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Tert-butyl hydroperoxide stimulated apoptosis independent of prostaglandin E₂ and IL-6 in the HTR-8/SVneo human placental cell line

Rita Loch-Caruso, PhD^{1,*}, Cassandra S. Korte, PhD^{1,2,*}, Kelly A. Hogan, PhD^{1,3}, Sarah Liao, MPH^{1,4}, Craig Harris, PhD¹

¹Department of Environmental Health Sciences, School of Public Health, University of Michigan, Ann Arbor, MI 48109-2029 USA

²Current address: College of Arts and Sciences, Lynn University, Boca Raton, FL 33431 USA

³Current address: Mayo Clinic, Rochester, MN 55905 USA

⁴Current address: Ardent Mills, LCC, Denver, CO, 80202 USA

Abstract

Significant gaps exist in our knowledge of how cellular redox status, sometimes referred to as oxidative stress, impacts placental trophoblasts. The present study used *tert*-butyl hydroperoxide (TBHP) as a known generator of reactive oxygen species (ROS) in the extravillous trophoblast cell line HTR-8/SVneo to examine the role of cellular redox disruption of prostaglandin E₂ (PGE₂) and the cytokine IL-6 in cell death. Cells were exposed to 0, 12.5, 25 or 50 μ M TBHP for 4, 8 and 24 h to ascertain effects on cell viability, caspases 3/7 activity, PGE₂ release, *PTGS2* mRNA expression, and IL-6 release. Experiments with inhibitors included the cyclooxygenase inhibitor indomethacin, mitogen-activated protein kinase inhibitors (PD169316, U0126, or SP600125), or treatments to counter expected consequences of TBHP-stimulated generation of ROS (deferoxamine [DFO], butylated hydroxyanisole [BHA], and N,N'-diphenyl-1,4-phenylenediamine [DPPD]) using 24-h exposure to 50 μ M TBHP. Cell viability, measured by ATP content, decreased 24% relative to controls with a 24-h exposure to 50 μ M TBHP, but not at lower TBHP concentrations nor at earlier time points. Exposure to 50 μ M TBHP increased caspase 3/7 activity, an indicator of apoptosis, after 8 and 24 h. Antioxidant treatment markedly reduced TBHP-stimulated caspase 3/7 activity, PGE₂ release, and IL-6 release. TBHP-stimulated IL-6 release was blocked by PD169316 but unaltered by indomethacin. These data suggest that TBHP-stimulated IL-6 release and caspase 3/7 activation were independent of PGE₂ yet were interrupted

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Corresponding author: Rita Loch-Caruso, PhD, Department of Environmental Health Sciences, School of Public Health, University of Michigan, Ann Arbor, MI 48109-2029 USA, Phone: 734-936-1256, Fax: 734-936-7283, rlc@umich.edu.

*These authors contributed equally to this manuscript

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CONFLICT OF INTERESTS

The Authors declare that there is no conflict of interest.

by treatments with known antioxidant properties, providing new insight into relationships between PGE₂, IL-6 and apoptosis under conditions of chemically-induced cellular oxidation.

Keywords

oxidative stress; reactive oxygen species; trophoblasts; apoptosis; prostaglandins

INTRODUCTION

Nutrient and oxygen exchange between mother and fetus relies on the establishment of the placental maternal-fetal interface. The extravillous trophoblasts are critical for placentation success with their proliferation, migration, and invasion of maternal uterine tissues. These trophoblast functions are regulated by factors such as hormones, prostaglandins, and cytokines [1, 2]. Disrupted regulation of extravillous trophoblast functions during placentation can lead to placental insufficiency with adverse consequences for the mother and offspring [3, 4].

Change in cellular redox status has emerged as an important factor required for normal placental remodeling as well as a mechanism of abnormal placentation, as recently reviewed [5]. Altered cellular redox states are often referred to as oxidative stress, defined as the cellular condition in which the balance of oxidants and antioxidants is disrupted to favor oxidation [6]. Trophoblast responses to pro-oxidant conditions include inhibited migration and invasion, as well as apoptosis [7–9].

In addition to its role in placental remodeling, disrupted cell redox regulation is a common mechanism of toxicant-stimulated cellular injury. Recent reports that include human, rodent, and cell culture approaches support oxidative stress as a critical mechanism by which chromium injures trophoblasts [10]. Moreover, studies with trophoblast cell lines support a role for excess oxidation of cellular molecules in apoptosis, pro-inflammatory cytokine release, and/or prostaglandin pathway activation by several toxicants [11–16]. In addition, oxidative stress induced by cigarette smoke extract is linked to cellular senescence in fetal membranes [17] and isolated placental cells [18]. However, the former toxicants can stimulate a diverse range of cellular responses beyond oxidation of cellular molecules. Moreover, there are significant gaps in our understanding of the sequence of events and inter-relationships between prostaglandins, cytokines, and apoptosis in trophoblast responses to changes in cellular redox status.

To understand better how trophoblasts respond to conditions of chemically-induced cellular oxidation, we assessed relationships between prostaglandin, cytokine, and apoptosis responses in the human extravillous trophoblast HTR-8/SVneo cell line [19] exposed to the cell membrane permeable toxicant *tert*-butyl hydroperoxide (TBHP) [20]. The present study tests the hypothesis that pro-oxidant conditions increase cell death in trophoblasts through redox-sensitive sequential stimulation of apoptosis by PGE₂ and IL-6.

METHODS

Reagents

Cell culture reagents including RPMI 1640 medium supplemented with glutamine but without phenol red, heat-inactivated fetal bovine serum (FBS), penicillin/streptomycin (10,000 units/mL and 10,000 µg/mL, respectively), 0.25% trypsin-EDTA, 10 mM non-essential amino acids in Minimal Essential Medium, Opti-MEM I Reduced Serum Medium supplemented with L-glutamine but without phenol red, Hank's Balanced Salt Solution (HBSS) with calcium and magnesium but without phenol red, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) were from Gibco/ThermoFisher Scientific (Carlsbad, CA). *Tert*-butyl hydroperoxide (TBHP; 70% in water), deferoxamine mesylate salt (DFO), *N,N'*-diphenyl-*p*-phenylenediamine (DPPD), butylated hydroxyanisole (BHA), indomethacin, dimethyl sulfoxide (DMSO), Tween 20, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). The inhibitor PD169316 was purchased from EMD Millipore (Billerica, MA). The inhibitors U0126 and SP600125 were from Enzo Life Sciences (Farmingdale, NY). QIAshredder columns, RNeasy kits, RT² First Strand Kits, RT2 SYBR Green qPCR Mastermix, prostaglandin-endoperoxide synthase 2 (PTGS2) primers, and Cignal Antioxidant Response Reporter (luc) Kit were purchased from Qiagen (Germantown, MD).

Cell line

The first trimester-derived human extravillous trophoblast cell line HTR-8/SVneo was provided by Dr. Charles H. Graham, Queen's University, Kingston, Ontario, Canada [19]. HTR-8/SVneo cells were maintained as previously described [14] and cultured between passages 73–87 to 70–80% confluence in a humidified environment at 37 °C with 5% CO₂ in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Authentication and female sex of the HTR-8/SVneo cell line was confirmed after completion of the experiments by comparing the short tandem repeat (STR) genetic profile of our cells to the STR profile published by the American Tissue Culture Collection (ATCC) (STR analysis was performed by the University of Michigan DNA Sequencing Core).

Cell culture and treatments

For TBHP concentration- and time-course experiments, 500,000 cells/well were seeded in 6-well plates. After a 24-h acclimation, cells were treated for 4, 8 or 24 h with 12.5, 25, or 50 µM TBHP diluted in culture medium. These exposure conditions allowed us to establish a TBHP time-course and concentration-response for measured endpoints, selecting the maximum exposure condition based on our prior finding that 50 µM TBHP exposure for 24 h increased oxidation of the DNA base thymine in HTR-8/SVneo cells [14]. Additional treatments were used in some experiments to counter expected consequences of TBHP-stimulated generation of free radicals and ROS, including pretreatment of cells for 1 h with 1 mM deferoxamine (DFO) prior to a 24-h exposure with 50 µM TBHP, or treatment with 1 µM butylated hydroxyanisole (BHA) or 25 µM *N,N'*-diphenyl-1,4-phenylenediamine (DPPD) during 24 h of co-exposure with 50 µM TBHP. For experiments that assessed the

role of prostaglandin synthesis, cells were co-exposed to 50 μ M TBHP and either 0.1% DMSO (solvent control) or 10 μ M indomethacin, a nonspecific inhibitor of cyclooxygenase. In experiments with MAPK inhibitors, cells were pretreated for 1 h with 0.1% DMSO (solvent control), 10 μ M PD169316 (p38 MAPK inhibitor), 5 μ M U0126 (ERK 1/2 inhibitor), or 5 μ M SP600125 (JNK inhibitor), and then exposed to 50 μ M TBHP for 24 h. The antioxidant concentrations used were based on prior reports of effective concentrations for DFO [21–23], BHA [24] and DPPD [21–23]. Likewise, inhibitor concentrations were from published reports of effective concentrations for indomethacin [25, 26], PD169316 [27], U0126 [28] and SP600125 [29]. All treatments were performed in triplicate in at least three separate experiments.

Measurement of reactive oxygen species

The dichlorofluorescein (DCF) assay was used to detect formation of reactive oxygen species (ROS) under fluorescent microscopy. HTR-8/SVneo cells were seeded at 100,000 cells/well in 12-well plates and treated for 3 h at 37 °C with medium or 50 μ M TBHP dissolved in medium. The treatment medium was removed and cells were washed with HBSS, followed by incubation with 100 μ M carboxy- H_2 DCFDA in HBSS for 1 h at 37 °C. Visualization of fluorescence occurred on an EVOS digital inverted fluorescence microscope (470 nm excitation and 525 nm emission). Brightness and contrast were adjusted uniformly and across all images. Three experiments were performed in replicates of 4 for each treatment group.

Assay of cellular viability

Cell viability was measured using the luminescence Cell Titer-Glo (Promega, WI) assay according to the manufacturer's protocol. This assay utilizes cellular ATP in a luciferase reaction to quantify live cells. Briefly, HTR-8/SVneo cells were seeded at a density of 20,000 cells/well in 96-well white, clear-bottom plates. The following day, cells were treated at various time points (4–24 h) with varied concentrations of TBHP (12.5–50 μ M). Cells treated with camptothecin (4 μ M) for 24 h served as a positive control. Luciferase activity was measured using the Glomax Multi Plus Detection System (Promega, WI).

Measurement of caspase 3/7 activity

Apoptosis was determined using the Caspase-Glo 3/7 Assay kit (Promega) which quantifies the activity of two downstream enzymes in the apoptotic pathway. HTR-8/SVneo cells were seeded at a density of 10–15,000 cells/well in 96-well, clear-bottom plates. Cells were treated with TBHP (12.5–50 μ M) or the positive control camptothecin (4 μ M) (data not shown) for 4 or 8 h, and then assayed on a luminometer according to manufacturer's instructions.

Detection of reactive oxygen species

The dichlorofluorescein (DCF) assay [30] was used to verify that treatment with TBHP produced ROS and free radicals. HTR-8/SVneo cells were seeded at 100,000 cells/well in 12-well plates and treated for 3 h at 37 °C with or without 50 μ M TBHP in medium. Three experiments were performed in replicates of 4 for each treatment group. Treatment medium

was removed and cells were washed with HBSS, followed by incubation with 100 μ M 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) in Hanks Balanced Salt Solution (HBSS) for 1 h at 37 °C. Fluorescence was visualized on an EVOS digital inverted fluorescence microscope (470 nm excitation and 525 nm emission). Brightness and contrast were adjusted uniformly and across all images.

Secretion of PGE₂

Quantification of prostaglandin content was performed by enzyme immunoassay (EIA) according to manufacturer's instructions (Cayman Chemical, Ann Arbor). Following conclusion of treatment, cell culture medium was collected, clarified by centrifugation, aliquoted, and stored at -80 °C until analysis. Values below the assay limit of detection, the lowest non-zero value on the back-calculated standard curve, were assigned a value of one-half the limit of detection. All samples were analyzed in triplicate.

Secretion of IL-6

Secreted IL-6 was measured in medium from cultured HTR-8/SVneo cells by the University of Michigan Immunologic Monitoring Core using commercially available ELISA kits (R&D Systems, Minneapolis, MN). Samples were diluted as necessary and results reported as pg/mL. Values below the limit of detection (LOD) were assigned a value of one-half the LOD.

Gene expression analysis

Gene expression analysis was performed on RNA that was isolated using RNeasy columns and reverse transcribed using RT² first strand kits according to manufacturer's instructions (Qiagen, Germantown, MD). Quantitative RT-PCR was performed on a Bio-Rad CFX Connect Real-Time System C1000 thermal cycler. The mRNA expression levels were estimated using primers provided with the Qiagen RT² qPCR Primer Assay for human PTGS2 (cat no. PPH01136F-200; RefSeq no. NM_000963) and human B2M (cat. no. PPH01094E-200; RefSeq no. NM_004048). The PCR was performed according to the manufacturer's instructions (Qiagen). Briefly, reactions were set up for each sample consisting of: 1 μ l cDNA synthesis reaction product, 1 μ l RT² qPCR Primer Assay reagent (10 μ M stock), 12.5 μ l RT² SYBR Green Mastermix, and 10.5 μ l RNase-free water for a total volume of 25 μ l. The reaction conditions were: 10 min at 95 °C for HotStarTaq DNA Polymerase activation, followed by 95 °C for 15 sec and 70 °C for 30 sec for 40 cycles. PTGS2 mRNA expression was normalized to B2M. Fold changes in PTGS2 mRNA expression was quantified using the Ct method on CFX manager software v 3.0 (Bio-Rad, Hercules, CA). All samples were assayed in triplicate.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey's or Dunnett's post-test or two-way ANOVA with Bonferroni post-test using Prism statistical package (San Diego, CA).

RESULTS

Reactive Species Production

TBHP treatment increased generation of intracellular ROS/radicals as visualized with fluorescence microscopy and the indicator dye carboxy-H₂DCFDA. HTR-8/SVneo cells treated with 50 μ M TBHP for 3 h exhibited increased intracellular fluorescence compared with non-treated controls (Figures 1B and A, respectively). Representative microscopy images are shown from three experiments performed with 4 replicates for each treatment group. These results confirm our prior studies in HTR-8/SVneo cells using TBHP concentrations ranging from 25 to 100 μ M for durations ranging from 4 to 24 h [12, 14, 15].

Cell Viability

Cell viability, as assessed by total ATP content, was reduced 24% relative to time-matched control by exposure to 50 μ M TBHP for 24 h ($p < 0.007$) but not at lower TBHP concentrations (12.5 and 25 μ M TBHP) nor at earlier time points (4 and 8 h), reflecting a significant treatment-time interaction (ANOVA interaction effect, $p = 0.026$) (Figure 2A). The increased values at 24 h with control, 12.5 μ M TBHP, and 25 μ M TBHP treatments relative to earlier time points reflect cell replication ($p < 0.05$), and this was not detected for cultures exposed to 50 μ M TBHP.

Activation of Apoptosis Pathway

Caspases 3/7 activity was used to assess activation of the apoptotic pathway. TBHP significantly increased activity of caspases 3/7 in time- and concentration-dependent manners (ANOVA time, treatment, and time x treatment interactions, $p < 0.0001$; Figure 2B). Relative to time-matched controls, activity was 5.3 fold and 5.9 fold greater in cells exposed to 50 μ M TBHP for 8 h and 24 h, respectively ($p = 0.004$), and 2.5 fold higher in cells exposed to 25 μ M TBHP for 24 h ($p < 0.01$). No change in caspases 3/7 activity was observed after 4 h of exposure to 12.5, 25, or 50 μ M TBHP. Time-dependent increases were observed with 50 μ M TBHP at 8 h compared with 4 h, and at 24 h compared with 4 h and 8 h exposures ($p = 0.005$). Treatment with 25 μ M TBHP for 24 h significantly increased activity compared with 4 h ($p = 0.002$) but not compared with 8 h of exposure.

Because TBHP is known to generate free radicals and peroxides intracellularly [20], we evaluated TBHP-stimulated caspase activity following pretreatment with DFO, an iron chelator that can inhibit iron-dependent generation of reactive oxygen species [31] and interrupt iron-independent lipid peroxidation [32]. DFO pretreatment (1 mM for 1 h) prevented TBHP-stimulated caspase activity measured after 8 h and 24 h of exposure to 50 μ M TBHP (Figure 2C; $p < 0.001$, compared with TBHP alone at same time point).

Additionally, we assessed the role of prostaglandin synthesis in the TBHP-stimulated caspase response. Treatment with the cyclooxygenase inhibitor indomethacin (10 μ M) for 8 or 24 h had no significant effect on TBHP-stimulated activation of caspase 3/7, with large increases in caspase 3/7 activity observed at both time points in cells exposed to TBHP, regardless of the presence or absence of indomethacin (Figure 2D; $P < 0.04$).

Prostaglandin Responses

TBHP stimulated release of PGE₂ in a concentration-dependent manner that was independent of exposure duration (ANOVA treatment effect, $p<0.0001$; ANOVA treatment x time interaction effects, ns; Figure 3A). At 4 h, the earliest time point assessed, 50 μ M TBHP significantly increased PGE₂ in media to concentrations 7.2 fold higher than in control cultures ($p=0.002$), and this increase was significant relative to 12.5 μ M TBHP ($p=0.0005$) and 25 μ M TBHP treatments ($p=0.008$) at 4 h, also. A similar pattern of response was observed after 24 h of exposure to TBHP. However, after 8 h of exposure only 25 μ M TBHP stimulated a statistically significant response ($p=0.02$). Treatment with 12.5 μ M TBHP failed to stimulate significant increases in PGE₂ at any of the time points assayed.

Next, we evaluated mRNA expression of PTGS2, an enzyme critical for PGE₂ synthesis. Similar to PGE₂ release, expression of PTGS2 mRNA was modified by concentration and duration of exposure to TBHP (ANOVA treatment effect, $p<0.0001$; ANOVA time effect, $p=0.05$; ANOVA treatment x time interaction effects, ns; Figure 3B). Expression of PTGS2 mRNA increased as early as 4 h after exposure to 50 μ M TBHP to levels averaging 10.6-fold higher than controls ($p<0.001$), and remained elevated for 8 h ($p<0.02$). By 24 h, the effect of 50 μ M TBHP on PTGS2 mRNA was not significantly different compared with time-matched control, and had declined significantly compared with 4 h exposure to 50 μ M TBHP ($p=0.006$). Lower concentrations of TBHP (12.5 μ M and 25 μ M) failed to increase PTGS2 mRNA expression significantly at any time point measured.

To investigate the role of PTGS2 further, we assessed PGE₂ release in cells exposed to TBHP (50 μ M) for 4 and 24 h in the absence or presence of the cyclooxygenase inhibitor indomethacin (10 μ M). Significant effects were observed for TBHP treatment, indomethacin treatment, and interaction between TBHP and indomethacin (ANOVA, $p<0.0001$; Figure 3C). Exposure to TBHP alone increased PGE₂ in the medium significantly compared to time-matched controls at 24 h ($p<0.0001$) but the increase at 4 h was not statistically significant ($p=0.052$, ns). Indomethacin reduced the average TBHP-stimulated PGE₂ concentration at 24 h from 32.3 pg/ml in cultures exposed to TBHP alone to 11.7 pg/ml in cultures co-exposed to TBHP and indomethacin ($p<0.0001$). However, indomethacin co-treatment did not completely prevent TBHP-stimulated PGE₂ release at 24 h, and PGE₂ concentrations were significantly elevated compared with controls ($p<0.05$).

To assess the role of ROS and free radicals in TBHP-stimulated PGE₂ release, cells were co-treated with 50 μ M TBHP and either BHA or DPPD, free radical scavengers that have been used as antioxidants in foods and other products [33, 34] and that have been shown to interrupt lipid peroxidation of cell membranes [22, 21]. Co-treatment with 1 μ M BHA or 25 μ M DPPD prevented TBHP-stimulated PGE₂ release as measured by PGE₂ concentration in culture media after 24 h of exposure (ANOVA TBHP treatment, antioxidant treatment, and interaction effects, $p<0.0001$; Figure 3D). Specifically, cultures exposed to TBHP without BHA or DPPD had 19.2 pg PGE₂/ml in the medium, whereas negligible PGE₂ release was observed in controls and in cultures treated with BHA or DPPD in the presence or absence of TBHP ($p<0.0001$).

Release of IL-6

To explore TBHP effects on a pro-inflammatory response, we assessed the ability of TBHP to stimulate cellular release of IL-6. Release of IL-6 was increased in a treatment and time-dependent manner (ANOVA treatment, time, and interaction effects, $P < 0.002$) with a significant increase observed only after a 24-h exposure to 50 μM TBHP compared with TBHP exposures at earlier time points (4 and 8 h) and compared with lower TBHP concentrations (0, 12.5, and 25 μM) at 24 h ($P < 0.001$) (Figure 4A). Next, we investigated the effect of DFO on IL-6 release stimulated by 50 μM TBHP after 24 h. Pretreatment with DFO (1 mM for 1 h) prevented TBHP-stimulated IL-6 release, with IL-6 levels 4.2-fold higher in cultures treated with TBHP alone compared with cultures pretreated with DFO ($P = 0.0002$; Figure 4B). BHA and DPPD were evaluated in an experiment separate from DFO. These latter treatments reduced but did not completely block TBHP-stimulated IL-6 release (ANOVA TBHP treatment effect, $p < 0.0001$; antioxidant effect, $P < 0.05$; and interaction effect, $P < 0.05$; Figure 4C). Co-treatment with BHA significantly suppressed TBHP-stimulated IL-6 compared with TBHP alone (52.3 ± 3.7 vs. 81.0 ± 10.6 pg/ml, respectively; $P < 0.02$). Similarly, co-treatment with DPPD suppressed TBHP-stimulated IL-6 release to 51.6 ± 4.1 pg/ml; $P < 0.02$). Although reduced, TBHP in the presence of BHA and DPPD still stimulated significant increases of IL-6 concentrations in media 2.3 and 2.2 fold, respectively, compared with vehicle controls ($P < 0.02$). Treatment with BHA or DBBP without TBHP had no significant effect on IL-6 release.

Because TBHP-stimulated PGE_2 release preceded IL-6 release, we evaluated whether IL-6 release is dependent on PGE_2 by inhibiting PGE_2 synthesis with the cyclooxygenase inhibitor indomethacin. Pretreatment with 10 μM indomethacin failed to block IL-6 release in cells stimulated with 50 μM TBHP, with similar levels of IL-6 observed in cultures exposed to TBHP with or without indomethacin that were elevated at least 4.2-fold compared with controls (Figure 4D; $p < 0.0001$).

We used inhibitors to examine the role of specific MAPK pathways on TBHP-stimulated IL-6 release. Pretreatment for 1 h with the p38 MAPK inhibitor PD169316 (10 μM) blocked TBHP-stimulated IL-6 release (Figure 4E; $p = 0.006$). However, 1 h pretreatment with ERK1/2 or JNK inhibitors (5 μM U0126 and 5 μM SP600125, respectively) had no significant effects on TBHP-stimulated IL-6 release (IL-6 concentrations remained increased).

DISCUSSION

The present study offers new insight into relationships between prostaglandins, IL-6 and apoptosis under conditions of chemically-induced cellular oxidation in placental cells. Specifically, we show that TBHP stimulated activation of the apoptotic caspases 3 and 7 independent of IL-6 and PGE_2 release. In addition, our data indicate that TBHP stimulated IL-6 release independent of prostaglandin synthesis and release.

Oxidative stress has emerged as an important factor in normal human placentation as well as a mechanism of disrupted placental development, integrity and function, as recently reviewed [5]. Oxidative stress is a term that describes “a disruption of redox signaling and

control” in the cell [6], commonly associated with the cellular condition in which the balance of oxidants and antioxidants is modified to favor oxidation [35]. Conditions or treatments that favor cellular oxidation inhibit trophoblast cell migration and invasion for placentation, and stimulate trophoblast cell death [7–9]. Although studies support a role for cellular oxidation in placental cell apoptosis, pro-inflammatory cytokine release, and prostaglandin pathway activation by toxicants [11–16], the interplay between these responses remains unresolved. The present study used TBHP as a prototypic pro-oxidant to study these responses under conditions of chemically-induced cellular oxidation. TBHP is a well-characterized cell membrane-permeable pro-oxidant that generates free radicals, peroxides and other ROS intracellularly [20]. The generation of ROS/radicals in HTR-8/SVneo cells was validated, consistent with prior studies with HTR-8/SVneo cells [12, 14, 15] and non-placental cells [21, 36–40].

TBHP stimulated PGE₂ release and upregulated *PTGS2* mRNA expression by 4 h of exposure, preceding increased caspase 3/7 activity at 8 h and increased IL-6 release at 24 h. Each of the aforementioned responses were inhibited by treatments expected to interrupt ROS activity (DFO, BHA, and DPPD) [21–24], consistent with underlying cellular redox-related mechanisms. However, the inhibitor indomethacin failed to prevent TBHP-stimulated IL-6 release and activation of caspases 3 and 7, suggesting that these responses were independent of cyclooxygenase-derived prostaglandins despite the temporal sequence of events. Although PGE₂-stimulated IL-6 release was reported in human term placental explants, exogenous PGE₂ was added to the culture media in that prior study [41]. The ability of indomethacin to block PGE₂ release in the HTR-8/SVneo cells indicates the efficacy of the inhibitor treatment used in our study, in agreement with prior reports [25, 26].

Treatments that increase intracellular levels of ROS up-regulate *PTGS2* expression and increase release of PGE₂ in a variety of cells, as reviewed [42]. Of particular relevance, the results with TBHP are similar to a prior report that a flame retardant chemical, brominated diphenyl ether-47, increases generation of reactive species and up-regulates *PTGS2* mRNA expression within 4 h of exposure in HTR-8/SVneo cells, and these responses are inhibited by the antioxidant α -tocopherol [43]. In other placental cell types, hydrogen peroxide stimulates PGE₂ release in amnion epithelial cells, amnion mesenchymal cells, and amnion explants isolated from human extraplacental membranes [44]. Furthermore, using N-acetyl-cysteine with lipopolysaccharide stimulation, Lappas and colleagues linked reactive oxygen species to prostaglandin release in studies in human amnion and choriodecidua explant cultures [45].

Consistent with TBHP-stimulated IL-6 release, previous studies support a link between increased cellular oxidation and increased IL-6 secretion in HTR-8/SVneo cells treated with the trichloroethylene metabolite *S*-(1,2-dichlorovinyl)cysteine [15] and brominated flame retardants [12, 13]. However, TBHP-stimulated release of IL-6 in the present study differed from PGE₂, increasing only after 24 h of exposure to the cytotoxic concentration of 50 μ M TBHP but not at lower TBHP concentrations and shorter durations of exposure. This pattern suggests that IL-6 may be a response, rather than a mediator, to TBHP-induced cytotoxicity. In addition to being a pro-inflammatory cytokine, IL-6 is considered a marker of the senescence-associated secretory phenotype (SASP) [46]. As such, the increased IL-6 we

observed may be an indication of oxidative stress-related cellular senescence, as suggested by Dixon et al. [47] for primary amnion epithelial cells exposed to lipopolysaccharide, TNF- α , and cigarette smoke extract. Furthermore, prevention of the TBHP-stimulated IL-6 release by the p38 MAPK inhibitor PD169316 is consistent with the SASP model proposed by Dixon et al. [47].

TBHP-stimulated cytotoxicity was detected in the HTR-8/SVneo cells after 24 h of exposure to 50 μ M TBHP but not at lower TBHP concentrations nor with shorter durations of exposure. Increased cell viability, as measured by ATP content, occurred at 24 h with lower TBHP concentrations similar to controls due to continued cell growth (not as a result of treatment). Because activation of apoptotic executor caspases 3 and 7 preceded cytotoxicity temporally, the data support apoptosis as a mechanism of cytotoxicity. The TBHP time-course, concentration-response, and inhibition by DFO for cytotoxicity and apoptotic pathway activation are similar to those of a prior TBHP study in a lung carcinoma cell line [37].

If the responses observed in HTR-8/SVneo cells were to occur in women with exposures that disrupt cellular redox regulation in placenta, there could be consequences for pregnancy. Whereas regulated apoptosis removes damaged cells to maintain a healthy placenta, abnormally increased apoptosis of placental cells is associated with pregnancy pathologies such as pre-eclampsia and fetal growth restriction, as reviewed [48, 49]. Furthermore, because PGE₂ has critical roles in placental function, particularly for trophoblast attachment and migration during early placentation, disrupted regulation of its synthesis and release could have implications for placental function and pregnancy health [1, 2, 50]. Likewise, elevated IL-6 concentrations in amniotic and cervical fluids are associated with preterm birth, as recently reviewed [51–53], and antibody to IL-6 receptor significantly prolongs gestation in an inflammatory model of preterm birth in mice [54], further implicating a role for IL-6 in preterm birth.

In summary, our data show that TBHP activated apoptotic caspases in a manner independent of PGE₂ and IL-6 release in trophoblasts. Furthermore, our results suggest that TBHP-stimulated IL-6 release was independent of prostaglandin synthesis, and IL-6 release may reflect senescence/cytotoxicity, separating the prostaglandin and proinflammatory cytokine responses. Because PGE₂ and IL-6 are important regulators of trophoblast function and loss of trophoblasts to apoptosis could potentially compromise placental development and function, changes in cellular redox status elicited by chemical pro-oxidants such as TBHP may compromise placental health by disrupting these pathways. Pregnant women are exposed to wide range of chemicals [55], many of which can stimulate ROS generation, suggesting that some environmental exposures may increase a woman's risk for pregnancy complications by disrupting placental cell processes sensitive to cellular redox regulation.

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Figure 1. TBHP-stimulated generation of intracellular reactive oxygen species visualized with 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-DCF) in HTR-8/SVneo cells. A) Control (untreated) cells. B) Cells after treatment with 50 μ M TBHP for 3 h. Three experiments were performed in replicates of 4 for each treatment group. Representative images shown.

Figure 2.

TBHP-stimulated cytotoxicity in HTR-8/SVneo cells. **A)** Time- and concentration-dependent effects on cell viability as assessed by ATP content in cells exposed to 0 (Control), 12.5, 25, or 50 μ M TBHP for 4, 8, or 24 h. N=3 experiments. **B)** Time- and concentration-dependent effects on caspase 3/7 activity in cells exposed to 0 (Control), 12.5, 25, or 50 μ M TBHP for 4, 8, or 24 h. N=4 experiments. **C)** Effect of deferoxamine (DFO) on TBHP-stimulated caspase 3/7 activity. Cells were pretreated with 1 mM DFO for 1 h, and then exposed to 50 μ M TBHP or untreated (Control) for 8 or 24 h. N=4 experiments. **D)** Lack of effect of indomethacin on TBHP-stimulated activation of caspase 3/7. Cells were treated with 50 μ M TBHP or vehicle (0.1% DMSO for indomethacin) in the presence or absence of 10 μ M indomethacin for 8 or 24 h. N=2 independent experiments. For all graphs, data are presented as the mean $\pm$ SD of independent experiments with 3 replicates per treatment in each experiment. *P<0.04, **P<0.007, ***P 0.0005 for comparisons within a time point. #P<0.04, ##P 0.003, ###P 0.0005, compared to same treatment at earlier time point(s).

Figure 3.

TBHP-induced effects on PGE₂ in HTR-8/SVneo cells. Time- and concentration-dependent effects on **A**) PGE₂ release (concentration in media) and **B**) *PTGS2* mRNA expression in cells exposed to 0 (Control), 12.5, 25, or 50 μ M TBHP for 4, 8, or 24 h. *** P 0.0008, ** P =0.008, * P <0.02, for comparisons shown within a time point. ## P =0.006, compared with same treatment at 4 h. **C**) PGE₂ release (concentration in media) for cells treated for 4 h or 24 h with or without the PTGS inhibitor indomethacin (10 μ M) concurrent with 50 μ M TBHP or vehicle (0.1% DMSO for indomethacin; Control). *** P <0.0001, compared with all other groups at both time points. # P <0.05, compared with Control and Indomethacin (only) treatment groups at 8 h and 24 h. **D**) PGE₂ release (concentration in media) for cells treated for 24 h with 50 μ M TBHP in the presence of 1 μ M butylated hydroxyanisole (BHA) or 25 μ M *N,N'*-diphenyl-*p*-phenylenediamine (DPPD). *** P 0.0001, compared to all other treatment groups. For all graphs, data are presented as the mean $\pm$ SD of 3 independent experiments with 3 replicates per treatment in each experiment.

Figure 4.

IL-6 release (concentration in media) in HTR-8/SVneo cells exposed to TBHP. **A**) Time and concentration-dependent TBHP-stimulated IL-6 release in cells exposed to 0 (Control), 12.5, 25, or 50 μ M TBHP for 4, 8, or 24 h. N=3 experiments. ***P 0.007, compared to all other treatment groups. **B**) IL-6 release in cells pretreated with 1 mM deferoxamine (DFO) prior to 24-h exposure to 50 μ M TBHP. N=4 experiments. ***P 0.002, compared to all other treatment groups. **C**) IL-6 release in cells co-treated with 50 μ M TBHP concurrent with 1 μ M BHA, 25 μ M DPPD, or vehicle (0.1% DMSO; Control) for 24 h. N=3 experiments *P<0.03, compared to all other treatment groups. ###P<0.0001, for comparisons shown. **D**) IL-6 release in cells co-treated for 24 h with 10 μ M indomethacin and 50 μ M TBHP or vehicle (0.1% DMSO for indomethacin; Control). N=3 experiments. ***P<0.0001, for comparisons shown. **E**) IL-6 release in cells pretreated with 10 μ M PD169316 (p38 MAPK

inhibitor), 5 μ M U0126 (ERK 1/2 inhibitor), or 5 μ M SP600125 (JNK inhibitor) for 1 h prior to a 24-h exposure to 50 μ M TBHP. Open bars represent cells treated with inhibitor vehicle (0.1% DMSO; Control) and filled bars represent treatment with TBHP. N=3 experiments.

*P<0.05, compared with the (Solvent) Control group that received the same inhibitor pretreatment but no TBHP treatment. #P=0.006, comparing TBHP treatment with and without inhibitor. For all graphs, data are presented as the mean $\pm$ SD of independent experiments with 3 replicates per treatment in each experiment.