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Protective Effect of (±)α-Tocopherol on Brominated Diphenyl Ether-47-Stimulated Prostaglandin Pathways in Human Extravillous Trophoblasts *In Vitro*

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Abstract

Brominated diphenyl ether (BDE)-47 is a prevalent flame retardant chemical found in human tissues and is linked to adverse pregnancy outcomes in humans. Because dysregulation of the prostaglandin pathway is implicated in adverse pregnancy outcomes, the present study investigates BDE-47 induction of prostaglandin synthesis in a human extravillous trophoblast cell line, HTR-8/SVneo, examining the hypothesis that BDE-47 increases generation of reactive oxygen species (ROS) to stimulate the prostaglandin response. Treatment with 20 μM BDE-47 significantly increased mRNA expression of prostaglandin-endoperoxide synthase 2 (PTGS2) at 4, 12 and 24 h, and 24-h treatment significantly increased cyclooxygenase (COX)-2 cellular protein expression and prostaglandin E2 (PGE2) concentration in culture medium. The BDE-47-stimulated PGE2 release was inhibited by the COX inhibitors indomethacin and NS398, implicating COX activity. Exposure to 20 μM BDE-47 significantly increased ROS generation as measured by carboxydichlorofluorescein fluorescence, and this response was blocked by cotreatment with the peroxyl radical scavenger (±)-α-tocopherol. (±)-α-Tocopherol cotreatment suppressed BDE-47-stimulated increases of PGE2 release without significant effects on COX-2 mRNA and protein expression, implicating a role for ROS in post-translational regulation of COX activity. Because prostaglandins regulate trophoblast functions necessary for placentation and pregnancy, further investigation is warranted of BDE-47 impacts on trophoblast responses.

Keywords

Polybrominated diphenyl ethers (PBDEs); HTR-8/SVneo cells; human placental cells; prostaglandins; α-tocopherol; cyclooxygenase (COX)-2

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Conflict of interest

The authors declare that there are no conflicts of interest.

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1. 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 (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.*, 1997a).

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). 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.*, 2007d; Jovanovic *et al.*, 2010; Jovanovic and Vicovac, 2009; Nicola *et al.*, 2005d).

Prostaglandins are small lipid molecules synthesized from membrane phospholipids in response to various physiological and pathological stimuli (Nicola *et al.*, 2005d). Of these, prostaglandin E2 (PGE2) is one of the most extensively studied prostaglandins, and has been shown to play critical roles in processes required in successful pregnancy, for example, implantation (Psychoyos *et al.*, 1995; Yee *et al.*, 1993), immunoprotection of the semiallogenic conceptus (Parhar *et al.*, 1988), and parturition (Keelan *et al.*, 2003). 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.*, 2007a; 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.*, 2009a). 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 PBDEs distribute to human placenta (Frederiksen *et al.*, 2009), extraplacental membranes (Miller *et al.*, 2009b), 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 cyclooxygenase (COX)-2 expression (Peltier *et al.*, 2012).

Our previous study showed that treatment with BDE-47, one of the most prevalent congeners found in human tissues (Hites, 2004), stimulates production of the proinflammatory cytokine IL-6 via a reactive oxygen species (ROS)-mediated mechanism in the first trimester EVT human placental cell line HTR-8/SVneo (Park *et al.*, 2014b). Although inappropriate activation of prostaglandin pathways may lead to placental dysfunction, there is a paucity of reports on PBDE-stimulated prostaglandin release in first trimester placenta. 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 ROS has been shown to modulate prostaglandin pathways in various experimental models including murine placenta (Basu, 1999; Davidge, 1998; Wentzel *et al.*, 1999; White *et al.*, 2002).

The present study examines the hypothesis that BDE-47 stimulates PGE2 production in human placental cells via a ROS-mediated mechanism. This work was performed with the HTR-8/SVneo cell line (Graham et al, 1993). The HTR-8/SVneo cell line was derived from first trimester placentae and has provided a useful cell culture model for studies of EVT cellular responses (Liu *et al.*, 2012; Wang *et al.*, 2012; Weber *et al.*, 2013) and initial investigations of toxicant actions on EVTs (Park *et al.*, 2014a; Tetz *et al.*, 2013a).

2. Materials and Methods

2.1. 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 1640 medium, fetal bovine serum (FBS), OptiMem 1 reduced-serum medium, Hank's balanced salt solution (HBSS), and 0.25% trypsin/EDTA solution and penicillin/streptomycin (P/S) were from Invitrogen Life Technologies (Carlsbad, CA, USA). The PGE2 ELISA kit and arachidonic acid were 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). The NP-40 substitute, IGEPAL 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).

2.2. 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). Cytotrophoblast cells isolated from first trimester human placentae were immortalized with simian virus 40 large T antigen to generate the HTR-8/SVneo cell line (Graham et al, 1993). 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 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 experiments. BDE-47 concentrations were selected to include concentrations relevant to human exposure (Doucet *et al.*, 2009) and previously shown by us to increase generation of ROS in the HTR-8/SVneo cells (Park *et al.*, 2014b). The final concentration of DMSO in medium was 0.7 % (v/v).

2.3. Carboxydichlorofluorescein assay

Stimulation of ROS generation was assessed using carboxydichlorofluorescein (cDCF) fluorescence in a variation of the dichlorofluorescein (DCF) assay. We used cDCF instead of DCF because the additional negative charges on cDCF improve cell retention of the probe. Because artifactual results can occur in the cDCF assay due to interactions with toxicants (Tetz *et al.*, 2013b), we confirmed that there was no increased cDCF fluorescence by BDE-47 in cell-free medium (data not shown). The HTR-8/SVneo cells were seeded at a density of 2.4×10^5 cells 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 were 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 a 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 a Molecular Devices SpectraMax Gemini M2e plate reader at an excitation wavelength of 492 nm and emission wavelength of 522 nm.

2.4. Prostaglandin E2 assay

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 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 a 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 in culture medium was measured by sandwich ELISA following the manufacturer's protocols. To probe ROS-mediated activation of prostaglandin pathways by BDE-47, HTR-8/SVneo cells were co-treated for 24 h with 20 μM (±)-α-tocopherol, a peroxyl radical scavenger. Concentrations of PGE2 in the medium were analyzed by ELISA as described above and expressed as pg/ml.

2.5. 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 (qRT-PCR) 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 a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). A housekeeping gene, β-microglobulin, was co-amplified as an internal control. Analysis by 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, then 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.

2.6. 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).

2.7. Statistical analysis

Statistical analysis was performed with Sigma Plot 11.0 software (Systat Software Inc., San Jose, CA, USA). After determining acceptable homogeneity of variance and normality ($P<0.05$), data were analyzed either by one-way analysis of variance (ANOVA) or two-way ANOVA. If significant effects were detected, the ANOVA was followed by Tukey post-hoc comparison of means. A P value <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.

3. Results

3.1. 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 level in HTR-8/SVneo cells exposed to BDE-47 concentrations ranging from 5 to 20 μM. The BDE-47 concentrations were selected based on prior findings that IL-6 and IL-8 release were stimulated with 15 and 20 μM BDE-47, and superoxide production was increased with 10, 15, 20 μM BDE-47 in HTR-8/SVneo cells (Park *et al.*, 2014b). Treatment with 20 μM BDE-47, but not lower concentrations, significantly increased mRNA expression of PTGS2, the gene for COX-2, compared to the solvent control at 4, 12 and 24 h by 5.3-fold, 4.5-fold, and 4.7-fold, respectively ($P<0.05$, Fig. 1A). mRNA expression of PTGES, the gene for prostaglandin E synthase (PGES), was suppressed 66% with 20 μM BDE-47 treatment, but not lower BDE-47 concentrations, compared with solvent control after 24 h ($P<0.05$, Fig. 1B). In addition, mRNA expression of HPGD, the gene for the prostaglandin catabolic enzyme 15-hydroxyprostaglandin dehydrogenase, was reduced by 66% and 44% with 15 and 20 μM BDE-47, respectively, after 24 h ($P<0.05$, Fig. 1C), with no statistically significant treatment effects at lower BDE-47 concentrations. There were no statistically significant differences between non-treated controls and solvent controls at any time point.

3.2. Effects of BDE-47 on PGE2 release

Because 20 μM BDE-47 induced a rapid increase of PTGS2 mRNA that was sustained for 24 h, we next evaluated stimulation of PGE2 release by 15 and 20 μM BDE-47. Treatment of HTR-8/SVneo cells for 24 h with 20 μM BDE-47 induced a significant 1.8-fold increase in PGE2 concentration in culture medium compared to the solvent control ($P<0.05$, Fig. 2A) whereas the effect at 15 μM BDE-47 was not statistically significant, in agreement with

BDE-47-stimulated PTGS2 mRNA expression shown in Fig. 1A. Co-treatment for 24 h with 10 μM indomethacin, a nonspecific COX inhibitor, or 5 μM 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 ($P<0.05$, Fig. 2B), indicating that BDE-47-induced PGE2 release was dependent on COX activity. Notably, the NS-398-mediated PGE2 decrease was similar to the 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 or 15 μM BDE-47 significantly alter PGE2 release.

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

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

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

To investigate the role of ROS in BDE-47-induced PGE2 production, HTR-8/SVneo cells were co-treated with 20 μM BDE-47 and 20 μM (±)-α-tocopherol for 24 h. As shown in Fig. 3, (±)-α-tocopherol cotreatment reduced PGE2 release stimulated by 20 μM BDE-47, with PGE2 concentrations in culture medium decreased 22.5% compared to cultures exposed to BDE-47 without (±)-α-tocopherol pretreatment (Fig. 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.

3.5. Effects of (±)-α-tocopherol treatment on COX-2 expression

To test whether suppression of BDE-47-induced PGE2 release by (±)-α-tocopherol cotreatment stems from changes in mRNA or protein expression for COX-2, qRT-PCR or western blot were conducted. Although 24-h 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 ($P<0.05$), the mRNA expression of PTGS2 was not significantly changed with (±)-α-tocopherol cotreatment compared to non-(±)-α-tocopherol-treated groups (Fig. 4), suggesting that ROS do not regulate COX-2 expression at the transcription level. Likewise, treatment for 24 h with 20 μM BDE-47 increased COX-2 protein abundance by 2.0-fold compared with control (Fig. 5B; $P<0.05$), but 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.

4. Discussion

PGE2 is a pro-inflammatory mediator of critical trophoblast functions during placentation (Biondi *et al.*, 2006; Horita *et al.*, 2007a; Nicola *et al.*, 2005a; Zhou *et al.*, 1997a). The present study demonstrated that BDE-47, a prevalent flame retardant chemical in the environment and in human tissue samples, stimulated 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 PGE2 release was regulated by 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 BDE-47-stimulated PGE2 release, it is suggested that BDE-47-mediated PGE2 production was mainly dependent on COX-2 activity in HTR-8/SVneo cells.

Stimulated PGE2 release could result from changes in mRNA transcription or protein synthesis (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 the increased PGE2 release 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 of 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 (Supplementary Fig. 2). An alternative explanation may involve isoforms of PGES, because there are three different PGES isotypes 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 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 regulation 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 regulating BDE-47 stimulated 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 PGE2 release without changing its expression, implicating post-translational regulation of COX activity by ROS. This explanation is consistent with previous reports that α-tocopherol inhibits PGE2 production and COX activity with 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) 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.*, 2014b) is consistent with the potential production of ONOO in BDE-47-treated HTR-8/SVneo cells. However, further study will be needed 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 phospholipase A2 (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 PGE2 release completely, supporting additional mechanisms. Further study will be needed to better understand the mechanisms for modulatory effects by vitamin E on COX activity.

COX-2 is induced in response to various stimuli including oxidative stress, pro-inflammatory cytokines, growth factors, oncogenes and tumor promoters while negatively regulated by glucocorticoids, interleukin (IL)-4, IL-13, and IL-10 (Surh *et al.*, 2004). The precise molecular mechanism underlying COX-2 expression is not fully elucidated, but roles of cellular signaling pathways mediated via kinases such as mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), phosphatidyl-ionositol-3-kinases (PI3K), Akt/PKB are reported (Surh, 2003). In addition, the promoter region for COX-2 gene contains binding sites for various transcription factors such as nuclear factor kappa B (NF-kB), nuclear factor for IL-6 (NF-IL6), nuclear factor of activated T-cells (NFAT), cAMP response element-binding protein (CREB), activating protein (AP)-2, and specificity protein (SP)-1 (Dannenberg *et al.*, 2001; Kosaka *et al.*, 1994; Shao *et al.*, 2000). Therefore, activation of kinase signaling pathways and transcription factors, either alone or in combination, results in increased COX-2 expression. This may explain why COX expression is not entirely dependent on ROS formation in the present study. Although there are limited studies on PBDE effects on cellular signaling pathways, it is reported that a commercial PBDE mixture DE-71 and PBDE congeners such as BDE-47, 77, 99, and 209 stimulates PKC translocation, PKC phosphorylation, and ERK phosphorylation *in vitro* (Fan *et al.*, 2010; Li *et al.*, 2012; Madia *et al.*, 2004). Further investigation on the interactions among kinase signaling pathways, transcription factors, and prostaglandin pathways will lead us toward a better understanding of the mechanisms associated with PGE2 production and COX expression stimulated by BDE-47 in gestational compartments.

Our findings implicate PGE2 as a potential target of 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.*, 2007a; 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 few reports 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 is 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.

BDE-47 concentrations used in our study range from 5 to 20 μM. 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 are estimated to range from 1.34 μM to 5.4 μM. Because concentrations of PBDEs in human placentae have been reported as high as ~8 μM (Doucet *et al.*, 2009), the effects observed in the present study with 20 μM BDE-47 in the prepared exposure medium may have relevance for human exposures, albeit at the high end of the exposure range. However, 20 μM was the only effective concentration in most end points except that 15 μM BDE-47 significantly suppressed HPGD mRNA expression (Fig. 1C), failing to show concentration-dependent responses. 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. We also observed anti-inflammatory effects of higher DMSO concentrations (0.75–1%) (Park *et al.*, 2014b), limiting the maximum concentration of DMSO and BDE-47 by 0.7% and 20 μM, respectively, in our study.

We have to be cautious in interpreting our results because overproduction of 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 roles of PGE2 have been implicated in regulating trophoblast function (Biondi *et al.*, 2006; Horita *et al.*, 2007d; Nicola *et al.*, 2005d), there are complex interactions between trophoblasts and a number of autocrine and paracrine factors such as growth factors, growth factor-binding proteins, proteoglycans, other cytokines/chemokines, integrins, adhesion and proteolytic molecules during trophoblast invasion and placentation (Anton *et al.*, 2012; Chakraborty *et al.*, 2002; Lala and Chakraborty, 2003). Moreover, 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.

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.*, 1997d). 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 PGE2 has been shown to regulate EVT proliferation, migration, and invasion during first trimester of pregnancy (Biondi *et al.*, 2006; Horita *et al.*, 2007d; Nicola *et al.*, 2005d), overproduction of PGE2 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.

5. Conclusions

In conclusion, this is the first study to show that treatment with BDE-47, a predominant flame retardant chemical found in human tissues, stimulated expression of COX-2, leading to increased conversion of arachidonic acid to PGE2 in human first trimester placental cells. In addition, (±)-α-tocopherol cotreatment reduced BDE-47-stimulated PGE2 release without affecting mRNA and protein expression of COX-2, implicating post-translational regulation of COX activity by ROS. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BDE-47	brominated diphenyl ether-47
carboxy-H2DCF-DA	6-carboxy dichlorodihydrofluorescein diacetate
COX-2	cyclooxygenase-2
cPGES	cytosolic prostaglandin E synthase
DCF	dichlorofluorescein
cDCF	carboxydichlorofluorescein
DMSO	dimethyl sulfoxide
HPGD	gene for 15-hydroxyprostaglandin dehydrogenase

mPGES-1	membrane-bound prostaglandin E synthase-1
mPGES-2	membrane-bound prostaglandin E synthase-2
NO	nitric oxide
ONOO	peroxynitrite
PBDE	polybrominated diphenyl ether
PGE2	prostaglandin E2
PGES	prostaglandin E synthase
PLA2	Phospholipase A2
PTGES	gene for prostaglandin E synthase
PTGS2	gene for prostaglandin-endoperoxide synthase 2 or COX-2
ROS	reactive oxygen species
TBHP	tert-butyl hydroperoxide
TXB2	thromboxane B2

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Highlights

- BDE-47 stimulated PGE2 release and COX-2 expression.
- BDE-47 resulted in differential expression of genes relevant to PGE2 pathways.
- (±)-α-tocopherol suppressed BDE-47-stimulated increases of PGE2 without affecting COX-2 mRNA and protein expression.

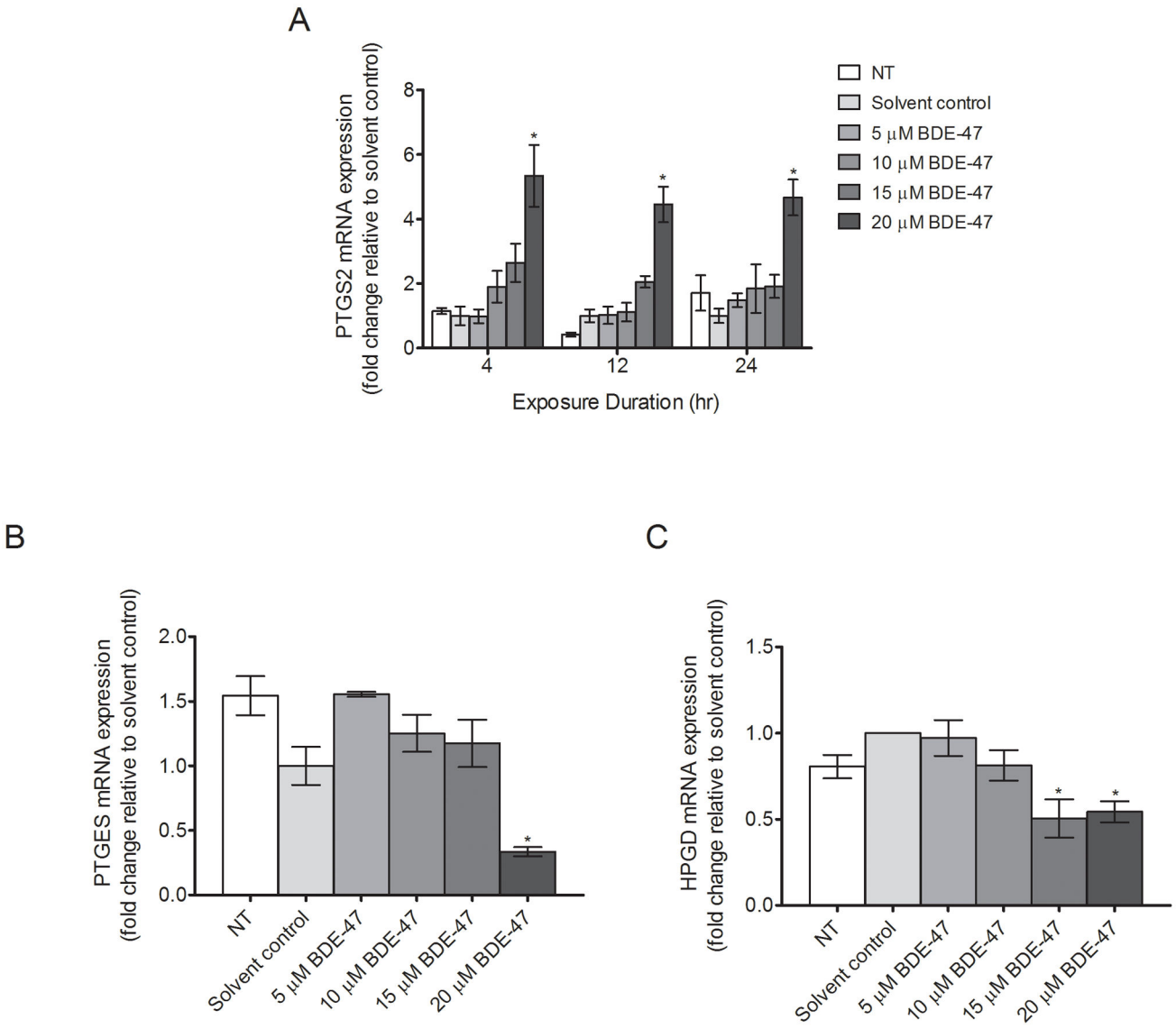

Fig. 1.

Concentration-dependent effects of BDE-47 on mRNA expression of PTGS2, PTGES, and HPGD in HTR-8/SVneo cells. Cells received no treatment (non-treated control, NT), or were treated with solvent control (DMSO, 0.7% v/v) or BDE-47 (5, 10, 15 or 20 μM), and then mRNA expression of target genes was quantified by qRT-PCR. A) Time-course and concentration-dependent response of BDE-47 on PTGS2 mRNA expression after 4, 12, or 24 h. B) BDE-47 concentration-dependent effects on PTGES mRNA expression after 24 h. C) BDE-47 concentration-dependent effects on HPGD mRNA expression after 24 h. *P<0.05, significantly different compared to solvent control within each time point (A) or experiment (B and C).

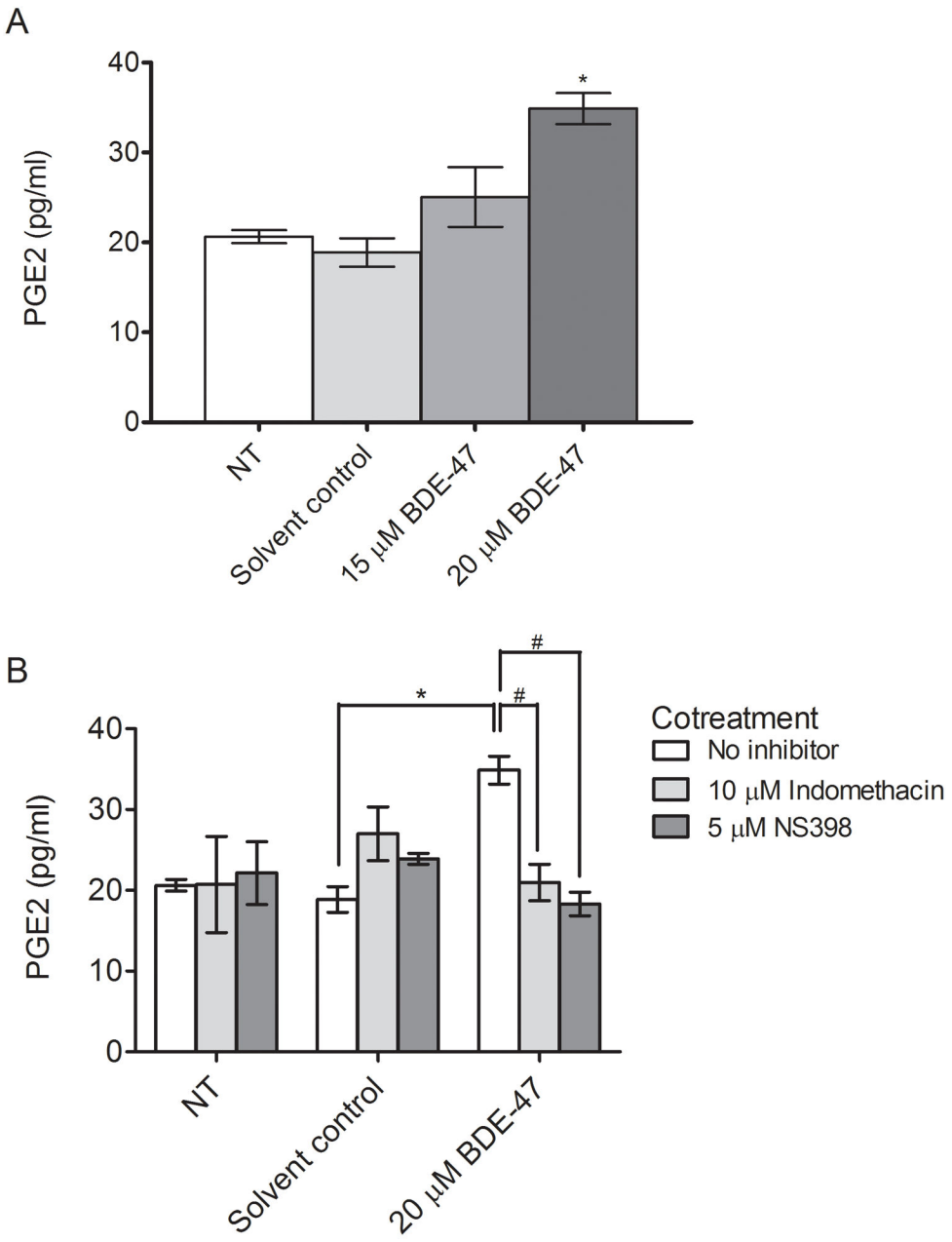

Fig. 2.

BDE-47 effects on COX activity in HTR-8/SVneo cells. COX activity was inferred by quantification of PGE2 in the culture medium from cells stimulated with exogenous arachidonic acid after BDE-47 treatment. A) PGE2 concentrations in medium of cells that were non-treated (NT, control), or treated with solvent control (DMSO, 0.7% v/v), 15 μM BDE-47, or 20 μM BDE-47 for 24 h. B) PGE2 concentrations in medium of cells that were non-treated (NT, control), or treated with solvent control (DMSO, 0.7% v/v) or 20 μM 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. *P<0.05, significantly

different compared to solvent control with no (±)-α-tocopherol cotreatment. #P<0.05, significantly different from each other.

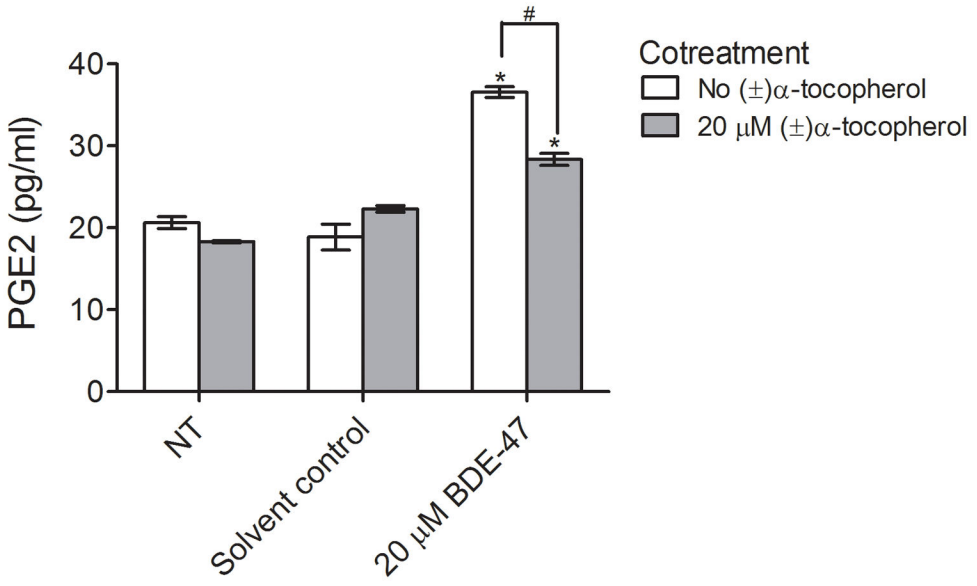

Fig. 3.

(±)-α-Tocopherol effects on BDE-47-stimulated COX activity in HTR-8/SVneo cells. COX activity was inferred by quantification of PGE2 concentrations in the culture medium of cells stimulated with exogenous arachidonic acid after BDE-47 treatment. Cells received no BDE-47 treatment (non-treated control, NT), or were treated with solvent control (DMSO, 0.7% v/v) or 20 μM BDE-47 for 24 h in the absence or presence of 20 μM (±)-α-tocopherol. *$P<0.05$, significantly different compared to solvent control with no (±)-α-tocopherol cotreatment. #$P<0.05$, statistically significantly different from each other.

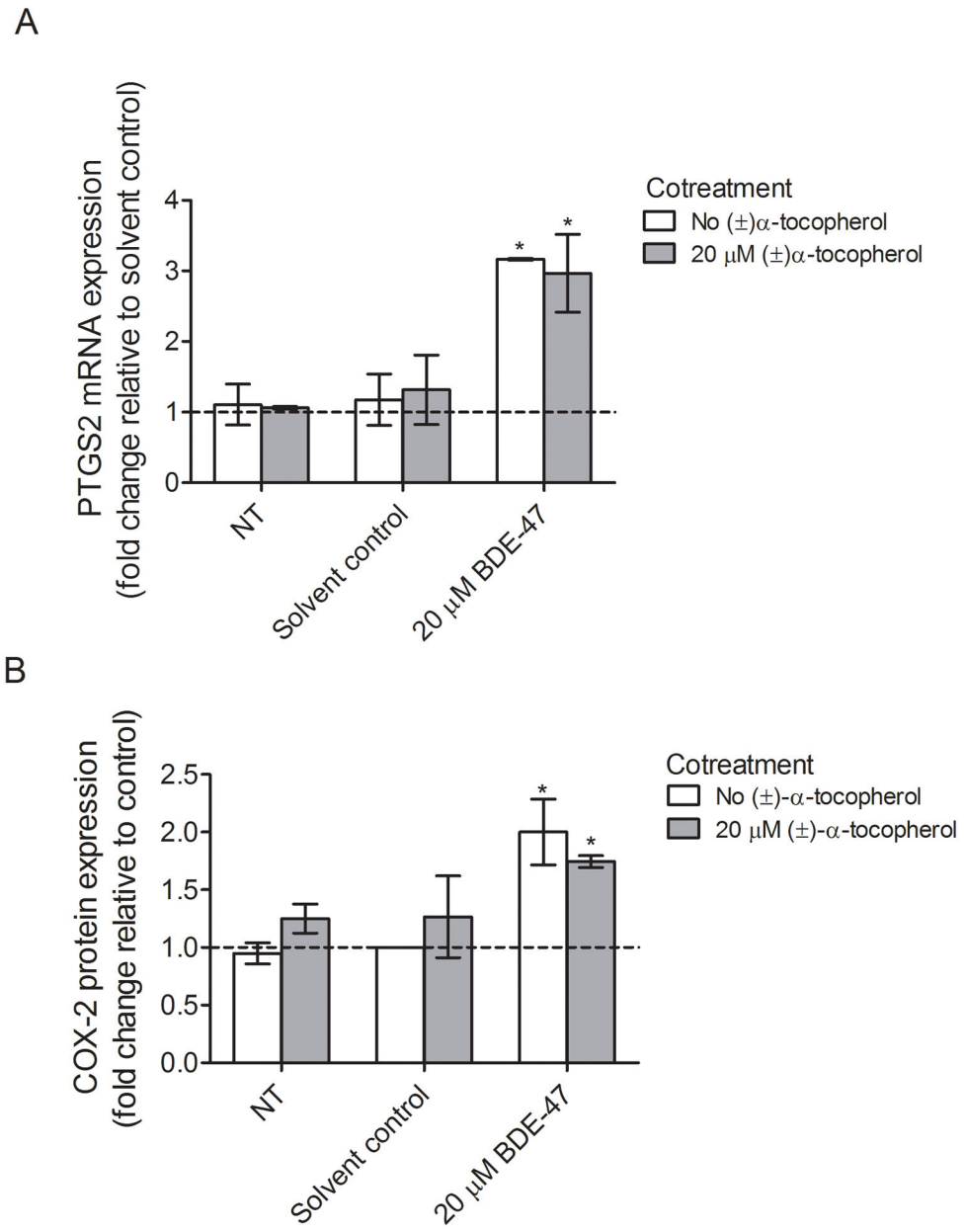

Fig. 4.

(±)-α-Tocopherol effects on BDE-47-stimulated PTGS2 mRNA expression and COX-2 protein expression in HTR-8/SVneo cells. Cells received no BDE-47 treatment (non-treated control, NT), or were treated with solvent control (DMSO, 0.7% v/v) or 20 μM BDE-47 for 24 h in the absence or presence of 20 μM (±)-α-tocopherol. A) Fold changes in PTGS2 mRNA expression relative to control (dashed line, Solvent control with no (±)-α-tocopherol cotreatment) *P<0.05, statistically significantly different compared to solvent control with no (±)-α-tocopherol cotreatment. 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. *P<0.05, statistically

significantly different compared to control (dashed line, Solvent control with no (±)-α-tocopherol cotreatment).

Table 1

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

Treatment	cDCF fluorescence intensity
Non-treated control	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/SVneo cells were non-treated (non-treated control), or were treated with DMSO (0.7% v/v, solvent control), 15 or 20 μM BDE-47, or 100 μM *tert*-butyl hydroperoxide (TBHP, positive control) in the absence or presence of (±)-α-tocopherol for 4 h.

*P<0.05, significantly different compared to solvent control.

#P<0.05, significantly different compared to 20 μM BDE-47-treated group.