression of redox-sensitive genes compared to solvent control, as assayed with a commercial Oxidative Stress PCR Array. Treatment of HTR-8 cells with 5, 10, 15, and 20 μM BDE-47 for 24 h increased intracellular GSH levels compared to solvent control, consistent with increased mRNA expression of genes related to GSH synthesis. At 24 h, 20 μM BDE-47 induced significant increases in the Nrf2 activity. To test the protective role of Nrf2 activation on BDE-47-stimulated inflammation, HTR-

8/SVneo cells were pretreated with *tert*-butyl hydroquinone (tBHQ) or sulforaphane,known Nrf2 inducers which induced cellular antioxidant defenses of the HTR-8/SVneo cells, and then treated with BDE-47. Pretreatment with tBHQ or sulforaphane reduced BDE-47 stimulated pro-inflammatory IL-6 release, 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 inflammation 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. Further investigation about the mechanisms of the anti-inflammatory effect mediated by Nrf2 induction is warranted.

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 (PE), 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 are associated with the pathophysiology of PE 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 beeb 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) prevents LPS-stimulated parturition, fetal death in mice and LPS-induced release of pro-inflammatory cytokines from human extraplacental membranes *in vitro* (Buhimschi *et al.*, 2003b; 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 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 PE and IUGR outcomes (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.*, 2003a; 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), the present study aimed to investigate the protective roles of Nrf2 on BDE-47-induced inflammatory responses in the HTR-8/SVneo cell model.

Materials and Methods

Chemicals and assay kits. BDE-47 was purchased from AccuStardard (New Haven, CT, USA). dimethyl sulfoxide (DMSO), tBHQ, and sulforaphane 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 (Germantown, MD). Dual Luciferase, GSH-Glo™ Glutathione Assays were purchased from Promega (Madison, WI). 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% P/S immediately prior to initiating the experiment. The final concentration of DMSO in medium was 0.7 % (v/v).

Measurement of intracellular gluthathione 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). 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. To assay for GSH, the culture medium was removed and 100 μl of GSH-Glo^{TM} Reagent was added to each well. After a 30 min-incubation, 100 μl of Luciferin Detection Reagent was added to each well, followed by a additional 15-min incubation. The plate was then read in a luminometer. 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.

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 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. After incubation, cell lysates were collected and homogenized using QIA shredder (Qiagen; Valencia, CA). Total RNA was extracted from homogenized lysates using RNeasy mini plus kit (Qiagen; Valencia, CA), and cDNA was synthesized from 1 μg of total RNA using iScript cDNA synthesis kits (Bio-Rad; Hercules, CA) 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 ΔCT values (gene of interest CT values – Average of all housekeeping gene CT values) using the ΔΔCT method. Mean ΔCT 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 μM 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). 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-micoglobulin mRNA levels and presented as fold change compared to solvent controls.

Measurement of ARE reporter activity. Nuclear factor (erythroid-derived 2)-like 2 (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 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% NEAA and 3% FBS. After transfection for 6 h at 37°C, transfection complex was replaced with the 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-κB reporter activity. NF-κB 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-κB 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-κB 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% P/S and pretreated with tBHQ for 1 h or

sulforaphane for 24 h prior to 20 μM 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 measured 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 ± 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), we quantified intracellular GSH concentration in HTR-8/SVneo cells after 24-h treatment with BDE-47. Treatment with 5, 10, 15 and 20 μM 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 μM BDE-47 resulted in significantly increased GSH production compared to treatment with 5 μM BDE-47, indicating a concentration-dependent response (Figure 4.1A; $P<0.05$). 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 BDE-47. Treatment with 20 μM BDE-47 stimulated GSH production after 24 h (51% compared to solvent control), as observed in the prior experiment, but there were no statistically significant changes in GSH levels at 0.5 or 4 h (Figure 4.1B; $P<0.05$) (Figure 4.2B). Treatment with 50 μM BSO, included as a positive control, significantly decreased GSH at 4 h and almost completely depleted GSH after 24 h.

Effect of BDE-47 treatment on ARE reporter activity

To investigate possible explanation for the increased cellular GSH observed with BDE-47, we evaluated BDE-47-stimulated Nrf2 activation using an ARE reporter activity assay. After 24 h treatment, 10 and 20 μM BDE-47 increased ARE activity by 1.7fold and 2-fold, respectively, compared to solvent control, indicating Nrf2 activation (Figure 4.2; $P<0.05$). We did not observe 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 the 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 μM BDE-47 treatment compared to solvent control (for complete mRNA array data, see Appendix, Table 1). Changes in expression of the array-identified genes were then examined by qRT-PCR. Consistent with the array results, treatment with 20 μM 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 4.3A, $P<0.05$). The mRNA expression of genes involved in GSH redox cycling was also induced by 20 μM BDE-47 treatment, with SLC7A11, SRXN1, GCLM, and GPX1 increased 3-fold, 1.8-fold, 1.5-fold and 1.7-fold, respectively (Figure 4.3B, $P<0.05$). BDE-47 suppressed mRNA expression of GSR to 0.7-fold relative to solvent control (Figure 4.3B, $P<0.05$). We did not observe any significant changes in mRNA expression with 15 μM 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 μM 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 μM tBHQ , Fig. 4.4A; $P<0.05$). Similarly, 10 μM sulforaphane increased ARE activity by 1.8-fold compared with controls not exposed to sulforaphane (No BDE-47 with 0 μM sulforaphane, Fig. 4.4B; $P<0.05$). No statistically significant changes were observed with 5 μM tBHQ, or with 5 and 7.5 μM sulforaphane. These results identified 20 and 50 μM tBHQ, and 10 μM 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. μMPretreatment with 0, 5, 20, and 50 μM tBHQ followed by 20 μM 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 μM tBHQ) (Figure 4.4A; $P<0.05$). Treatment with 50 μM tBHQ increased ARE activity significantly higher than 0, 5 μM tBHQ and 10 μM tBHQ within the 20 μM BDE-47 treated group, showing a concentration-dependant reponse. BDE-47-treated cells always showed significantly higher ARE activity compared to cells with no BDE-47 and the same tBHQ concentration (Figure 4.4A; $P<0.05$). Similarly, pretreatment with 0, 5, 7.5, and 10 μM sulforaphane followed by 20 μM BDE-47 treatment increased ARE activity 1.6-fold, 1.8-fold, 2-fold, and 3-fold, respectively, in BDE-47-treated cells compared to control (No BDE-47 with 20 μM tBHQ) (Figure 4.4B; $P<0.05$). Treatment with 10 μM sulforaphane resulted in significantly increased activity compared to 5 and 7.5 μM sulforaphane, showing a concentration-dependent increase (Figure 4.4B; $P<0.05$). 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 μM tBHQ increased GSH production 20% and 37%, respectively, compared to control (No BDE-47 with 0 μM tBHQ) (Figure 4.5; P<0.05). In 20 μM BDE-47-treated cells, pretreatment with 0, 10, 20 and 50 μM tBHQ increased GSH production by 17%, 27%, 40%, and 63%, respectively, compared to control (No BDE-47 with 0 μM tBHQ) (Figure 4.5; P<0.05). tBHQ induced GSH production in a concentration-dependent manner, such that pretreatment with 50 μM tBHQ significantly increased GSH compared with pretreatment with 10 and 20 μM tBHQ in those cells subsequently exposed to 20 μM BDE-47 (Figure 4.5; P<0.05). BDE-47-treated cells showed significantly higher ARE activity compared to no BDE-47-treated cells (Figure 4.5; P<0.05). These findings suggeset that cells pretreated with tBHQ may have an augmented defensive capacity against BDE-47 treatment with increased GSH production.

Effect of Nrf2 inducers on expression of HMOX1 and GCLM

To test activation of Nrf2 pathways by Nrf2 inducers further, mRNA expression of the antioxidant genes HMOX1 and GCLM was quantified in HTR-8/SVneo cells after Nrf2 induction by pretreatment with tBHQ followed by treatment with BDE-47. 20 μM BDE-47 treatment resulted in significantly increased mRNA expression of HMOX1 and GCLM compared to control (No BDE-47 with 0 μM tBHQ at all tBHQ concentrations (0, 10, 20 and 50 μM) (Figure 4.6 A and B; P<0.05). Pretreatment with 50 μM tBHQ significantly increased

HMOX1 mRNA expression by 68% compared to no pretreatment in BDE-47-treated cells (Figure 4.6A; $P<0.05$). However, mRNA expression of GCLM was not statistically significantly increased (Figure 4.6B; $P=0.062$). 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 be able to protect themselves from BDE-47-stimulated oxidative damage.

Effect of Nrf2 inducers on IL-6 production

To investigate the roles of Nrf2 induction on the regulation of BDE-47-stimulated IL-6, HTR-8/SVneo cells were pretreated with tBHQ or sulforaphane prior to exposure to BDE-47. Treatment with BDE-47 increased IL-6 release in HTR-8/SVneo cells compared to solvent controls (No BDE-47) regardless of tBHQ or sulforaphane pretreatment (Figures 4.7A and Figure 4.7B, respectively; $P<0.05$). Notably, pretreatment with 50 μM tBHQ significantly suppressed BDE-47-stimulated IL-6 release by 55% from HTR-8/SVneo cells compared to 20 μM BDE-47 with 0 μM tBHQ (Figure 4.7A; $P<0.05$). Similarly, pretreatment with 10 μM sulforaphane decreased BDE-47-induced IL-6 release by 65 % (Figure 4.8B; $P<0.05$) compared to 20 μM BDE-47 with 0 μM sulforaphane. We did not observe any significant changes in solvent controls (No BDE-47) with tBHQ or sulforaphane pretreatment.

Effect of tBHQ on NF-κB transactivation

It is implicated that Nrf2 activation exhibits its anti-inflammatory effect partly via suppression of an inflammatory transcription factor NF-κB (Jin, *et al*., 2011). To examine the possible involvement of NF-κB in BDE-47 mediated IL-6 release and suppression of IL-6 release by Nrf2 inducion, NF-κB reporter activity was measured. Treatment with 20 μM BDE-47 increased NF-

κB reporter activity 3.4-fold compared to control (No BDE-47 with 0 μM tBHQ) (Figure 4.8; $P<0.05$). Pretreatment with tBHQ suppressed the BDE-47-stimulated activation of NF-κB by 32% (Figure 4.8; $P<0.05$). Treatment with tBHQ alone had no statistically significant effect compared with solvent controls not exposed to tBHQ (No BDE-47 treatment groups; Figure 4.8).

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). During pregnancy, BDE-47 accumulates in human placenta (Frederiksen, *et al*., 2009; Miller, *et al*., 2009), extraplacental membranes (Miller, *et al*., 2009), and amniotic fluid (Miller, *et al*., 2012). Previously, we showed that BDE-47 directly stimulates 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 stimulated Nrf2-mediated oxidative stress responses in HTR-8/SVneo cells, resulting in differential expression of redox-sensitive genes, transactivation of Nrf2 and NF-κB, and augmentation of GSH. Especially, we report novel findings that induction of Nrf2 activity by Nrf2 inducers suppressed BDE-47-stimulated proinflammatory IL-6 release and NF-κB reporter activity in HTR-8/SVneo cells, implicating crosstalk between Nrf2 and NF-κB pathways.

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. We observed stimulated mRNA expression of HMOX1, the gene for the antioxidant and anti-inflammatory enzyme heme oxygenase (HO)-1 (Tjoa, *et al*., 2003). HO-1 cleaves the α-methene bridge of heme moiety to produce equimolar amounts of carbon monoxide (CO), bilirubin and

iron (Abraham and Kappas, 2008; Ndisang *et al.*, 2004). CO and bilirubin are known to alleviate apoptosis, necrosis, inflammation and oxidative stress (Bainbridge *et al.*, 2006; Baranano *et al.*, 2002; Jadhav *et al.*, 2008; Ndisang and Jadhav, 2009; Stocker *et al.*, 1987), while iron enhances the synthesis of the antioxidant ferritin (Balla *et al.*, 1992; Hintze and Theil, 2005). Several lines of evidence suggest that HO-1 is a key regulator during pregnancy (Vince, *et al*., 1995). For example, HO-1 polymorphisms have been associated with incidence of idiopathic recurrent miscarriages in women (Denschlag *et al.*, 2004). In addition, 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), further suggesting that HO expression is required to support successful pregnancy in humans. Based on the observed increase in HMOX1 expression with BDE-47 and previously published reports, we suggest that HMOX1 may play a protective role against BDE-47-stimulated oxidative stress and inflammation in placental cells.

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.*, 2007; Nicola *et al.*, 2005). Given the critical roles for prostaglandins in pregnancy, further study could investigate the effects of BDE-47 on PGE2 production and trophoblast cellular function.

Expression of DHCR24, the gene for 3β-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; Tetz *et al.*, 2013). Moreover, DHCR24 expression was downregulated in the IUGR placentas (Diplas *et al.*, 2009). The increased mRNA expression of PRNP, the gene for prion protein, is consistent with BDE-47-stimulated ROS production because PNRP is known to protect cells from oxidative damage and to prevent apoptosis (Liang *et al.*, 2006; Watt *et al.*, 2005). Potential roles for PRNP during pregnancy are largely unknown, but it was recently reported that PRNP is highly expressed in placentas from preeclamptic pregnancies (Hwang *et al.*, 2010). Whether the BDE-47-stimulated DHCR24 and PRNP gene responses observed in the present study are relevant to human pregnancy requires additional experiments beyond the scope of the present study.

Expression of genes involved in GSH redox cycling such as SLC7A11, SRXN1, GSR, GCLM, and GPX1 was also differentially regulated with BDE-47 treatment in HTR-8/SVneo cells. SLC7A11 encodes an amino acid antiporter that mediates the exchange of extracellular L-cystine and intracellular L-glutamate across the cellular plasma membrane, which is critical to glutathione production and oxidative protection (Lewerenz *et al.*, 2013). SRXN1 codes for sulfiredoxin 1, which plays a role in reduction of oxidative modification on proteins (Findlay *et al.*, 2005), and GPX1 is the gene for gluthathione peroxidase 1, which catalyzes the reduction of hydroperoxides and lipid peroxides using GSH as a reductant (Chance *et al.*, 1979). Increased expression of SRNX1 and GPX1 in the present study may implicate increased oxidation of proteins and lipids in BDE-47-treated cells by BDE-47-stimulated ROS generation (Park, *et al*., 2014), although further studies should be conducted to measure oxidation of proteins and lipids. Expression of SLC7A11 was stimulated with BDE-47 treatment, suggesting increased cellular uptake of cystine as a substrate for intracellular GSH production. 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). Expression of GCLM was stimulated with BDE-47 treatment, consistent with the augmented GSH concentrations observed in HTR-8/SVneo cells. Gluthathione reductase (GSR) is an antioxidant enzyme that catalyzes the reduction of GSH disulfide by NADPH (Harvey *et al.*, 2009), leading to increased availability of reduced GSH. mRNA expression of GSR was reduced by BDE-47 treatmentalso in agreement with the changes in cellular redox status. 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.

Consistent with array results showing differential expression of redox-sensitive genes, the present study found increased ARE reporter activity in BDE-47-exposed HTR-8/SVneo cells, indicating Nrf2 induction in response to BDE-47. To defend against exogenous toxicants and injury, cells express a variety of cytoprotective and detoxifying enzymes regulated by redox-sensitive transcription factors that bind to the antioxidant response element (ARE) in the promoter. Nrf2 is a well-known redox-sensitive transcription factor that binds to ARE and activates the ARE-mediated gene expression (Itoh *et al.*, 1997; Motohashi and Yamamoto, 2004; Osburn *et al.*, 2006). BDE-47 stimulates increased ROS generation (Park, *et al*., 2014), and ROS can oxidize cysteine residues on Nrf2 inhibitor Keap1, leading to conformational changes in Keap1, Nrf2 release, and translocation of Nrf2 to activate ARE-dependent gene expression (Rushmore *et al.*, 1991) as a defensive mechanism to protect cells from ROS and inflammation. 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 EVTs from placentae with IUGR or PE (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 PE (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.

Our results clearly show that pretreatment with the Nrf2 inducer tBHQ or sulforaphane suppressed BDE-47-stimulated IL-6 production in HTR-8/SVneo cells. In addition, induction of Nrf2 with tBHQ pretreatment suppressed BDE-47-stimulated NF-κB transactivation. NF-κB 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-κB activity and IL-6 release, NF-κB is well known to regulate the transcription of IL-6 (Blackwell and Christman, 1997; Reuter, *et al*., 2010). As such, decreased NF-κB activity partially explains the anti-inflammatory effect of tBHQ in the present study. Consistent with our findings, 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). For example, tBHQ has shown to decrease NF-κB activation, inflammatory cytokines production (TNF-α, IL-1β, and IL-6), COX-2 expression, and PGE2

release *in vivo* and *in vitro* (Jin *et al.*, 2010; Khodagholi and Tusi, 2011; Koh *et al.*, 2009). Another Nrf2 inducer, sulforaphane, has shown to reduce DNA binding of NF-κB and decrease generation of nitric oxide (NO), PGE2 and TNF-α in Raw 264.7 murine macrophages (Heiss *et al.*, 2001). 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). 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-κB signaling pathway (Jin, et al., 2011; Khodagholi and Tusi, 2011). The augmented GSH concentrations and increased antioxidant gene expression observed in the present study are consistent with a role of Nrf2 inducers on increased cellular antioxidative capacity in previous studies (Alfieri *et al.*, 2011; Hara *et al.*, 2003). In addition, our results showed that suppression of BDE-47-stimulated NF-κB transactivation by tBHQ pretreatment, implicating cross talk between the Nrf2 and NF-κB signaling pathways.

The Nrf2 and NF-κB 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-κB binding to DNA. In addition, Nrf2 target genes such as HO-1, NQO1, and thioredoxin (TRX) are able to influence NF-κB activity (Wakabayashi, *et al*., 2010). Moreover, Nrf2 interferes with NF-κB inflammatory signaling pathways through the

maintenance of cellular redox status because NF-κB is activated in the oxidizing environment (Kabe *et al.*, 2005). Our results are in agreement with this model of Nrf2 and NF-κB interactions, showing that tBHQ stimulated mRNA expression of HMOX1, the gene for HO-1, and augmented GSH production concomitant with suppression of NF-κB 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-κB activity, and, in turn, decreasing IL-6 production in HTR-8/SVneo cells. In addition, expression of the Nrf2 target protein HO-1 may modulate NF-kB activity through the action of bilirubin and free iron that are produced by HO-1 activity (Alam *et al.*, 1999; Jun *et al.*, 2006; Pae *et al.*, 2006; Seldon *et al.*, 2007; Soares *et al.*, 2004; Tenhunen *et al.*, 1968). Further studies could investigate the effect of HO-1 induction or its products on inflammation induced by BDE-47 treatment in HTR-8/SVneo cells. However, we shoud 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 dependance of the anti-inflammatory effect on Nrf2 should be tested using genetic knockdown approaches such as RNAi.

In summary, BDE-47, a predominant flame retardant chemical found in human tissues, activates Nrf2-dependent oxidative stress responses in human first trimester EVTs 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-κB 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 EVTs 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.

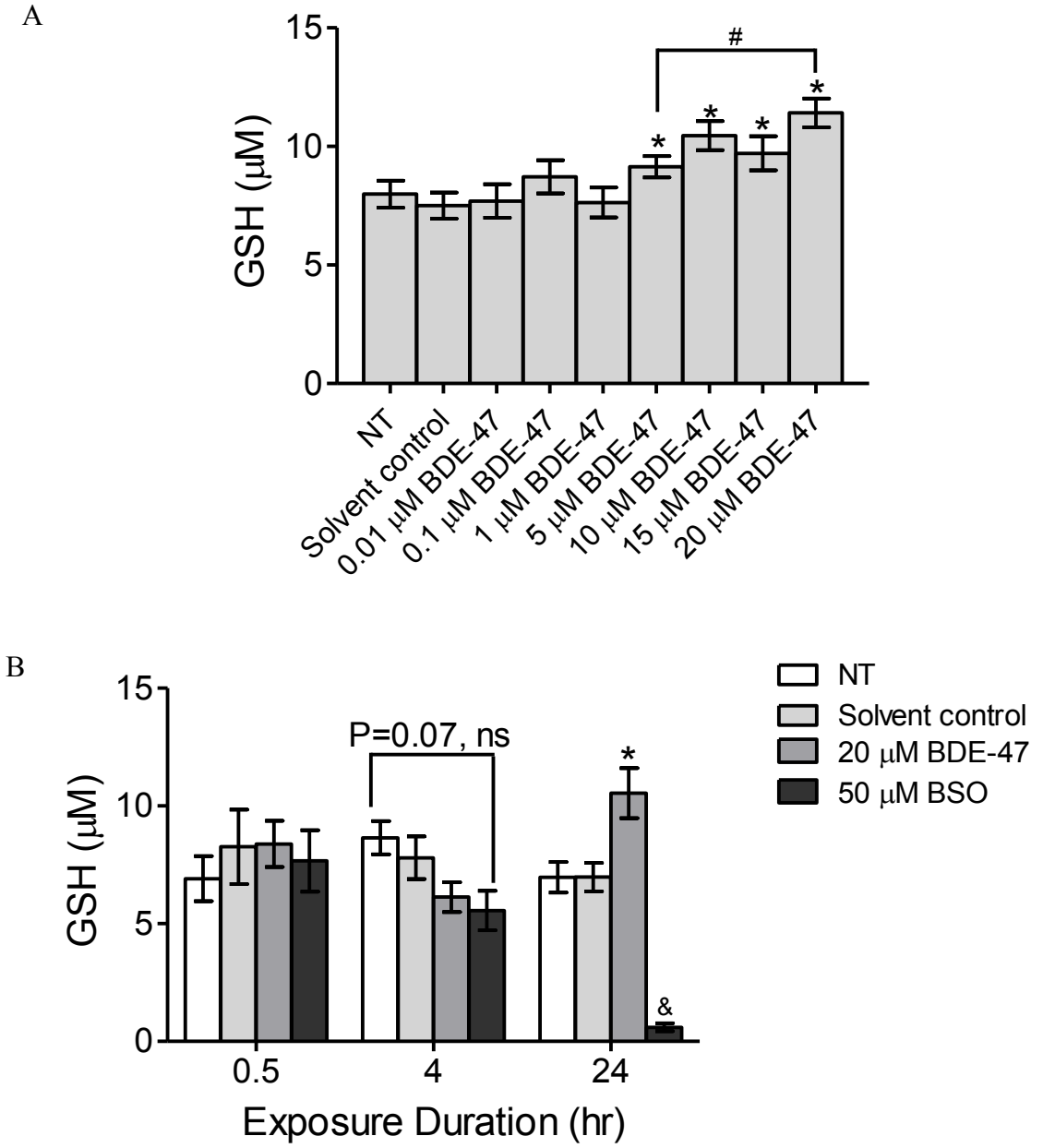

Figure 4.1. BDE-47-stimulated intracellular GSH production in HTR-8/SVneo cells.
A) HTR-8/SVneo cells were exposed to NT (non-treated control), solvent control (0.07% v/v DMSO) or BDE-47 treatment for 24 h, and then GSH levels were quantified. B) Time-course of GSH levels. Bars represent means±SEM (n=3 experiments). Each experiment was performed in triplicate.*P<0.05, significant 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.

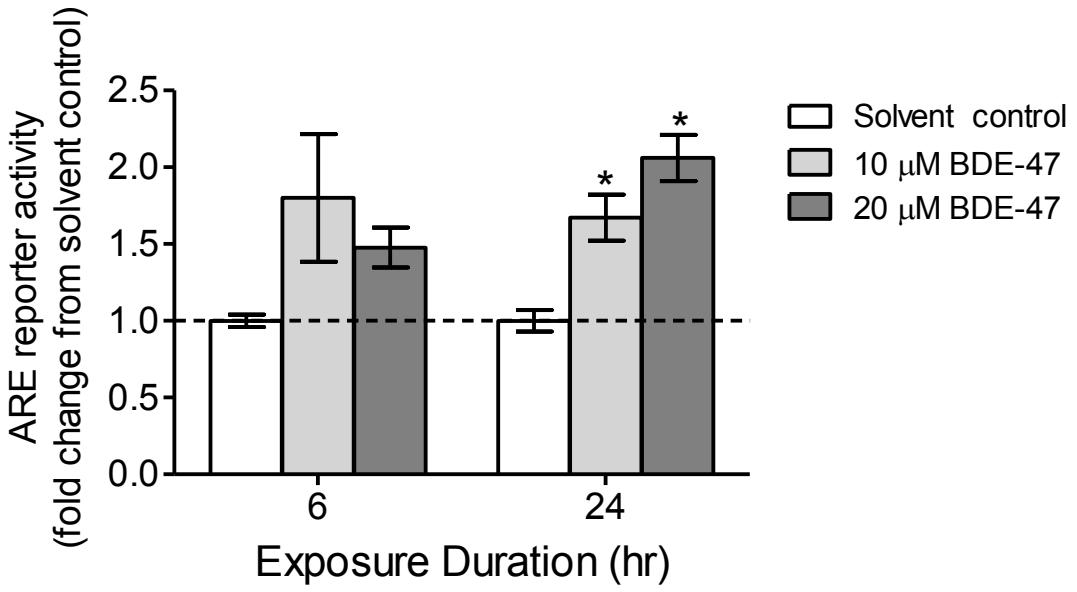

Figure 4.2. BDE-47-stimulated ARE reporter activity in HTR-8/SVneo cells.
HTR-8/SVneo cells were exposed to solvent control (0.7% v/v DMSO) or BDE-47 treatment for 6 or 24 h, and then ARE reporter activity was assessed. Data are presented as means±SEM fold change over solvent control (dashed line) for 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.

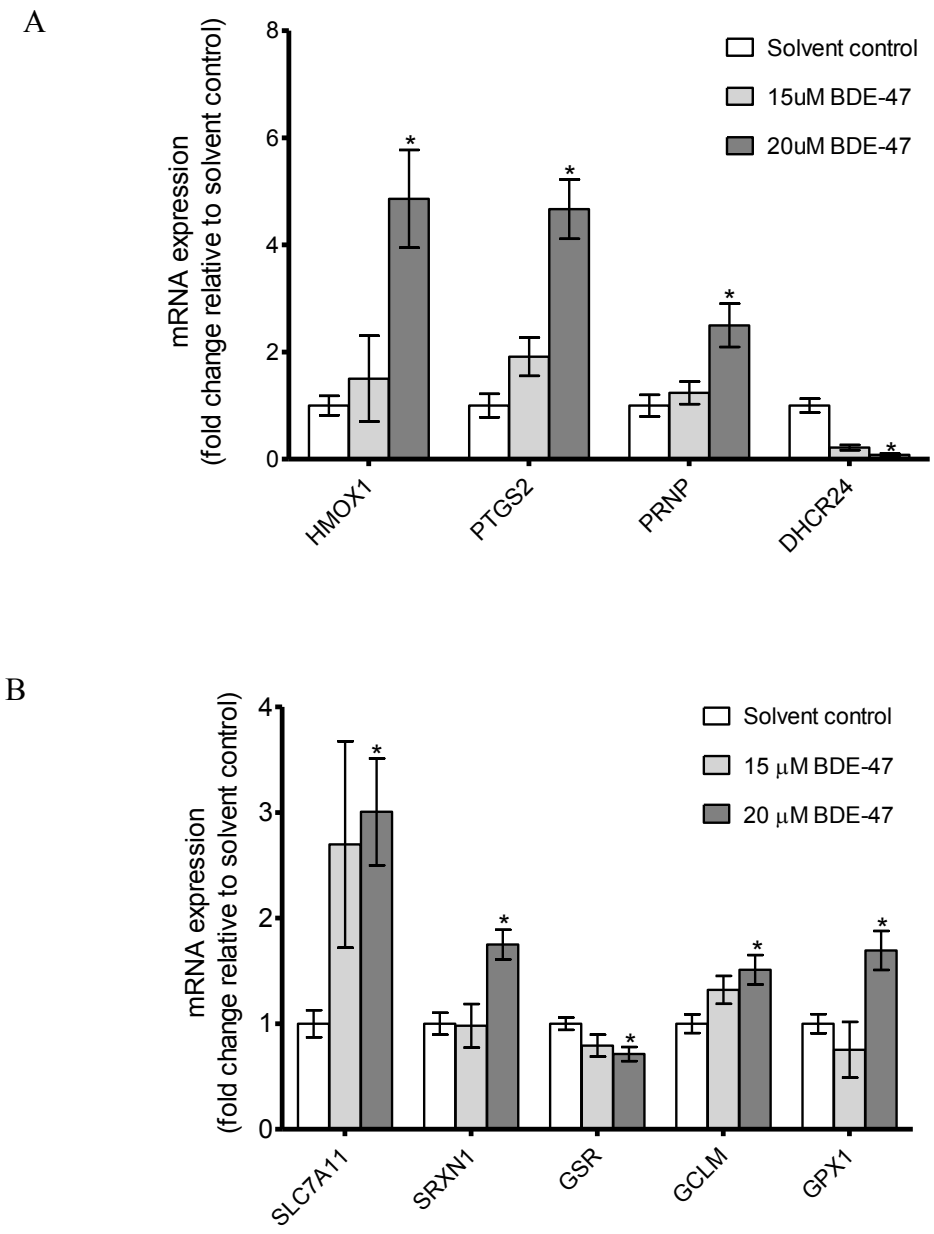

Figure 4.3. BDE-47 effects on HTR-8 cell mRNA expression of genes previously identified with a targeted gene expression array.
A) mRNA expression of HMOX1, PTGS2, PRNP, and DHCR24. B) mRNA expression of SLC7A11, SRXN1, GSR, GCLM, and GPX1. HTR-8/SVneo cells were exposed to solvent control (0.7% v/v DMSO) or BDE-47 treatment for 24 h. Then, mRNA expression of redox-sensitive genes was quantified by qRT-PCR. Bars represent means±SEM (n=3 experiments). Each experiment was performed in triplicate.*P<0.05, significant compared to solvent control.

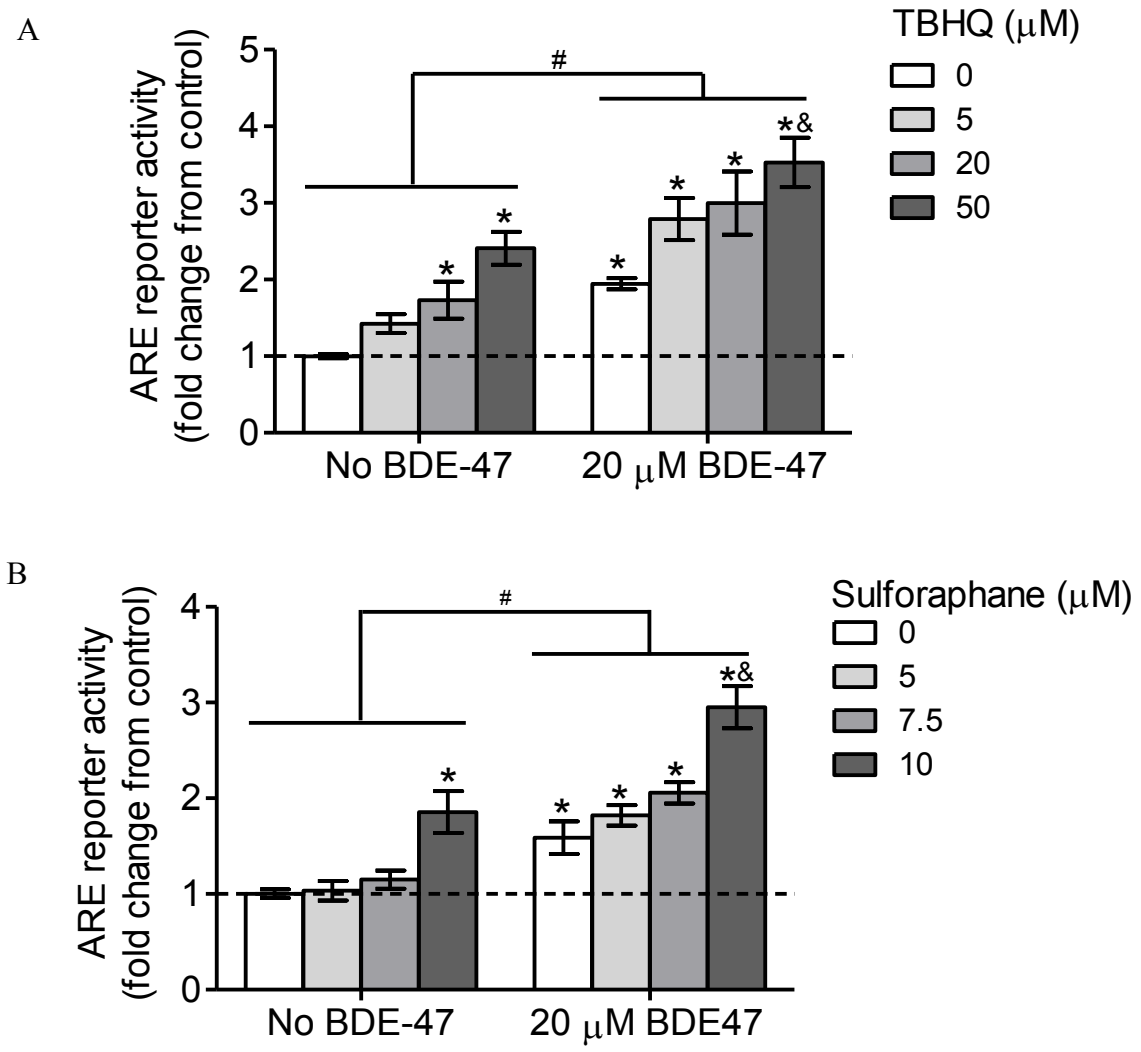

Figure 4.4. Effect of pretreatment with Nrf2 inducers on BDE-47-stimulated ARE reporter activity in HTR-8/SVneo cells.

A) Cells were pretreated with TBHQ for 1 h prior to subsequent incubation without or with BDE-47 for 24 h. Then, ARE reporter activity was quantified. B) Cells were pretreated with sulforaphane for 24 h h prior to subsequent incubation without or with BDE-47 for 24 h. Then, ARE reporter activity was measured. Data are presented as means±SEM fold change over control (dashed line, No BDE-47 with 0 μM TBHQ). 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). Each experiment was performed in triplicate. *$P<0.05$, compared to control. $^{\&}P<0.05$, compared to 0, 5, and 10 μM TBHQ within 20 μM BDE-47 treatment (A) or compared to 0, 5, and 7.5 μM sulforaphane within 20 μM BDE-47 treatment (B). $^{\#}P<0.05$, significantly different from each other.

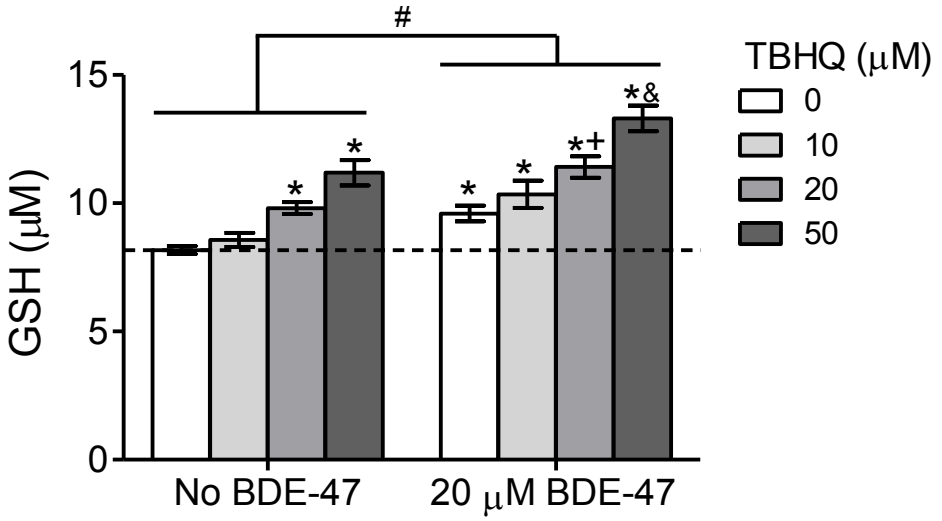

Figure 4.5. Effect of pretreatment with the Nrf2 inducer TBHQ on BDE-47-stimulated GSH production in HTR-8/SVneo cells.
Cells were pretreated with TBHQ, and then exposed to solvent control (No BDE-47, 0.7% v/v DMSO) or BDE-47 for 24h. GSH levels were quantified using a luminescence-based assay (n=3 experiments). Each experiment was performed in triplicate. *P<0.05, compared to control (dashed line, No BDE-47 with 0 μM TBHQ). $^{+}$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 from each other.

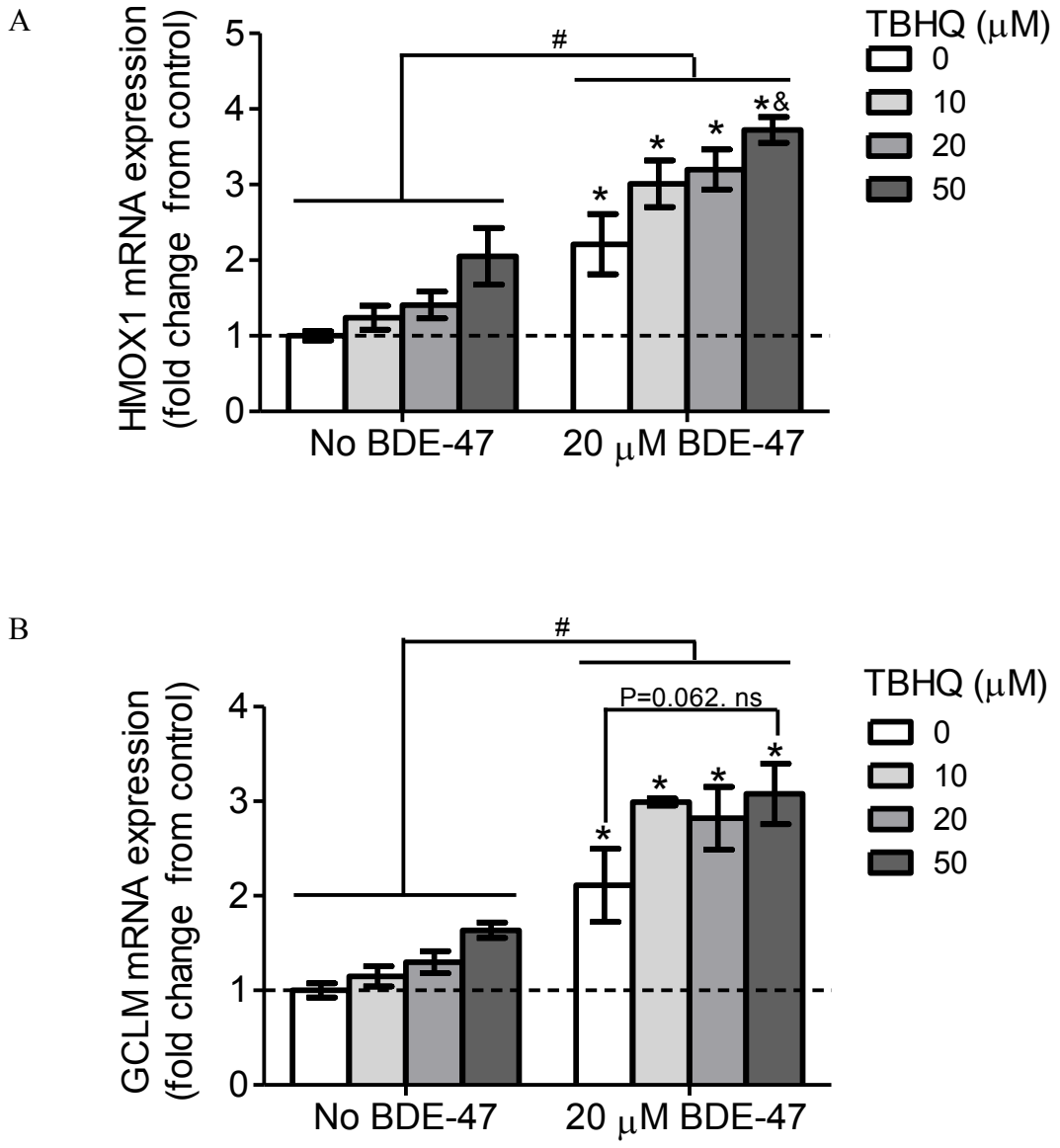

Figure 4.6. Effect of pretreatment with the Nrf2 inducer TBHQ on mRNA expression of antioxidant genes.

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 24h. 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). A) mRNA expression of HMOX1. B) mRNA expression of GCLM. *P<0.05, compared to control. $^{\#}$P<0.05, significantly different from each other. $^{\&}$P<0.05, compared to 20 μM BDE-47 with 0 μM TBHQ.

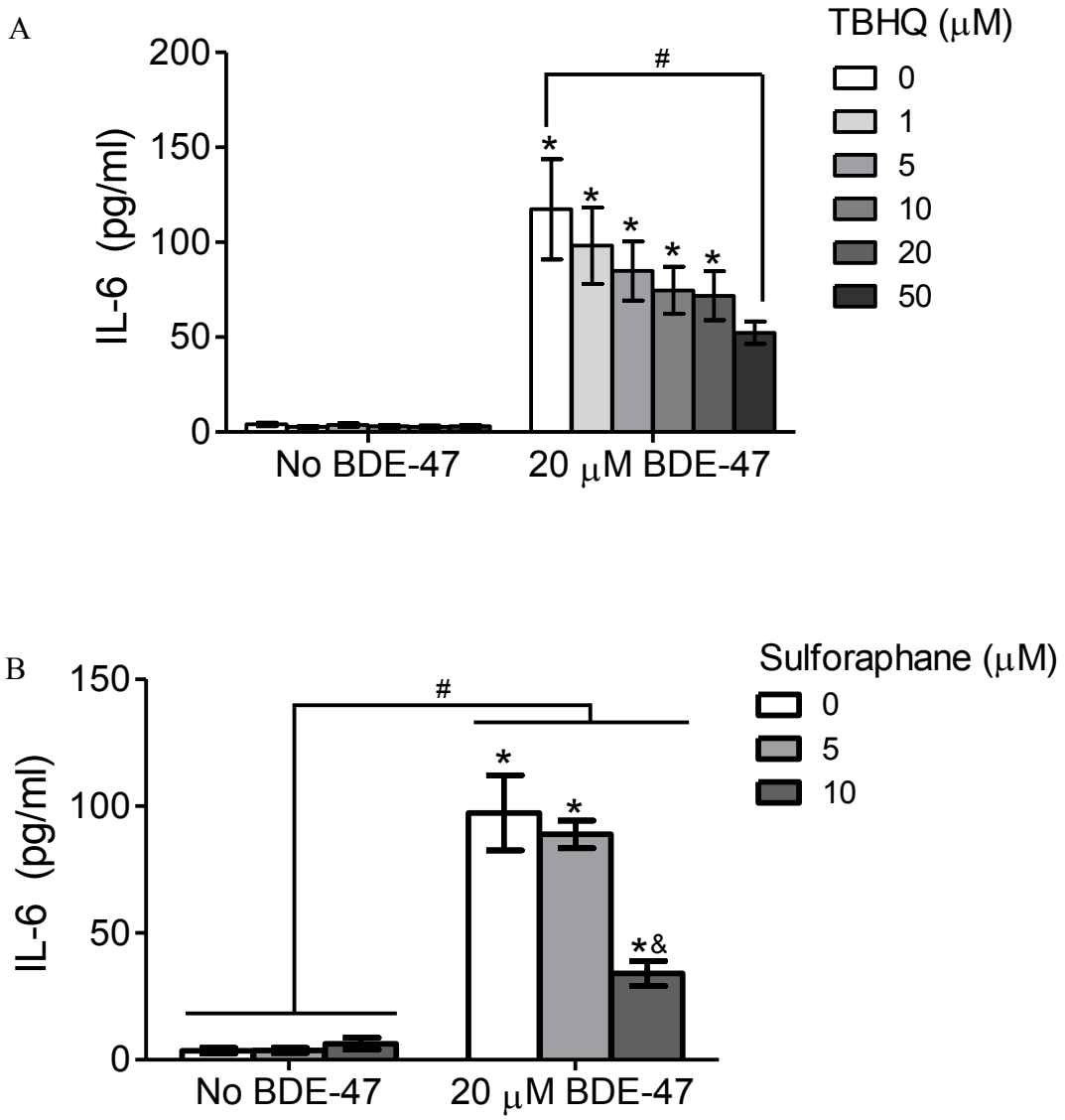

Figure 4.7. Effect of pretreatment with Nrf2 inducers on BDE-47-stimulated IL-6 release from HTR-8/SVneo cells.

A) HTR-8/SVneo cells were pretreated with TBHQ for 1 h prior to treatment with BDE-47 for 24 h. B) After treatment with sulforaphane for 24 h, cells were exposed to solvent control (No BDE-47, 0.7% v/v DMSO) or BDE-47 for 24h. Then, IL-6 levels were quantified using ELISA. Bars represent means±SEM (n=3 experiments). Each experiment was performed in triplicate. *P<0.05, compared to No BDE-47 with 0 μM TBHQ or sulforaphane. $^{\#}$P<0.05, significantly different from each other. $^{\&}$P<0.05, compared to 20 μM BDE-47 with 0 or 5 μM sulforaphane.

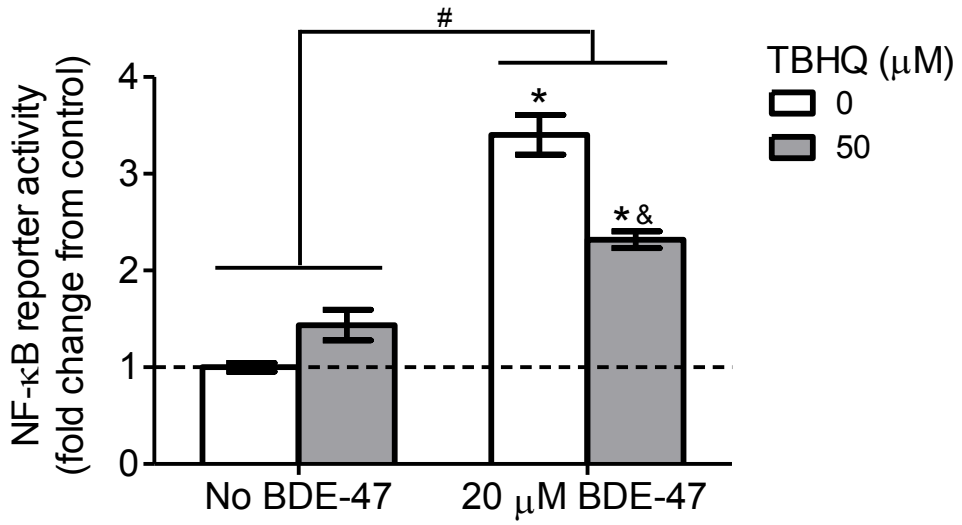

Figure 4.8. Effect of pretreatment with TBHQ on BDE-47-induced NF-κB reporter activity in HTR-8/SVneo cells.

HTR-8/SVneo cells were pretreated with TBHQ for 1 h prior to treatment with BDE-47 for 24 h. After incubation with BDE-47, NF-κB reporter activity was quantified. Data are presented as means±SEM fold change over control (dashed line, No BDE-47 with 0 μM TBHQ). 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). *$P<0.05$, compared to control. $^{\#}P<0.05$, significantly different from each other. $^{\&}P<0.05$, compared to 20 μM BDE-47 with 50 μM TBHQ.

Appendix 4.1. Oxidative stress response array[1]

Symbol	Description	4 h		24 h	
		Fold change	P value	Fold change	P value
ALB	Albumin	0.79	0.85728	0.46	0.24648
ALOX12	Arachidonate 12-lipoxygenase	1.07	0.91189	1.16	0.72132
AOX1	Aldehyde oxidase 1	0.81	0.52586	2.95	0.00028
APOE	Apolipoprotein E	0.87	0.66628	0.56	0.01941
ATOX1	ATX1 antioxidant protein 1 homolog (yeast)	0.97	0.71154	0.68	0.00007
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	1.00	0.96417	1.14	0.10141
CAT	Catalase	1.01	0.91189	0.81	0.00109
CCL5	Chemokine (C-C motif) ligand 5	0.56	0.54991	1.94	0.31119
CCS	Copper chaperone for superoxide dismutase	1.10	0.47196	1.30	0.01038
CYBB	Cytochrome b-245, beta polypeptide	NA	NA	NA	NA
CYGB	Cytoglobin	0.70	0.33950	0.95	0.81152
DHCR24	24-dehydrocholesterol reductase	0.95	0.74263	0.26	0.00000
DUOX1	Dual oxidase 1	0.93	0.88184	1.61	0.14186
DUOX2	Dual oxidase 2	0.75	0.53378	1.18	0.58108
DUSP1	Dual specificity phosphatase 1	3.04	0.05355	1.43	0.26027
EPHX2	Epoxide hydrolase 2, cytoplasmic	0.58	0.29158	0.66	0.15208
EPX	Eosinophil peroxidase	0.78	0.63692	0.84	0.58108
FOXM1	Forkhead box M1	0.94	0.63692	0.88	0.13214
FTH1	Ferritin, heavy polypeptide 1	1.02	0.90003	1.74	0.00016
GCLC	Glutamate-cysteine ligase, catalytic subunit	0.83	0.54750	1.54	0.05160
GPX1	Glutathione peroxidase 1	1.01	0.90003	1.85	0.00001
GPX2	Glutathione peroxidase 2 (gastrointestinal)	0.95	0.91189	1.15	0.72132
GPX3	Glutathione peroxidase 3 (plasma)	1.05	0.47196	0.62	0.00000
GPX4	Glutathione peroxidase 4 (phospholipid hydroperoxidase)	1.04	0.65208	0.63	0.00001
GPX5	Glutathione peroxidase 5 (epididymal androgen-related protein)	NA	NA	NA	NA
GSR	Glutathione reductase	1.35	0.29964	2.08	0.00141
GSS	Glutathione synthetase	1.01	0.95134	1.22	0.03016
GSTP1	Glutathione S-transferase pi 1	1.12	0.36484	1.04	0.58108
GSTZ1	Glutathione transferase zeta 1	1.04	0.74263	1.02	0.74909
HSPA1A	Heat shock 70kDa protein 1A	0.39	0.29158	1.03	0.92751
KRT1	Keratin 1	NA	NA	NA	NA
LPO	Lactoperoxidase	NA	NA	NA	NA
MB	Myoglobin	1.23	0.87559	0.63	0.56381
MBL2	Mannose-binding lectin (protein C) 2, soluble	NA	NA	NA	NA
MPO	Myeloperoxidase	NA	NA	NA	NA
MPV17	MpV17 mitochondrial inner membrane protein	0.93	0.29964	1.04	0.36442

MSRA	Methionine sulfoxide reductase A	0.86	0.41372	1.61	0.00162
MT3	Metallothionein 3	NA	NA	NA	NA
NCF1	Neutrophil cytosolic factor 1	0.40	0.29964	0.51	0.22023
NCF2	Neutrophil cytosolic factor 2	0.91	0.84078	1.20	0.52355
NOS2	Nitric oxide synthase 2, inducible	0.76	0.41372	1.10	0.67158
NOX4	NADPH oxidase 4	0.74	0.65208	0.91	0.80238
NOX5	NADPH oxidase, EF-hand calcium binding domain 5	1.51	0.42014	2.24	0.12118
NUDT1	Nudix (nucleoside diphosphate linked moiety X)-type motif 1	0.95	0.65208	0.97	0.68372
PDLIM1	PDZ and LIM domain 1	0.89	0.42014	0.66	0.00137
PRDX1	Peroxiredoxin 1	0.92	0.66628	0.79	0.09428
PRDX2	Peroxiredoxin 2	0.91	0.29964	0.71	0.00016
PRDX3	Peroxiredoxin 3	0.96	0.76440	0.83	0.08061
PRDX4	Peroxiredoxin 4	1.07	0.76440	1.69	0.00322
PRDX5	Peroxiredoxin 5	0.98	0.81849	1.08	0.13692
PRDX6	Peroxiredoxin 6	0.98	0.75656	1.15	0.00511
PRNP	Prion protein	1.06	0.71154	2.43	0.00001
PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	0.02	0.06755	0.02	0.00621
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	2.18	0.15622	2.90	0.00353
RNF7	Ring finger protein 7	1.15	0.54750	1.98	0.00089
SELS	Selenoprotein S	1.17	0.15622	1.68	0.00001
SEPP1	Selenoprotein P, plasma, 1	0.75	0.74263	2.27	0.14876
SFTPD	Surfactant protein D	0.59	0.29158	1.07	0.76998
SIRT2	Sirtuin 2	0.89	0.42596	0.95	0.61995
SOD1	Superoxide dismutase 1, soluble	0.98	0.90003	0.92	0.32389
SOD2	Superoxide dismutase 2, mitochondrial	0.88	0.29964	1.04	0.59632
SOD3	Superoxide dismutase 3, extracellular	NA	NA	NA	NA
SQSTM1	Sequestosome 1	1.10	0.42596	2.66	0.00000
SRXN1	Sulfiredoxin 1	0.90	0.41372	2.92	0.00000
TPO	Thyroid peroxidase	NA	NA	NA	NA
TTN	Titin	NA	NA	NA	NA
TXNRD2	Thioredoxin reductase 2	1.03	0.88184	1.09	0.56381
UCP2	Uncoupling protein 2 (mitochondrial, proton carrier)	0.81	0.29158	0.66	0.00170
AKR1C2	Aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	1.20	0.84078	2.81	0.07325
BAG2	BCL2-associated athanogene 2	1.02	0.87559	1.48	0.00013
FHL2	Four and a half LIM domains 2	1.63	0.03733	1.91	0.00023
GCLM	Glutamate-cysteine ligase, modifier subunit	0.93	0.54750	1.90	0.00001
GLA	Galactosidase, alpha	0.92	0.56400	1.11	0.26027
HMOX1	Heme oxygenase (decycling) 1	1.15	0.33950	4.72	0.00000

HSP90AA1	Heat shock protein 90kDa alpha (cytosolic), class A member 1	0.91	0.29964	1.68	0.00001
LHPP	Phospholysine phosphohistidine inorganic pyrophosphate phosphatase	0.92	0.54991	0.91	0.31780
NCOA7	Nuclear receptor coactivator 7	1.16	0.29158	1.59	0.00016
NQO1	NAD(P)H dehydrogenase, quinone 1	0.83	0.29964	1.23	0.09026
PTGR1	Prostaglandin reductase 1	0.91	0.29158	0.88	0.02136
SLC7A11	Solute carrier family 7 (anionic amino acid transporter light chain, xc- system), member 11	1.25	0.29964	6.71	0.00000
SPINK1	Serine peptidase inhibitor, Kazal type 1	NA	NA	NA	NA
TRAPPC6A	Trafficking protein particle complex 6A	1.05	0.71154	1.13	0.15208
TXN	Thioredoxin	1.01	0.94674	1.61	0.00016
TXNRD1	Thioredoxin reductase 1	0.90	0.65208	1.97	0.00123

[1]HTR-8 cells were treated with DMSO (0.7% v/v; solvent control) or 20 μM MEHP for 4 or 24 h (n=3 experiments). The Oxidative Stress Response qRT-PCR Array (SABiosciences; Valencia, CA) was used to explore changes in gene expression as described in the "Materials and Methods" section. Fold changes and p-values are shown for all genes and time points tested. NA= CT value of above 40 for one or more experiments, suggesting low level expression of that gene.

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CHAPTER 5. **DISCUSSION**

Polybrominated diphenyl ethers (PBDEs) are synthetic flame-retardants that have been used in textiles, plastics, building materials and insulation (Miller *et al.*, 2009). Due to widespread use and bioaccumulation of PBDEs, human exposure to PBDEs increased exponentially over recent decades (Hites, 2004). Despite distribution of PBDEs to human gestational compartments during pregnancy (Frederiksen *et al.*, 2009a; Frederiksen *et al.*, 2009b; Miller, *et al*., 2009; Miller *et al.*, 2012), studies of mechanisms by which PBDEs act on gestational tissues during pregnancy are limited. This thesis explores mechanisms by which BDE-47-stimulated reactive oxygen species (ROS) may activate inflammatory pathways in human first trimester placental cells. As such, this is the first study to show that BDE-47, a prevalent PBDE congener, induced oxidative stress responses such as ROS formation, modification of redox-sensitive gene expression and activation of Nrf2, a master regulator of oxidative stress, in a human first trimester extravillous trophoblast (EVT) cell line, HTR-8/SVneo (Figure 5.1). In addition, BDE-47 stimulated production of inflammatory mediators including the cytokines interleukin (IL)-6, IL-8, and prostaglandin E2 (PGE2) in HTR-8/SVneo cells (Figure 5.1). Finally, our results demonstrated signal transduction crosstalk between the oxidative stress and inflammatory pathways, showing that BDE-47-stimulated IL-6 and PGE2 release were suppressed by treatment with antioxidants and/or Nrf2 inducers (Figure 5.1).

Cytokines and prostaglandins are considered key players in reproductive processes including the establishment and maintenance of pregnancy, and the initiation of labor (Bowen *et al.*, 2002; Gibb, 1998; Goldenberg and Andrews, 1996; Gomez *et al.*, 1997; Hansen *et al.*, 1999; Khan *et al.*, 2008; Kniss, 1999; Mitchell *et al.*, 1995; Orsi, 2008). There have been extensive studies suggesting that improper regulation of the inflammatory networks may lead to

adverse pregnancy outcomes such as miscarriage, preeclampsia, intrauterine growth restriction (IUGR) and preterm labor (Orsi and Tribe, 2008; Tjoa *et al.*, 2004). However, studies about the roles of cytokines and prostaglandins in first trimester placenta are limited because many studies focused on their roles in the biological processes of birth, including dynamic cervical remodeling (Norman *et al.*, 2007), uterine contractility (Baggia *et al.*, 1996), and gestational membrane rupture (Keelan *et al.*, 2003). In addition, increased production of cytokines and prostaglandins has been used as an indicator of pathologic activation of pro-inflammatory pathways by bacterial vaginosis (Flynn *et al.*, 1999; Leitich *et al.*, 2003) or intrauterine infection (Goldenberg *et al.*, 2008; Romero *et al.*, 2001) in association with increased risk of preterm labor. Although adverse birth outcomes often manifest during the later stages of pregnancy, there is a growing body of literature suggesting that adverse obstetrical outcomes may result from abnormal placentation and placental dysfunction occurring in early pregnancy (Anton *et al.*, 2012; Ness and Sibai, 2006).

It has been suggested that defects in EVT cellular function involving dysregulation of inflammatory mediators in placenta contribute to the placental dysfunction seen in IUGR, preeclampsia, and preterm birth (Arechavaleta-Velasco *et al.*, 2002; Goldenberg *et al.*, 2000; Goncalves *et al.*, 2002; Lamont, 2003; von Dadelszen and Magee, 2002; von Dadelszen *et al.*, 2003). Histologic examination found evidence of localized inflammation (histologic chorioamnionitis) in 85% of placentae from spontaneous preterm births delivered at 28 weeks gestation (Yoon *et al.*, 2000), with higher rates of placental ischemia and abnormal placentation compared with controls at term (Germain *et al.*, 1999; Kim *et al.*, 2003). A recent study reported that LPS increases production of IL-8 and IL-6 and decreases invasion activity in HTR-8/SVneo cells (Anton, et al., 2012). Moreover, a few studies showed that dysregulation of IL-6, IL-8, and

PGE2 changed trophoblast cellular functions such as proliferation, migration, and invasion *in vitro* (Biondi *et al.*, 2006; Dubinsky *et al.*, 2010; Horita *et al.*, 2007b; Jovanovic *et al.*, 2010; Jovanovic and Vicovac, 2009; Nicola *et al.*, 2005a). Our results clearly show that treatment with BDE-47 stimulated proinflammatory cytokines IL-6 and IL-8 at protein and mRNA levels in HTR-8/SVneo cells. Moreover, BDE-47 treatment induced COX activity leading to increased PGE2 release from HTR-8/SVneo cells. Although interaction between BDE-47 exposure and inflammation have been implicated in limited studies (Ashwood *et al.*, 2009; Peltier *et al.*, 2012), this study is the first to show the activation of inflammatory pathways by BDE-47 in human first trimester placental cells.

In the present study, we proposed ROS as a critical regulator of BDE-47-stimulated inflammatory pathways in human placental cells. Our findings showed that treatment with BDE-47 stimulated ROS in HTR-8/SVneo cells, and that BDE-47-induced IL-6 and PGE2 production was suppressed by antioxidant treatment, indicating that ROS play a role in the initiation of inflammatory responses in response to BDE-47 exposure. It has been reported that ROS can regulate signal transduction pathways in mammalian cells as second messengers (Khan and Wilson, 1995). Similar to our results, ROS have been shown to activate production of IL-6 and IL-8 (Frossi *et al.*, 2003; Verhasselt *et al.*, 1998), and cyclooxygenase (COX) expression and/or activity (Feng *et al.*, 1995; Kiritoshi *et al.*, 2003; Martinez-Revelles *et al.*, 2013). Although mechanisms of regulation of inflammatory response by ROS are not fully understood, involvement of the redox-sensitive transcription factor NF-κB has been implicated (Reuter *et al.*, 2010). NF-κB plays a crucial role in immune and inflammatory response, regulating expression of a large number of genes, including those for cytokines, growth factors, adhesion molecules, immunoreceptors, and acute-phase proteins (Blackwell and Christman, 1997). Of direct

relevance to the present study, NF-κB has been shown to regulate expression of IL-6, IL-8 (Blackwell and Christman, 1997; Reuter, *et al*., 2010) and COX gene expression (Chiang *et al.*, 2003; Crofford *et al.*, 1997). Several lines of evidence suggest a role for ROS in activation of NF-κB, and antioxidants inhibit NF-κB activation *in vitro* and *in vivo* (Blackwell and Christman, 1997). Our results showed that NF-κB reporter activity increased with BDE-47 treatment concomitant with increases in IL-6, IL-8, COX-2 expression, and COX activity, implicating the involvement of NF-κB on BDE-47-stimulated inflammatory responses. However, our findings are correlative only, and further study will be needed to demonstrate the causal relationship between NF-κB activation and the observed inflammatory responses.

In addition to NF-κB, other redox-sensitive transcription factors may play a role in the regulation of BDE-47-stimulated inflammatory pathways in the present study. The promoter region of the IL-6 and IL-8 genes contains multiple regulatory elements including NF-κB, antioxidant response element (ARE)/ARE-like element, and activator protein (AP)-1 (Dendorfer *et al.*, 1994; Roebuck, 1999). The promoter region of PTGS2 gene also contains various *cis*-acting elements such as NF-κB, AP-1, cAMP response element (CRE), and NF-IL6 (Inoue *et al.*, 1995). Our findings showed that ARE reporter activity increased with BDE-47 treatment coinciding with increases in IL-6, IL-8, and COX-2 expression in HTR-8/SVneo cells. Although the protective role of Nrf2 activation against oxidative stress and inflammation is well established, it is suggested that Nrf2 may play a role in activation of inflammatory genes. For example, it is reported that Nrf2 induces IL-6 and IL-8 expression via binding to ARE *in vivo* and *in vitro* (Wruck *et al.*, 2011; Zhang *et al.*, 2005). Moreover, PTGS2 is a known Nrf2 target gene (Tufekci *et al.*, 2011) and its expression is regulated by Nrf2/ARE signaling pathways in various experimental models (Luo *et al.*, 2011; Rojo *et al.*, 2010; Wang *et al.*, 2010). Further

study is warranted to examine the role Nrf2 in the activation of BDE-47-stimulated inflammation. Furthermore, the involvement of other redox-sensitive transcription factors such as AP-1 could be investigated, considering the observed ROS formation by BDE-47 treatment (Park *et al.*, 2014).

ROS overproduction has been a common contributing factor to toxicity of PBDEs as detected with *in vitro* systems (He *et al.*, 2008; Reistad and Mariussen, 2005; Shao *et al.*, 2008; Yan *et al.*, 2011). The present study provides new information that BDE-47 increased ROS generation in the human trophoblast cell line HTR-8/SVneo. Moreover, BDE-47 decreased mitochondrial membrane potential, indicating mitochondrial dysfunction (Brand and Nicholls, 2011). Because mitochondrial defects can lead to enhanced mitochondrial production of ROS (Sohal *et al.*, 1995), we suggest that the BDE-47-stimulated ROS in HTR-8/SVneo cells may have originated from mitochondria (Park, *et al*., 2014). Similar to our results, a few studies reported reduced mitochondrial membrane potential (Shao, *et al*., 2008; Yan, *et al*., 2011), implicating the involvement of mitochondria on BDE-47-stimulated ROS. However, the mechanisms of BDE-47-stimulated ROS formation is still not clear. Specifically, the mitochondrial origin of BDE-47-stimulated ROS has not been verified experimentally yet. In addition, we don't know whether BDE-47-stimulated ROS formation is a direct result from its exposure or a secondary response followed by signaling cascades. Moreover, not BDE-47, but its reactive metabolites may play a role in the activation of BDE-47-stimulated responses. It was recently reported that hydroxylated PBDEs may be more toxic than the parent PBDEs (Dingemans *et al.*, 2008; Feo *et al.*, 2013). Oxidative stress by hydroxylated BDE-47, such as formation of ROS and DNA damage, has also been reported (An *et al.*, 2011; Ji *et al.*, 2011). Finally, BDE-47-stimulated ROS could originate from multiple sources, not a single source. In

fact, Reistad and Mariussen (2005) reported that BDE-47 enhanced the production of ROS, potentially through NADPH oxidase activation in human granulocytes, suggesting complex pathways of BDE-47-mediated ROS formation.

In the present study, we explored the ability of Nrf2 to inhibit BDE-47-stimulated inflammatory responses in HTR-8/SVneo cells. Nrf2 is a well-known redox-sensitive transcription factor that binds to antioxidant response element (ARE) and activates ARE-mediated gene expression (Itoh *et al.*, 1997; Motohashi and Yamamoto, 2004; Osburn *et al.*, 2006), resulting in the upregulation of phase II detoxification enzymes and antioxidant enzymes (Kensler *et al.*, 2007). Therefore, activation of Nrf2 pathways is an important cellular defense mechanism against exogenous toxins and injury. Our results clearly showed that treatment with BDE-47 stimulated Nrf2-mediated oxidative stress responses via differentially regulating expression of redox-sensitive genes, augmenting intracellular GSH production, and stimulating Nrf2 reporter activity in HTR-8/SVneo cells. These findings not only confirm the previous data showing BDE-47-stimulated ROS formation, but also implicate a protective role of Nrf2-signaling pathways in response to inappropriate inflammatory responses by BDE-47 treatment. In addition, there is a growing body of literature reporting anti-inflammatory effect of Nrf2 activation *in vivo* and *in vitro* (Jin *et al.*, 2011; Jin *et al.*, 2008; Jin *et al.*, 2009; Khodagholi and Tusi, 2011; Khor *et al.*, 2006; Koh *et al.*, 2009; Rangasamy *et al.*, 2004; Rangasamy *et al.*, 2005; Thimmulappa *et al.*, 2006). Therefore, we tested the hypothesis that activation of Nrf2 by Nrf2 inducers may show an anti-inflammatory effect on BDE-47-stimulated pro-inflammatory cytokine production in HTR-8/SVneo cells. Indeed, our results, for the first time, showed that pretreatment with Nrf2 inducers such as *tert*-butylhydroquinone (tBHQ) or sulforaphane resulted in suppression of BDE-47-stimulated IL-6 release from HTR-8/SVneo cells via upregulating

Nrf2 reporter activity, increasing intracellular GSH production, increasing expression of antioxidant enzymes, and downregulating NF-κB reporter activity, implicating interplay between the NF-κB and Nrf2 pathways.

Among a wide range of natural and synthetic small molecules that are potent inducers of Nrf2 activity (Khodagholi *et al.*, 2010; Stewart *et al.*, 2003; Tusi *et al.*, 2010), tBHQ is one of the most common. tBHQ has a variety of other pharmacological activities including antioxidant and anti-inflammatory potential (Koh, *et al*., 2009). Although the precise mechanism regarding the anti-inflammatory ability of tBHQ remains elusive, the prevailing theory has been that Nrf2 interferes with inflammatory signaling pathways by inhibiting NF-kB activation through the maintenance of cellular redox status. Activation of the NF-kB signaling pathway has been shown to be responsive to excess ROS and is important in the generation of inflammation (Thimmulappa, *et al*., 2006). The antioxidant transcription factor Nrf2 plays an important role in limiting ROS levels and thereby suppressing the redox-sensitive NF-kB signaling pathway involved in inflammation (Itoh, *et al*., 1997; Lee and Johnson, 2004). It is therefore implied that tBHQ may play an important role in anti-inflammation by a mechanism of augmentation of cellular antioxidative responses via up-regulation of the Nrf2 signaling pathway, resulting in decreased pro-inflammatory cytokine production via inactivation of NF-kB signaling pathway (Jin, *et al*., 2011). Consistent with this theory, anti-inflammatory effects of tBHQ have been reported with the evidence of stabilization of Nrf2, suppression of NF-κB expression and activity, and suppression of phosphorylation of MAP kinases (p38, ERK1/2, and JNK) (Jin, *et al*., 2011; Jin *et al.*, 2010; Jin, *et al*., 2009; Khodagholi and Tusi, 2011; Koh, *et al*., 2009). In addition, our results showed that pretreatment with tBHQ suppressed BDE-47-stimulated IL-6 release from HTR-8/SVneo cells while upregulating Nrf2 reporter activity, intracellular GSH

production, and expression of antioxidant enzymes, and downregulating NF-κB reporter activity. Although our study did not investigate changes in cellular redox status by tBHQ treatment, tBHQ-stimulated increased cellular production of GSH suggests that tBHQ may help maintain cellular redox status by augmenting GSH levels against BDE-47-stimulated ROS. However, the mechanism by which tBHQ activates Nrf2 is not entirely understood. It has been shown that ROS can activate ARE by stimulating nuclear translocation of the transcription factor Nrf2 (Itoh *et al.*, 1999; Sian *et al.*, 1994). tBHQ-induced ROS production may be a consequence of redox cycling from a fully reduced form to an oxidized form as a semiquinone anion radical (Gharavi *et al.*, 2007). Oxidative stress-dependent Nrf2 activation by tBHQ has been reported in a few studies, showing that antioxidant treatments inhibited tBHQ-induced gene induction in certain cell types (Hara *et al.*, 2003; Itoh, *et al*., 1999; Pinkus *et al.*, 1996). On the other hand, it is reported that Nrf2-dependent activation of ARE by tBHQ is mediated via an oxidative stress-independent pathway in IMR32 cells and SH-SY5Y cells (Sian, *et al*., 1994). The oxidative stress-independent ARE activation mediated by Nrf2 is shown to be mediated via various kinase pathways such as phosphatidylinositol-3-kinase and p38 (Kang *et al.*, 2001; Lee *et al.*, 2001; Yu *et al.*, 2000). An explanation for cell-specific differences may be due to variations in subcellular compartmentalized redox status (Imhoff and Hansen, 2010). It is suggested that redox status changes in specific subcellular compartments, and each compartment may dictate different steps in the Nrf2 pathway (Hansen *et al.*, 2004; Imhoff and Hansen, 2010). Although we observed tBHQ-mediated ROS formation in HTR-8/SVneo cells (data not shown), we have not tested the hypothesis that tBHQ-mediated Nrf2 activation is ROS-dependent because this was not directly relevant to the objectives of this study. Rather, this study utilized tBHQ as a tool to induce Nrf2 and thereby inactivate NF-κB as a means to test the role of NF-κB in the BDE-47-

stimulated inflammatory response. To better understand the mechanism, further investigation using antioxidants treatment and measuring redox status in subcellular compartments will be needed.

In addition to tBHQ, we used another Nrf2 inducer, sulforaphane, to investigate the role of NF-κB in BDE-47-stimulated cytokine release. Sulforaphane is an isothiocyanate derived from cruciferous vegetables (Keum, 2011). Cytoprotective and anti-inflammatory effects of sulforaphane have been reported (Heiss *et al.*, 2001; Juge *et al.*, 2007). 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), causing a conformational change of Keap1 (Imhoff and Hansen, 2010; Keum, 2011). It is reported that the induction of Nrf2 by sulforaphane is regulated by posttranslational stabilization, attenuating ubiquitinylation and proteosomal degradation of Nrf2 and decreasing protein expression level of Keap1 (Keum *et al.*, 2006; Nguyen *et al.*, 2003). In addition, sulforaphane may affect the activity of a variety of intracellular kinases to phosphorylate Nrf2 proteins, which dictates the nucleocytoplasmic trafficking of Nrf2 or modulates the Nrf2 protein stability (Rada *et al.*, 2011; Rojo *et al.*, 2008). Our results showed that sulforaphane pretreatment induced Nrf2 transactivation and suppressed BDE-47-stimulated IL-6 production in HTR-8/SVneo cells (Figure 4.7B). Although sulforaphane stimulated induction of Nrf2-regulated genes such HO-1, NQO1 and TXR1(Juge, *et al*., 2007), further investigation will be necessary to confirm sulforaphane-mediated Nrf2 activation and to elucidate mechanisms for the anti-inflammatory effect by sulforaphane observed in this study. Unlike pretreatment with tBHQ, we did not observe ROS formation with sulforaphane pretreatment in the HTR-8/SVneo cells, implicating different mechanisms on Nrf2 activation by each inducer (data not shown).

We have to be cautious in interpreting our results because overproduction of IL-6, IL-8, and PGE2 alone may not accurately represent the response of trophoblast cells during an inflammatory state nor the impact of BDE-47 exposure on trophoblast cellular function in vivo. Although changes in inflammatory mediators play an important role in regulating trophoblast function, there are complex interactions between these 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). In addition, the impact of BDE-47-stimulate inflammatory responses on trophoblast cellular function and invasion should be investigated further to confirm potential relevance of our findings to placentation and pregnancy.

Another limitation of the present study is that the results of in vitro experiments using a transformed cell line may not accurately reflect responses of primary extravillous trophoblast cells. It has been reported that HTR-8/SVneo cells have a similar phenotype compared to their primary counterparts (Biondi *et al*., 2006; Graham *et al*., 1993; Jovanović *et al*., 2010). For example, HTR-8/SVneo cells retain migratory capability and express specific placental trophoblast markers including HLA-G, cytokeratin-7, and α5β1 integrin up to passage number 105 (Biondi, *et al*., 2006; Khan *et al.*, 2011). However, it has been reported that HTR-8/SVneo cells may have a different transcriptomic and epigenetic profile compared to primary extravillous trophoblast cells (Bilban *et al.*, 2010; Novakovic *et al.*, 2011). To address this issue, further investigation using primary trophoblasts or placental tissues will be needed to validate the potential relevance of our results to pregnancy.

Finally, using pharmacological Nrf2 inducers may impact multiple cellular mechanisms other than Nrf2 signaling pathways. For example, tBHQ or sulforaphane may bind in a non-specific manner to other proteins and affect other redox-sensitive transcription factors and protein kinases (Reuter, *et al*., 2010). 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. However, 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 antiinflammatory effect in response to BDE-47, . Although our findings implicate Nrf2 activation as a potential therapeutic target against toxicant-induced inflammatory responses in human placental cells, further investigation using genetic knockdown and overexpression of Nrf2 is warranted to understand the role of Nrf2 on BDE-47-stimulated responses more accurately.

Despite these limitations, our findings suggest potential adverse impacts of PBDE exposure during pregnancy. Invasion of EVTs 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 (Zhou *et al.*, 1997a; Zhou *et al.*, 1997b). The present study used HTR-8/SVneo, a human first trimester EVT cell line as a model to study the effects of BDE-47 treatment. Because IL-6, IL-8, and PGE2 have been shown to regulate EVT proliferation, migration, and invasion during first trimester of pregnancy (Biondi, *et al*., 2006; Horita *et al.*, 2007a; Jovanovic, *et al*., 2010; Jovanovic and Vicovac, 2009; Nicola *et al.*, 2005b), overproduction of these mediators 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 conclusion, the present study provided the first experimental data to support a mechanism by which PBDE exposure could contribute to increased risk for adverse birth outcomes. 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. By demonstrating that a common toxicological effect, oxidative stress, activates inflammatory pathways associated with impaired trophoblast function and placental dysfunction, these data provide support for a plausible biological explanation for environmental contaminant exposure associations with adverse obstetrical outcomes. . Given that multiple antioxidant trials for preventing adverse birth complications have not been successful (Polyzos *et al*., 2007; Rumbold *et al*., 2008), the activation of Nrf2 antioxidant signaling pathways using various synthetic/natural Nrf2 inducers may be a novel approach leading to augmentation of intracellular antioxidant capacity (Polyzos *et al.*, 2007; Rumbold *et al.*, 2008). Therefore, this research contributes new information for potential interventions to reduce adverse obstetrical outcomes originating from abnormal placental function, with possible economic, societal and public health benefits.

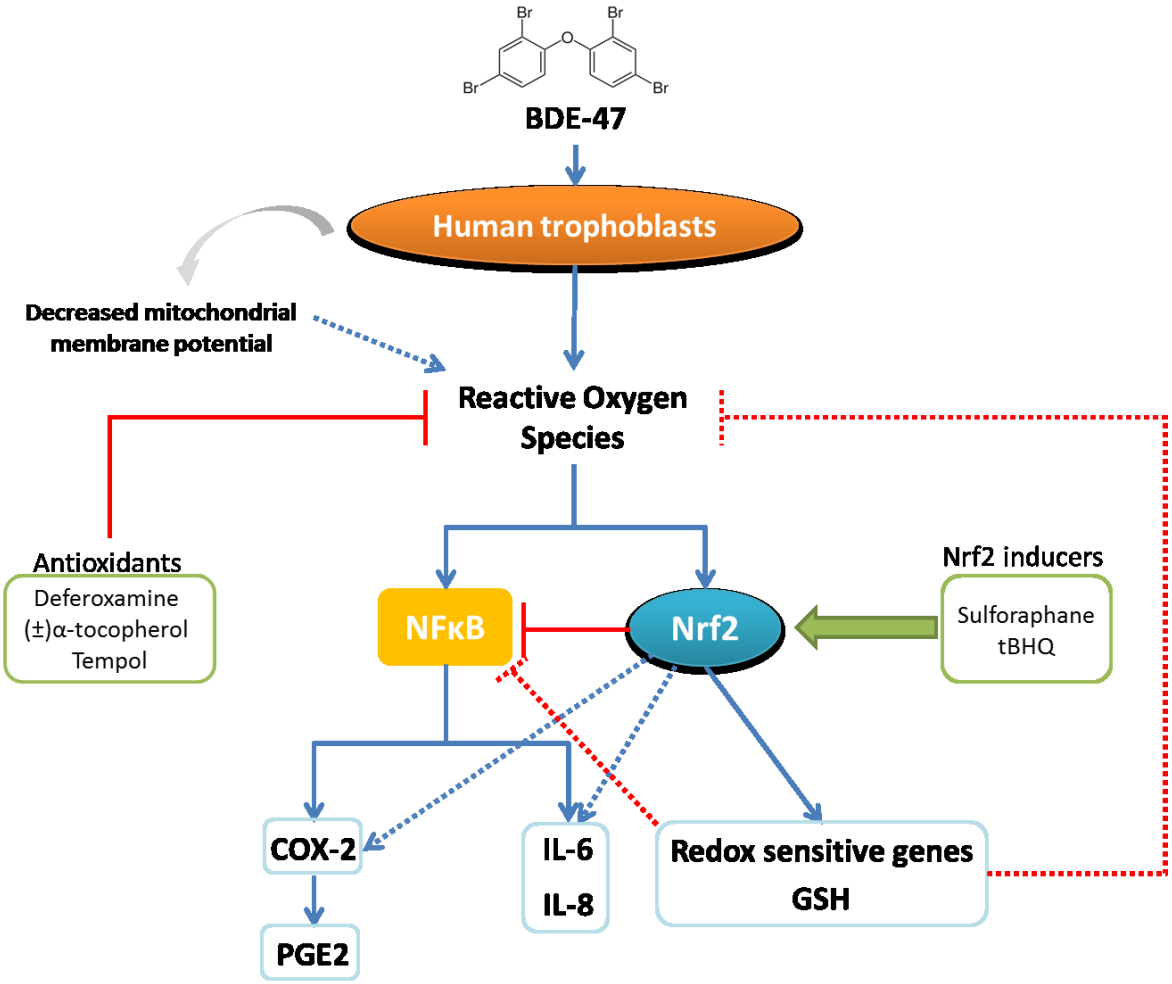

Figure 5.1. Proposed model of BDE-47 effects in human placental trophoblast cells. BDE-47 stimulates production of reactive oxygen species (ROS) with the evidence of decreased mitochondrial membrane potential. BDE-47-induced ROS activates a redox-sensitive transcription factor NF-kB leading to increased expression of COX-2 and stimulated production of proinflammatory PGE2, IL-6, and IL-8. Suppression of IL-6 and PGE2 release by antioxidant treatments suggests that BDE-47-stimulated ROS play a role in the initiation of proinflammatory responses. BDE-47-stimulated ROS also activate the antioxidative transcription factor Nrf2, resulting in differential expression of antioxidant and detoxifying enzymes and increased GSH production. Pretreatment with the Nrf2 inducers tBHQ and sulforaphane suppressed BDE-47-induced NF-kB activity and IL-6 release by stimulating Nrf2 activity, expression of antioxidant genes, and GSH production, suggesting the protective role of Nrf2 on BDE-47-stimulated inflammatory responses in human trophoblasts. The dotted lines represent relationships that are inferred, but are not shown in the present study.

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