# TERT-BUTYL HYDROPEROXIDE STIMULATES PARTURITION-ASSOCIATED PATHWAYS IN A HUMAN PLACENTAL CELL LINE

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Toxicology)
in the University of Michigan
2013

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2013

*For Kevin*

# ACKNOWLEDGEMENTS

I gratefully acknowledge all the support I've been given during my time at the University of Michigan. I would like to extend special thanks to my mentor Dr. Rita Loch-Caruso. Rita has guided me through this process with kindness, patience, and understanding. This dissertation wouldn't have been possible without the many thoughtful conversations we've had over the years. I would also like to thank my committee members, Dr. Craig Harris, Dr. Peter Mancuso, and Dr. Jeff Martens for their guidance, constructive criticism, and advice.

I would like to thank several individuals for their help along the way. Thank you to Joel Whitfield of the University of Michigan Immunologic Monitoring Core, Sarah Liao of the Caruso lab, and Joe Jilek of the Harris lab for their assistance in conducting several experiments. Thank you to Lucas Korte for collaborating with me in making my diagrams. A special thank you to Dr. Lauren Tetz for being my lab mate, teacher, and, most of all, my friend.

I would also like to thank my family and friends for supporting me through this challenging quest. Thank you to members of the Caruso lab, past and present, for their providing a thoughtful sounding board.

This work would not have been possible without funding from the Environmental Toxicology and Epidemiology Training Grant and the Rackham Graduate School. I'd also like to acknowledge additional funding was provided to Dr. Rita Loch-Caruso from the NIH and Superfund Research Program.

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## ABSTRACT

Preterm birth is a leading cause of infant mortality and morbidity. Increased understanding of the etiology of preterm birth is needed to accelerate medical and public health interventions. Parturition is associated with markers of the damaging effects of reactive oxygen species (ROS). Whereas roles for prostaglandins, cytokines, and apoptosis in parturition have been described, the effect of ROS on these parturition pathways is unclear. Using the human placental trophoblast cell line HTR-8/SVneo, we investigated the effect of the lipophilic ROS-producing chemical *tert*-butyl hydroperoxide (TBHP) on trophoblast apoptosis, prostaglandin production, and cytokine release.

Exposure to TBHP stimulated significant, though modest, increases in redox-related responses including production of ROS, glutathione redox potential ($E_h$), reporter activity at the antioxidant response element (ARE), and expression of the antioxidant genes thioredoxin reductase 1 and glutaredoxin 2. Under our treatment conditions, TBHP significantly increased caspase 3/7 activity and decreased cell viability, indicative of apoptosis. Apoptotic responses were inhibited by the iron-chelating antioxidant, deferoxamine. Likewise, TBHP stimulated release of prostaglandin $E_2$ ($PGE_2$) and mRNA expression of prostaglandin-endoperoxide synthase 2 (PTGS2), an enzyme in the prostaglandin synthetic pathway. In addition, TBHP increased release of the cytokine interleukin-6 (IL-6) and reporter activity of the proinflammatory transcription factor nuclear factor-kappaB (NF-kappaB). Cotreatment with the PTGS inhibitor indomethacin

or the antioxidants butylated hydroxyanisole (BHA) and diphenyl phenylenediamine (DPPD) blocked the TBHP-stimulated $PGE_2$ response. Similarly, TBHP-stimulated IL-6 release was blocked by the antioxidants BHA, DPPD, and deferoxamine.

We next examined the redox-sensitive mitogen activated protein kinases (MAPKs) as an upstream pathway for the TBHP-stimulated responses. Treatment with TBHP increased phosphorylation of p38 MAPK, indicative of activation, and pretreatment with the p38 MAPK inhibitor PD169316 blocked the TBHP-stimulated increases in IL-6 as well as $PGE_2$ and PTGS2, suggesting dependence of these responses on p38 MAPK activity.

In summary, these results suggest that exposure to an ROS-producing chemical stimulates responses in human placental cells linked to parturition. Because exposure to some environmental pollutants causes cellular ROS formation, these findings suggest a plausible mechanism that may underlie associations between pollutant exposures and increased risk for preterm birth.

# CHAPTER 1

# INTRODUCTION

## Preterm birth – a significant public health problem

Birth that is preterm, or before 37 weeks of gestation, results in sequelae that include delayed cognitive development (Colvin et al. 2004), pulmonary deficiencies (Baraldi and Filippone 2007), sensory impairments (Colvin et al. 2004), and behavioral disorders (Johnson 2007; Saigal and Doyle 2008). Furthermore, complications, such as those of impaired cognition, can persist into adulthood (Hack 2009). Preterm birth also is the leading cause of infant mortality: over one-third of infant deaths in the United States can be attributed to preterm birth (Callaghan et al. 2006). Moreover, preterm birth is prevalent: in 2010, over 475,000 infants were born preterm, accounting for 12.0% of all deliveries (Martin et al. 2012). In addition to its effect on the public health, early birth is also costly to society. In 2005, the economic cost of preterm birth was estimated at over $26 billion annually (Behrman and Butler 2007). With the exception of progesterone administration (Merlob et al. 2012), clinical interventions to prevent preterm birth have proven largely unsuccessful, in part due to of a lack of understanding about the etiology of preterm birth (Behrman and Butler 2007). In 2007, the Institute of Medicine released a report recommending more research to understand mechanisms involved in the pathogenesis of preterm birth and thusly accelerate development of measures to avoid preterm birth (Behrman and Butler 2007).

## Parturition

Parturition is characterized by three major physiologic events: coordinated myometrial contractions, cervical ripening, and rupture of the extraplacental membranes (Figure 1.1). Two biochemical mediators, matrix metalloproteinases (MMPs) and prostaglandins, are largely responsible for driving the events of labor. Matrix metalloproteinases are protease enzymes whose collagenolytic activity is involved in a variety of biological processes including cellular migration (Librach et al. 1991), activation of cytokines (D'Haese 2000; Van den Steen et al. 2000), and unmasking of ligands that initiate apoptosis (Kayagaki et al. 1995). During parturition, MMP protease activity degrades the extracellular matrix of the cervix and membranes leading to softening of the cervix and membrane ruptures (Gonzalez et al. 2011; Uchide et al. 2000).

Prostaglandins are lipid-signaling molecules that elicit a variety of functions including regulation of vascular tone (Félétou et al. 2011), pulmonary smooth muscle tone (Tilley et al. 2003), and platelet aggregation (Kuriyama 2010). Prostaglandins are produced by a series of step-wise biochemical reactions. First, arachidonic acid is released from the phospholipid bilayer by phospholipase $A_2$ ($PLA_2$) (Balsinde et al. 1999). Arachidonic acid is then metabolized by prostaglandin endoperoxide synthase (PTGS, also known as cyclooxygenase) to produce prostaglandin $H_2$ ($PGH_2$) (Chandrasekharan and Simmons 2004). Finally, prostaglandins are further isomerized by respective prostaglandin synthases. In the case of the bioactive prostaglandin $PGE_2$, $PGH_2$ is isomerized by prostaglandin E synthase (PTGES) to produce $PGE_2$ (Park et al. 2006). Prostaglandins can be further catabolized to biologically inactive keto-metabolites by 15-hydroxyprostaglandin dehydrogenase (HPGD) (Tai et al. 2006). Of relevance to

this dissertation, prostaglandins initiate myometrial contractions during parturition (Bailie et al. 2002; Parkington et al. 1999).

Preterm labor occurs with untimely activation of the pathways that generate mediators of normal term parturition (Behrman and Butler 2007). The placenta and extraplacental membranes, composed of amnion, chorion laeve, and decidua (Figure 1.1), are important sources for production of parturition mediators. For example, increased expression of MMP-9 is found in placenta and extraplacental membranes of women in both term and preterm labor (Xu et al. 2002). Likewise, expression of PTGS2, a key inducible enzyme in the synthesis of prostaglandins, is increased during labor at term or preterm in placenta (Cindrova-Davies et al. 2007; Hanna et al. 2006) and extraplacental membranes (Mijovic et al. 1998; Slater et al. 1998).

**Inflammation and parturition**

In addition to production of parturition-driving MMPs and prostaglandins, the placenta and extraplacental membranes are important sources of proinflammatory cytokines. During labor, the amnion and choriodecidua produce the cytokines interleukin (IL)-6 (Laham et al. 1996) and IL-1β (Elliott et al. 2001). Similarly, tumor necrosis factor (TNF)-α, IL-1β, (Cindrova-Davies et al. 2007b), and IL-6 (Steinborn et al. 1998) are also increased in placenta during labor. Importantly, these inflammatory cytokines initiate production of the parturition-promoting MMPs and prostaglandins, and the subsequent events of parturition (Romero et al. 2007). For example, exposure of extraplacental membranes to TNFα and IL-1β stimulates MMP-9 expression and decreases tensile strength of the tissue (Kumar et al. 2006). In addition, the cytokines TNFα, IL-1β, or IL-6 stimulate $PGE_2$ release from human amnion cells (Furuta et al. 2000). Further implicating

inflammatory pathways in labor initiation, administration of IL-1β to pregnant mice induces premature labor (Romero et al. 1991), while knockout of IL-6 in mice delays normal labor in mice (Robertson et al. 2010).

Inflammation due to infectious agents stimulates preterm birth. In point of fact, as many as 25-40% of preterm births can be attributed to intrauterine infection (Goldenberg and McClure 2010). Pregnancies complicated with bacterial vaginosis (Flynn 1999; Leitich et al. 2003) or intrauterine infection (Goldenberg et al. 2008; Romero et al. 2007) are at increased risk of preterm birth. Furthermore, administration of lipopolysaccharide (LPS) to pregnant mice stimulates early parturition (Elovitz et al. 2003; Kaga et al. 1996). Mechanistically, bacterial products, including LPS, engage toll-like receptors (TLRs) on the surface of the cell to stimulate cytokine production (Romero et al. 2007) that then signal to downstream labor effectors, inducing parturition (Figure 1.1).

**Inflammation, signal transduction, and transcription factors**

The mitogen activated protein kinases (MAPKs) are a prototypical class of signal transducers that regulate a wide variety of cellular functions (Zhang and Dong 2005). Three common pathways exist in mammals: extra-cellular signal-regulated protein kinases (ERKs), c-Jun $NH_2$-terminal kinases (JNKs), and p38 MAPK. Activation of MAPKs initiates inflammatory responses in a number of tissue and cell types (Zhang and Dong 2005), including human gestational tissues. MAPK pathway activity is required for cytokine, prostaglandin, and MMP release following stimulation of trophoblast, myometrium, and the extraplacental membranes with LPS or inflammatory cytokines (Bartlett et al. 1999; Lappas et al. 2007; Li et al. 2010; Renaud et al. 2009; Sooranna et al. 2005).

Similarly, nuclear factor-κB (NF-κB) transcriptional activity is required in many inflammatory processes (Vallabhapurapu and Karin 2009), including induction of parturition-promoting mediators in gestational tissues (Lappas and Rice 2007). The family of NF-κB transcription factors consists of five members: RelA, c-Rel, and RelB, which contain transcriptional activation domains. The remaining two members, NF-κB1 and NF-κB2, do not contain said domains. In unstimulated cells, inactive NF-κB is sequestered into the cytoplasm by inhibitor of κB (IκB). Upon exposure to various stimuli, IκB is degraded and NF-κB dimers are free to translocate to the nucleus where they activate transcription (Vallabhapurapu and Karin 2009). Inhibition of NF-κB activity prevents proinflammatory cytokine- and LPS-induced increases in cytokine release and prostaglandin synthesis in cultured gestational membrane cells and tissues (Ackerman et al. 2008; Keelan et al. 2009; Lappas et al. 2002) and delays labor onset in mice (Condon et al. 2004). Furthermore, nuclear translocation of RelA (also known as p65) in fetal membranes is increased in laboring women compared to non-laboring women (Vora et al. 2010).

**Reactive oxygen species and parturition**

Parturition is associated with biomarkers indicating cellular and tissue damage owing to reactive oxygen species (ROS). The ROS include the oxygen-containing radicals superoxide and hydroxyl radical, and the non-radical hydrogen peroxide, among others. Hallmark indicators of cellular oxidative damage include lipid peroxidation, protein carbonyl formation, and oxidation of DNA bases. Women in labor at term have increased cord blood levels of malondialdehyde (MDA) (Mocatta et al. 2004; Yaacobi et al. 1999) and lipid hydroperoxides (Mongelli et al. 1997; Rogers et al. 1998). Term parturition is also associated with increased maternal blood MDA (Nakai et al. 2000) and

susceptibility of maternal serum to lipid oxidation (Fainaru et al. 2002). Labored placenta exhibits increased lipid peroxidation (Diamant et al. 1980), 8-isoprostanes, 8-hydroxydeoxyguanosine (8-OHdG) (Hung et al. 2011), and hydroxynonenal (Cindrova-Davies et al. 2007b), all markers for the damaging effects of ROS.

In addition to association with term labor, markers for cellular damage due to ROS have been associated with adverse pregnancy outcomes. For example, preeclampsia is associated with protein nitrotryosine, malondialdehyde, and lipid hydroperoxides in the placenta (Hubel 1999). Besides preeclampsia, increased urinary excretion of 8-OHdG at midgestation is associated with shortened gestation length (Stein et al. 2008). The damaging effects of ROS also appear to be increased in preterm deliveries compared with term deliveries. One study found increased MDA in cord blood of preterm deliveries, but also found decreased protein carbonyl content (Mocatta et al. 2004), whereas a second study reported that protein carbonyl content, as well as MDA, were increased maternal and cord blood of preterm deliveries (Pathak et al. 2010). Furthermore, total placental isoprostanes are increased in preterm deliveries compared with term deliveries (Comporti et al. 2004).

Given the association between ROS and labor, it is important to understand whether ROS may be playing a mechanistic role in the activation of parturition-promoting pathways within the gestational compartment. However, there are rather few studies to implicate that ROS may be involved in mediating the events of labor. In mice, administration of the antioxidant N-acetylcysteine (NAC) blocks LPS-induced premature delivery (Buhimschi et al. 2003), and treatment of pregnant rats with diphenyl phenylenediamine (DPPD), another antioxidant, significantly increases gestation length

(Matsumoto et al. 2013). In human tissues, NAC blocks LPS-stimulated cytokine production from extraplacental membrane explants in vitro (Lappas et al. 2003).

**Reactive oxygen species and cellular signaling transduction relevant to parturition**

In addition to damaging effects on cellular constituents, ROS also act as signal transduction mediators (Nathan 2003; Paulsen and Carroll 2009; Reth 2002). For example, ROS may oxidize thiolate anions located on the cysteine residues of proteins. Similar to other types of posttranslational modifications like phosphorylation, cysteine oxidation can vastly affect protein function (Reth 2002). For instance, oxidation of cysteine resides within the catalytic domains of phosphatases blocks phosphatase activity. Inhibition of phosphatase results in dysregulation of MAPK phosphorylation and activity (Salmeen 2005). Several lines of evidence suggest that ROS may act as signal transduction mediators in inflammatory pathways. For example, exposure of T cells directly to hydrogen peroxide induces activation of NF-κB (Schreck et al. 1991). In addition, many antioxidants are also anti-inflammatory agents: N-acetyl cysteine and α-tocopherol block LPS-stimulated production of cytokines and nuclear translocation of NF-κB in rat Kupffer cells (Fox et al. 1997). Moreover, exposure of cells to pro-inflammatory stimuli induces production of ROS via the enzyme NADPH oxidase (Rhee et al. 2003). As such, ROS at the maternal-fetal interface may stimulate production of parturition-promoting inflammatory pathways involving prostaglandins and cytokines.

***Tert*-butyl hydroperoxide as a model ROS-producing chemical**

A variety of methods can be used to generate ROS in vitro in studies of gestational cells. For example, cells from the amnion have been treated with hydrogen peroxide (Kumar et al. 2004a; Kumar et al. 2004b), and myometrial cells have been treated with xanthine oxidase to produce superoxide (Warren et al. 2005). In addition,

placental explants have been exposed to hypoxia-reoxygenation (Hung et al. 2001; Cindrova-Davies et al. 2007a), which also generates ROS. Unlike glucose oxidase- and xanthine oxidase-containing model systems that utilize enzymatic reactions to generate ROS extracellularly, TBHP is lipid soluble and passes the plasma membrane and to enter cells (Ahmed-Choudhury et al. 1998). Principally, TBHP produces *tert*-butyl alkoxyl and *tert*-butyl peroxyl radicals in a series of iron-catalyzed reactions similar to the Fenton and Haber-Weiss reactions (Huang et al. 2005; Figure 1.2). These alkoxyl and peroxyl radicals may go on to react with cellular constituents, producing alterations in signal transduction or even cellular damage. In addition to direct, iron-catalyzed formation of ROS, TBHP may also indirectly shunt the cell towards production of ROS. As a hydroperoxide, TBHP is a substrate for the metabolizing enzyme glutathione peroxidase (Eklow et al. 1984; Huang et al. 2005). Using the cellular thiol glutathione (GSH) as a cofactor, TBHP is converted to *tert*-butyl alcohol by glutathione peroxidase (Figure 1.2). By effectively depleting cellular stores of GSH, TBHP diminishes the cellular reducing capacity. As a result, additional ROS produced by TBHP or other cellular processes may not be scavenged, and are thus free to react with further cellular constituents including lipids, proteins, or DNA. TBHP is commonly used as a model to study the effect of ROS on cellular functions. Moreover, recently, the Environmental Protection Agency released a report suggesting that a breakdown product of TBHP, *tert*-butyl alcohol, may be contaminating water near hydraulic fracturing sites (DiGiulio et al. 2011). The extent and health effects of human exposure to TBHP or its breakdown products in contaminated waters are unclear.

**The HTR-8/SVneo cell line as a model for in vitro study of placental cell responses**

The HTR-8/SVneo cell line was derived from first trimester human extravillous trophoblast cells and immortalized using the SV40 T antigen (Graham et al. 1993). These cells are phenotypically similar to extravillous trophoblasts in that they migrate in response to cytokines (Jovanović and Vićovac 2009; Jovanović et al. 2010) and prostaglandins (Nicola et al. 2005). However some differences between the epigenetic profiles of the cell line and primary cells have been noted (Novakovic et al. 2011). Because HTR-8/SVneo cells are easily available and widely studied, many aspects of their physiology have been described. In addition, the extravillous trophoblast cell may be an important target in dysfunctional pregnancies: it is well-established that aberrant extravillous trophoblast migration and spiral artery transformation are associated with preeclampsia (Merviel et al. 2004; Wang et al. 2009), and similar pathologies have been identified in the placental bed of preterm pregnancies (Kim et al. 2002; Kim et al. 2003). HTR-8/SVneo cells have also been used in several previous studies to elucidate mechanisms of signaling related to parturition (Allen et al. 2013; Wang et al. 2004; Wang et al. 2007).

**Environmental exposures and risk for preterm labor**

Limited studies suggest that environmental exposures may increase risk for preterm labor (see Ferguson et al. 2013 for a comprehensive review). For example, cigarette smokers are more likely to deliver preterm (Cnattingius 2004) and have increased systemic markers for the damaging effects of ROS and inflammation (Yanbaeva et al. 2007). Similarly, increased organochlorine pesticide exposures are associated with increased markers for ROS and increased rates of preterm delivery (Pathak et al. 2010). Other exposures, including lead and phthalates, have also been

associated with increased risk for preterm birth (Jelliffe-Pawlowski et al. 2006; Torres-Sánchez et al. 1999; Dietrich 1991; Meeker et al. 2009), and increased generation of ROS in various tissues (Ercal et al. 2001; Ferguson et al. 2011; Jomova and Valko 2011; Kasahara et al. 2002; Liu et al. 2010; Seo et al. 2004; Tetz et al. 2013). Because pregnant women are exposed to many chemicals through the ambient, home, and work environments, an improved understanding of environmental chemical impacts on gestational tissues is warranted.

**Hypothesis**

Damage due to ROS is associated with preterm birth, and ROS can act as signaling molecules to mediate inflammatory-type pathways important for parturition. Therefore this dissertation characterizes the mechanistic relationship between ROS and parturition-associated mediators. **We hypothesize that reactive oxygen species stimulate parturition mediators including prostaglandins, cytokines, and apoptotic caspases in human gestational cells and tissues**. To test this hypothesis, we used the prototypical pro-oxidant chemical *tert*-butyl hydroperoxide (TBHP) and the human placental trophoblast cell line HTR-8/SVneo. The specific aims of this dissertation are to: 1) demonstrate that TBHP stimulates cellular apoptosis; 2) demonstrate that TBHP stimulates prostaglandin production; 3) demonstrate that TBHP stimulates release of the parturition-associated cytokine IL-6 following exposure to TBHP in a human placental trophoblast cell line, HTR-8/SVneo. This dissertation is the first series of studies to examine the effect of low dose chemical oxidant on the parturition-specific mediators IL-6 and $PGE_2$.

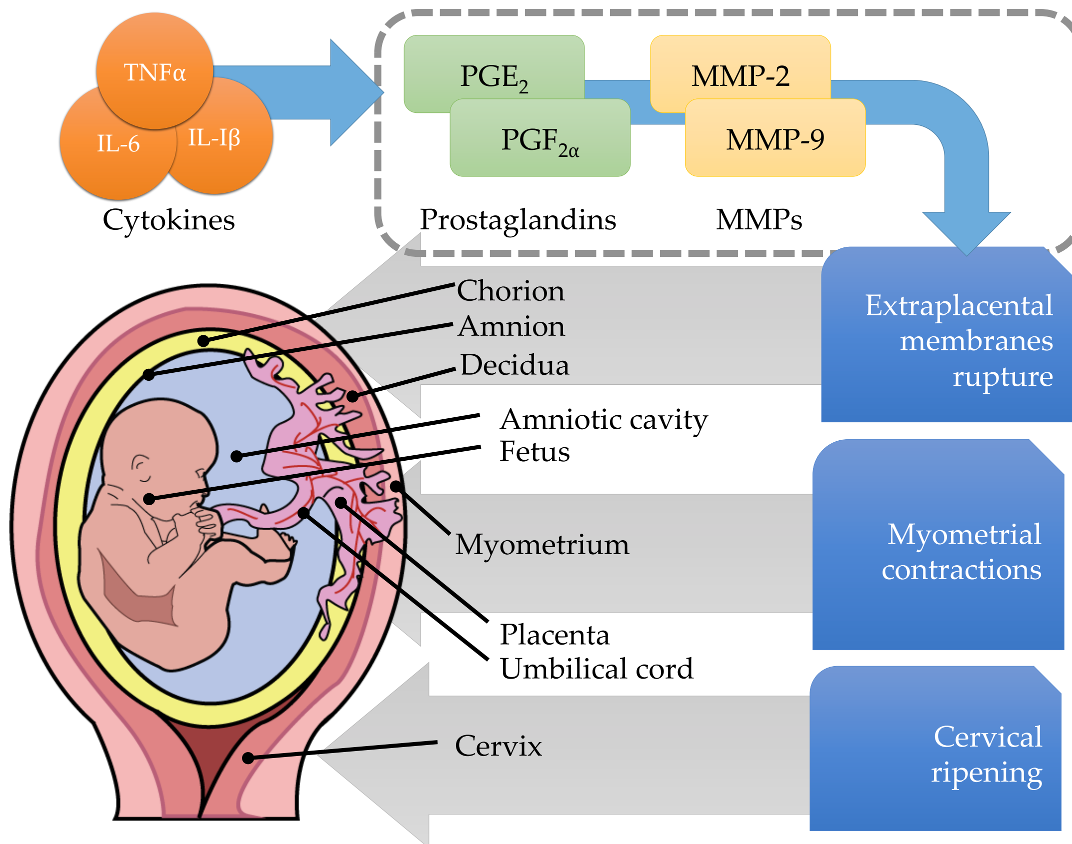
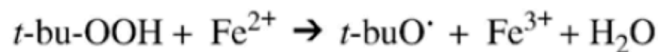
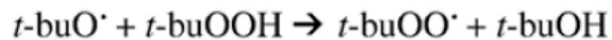
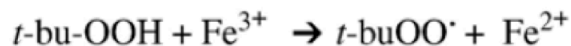



**Figure 1.1. The pregnant uterus and parturition.** Cytokines, including IL-1β, TNFα, and IL-6, stimulate synthesis of biochemical mediators prostaglandins, $PGE_2$ and $PGF_{2\alpha}$, and MMPs, MMP-2 and MMP-9. These biochemical mediators then drive the events of labor: rupture of the extraplacental membranes, contractions of the myometrium, and ripening of the cervix. Image generated in collaboration with Lucas J. Korte, 2013.







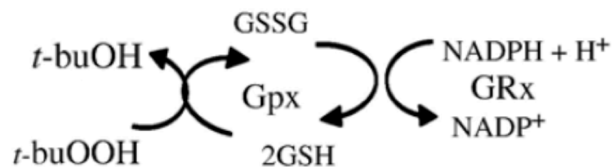



**Figure 1.2. Generation of free radicals by TBHP.** *Tert*-butyl hydroperoxide (TBHP; *t*-bu-OOH) produces *tert*-butyl alkoxyl radical (*t*-buO•) in reaction with ferrous iron ($Fe^{2+}$). Ferric iron ($Fe^{3+}$) reacts with TBHP to produce *tert*-butyl peroxyl radical (*t*-buOO•). Resultant peroxyl and alkoxyl radicals may then react with cellular constituents including DNA, protein, or lipids to produce alkyl radicals (R•). Because it is a hydroperoxide, TBHP is as a substrate for glutathione peroxidase (Gpx). Gpx converts TBHP to *tert*-butyl alcohol (*t*-buOH), in concert with oxidation of two glutathione (GSH) molecules to one glutathione disulfide (GSSG). Glutaredoxin (GRx) catalyzes the reduction of oxidized GSSG to GSH using NADPH as an electron donor. Image modified from Huang et al. 2005.

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# CHAPTER 2

## PLACENTAL CELL LINE APOPTOSIS IN RESPONSE TO *TERT*-BUTYL HYDROPEROXIDE

### INTRODUCTION

Preterm birth increases risk for neonatal morbidity (Callaghan et al. 2006) and mortality (Saigal and Doyle 2008). Cellular damage due to reactive oxygen species (ROS) has been associated with both preterm and term labor. Maternal urinary excretion of 8-hydroxydeoxyguanosine (8-OHdG), a marker for oxidative DNA damage, is associated with decreased gestation length (Stein et al. 2008). In addition, maternal blood and cord blood of preterm deliveries have increased malondialdehyde (MDA), indicating lipid peroxidation, and protein carbonyl content, a marker for protein oxidation, compared with women who labor at term (Pathak et al. 2010). Women in labor at term also have increased cord blood levels of MDA (Mocatta et al. 2004; Yaacobi et al. 1999) and lipid hydroperoxides (Mongelli et al. 1997) (Rogers et al. 1998).

In 2007 the Institute of Medicine released a report recommending that mechanistic studies are needed order to understand how the environment may be contributing to preterm birth (Behrman and Butler 2007). Several environmental chemicals have been linked to preterm birth, including organchlorine pesticides (Pathak et al. 2010), lead (Jelliffe-Pawlowski et al. 2006), and cigarette smoke (Cnattingius 2004). Because these chemicals also stimulate ROS in vivo (Pathak et al. 2010; Ahmed et al. 2008; Banerjee et al. 2001; Siddharth et al. 2012; Ercal et al. 2001; Jomova and Valko

2011; Liu et al. 2010; van der Vaart et al. 2004; Yanbaeva et al. 2007), we hypothesized that ROS may be playing a role in modulating the activation of pathways that drive parturition.

Apoptosis within the gestational compartment is associated with parturition; increases in placental trophoblast apoptosis has been identified in a mouse model of premature labor (Kakinuma et al. 1997). Furthermore, chorion trophoblast apoptosis has been implicated in fetal membrane weakening (Moore et al. 2006). Linking ROS to gestational cell apoptosis, antioxidants inhibit spontaneous chorion trophoblast apoptosis in vitro (Ohyama et al. 2001). Although formation of ROS has been linked to trophoblast apoptosis in past studies (Cindrova-Davies et al. 2007; Ohyama et al. 2001), there is little known about how a chemical oxidant may effect apoptosis pathways in human trophoblasts. As such, the objective of this study was to examine the effect of the pro-oxidant chemical *tert*-butyl hydroperoxide (TBHP) on apoptosis pathway activation and to characterize corresponding changes in redox-sensitive responses in the human placental cell line HTR-8/SVneo.

## MATERIALS AND METHODS

### Materials

We purchased RPMI 1640 medium supplemented with L-glutamine and 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, Hank's Balanced Salt Solution (HBSS) with calcium and magnesium but without phenol red, Opti-MEM I Reduced Serum Medium supplemented with L-glutamine and hydroxyethyl piperazineethanesulfonic acid (HEPES) and without phenol red, 10 mM non-essential

amino acids in minimal essential medium, and 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-$H_2$DCFDA) were from Life Technologies (Carlsbad, CA). *Tert*-butyl hydroperoxide (70% in water), deferoxamine mesylate salt, dimethyl sulfoxide, indomethacin, sulforaphane, and camptothecin 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). We purchased 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-$H_2$DCFDA) from Molecular Probes, Life Technologies (Carlsbad, CA). Antioxidant Response Cignal reporter assay kit, Attractene transfection reagent, QIAshredder columns, and RNeasy kits were purchased from Qiagen (Germantown, MD). Dual Luciferase, Caspase-Glo 3/7, and CellTiter-Glo luminescent assays were purchased from Promega (Madison, WI). We purchased iScript cDNA synthesis kits and SsoAdvanced SYBR Green Supermix from Bio-Rad (Hercules, CA). Primers were synthesized by Integrated DNA Technologies (Coralville, IA).

**Cell culture and treatments**

The HTR-8/SVneo cell line was utilized to study placental cell responses to ROS. HTR-8/SVneo cells are human extravillous trophoblast cells derived from first trimester placentae and were kindly provided as a gift from Dr. Charles H. Graham (Graham et al. 1993), Queen's University, Kingston, Ontario, Canada. HTR-8/SVneo cells between passages 73-87 were maintained at 37°C in a humidified incubator with 5% $CO_2$ in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin for 2-3 days until disaggregated by trypsinization. Unless otherwise noted,

HTR-8/SVneo cultures were allowed to reach 70-80% confluence 24 h after subculture before initiating an experiment.

*Tert*-butyl hydroperoxide was employed as a model pro-oxidant that crosses the plasma cell membrane to generate intracellular ROS (Ahmed-Choudhury et al. 1998). To treat HTR-8/SVneo cells, culture medium was exchanged with fresh medium containing TBHP (diluted directly in medium) or medium alone. For experiments with treatments that preceded TBHP exposure, medium was aspirated and cells were pretreated with 1 mM deferoxamine (DFO; dissolved directly into medium), 0.1% DMSO (vehicle control), PD169316, U0126, SP600125, or medium alone. One hour later, medium was aspirated, and cells were treated with TBHP or medium alone. For experiments that included indomethacin, cells were coexposed to 50 μM TBHP, 0.1% DMSO (vehicle control), or 10 μM indomethacin. We chose to treat with 50 μM TBHP because it was the effective concentration to elicit cellular responses, including apoptosis and prostaglandin (see Chapter 3) and cytokine (see Chapter 4) release. HTR-8/SVneo cells were exposed to 10 μM sulforaphane or 4 μM camptothecin as positive controls for Nrf activity (Kensler et al. 2013) and caspase activity (Tetz et al. 2013), respectively.

**Caspase 3/7 Activity Assay**

Caspase 3/7 activity was assayed as an indication of activation of the apoptotic pathway. HTR-8/SVneo cells were seeded at a density of 10-15,000 cells/well in 96-well white, clear bottom plates. Following treatment with TBHP, Caspase-Glo 3/7 reagent was added to each well, and incubated for 1 h at room temperature in the dark. Luminescence was read on a GloMax Multi Plus detection system (Promega) using the manufacturer's pre-loaded Caspase-Glo assay protocol.

**Viability Assay**

Cellular viability was assessed using the luminescent CellTiter-Glo Assay. In this assay, cellular ATP acts as a cofactor in the luciferase reaction to produce light. Thus, the amount of output light is proportional to the number of viable cells. HTR-8/SVneo cells were seeded at a density of 20,000 cells/well in 96-well white, clear-bottom plates. Following treatment of cells with TBHP or positive control (camptothecin), CellTiter-Glo reagent was added to wells and plates were mixed briefly on an orbital shaker. The plate was incubated at room temperature for 10 min in the dark and then read on a luminometer using manufacturer's programmed settings.

**Measurement of ROS**

Formation of cellular ROS following treatment with TBHP was assessed by dichlorofluorescein (DCF) assay. HTR-8/SVneo cells were seeded in 96-well black, clear-bottom plates. Twenty-four hours later, cells were 90-100% confluent and treated with 50, 100, or 200 μM TBHP for 2 h in replicates of 5 per treatment. Preliminary studies suggested no effect of treatment with lower doses (12.5 or 25 μM TBHP) at this treatment duration. Treatments were then removed and cells were washed with HBSS, followed by incubation with 100 μM carboxy-$H_2$DCFDA for 1 h at 37°C in HBSS. The probe was then removed by gentle aspiration, cells were washed again, and fresh HBSS was added to wells. The plate was read immediately from the bottom of the wells using a SpectraMax M2e microplate reader (Molecular Devices, Sunnyvale, CA) with excitation and emission wavelengths of 492 and 522 nm, respectively.

**Determination of cellular thiol concentrations**

TBHP effects on reduced gluathione (GSH) and oxidized glutathione (GSSG) and the glutathione redox potential ($E_h$) were measured in HTR-8/SVneo cells. The cells were seeded at a density of 1.5 x $10^6$ in 60 mm dishes. Diluted TBHP was added directly to culture medium on the cells in a time-staggered fashion, and all cells were lysed and collected at the same time on ice. Cellular GSH concentrations were analyzed by high-performance liquid chromatography (HPLC) as previously described (Harris and Hansen 2012). Briefly, the exposure medium was removed, then cells were washed two times with ice cold dPBS and lysed in buffer containing 5% perchloric acid, 0.2 M boric acid, and 10 µM γ-glutamyl glutamic acid standard. Lysates were collected with scraping, snap frozen on liquid nitrogen, and stored at -80°C until analysis. Thawed lysates were sonicated, precipitated protein was pelleted by centrifugation, and supernatants were transferred to fresh tubes. Samples were derivatized with dansyl chloride at room temperature overnight after addition of iodoacetic acid and saturated KOH in $KBO_4$. Samples were extracted with chloroform and injected into the HPLC instrument. Protein pellets were solubilized and digested with 250 mM NaOH at 4°C overnight. Total protein was quantified with the BCA assay.

**Transfections and reporter assays**

Nuclear factor (erythroid-derived 2)-like 2 (Nrf) 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. Suspensions of 20,000 HTR-8/SVneo cells were seeded into white, clear bottom 96-well plates containing

transfection reagent complexed with negative control, positive control, or ARE reporter constructs. Cells were allowed to adhere and transfect for 6 h in the incubator, and an 18-h recovery period before treating. Cells were then treated with 50 μM TBHP in triplicate for 24 h, 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 for cell number and transfection efficiency. Data are presented as the fold change in luciferase activity normalized to the control. Cells were maintained in Opti-MEM I supplemented with 1% NEAA and 3% FBS throughout the duration of transfection experiments.

**RNA isolation and quantitative real-time PCR**

Cells were seeded at a density of 500,000 cells/well in 6-well plates. Following removal of culture medium, cells were homogenized using Qiashredder columns, and then replicate homogenates were pooled and RNA was extracted using RNeasy columns according to manufacturer's instructions. One microgram of total RNA was reverse transcribed using iScript cDNA synthesis kits according to manufacturer's instructions. The following forward and reverse primer sequences were used: beta-2-microglobulin (B2M) 5'-TGGAGGCTATCCAGCGTACT-3' and 5'-CGGATGGATGAAACCCAG ACA-3'; glutaredoxin 2 (GLRX2) 5'-TCCTGTTCTTACTGTACAATGGCA-3' and 5'-TTGGAACAGTTCTTTCACCAGTC-3'; thioredoxin reductase 1 (TXNRD1) 5'-CCTGTCTGTGCAGAGGTATTCA-3' and 5'-TTTGCAGTCTTGGCAACAGC-3'. Quantitative RT-PCR reactions were performed on a Bio-Rad CFX Connect Real-Time

System or Bio-Rad CFX96 Real-Time System C1000 thermal cyclers according to SsoAdvanced SYBR Green Supermix directions. The housekeeping gene B2M was run in parallel reactions as a means of normalization. Fold changes in target mRNA expression was quantified using the efficiency-corrected $\Delta\Delta$Ct method (Yuan et al. 2006) on Bio-Rad CFX manager software. Efficiency was calculated from standard curves run in parallel for each experiment. All samples were assayed in triplicate.

**Statistics**

Data were analyzed by matched one or two-way analysis of variance (ANOVA) or paired t-tests where appropriate. Statistical analysis was performed using SigmaPlot software version 11.0 (Systat Software, Chicago, IL). The qRT-PCR data were analyzed using CFX software (Bio-Rad, Hercules, CA). The data subjected to analysis for qRT-PCR data were $\Delta$Ct values (Yuan et al. 2006).

## RESULTS

**Effect of TBHP treatment on HTR-8/SVneo caspase 3/7 activity**

Activity of the effector caspases 3/7 was measured as an indication of apoptosis (Slee et al. 2001). Although TBHP exposure failed to stimulate caspase activity at 4 h, treatment with 50 μM TBHP for 8 h stimulated a 5.3 fold increase (Figure 2.1A; $p<0.001$), and exposure for 24 h with 25 μM TBHP or 50 μM TBHP stimulated 2.5 fold and 5.9 fold increases, respectively, compared to time-matched controls ($p<0.05$). Moreover, the response at 24 h was concentration-dependent, with 50 μM TBHP stimulating significantly more activity than 25 μM TBHP ($p<0.001$). Camptothecin (4 μM), a positive control for apoptosis, stimulated significant increases over the solvent control at 8 and 24 h ($p<0.001$) to levels similar to those elicited by 50 μM TBHP.

To test the hypothesis that TBHP-stimulated caspase activity is dependent on free radical formation, cells were pretreated with the iron chelator DFO (1 mM). DFO pretreatment significantly decreased TBHP-stimulated caspase activity to levels near those of controls at 8 h and 24 h (Figure 2.1B; $p<0.001$, compared with TBHP alone).

**Cell Viability**

The effect of TBHP on cellular viability was evaluated using the CellTiter-Glo assay for ATP content. Treatment with 12.5, 25, or 50 μM TBHP at 4 and 8 h did not significantly affect cell viability (Figure 2.1C). In contrast, concentration-dependent decreases in viability of HTR-8/SVneo cells were observed after 24 h of exposure to 25 and 50 μM TBHP ($p<0.05$). The positive control treatment with camptothecin (4 μM) significantly reduced viability by 48% at 24 h treatment ($p<0.001$) but not with 4 or 8 h of treatment.

**Generation of cellular ROS**

The ROS-sensitive probe DCF was used to evaluate generation of cellular ROS as a result of TBHP exposure. Exposure to 200 μM TBHP for 2 h resulted in a significant increase of 76% in DCF fluorescence compared to the control (Figure 2.2; $p=0.011$). Exposure to 50 and 100 μM TBHP failed to significantly increase the fluorescent signal.

**TBHP effects on cellular glutathione redox couple**

We examined the cellular redox profile by measuring the concentrations of oxidized and reduced GSH, and then using the Nernst equation to derive the GSH/GSSG redox potential ($E_h$). Exposure of HTR-8/SVneo cells to 50 μM TBHP for 15, 30, 45, or 60 min resulted in no statistically significant alterations of cellular GSH content (Figure 2.3A). However, treatment for 15 and 30 min increased GSSG concentrations compared

with controls by 76% and 62%, respectively (Figure 2.3B; $p<0.05$). By 45 min of exposure, the TBHP-stimulated increase in GSSG was no longer statistically significant, although levels still appeared to be elevated. The GSH/GSSG redox potential also changed: exposure to TBHP for 15 min significantly increased it by 6.8 mV compared with control (Figure 2.3C; $p=0.037$). No statistically significant changes in the redox potential were observed at longer exposure durations with TBHP up to 24 h (data not shown).

**ARE reporter activity in response to TBHP**

Activity of the redox sensitive promoter element ARE was assessed in HTR-8/SVneo cells transfected with an ARE-reporter gene construct. Exposure to 50 μM TBHP resulted in increases of 15, 42, and 35% over control at 2, 6, and 8 h, respectively (Figure 2.4; $p<0.05$). Included as a positive control, 10 μM sulforaphane increased ARE activity by 41, 71, 91 and 209% over control at 2, 4, 6, and 8 h ($p<0.05$).

**Antioxidant gene mRNA expression**

We measured TBHP-induced changes in mRNA expression of the antioxidant genes TXNRD1 (Nordberg and Arnér 2001) and GLRX2 (Wu et al. 2011), previously shown to be induced in HTR-8/SVneo cells by the ROS-stimulating environmental chemical mono-2-ethylhexyl phthalate (Tetz et al. 2013). Cells treated with 25 μM TBHP for 4 h had a 23% increase in TXNRD1 mRNA expression (Figure 2.5A; $p<0.001$), whereas treatment with 50 μM TBHP stimulated significant increases of 30-44% at 4, 8, and 24 h ($p<0.05$).  Exposure to 50 μM TBHP resulted an increase in GLRX2 mRNA of 30% at 24 h but not 4 or 8 h compared with time-matched control (Figure 2.5B; $p<0.05$).

# DISCUSSION

Apoptosis, defined as programmed cell death, is characterized by cell membrane blebbing and DNA fragmentation (Elmore 2007). Hallmark indicators of cellular apoptosis are the activation of the effector caspases 3 and 7, which are responsible for protein cleavage that leads to the morphological changes observed during apoptosis (Porter and Jänicke 1999; Wyllie 2010). Here, we found that treatment with TBHP resulted in robust increases in caspase 3/7 activity and a subsequent decrease in cellular viability, suggesting induction of apoptosis.

Apoptosis of chorionic membrane trophoblasts (Ohyama and Oka 1998), placental stromal cells, and placental trophoblasts (Axt et al. 1999; Smith et al. 1997) have been reported in human tissues at term. In addition, apoptosis in the extraplacental membrane is associated with preterm premature rupture of the membrane (Fortunato et al. 2000) and onset of labor at term (Kumagai et al. 2001; Lei et al. 1996). Placental trophoblast apoptosis has also been associated with preterm birth (Balkundi et al. 2003; Kakinuma et al. 1997). In the chorion, apoptosis is thought to contribute to weakening of the extraplacental membranes and subsequent rupture of the membrane (Moore et al. 2006), whereas in the placenta, it may contribute to placental aging (Smith et al. 1997) and parturition (Axt et al. 1999). Here we found that treatment with 50 μM TBHP to HTR-8 cells increased caspase 3/7 activity by approximately 5 to 6 fold at 8 and 24 h of exposure. Notably, a 3-fold increase in apoptosis has been reported with term labor in chorionic trophoblasts (Harirah et al. 2012). Also similar to our study, a 2-fold increase in cleaved caspase 3 was observed in placental explants exposed to hypoxia-reoxygenation (Cindrova-Davies et al. 2007).

Consistent with results reported here, exposure of placental explants to hypoxia-reperfusion injury results in apoptosis, and the antioxidants vitamin C and E (Cindrova-Davies et al. 2007) and deferoxamine (Hung et al. 2002) inhibit this response. In the present study, treatment with deferoxamine, an iron chelator, blocked the TBHP-stimulated caspase activity. Because TBHP forms alkoxyl and peroxyl free radicals in iron-catalyzed reactions (Van der Zee et al. 1989), inhibition of the apoptotic response by deferoxamine suggests that the apoptotic response is dependent on free radical formation. Furthermore, we have previously shown that treatment of HTR-8/SVneo cells with 50 μM TBHP causes formation of oxidized thymine adducts (Tetz et al. 2013), suggesting the possibility of DNA damage-induced apoptosis (Wyllie 2010). However, we did not detect significantly increased ROS with 50 μM TBHP using the DCF assay, which can detect $H_2O_2$, hydroxyl, and alkoxyl radicals (Setsukinai et al. 2003), although the DCF assay increases were statistically significant with higher concentrations of TBHP. The latter finding suggests that the DCF assay may be insensitive for detection of low levels of ROS or that there may be an alternative mechanism to explain the inhibition by deferoxamine. Further studies, therefore, are needed to examine the role iron plays in TBHP-stimulated apoptosis.

The time-course and magnitude of ROS resulting from TBHP vary widely across cell types. Others have reported increases of approximately 1.9-4.5 fold in ROS with cells exposed to 50 μM TBHP at similar time points (Sardao et al. 2007; Silva et al. 2010). Increases of over 10 fold have been reported in primary rat hepatocytes exposed to 1 mM TBHP for 6 h (Perez et al. 2006). Previous studies show that the half-life of TBHP in culture (Dringen et al. 1998) and time to ROS formation (Sitozhevsky et al. 1997) occurs

quite quickly within cells, suggesting that the lack of ROS detection with 50 μM TBHP in the present study may be related to the time point assayed, 2 h. Alternatively, the failure to observe ROS with 50 μM TBHP, in contrast to treatment with 200 μM TBHP, may reflect a potential lack of sensitivity of DCF assay with this system. Indeed, previous studies in our lab have maximally detected increases of approximately 4 fold in DCF fluorescence upon treatment of HTR-8/SVneo cells with 180 μM mono-2-ethylhexyl phthalate, suggesting detection thresholds may be rather low (Tetz et al. 2013).

The tripeptide glutathione, the most abundant cellular thiol, plays a critical role in regulating the cellular redox homeostasis. Treatment with 50 μM TBHP resulted in no significant changes in reduced GSH in the HTR-8/SVneo cells over the time course examined. In contrast, in hepatocytes (Buc-Calderon et al. 1991; Martín et al. 2001), macrophages (Kanupriya et al. 2007), cardiomyocytes, and neuronal cells (Lam et al. 2011), TBHP induces marked oxidation of GSH. The reported decline in GSH is due, at least in part, to the metabolism of TBHP by glutathione peroxidase, with GSH required as an electron donor (Eklow et al. 1984). However, TBHP itself has also been reported to inhibit glutathione peroxidase activity (Toussaint et al. 1993). Peroxidase activity may be blocked by TBHP in HTR-8/SVneo cells, suggesting a possible explanation for the difference between studies that have observed decreases in GSH and the present report. In addition, we observed that concentrations of GSSG were increased and there was a subsequent, though modest, increase of +6.8 mV in the GSH/GSSG reduction potential. Typically, apoptosis is induced when the GSH/GSSG reduction potential changes to approximately -170 to -150 mV (Harris and Hansen 2012): however, the reduction potential at its most oxidizing with TBHP treatment was -227.7 mV. The biological

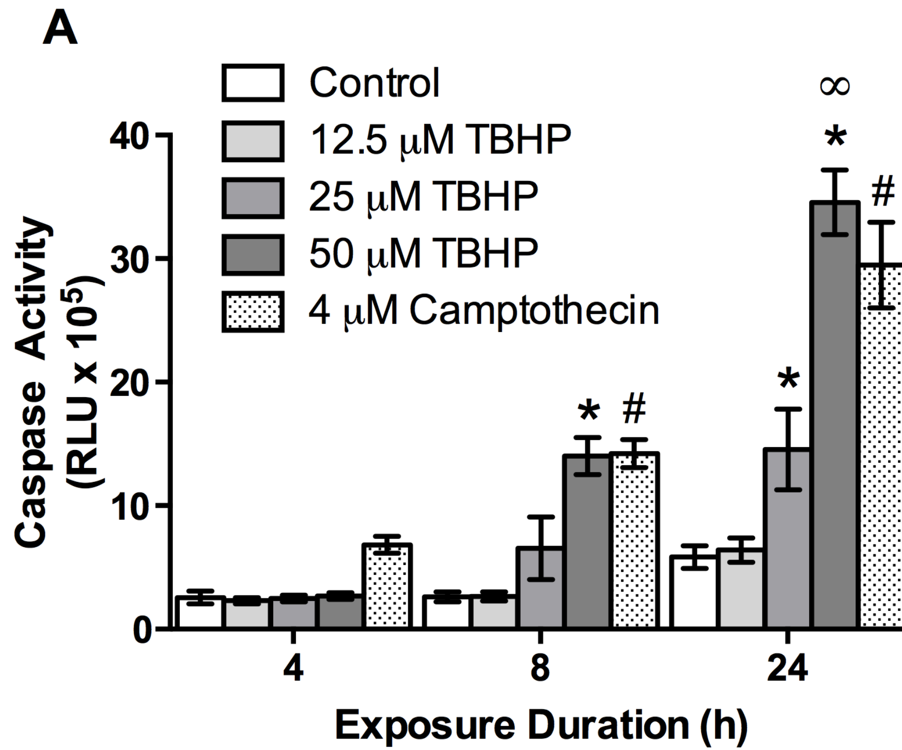
significance of low magnitude changes in the reduction potential is unclear, though it has been suggested that even mild changes in the reduction potential may have physiologic consequences because of the importance of redox-sensitive protein thiols in cellular functions (Dooley et al. 2004). Alternatively, TBHP-induced apoptosis may be occurring independently of GSH.

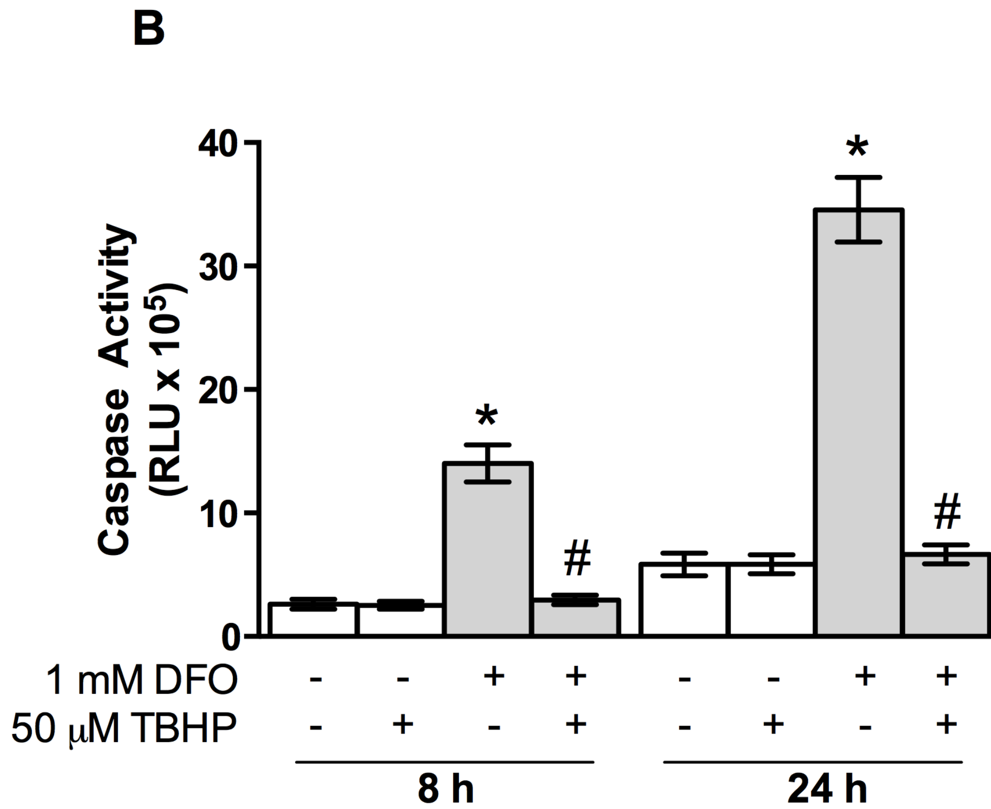
In addition, we examined TBHP effects on ARE-driven reporter gene expression, which can serve as a measure of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factor transactivation (Tan et al. 2007). Under reducing conditions, Nrf2 is targeted for ubiqutination and subsequent proteasomal degradation by Keap1. Upon oxidation or electrophilic attack of sulfhydryl groups on Keap1, Nrf2 then binds ARE and drives transcription of for ubiquitination, This response element is found in a number of genes, including enzymatic antioxidants (Motohashi and Yamamoto 2004). Notably, TBHP stimulated modest increases, less than 50%, in ARE activity. In accordance with TBHP-stimulated Nrf2 activation, Nrf2 nuclear translocation has been observed by immunofluorescence staining in endothelial cells treated with TBHP (Bitar et al. 2012). In contrast, the positive control sulforaphane induced more robust increases of approximately 2 fold over an 8 h time course in our study. Similar increases within this time course have been observed with sulforaphane in HepG2 cells (Hong et al. 2005) and human keratinocytes (Wagner et al. 2010), suggesting that HTR-8/SVneo cells are capable of mounting ARE-driven responses. The biological significance of lower-level induction of ARE activity in HTR-8/SVneo cells by TBHP is unclear. However, Nrf2 has established cytoprotective roles (Baird and Dinkova-Kostova 2011). Therefore, low level Nrf2 induction may not have been sufficient overcome the stress and resultant apoptosis

exacted by TBHP. Additionally, we also found modest increases in expression of transcripts for antioxidant genes TXNRD1 and GLRX2. Because transcription of the latter genes is linked to the ARE (Gallogly et al. 2009; Hintze et al. 2003), the modest increases in TBHP-stimulated ARE activity are consistent with the relatively low-level increases in the TXNRD1 and GLRX2 mRNA responses.

Compared to the robust increase in TBHP-stimulated caspase 3/7 activity, the more direct measures of cellular redox responses (ROS, GSSG, and antioxidant gene expression) are consistently modest. There are several possible explanations for these findings. First, it is possible that these indices are changing over time, but we have missed the window of more robust change. Second, there are a myriad of measures that are used to examine cellular responses to ROS, and we may have chosen ones that are not responsive to TBHP in this HTR-8/SVneo cell system. For example, because TBHP-stimulated $PGE_2$ (see Chapter 3) and IL-6 (see Chapter 4) are blocked by antioxidants that prevent lipid peroxidation (DPPD and BHA), measures of lipid peroxidation may accurately reflect the HTR-8/SVneo response to ROS more accurately. Third, TBHP may be stimulating ROS responses that are in fact very modest, and a modest change is responsible for driving the TBHP-stimulated downstream effects on apoptosis, prostaglandins (see Chapter 3), and cytokines (see Chapter 4). Because TBHP stimulated modest redox responses while still inducing apoptosis, such modest changes may have rather significant implications for the risk of adverse outcomes of pregnancy, like preterm birth. However, it is worth noting that the biological significance of such low-level changes in ROS generation, GSSG, and antioxidant gene expression, though consistent, may be limited or even result in no functional change within the cell.

In summary, we have shown that exposure to the prototypical chemical oxidant TBHP stimulated caspase 3/7 activity, a hallmark of apoptosis. Apoptosis of trophoblast cells may increase risk for preterm deliveries by weakening the structural integrity of the extraplacental membrane tissue or attachment of the placenta to the uterine wall. We also observed modest changes in redox-related responses involving ROS, GSSG, ARE activity, and antioxidant gene expression. Additional studies are needed to determine whether low level changes in cellular redox conditions such as those observed in our study can, in fact, mediate end points of pathophysiologic significance such as gestational cell apoptosis, resulting in significant impacts on pregnancy outcomes.


A
Control
12.5 μM TBHP
25 μM TBHP
50 μM TBHP
4 μM Camptothecin
Caspase Activity
(RLU x 10^5)
40
30
20
10
0
4
8
24
Exposure Duration (h)
∞
*
#


B
Caspase Activity
(RLU x 10^5)
40
30
20
10
0
1 mM DFO
50 μM TBHP
8 h
24 h
*
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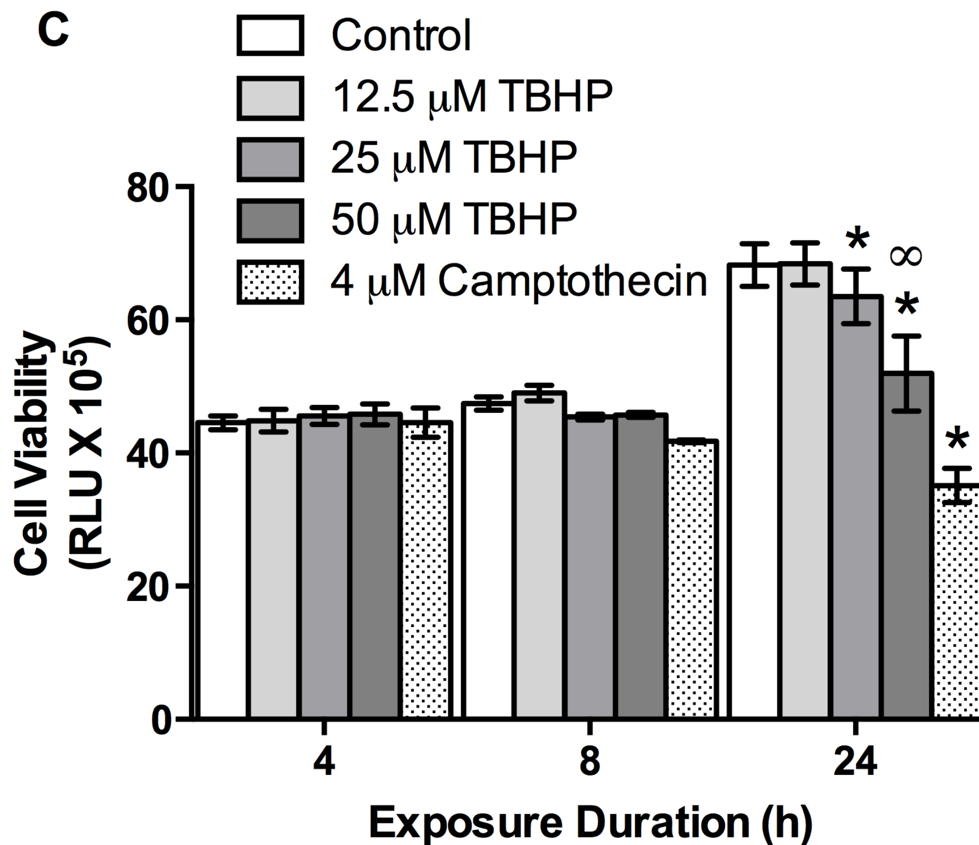



**Figure 2.1. Effects of TBHP on HTR-8/SVneo cell caspase 3/7 activity and viability.** A) Treatment effects of TBHP on caspase 3/7 activity. Cells were exposed to 0, 12.5, 25, or 50 μM TBHP for 4, 8, or 24 h. Caspase 3/7 activity was measured using a luminescence caspase assay. Camptothecin (in 0.1% DMSO vehicle) was included as a positive control for apoptosis. Data are presented as the mean ± SEM of 4 experiments. *, Significantly different compared to time-matched control ($p<0.05$). ∞, Significantly different compared with time-matched cells treated with 25 μM TBHP ($p<0.05$). #, Significantly different compared to time-matched camptothecin vehicle control ($p<0.05$). Camptothecin vehicle control (0.1% DMSO alone) had no significant effect on caspase activity (data are not shown). B) Deferoxamine (DFO) inhibits TBHP-stimulated caspase 3/7 activity. HTR-8/SVneo cells were pretreated with 1mM deferoxamine (DFO) for 1 h, and then exposed to 50 μM TBHP for 8 or 24 h. Filled bars represent treatment with TBHP and unfilled bars represent cells not treated with TBHP. Data are presented as mean ± SEM of 4 experiments. *, Significantly different compared with the untreated controls ($p<0.05$). #, Significantly different compared with TBHP alone treatment ($p<0.05$). C) Effect of TBHP on cellular viability. Cells were exposed to 0, 12.5, 25, or 50 μM TBHP for 4, 8, or 24 h. Cell viability was assessed by a luminescent ATP assay as described in the materials and methods. Camptothecin (in 0.1% DMSO vehicle) was included as a positive control. *, Significantly different compared to time-matched control ($p<0.05$). ∞, Significantly different compared with time-matched 25 μM TBHP-treated cells ($p<0.05$).

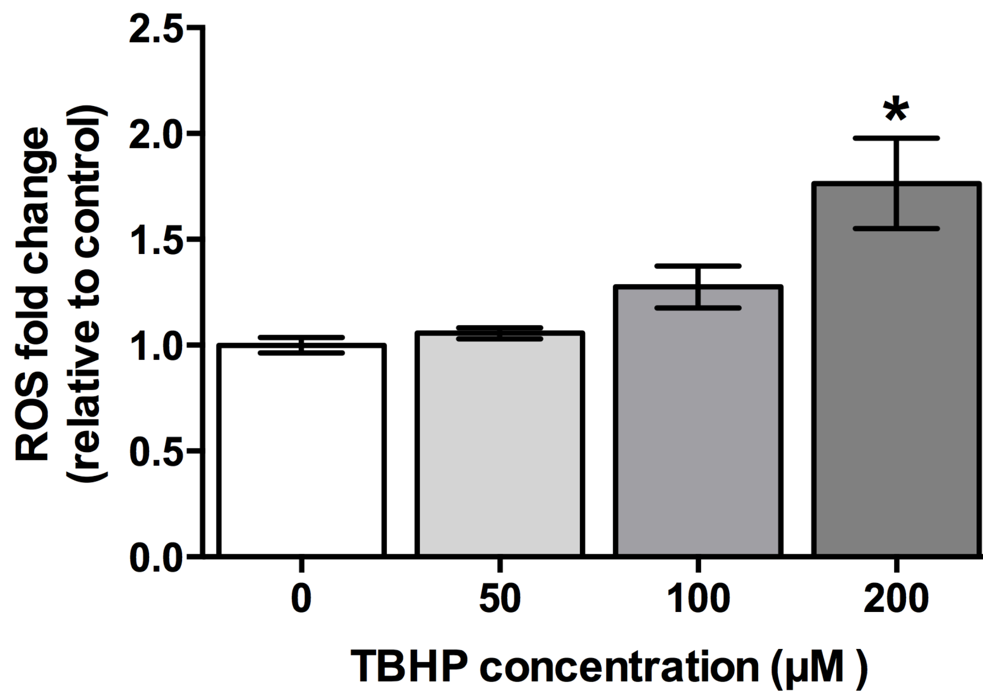
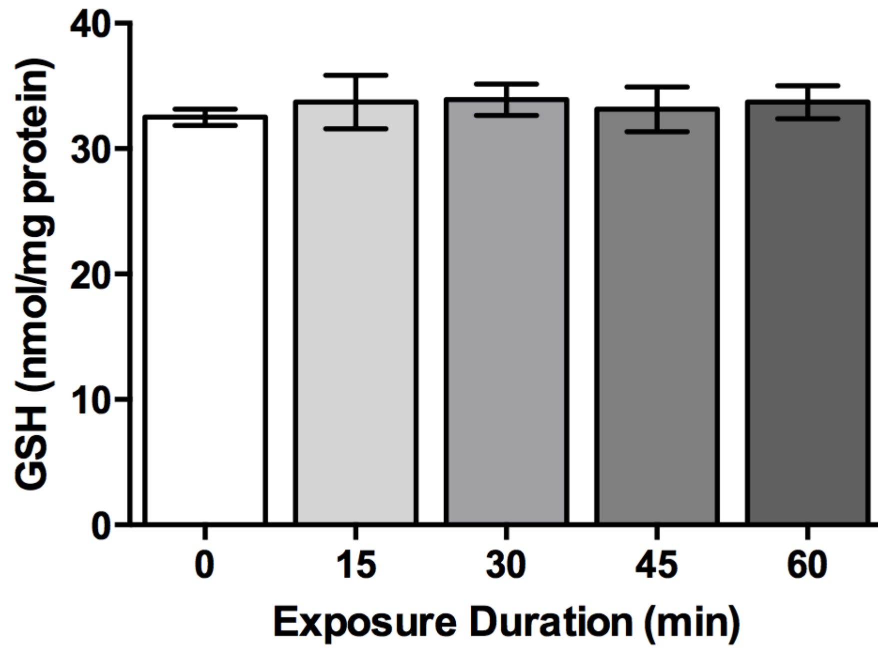



**Figure 2.2. Treatment effects of TBHP on reactive oxygen species (ROS) formation.** Cells were treated with 0, 50, 100, or 200 µM TBHP for 2 h. Detection of ROS was performed using the redox sensitive probe DCF as described in the materials and methods. Data are represented as mean ± SEM fold change in DCF fluorescence relative to the control of 3 experiments. Average control values were 77.0 ± 2.8 relative fluorescence units (RFU). *, Significantly increased compared to untreated control ($p<0.05$).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GSH (nmol/mg protein)
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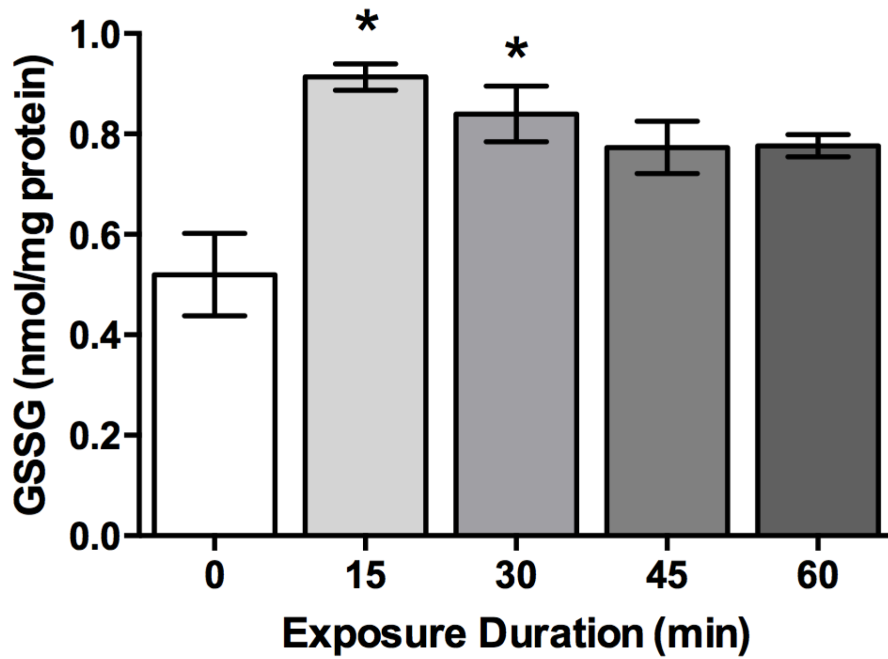
30
20
10
0
0
15
30
45
60
Exposure Duration (min)


B
GSSG (nmol/mg protein)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
0
15
30
45
60
Exposure Duration (min)

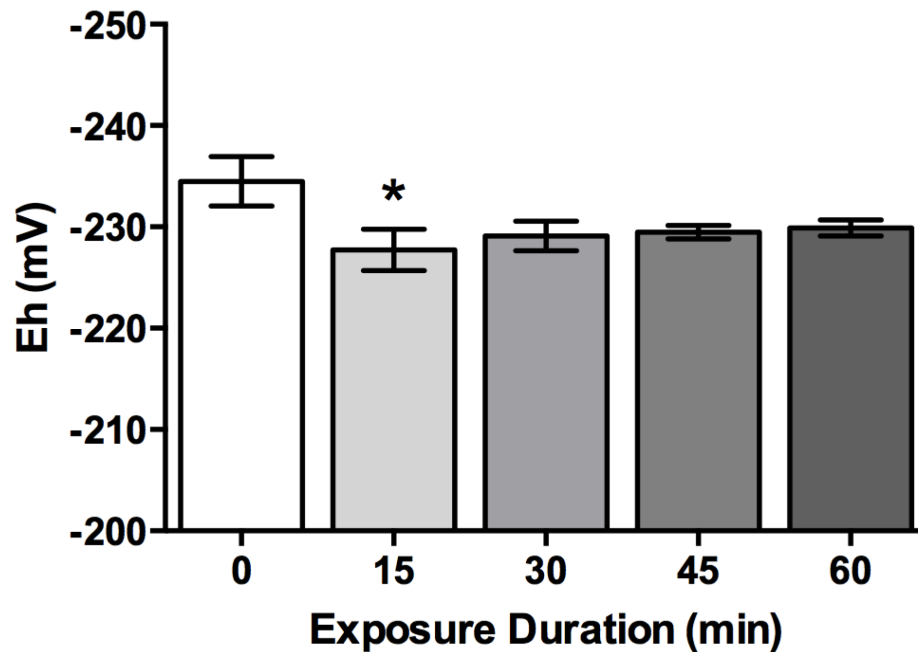



**Figure 2.3. Effect of TBHP on reduced and oxidized glutathione cellular concentrations and the gluathione redox potential (Eh).** HTR-8/SVneo cells were treated with 50 μM TBHP for 0, 15, 30, 45, or 60 min. A) Concentrations of cellular reduced glutathione (GSH) relative to total protein content. B) Concentrations of cellular oxidized glutathione (GSSG) relative to total protein content. C) Intracellular glutathione redox potential ($E_h$). Data are represented as mean ± SEM of 3 experiments. *, Significantly different compared to control (0 min time point) ($p<0.05$).

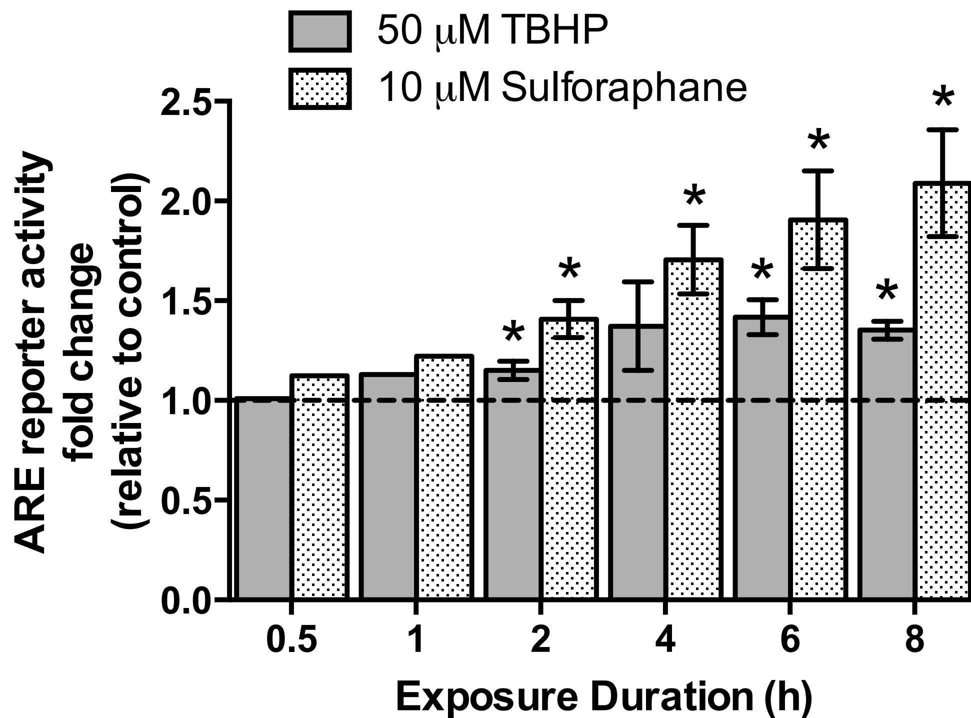



**Figure 2.4. Effect of TBHP on antioxidant response element (ARE) reporter activity.** HTR-8/SVneo cells were treated with 50 μM TBHP for 0.5, 1, 2, 4, 6, or 8 h. Sulforaphane (10 μM) was included as a positive control. Reporter assays were performed as described in the materials and methods. Data are presented as mean ± SEM fold change over the 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 control (cells that did not receive treatment) for each time point. For exposure durations of 0.5 and 1 h, n=1 experiment. For exposure times of 2, 4, and 6 h, n=4 experiments. For the 8 h time point, n=3 experiments. Because of low experimental number, statistical analysis was performed only at 2, 4, 6, and 8 h. *, Significantly different compared to time-matched control ($p<0.05$).

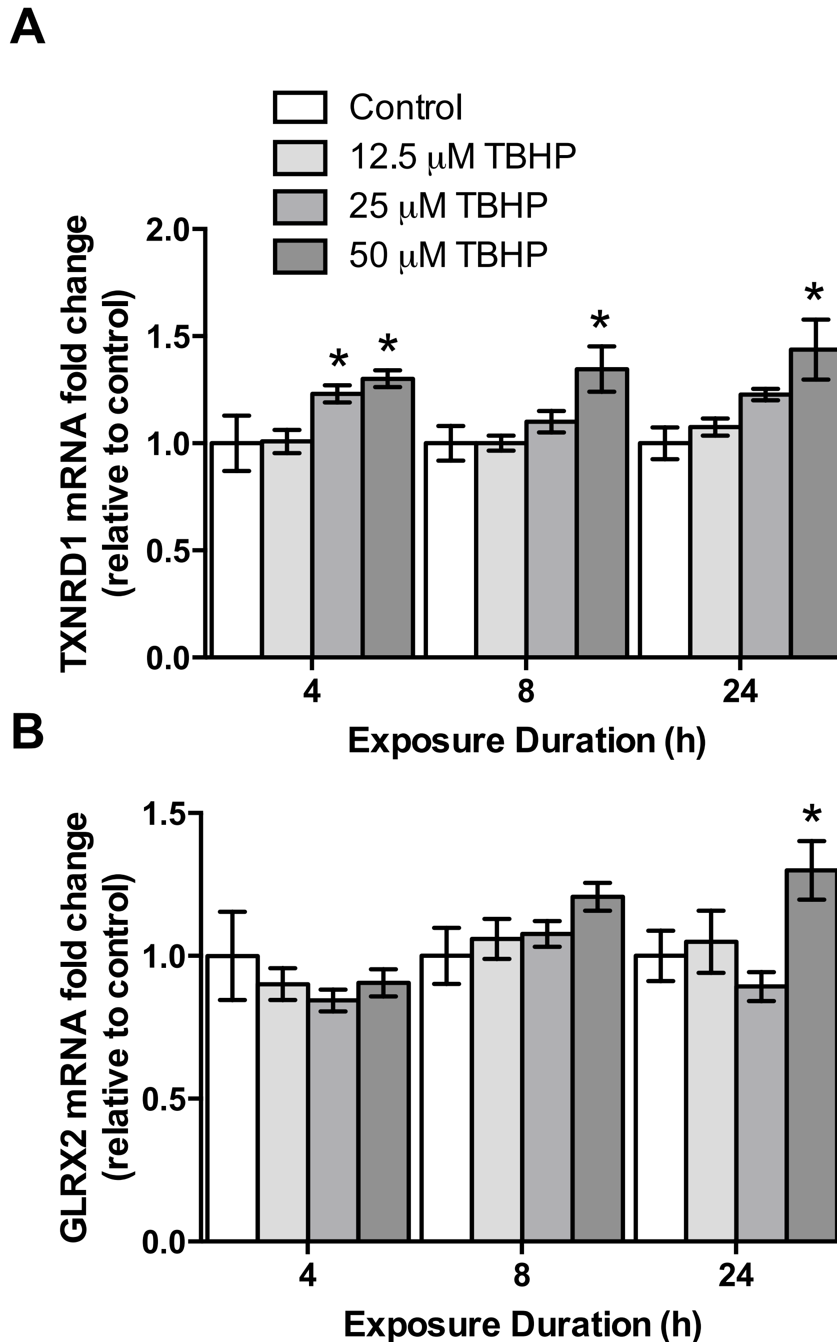



**Figure 2.5. Treatment effects of TBHP on TXNRD1 and GLRX2 mRNA expression.** HTR-8/SVneo cells were treated with 0, 12.5, 25, or 50 μM TBHP for 4, 8, or 24 h. A) Cellular TXNRD1 mRNA expression. B) Cellular GLRX2 mRNA expression. Data are represented as mean ± SEM of 4 experiments. Fold changes were calculated using the ΔΔCt method (Yuan et al. 2006). The target gene from each sample was first normalized to the housekeeping gene B2M, and then fold changes were calculated relative to the normalized control. *, Significantly increased compared to time-matched controls ($p<0.05$).

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# CHAPTER 3

## *TERT*-BUTYL HYDROPEROXIDE STIMULATES $PGE_2$ RELEASE FROM A HUMAN PLACENTAL CELL LINE

## ABSTRACT

Preterm birth is a significant public health problem. Although parturition is associated with biomarkers for damage due to exposure to reactive oxygen species (ROS) within gestational tissues, the impact of ROS on parturition-promoting pathways in gestational cells has received little attention within the literature. The present study examined the effect of oxidative insult on production of prostaglandins (PG), well-established mediators involved in parturition promotion, using the human extravillous trophoblast cell line HTR-8/SVneo and the model ROS-producing chemical *tert*-butyl hydroperoxide (TBHP). After 4, 8 or 24 h exposures to 12.5, 25 or 50 μM TBHP, $PGE_2$ release, mRNA expression of prostaglandin-endoperoxide synthase 2 (PTGS2), hydroxyprostaglandin dehydrogenase 15-(NAD) (HPGD), prostaglandin E synthase (PTGES), and PTGS1 expression were assayed. Treatment with 50 μM TBHP at 4 and 24 h, and 25 μM TBHP at 8 and 24 h, induced significant increases in $PGE_2$ release into the culture medium ($p<0.05$). This was also accompanied by significant increases in PTGS2 mRNA expression at 4, 8 and 24 h and a significant decrease in HPGD mRNA expression at 24 h. Cotreatment with the PTGS inhibitor indomethacin or the antioxidants butylated hydroxyanisole (BHA) and diphenyl phenylenediamine (DPPD) blocked the TBHP-stimulated $PGE_2$ response. Treatment with TBHP increased phosphorylation of

p38 MAPK, indicative of activation, and pretreatment with the p38 MAPK inhibitor PD169316 blocked the TBHP-stimulated increases in $PGE_2$ and PTGS2, suggesting dependence of the prostaglandin response on p38 MAPK activity. In summary, these results suggest that exposure to the ROS-producing chemical TBHP stimulates responses in human placental cells linked to parturition. Because ROS are a common effector in toxic responses, these findings suggest a potential mechanism by which environmental pollutant exposures increase risk for preterm birth.

## INTRODUCTION

Preterm birth is defined as birth before 37 weeks of gestation (World Health Organization 2010). In the United States, it is the leading cause of infant mortality (Callaghan et al. 2006) and accounts for approximately 12% of births (Martin et al. 2012). Furthermore, untimely birth also increases risk for multiple adverse outcomes including respiratory impairments (Baraldi and Filippone 2007) and neurodevelopmental deficits (Colvin et al. 2004). Although infection during pregnancy is acknowledged as a leading cause of preterm birth (Goldenberg and McClure 2010), the etiology is unknown in about half of all cases of preterm birth (Muglia and Katz 2010). Therefore, it has been recommended that additional research is needed to understand the causes of preterm birth (Behrman and Butler 2007). Suggesting that the environment may impact initiation of preterm birth, environmental chemical exposures, including lead (Dietrich 1991; Jelliffe-Pawlowski et al. 2006; Torres-Sánchez et al. 1999), organochlorine pesticides (Pathak et al. 2009; Pathak et al. 2010), and phthalates (Meeker et al. 2009), have been associated with its increased risk.

A potential mechanism, common to many environmental chemicals, that may increase risk for preterm birth is through formation of reactive oxygen species (ROS). Within the cell, ROS can act as second messenger signaling molecules (Poole and Nelson 2008). Oxidation of sensitive thiol residues on proteins can act as a switch, regulating protein activity, and resulting in modulation of signal transduction kinase and transcription factor activity (Poole and Nelson 2008). Reactive oxygen species increase production of prostaglandins and expression of prostaglandin-endoperoxide synthase 2 (PTGS2), the inducible isoform of the PTGS enzymes, in a variety of non-gestational cells and tissues (Adderley and Fitzgerald 1999; Li et al. 2002; Nakamura and Sakamoto 2001), by stimulating mitogen-activated protein kinases (MAPK) and transcription factors including nuclear factor-κB (Adderley and Fitzgerald 1999). Prostaglandins are lipid-signaling molecules that are increased during labor in all mammalian species (Young 2001) and stimulate myometrial contractions, cervical ripening, and membrane ruptures (Olson and Ammann 2007). They are, therefore, critical mediators to the progression of parturition.

Markers for cellular damage due to ROS have been associated with adverse pregnancy outcomes, including preterm birth. Increased urinary excretion of 8-hydroxydeoxyguanosine (8-OHdG), a marker for ROS-induced DNA damage, is associated with decreased gestation length and low birthweight (Stein et al. 2008). Another study finds similar associations between malondialdehyde and protein carbonyl levels in maternal blood and cord blood and preterm birth (Pathak et al. 2010). In addition, it has been speculated that iron-derived ROS due to bleeding during pregnancy may cause preterm birth (Sakata et al. 2008).

Although it has been suggested that preterm birth might be associated with conditions of oxidative stress (Pathak et al. 2010), the mechanistic links between ROS and parturition-promoting pathways within the gestational compartment has received relatively limited attention. The purpose of this study is to examine the effect of a prototypical pro-oxidant chemical, *tert*-butyl hydroperoxide (TBHP), on prostaglandin production from a human placental trophoblast-derived cell line, HTR-8/SVneo.

## MATERIALS AND METHODS

### Materials

*Tert*-butyl hydroperoxide (TBHP; 70%), *N,N′*-diphenyl-p-phenylenediamine (DPPD), butylated hydroxyanisole (BHA), indomethacin, dimethyl sulfoxide (DMSO), Trizma, sodium chloride, Tween 20, Ponceau S solution, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), and 2-mercapthoethanol were 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). Heat-inactivated fetal bovine serum (FBS), RPMI 1640 medium supplemented with L-glutamine and without phenol red, Opti-MEM I Reduced Serum Medium supplemented with L-glutamine and hydroxyethyl piperazineethanesulfonic acid (HEPES) and without phenol red, penicillin/streptomycin (10,000 units/mL and 10,000 μg/mL, respectively), and Dulbecco's phosphate-buffered saline (dPBS) were purchased from Gibco Life Technologies (Carlsbad, CA). We purchased prostaglandin enzyme immunoassay (EIA) kits from Cayman Chemical (Ann Arbor, MI). QIAshredder columns and RNeasy kits were from Qiagen (Germantown, MD). We used $RT^2$ First Strand Kits, $RT^2$ SYBR Green qPCR Mastermix, prostaglandin-endoperoxide synthase 1(PTGS 1),

PTGS2, prostaglandin E synthase (PTGES), hydroxyprostaglandin dehydrogenase 15-(NAD) (HPGD), and β-2-microglobulin (B2M) $RT^2$ qPCR Primer Assays from SABiosciences, Qiagen (Germantown, MD). IGEPAL CA-630 (NP-40 substitute) 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). We purchased antibodies for phospho-p38 MAPK (Thr180/Tyr182), total p38 MAPK, and β-tubulin, as well as alkaline phosphatase-linked secondary antibody, from Cell Signaling Technology (Beverly, MA). Enhanced chemifluorecence (ECF) substrate and PVDF membrane Hybond-P were from GE Healthcare Life Sciences (Pittsburgh, PA).

**Cell culture and treatment**

The human trophoblast cell line HTR-8/SVneo was kindly provided by Dr. Charles H. Graham, Queen's University, Kingston, Ontario, Canada. This immortalized cell line consists of first trimester extravillous trophoblast-derived cells transfected with the SV40 T antigen (Graham et al. 1993). HTR-8/SVneo cells are known to have similar phenotypes to their primary counterparts; they express HLA-G (Khan et al. 2011; Kilburn et al. 2000) antigens, produce human chorionic gonadotropin, urokinase-plasminogen activator (Graham et al. 1993), and matrix metalloproteinases (Jovanović and Vićovac 2009; Jovanović et al. 2010), and cellular migration is controlled in response to similar stimulation (Jovanović and Vićovac 2009; Jovanović et al. 2010; Nicola et al. 2005; Paiva et al. 2009).

HTR-8/SVneo cells were maintained as previously described (Tetz et al. 2013). Briefly, HTR-8/SVneo cells between passages 73-87 were incubated in a humidified environment at 37°C with 5% $CO_2$ in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were allowed to reach about 70-80% confluence 24 h after subculture before starting an experiment. For time course experiments, 500,000 cell/well were seeded in 6-well plates. For antioxidant and MAPK inhibitor experiments, HTR-8/SVneo cells were seeded at a density of 100,000 cells/well in 24-well plates. Cells were seeded at a density of $1x10^6$ in 60 mm dishes for western blotting experiments.

*Tert*-butyl hydroperoxide was used in these experiments as a model ROS-producing chemical because of its ability to cross the plasma membrane and generate ROS intracellularly (Ahmed-Choudhury et al. 1998). For time course experiments, culture medium was removed by aspiration and cells were treated with 12.5, 25, or 50 µM TBHP, diluted directly into culture medium. For experiments involving inhibitors, vehicle controls (0.1-0.4% DMSO) and no treatment controls were run in parallel to treatment groups. Cells were pretreated with the MAPK inhibitors PD169316 (10 µM), U0126 (5 µM), and SP600125 (5 µM) for 1 h before removal and subsequent exposure to TBHP. Concentrations of MAPK inhibitors were chosen based on efficacy in previous studies (Shaul and Seger 2004; Renaud et al. 2009). In experiments with antioxidants or indomethacin, cells were cotreated with TBHP. In western blotting experiments, cells were exposed to reduced serum to reduce basal phosphorylation (Shaul and Seger 2004) in OptiMEM I supplemented with 1% FBS for 18 h before treatment.

**Enzyme immunoassays**

Following conclusion of treatment, cell culture medium was collected, clarified by centrifugation, aliquoted, and stored at -80°C until analysis. In some experiments, experimental replicates were combined before analysis. Quantification of prostaglandin content was performed by enzyme immunoassay (EIA) according to manufacturer's instructions. 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.

**Quantitative real-time PCR**

Following removal of culture medium, cells were homogenized using Qiashredder columns, replicate homogenates were pooled, and RNA was extracted using RNeasy columns according to manufacturer's instructions. One microgram total RNA was reverse transcribed using $RT^2$ first strand kits according to manufacturer's instructions. Quantitative RT-PCR reactions were performed on a Bio-Rad CFX Connect Real-Time System or Bio-Rad CFX96 Real-Time System C1000 thermal cyclers according to $RT^2$ SYBR Green Mastermix directions. The housekeeping gene B2M was run in parallel reactions as a means of normalization. Fold changes in target mRNA expression was quantified using the ΔΔCt method (Yuan et al. 2006) on CFX manager software version 3.0 (Bio-Rad, Hercules, CA). All samples were assayed in triplicate.

**Western blot**

After treatments were removed, cells were washed twice with ice-cold dPBS, incubated with lysis buffer (0.5% IGEPAL, 250 mM NaCl, 50 mM tris-HCl, with 2X protease and phosphatase inhibitor tablets), cells were scraped, and cellular protein was collected. Lysates were clarified, aliquoted, and stored at -80°C until analysis. Total

protein was quantified by BCA assay. Protein was boiled in sample buffer, 25 μg protein was subjected to SDS-polyacrylamide gel electrophoresis, and electrotransfer to 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 wash, 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. Blots were stripped before reprobing for total p38 MAPK using stripping buffer (100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.4 mM Tris-HCl, pH 6.7) with constant agitation at 50°C for 30 min. Images shown are representative of 3 individual experiments. Densitometry was used to semi-quantitate data with ImageJ software (National Institutes of Health) as described previously (Miller 2010).

**Statistics**

Data were analyzed using SigmaPlot version 11.0 (Systat Software, Chicago, IL) by repeated-measures one-way or two-way analysis of variance (ANOVA) with Tukey post-hoc analysis, where appropriate. In cases where data were non-normal or had unequal variance, data were log-transformed before analysis. For qRT-PCR analysis, ΔCt values were the statistics utilized for analysis (Yuan et al. 2006).

## RESULTS

### Effect of TBHP stimulates on HTR-8/SVneo $PGE_2$ release and PTGS2 mRNA expression

HTR-8/SVneo cells were treated with 12.5, 25, or 50 μM TBHP for 4, 8, or 24 h. Treatment with 12.5 μM TBHP failed to stimulate significant increases in $PGE_2$ at any of the time points assayed (Figure 3.1A). Treatment with 50 μM TBHP stimulated significant increases of 7.2, 2.8, and 4.8 fold at 4, 8, and 24 h, respectively ($p<0.05$). Treatment with 25 μM TBHP significantly increased $PGE_2$ release by 3.1 fold at 8 h and 2.7 fold at 24 h ($p<0.05$). With 24 h exposure, the stimulated $PGE_2$ increase with 50 μM TBHP was significantly greater than the increase due to 25 μM ($p=0.010$).

Because prostaglandin production is rate-limited by PTGS2 activity, we assessed changes in PTGS2 mRNA expression following treatment with TBHP. Treatment with 12.5 and 25 μM TBHP failed to stimulate significant increases in PTGS2 mRNA (Figure 3.1B). However, treatment with 50 μM TBHP stimulated significant increases of 10.6, 7.6 and 3.2 fold increases compared with time-matched controls at 4, 8, and 24 h ($p<0.05$).

Next, we examined the effect of TBHP on mRNA expression of other enzymes in the $PGE_2$ metabolic pathway. Treatment with 50 μM TBHP stimulated a significant decrease in the mRNA expression in the prostaglandin catabolic enzyme HPGD with 24 h treatment (Figure 3.2; $p=0.02$). We also assessed TBHP treatment effects on mRNA expression of PTGS1 and PTGES, but observed no significant changes (see Appendix A.1).

### Effect of PTGS inhibitor on TBHP-stimulated $PGE_2$ release

We assessed dependence of TBHP-stimulated $PGE_2$ release on increased PTGS2 activity using indomethacin, a PTGS inhibitor (Vane et al. 1998). Indomethacin significantly blocked TBHP-stimulated $PGE_2$ release at 4 and 24 h ($p<0.05$), returning $PGE_2$ to levels near those observed with vehicle controls (Figure 3.3A and B; $p<0.05$). A 24 h coincubation of TBHP with the inhibitor vehicle (1% DMSO) decreased stimulated $PGE_2$ compared to controls incubated with TBHP alone ($p<0.05$). This is not altogether surprising considering that DMSO can act as an antioxidant (Repine et al. 1979; Vlahopoulos et al. 1999). We did not observe any significant changes in PTGS2 mRNA expression due to cotreatment with indomethacin (data not shown), consistent with a previous report that indomethacin does not exert its effect at the transcriptional level (Vane et al. 1998).

**Antioxidant effects on TBHP-stimulated $PGE_2$ release**

We assessed dependence of $PGE_2$ release on oxidative mediators by cotreating cells with TBHP and the antioxidants BHA or DPPD. Cotreatment with BHA or DPPD completely prevented TBHP-stimulated release of $PGE_2$ compared to the TBHP + vehicle control (Figure 3.4A and B; $p<0.05$).

**Effects of MAPK inhibitors on TBHP-stimulated $PGE_2$ release and PTGS2 expression**

The MAPKs are a family of redox-sensitive signal transduction enzymes (McCubrey et al. 2006) whose activity can mediate prostaglandin production (Smith et al. 2000). HTR-8/SVneo cells were pretreated with MAPK inhibitors and then treated with 50 μM TBHP for 4 or 24 h. Pretreatment with the p38 MAPK inhibitor PD169316 significantly decreased TBHP-stimulated $PGE_2$ at 4 and 24 h (Figure 3.5A and B;

$p<0.05$). Both the ERK 1/2 inhibitor U0126 and the JNK inhibitor SP600125 failed to significantly decrease TBHP stimulated $PGE_2$.

We also examined the effects of MAPK inhibitors on HTR-8/SVneo PTGS2 mRNA expression. Pretreatment with 10 μM PD169316 significantly blocked TBHP-induced PTGS2 mRNA expression compared with the TBHP + vehicle control (Figure 3.5C; $p<0.05$). Similar to observations with $PGE_2$ release, pretreatment with U0126 and SP600125 failed to significantly decrease PTGS2 mRNA. We also observed that pretreatment with PD169316 and SP600125 significantly decreased basal mRNA expression by 64 and 38% ($p<0.05$).

**p38 MAPK phosphorylation**

To document TBHP-stimulated activation of p38 MAPK, phosphorylation of p38 was assessed by western blot following treatment of HTR-8/SVneo cells with 50 μM TBHP. Treatment at all exposure durations, except 1 h, resulted in significant increases in phosphorylation of p38 (Figure 3.6A and B; $p<0.05$). There were no significant changes in total p38 (Figure 3.6A, data not shown).

## DISCUSSION

The role of ROS in stimulation of parturition-associated pathways in cells of human gestation has been scarcely studied. The purpose of this study is to examine the effect of the prototypical pro-oxidant chemical TBHP on parturition-promoting pathways involving prostaglandin synthesis in a human gestational cell model.

Because of its importance in the progression of labor (Olson and Ammann 2007), we studied $PGE_2$ release following exposure to TBHP. In accordance with previous studies with HTR-8/SVneo cells, basal levels of $PGE_2$ were very low or undetectable

(Horita et al. 2007; Nicola et al. 2005). Treatment with TBHP stimulated increases within 4 h of exposure and these increases were sustained through 8 and 24 h. Consistent with our findings, a different ROS-producing oxidant chemical, $H_2O_2$, stimulates $PGE_2$ increases from pig endothelium (Thengchaisri and Kuo 2003), mouse kidney cells (Soodvilai et al. 2007), and mouse embryonic stem cells (Lee et al. 2009). In contrast, exposure to $H_2O_2$ resulted in decreased prostanoid production, including $PGE_2$, from human vessel segments (Yamaja Setty et al. 1984). However, within the gestational compartment, less is known about the impact of oxidative insult on prostaglandin synthesis. $PGE_2$ is stimulated by $H_2O_2$ in human amnion epithelium, amnion fibroblast (Kumar et al. 2004b), and amnion-derived WISH cells (Kumar et al. 2004a). In rat corpus luteum, lipid hydroperoxides also induce $PGE_2$ release (Kodaman et al. 1994). Results presented here are consistent with the limited studies of cells derived from gestational tissues, and suggest that $PGE_2$ may be produced as a result of exposure to ROS-producing chemicals.

A key step in the biosynthesis of prostaglandins is the conversion of arachidonic acid to $PGH_2$, catalyzed by cyclooxygenases PTGS1 and PTGS2 (Chandrasekharan and Simmons 2004; Smith et al. 2000; Vane et al. 1998). $PGH_2$ is then isomerized to $PGE_2$ by prostaglandin E synthases (Park et al. 2006). We found increased mRNA expression of PTGS2 following treatment with TBHP. Consistent with our findings, expression of PTGS2, the inducible cyclooxygenase isoform (Dubois et al. 1998), is increased under with ROS in rat cardiomyocytes (Adderley and Fitzgerald 1999) and bovine luteal cells (Nakamura and Sakamoto 2001). Furthermore, observed increases in PTGS2 mRNA following TBHP were similar in magnitude to those found during labor: PTGS2 is

increased 50% in myometrium (Choi et al. 2007), 2 fold in placenta (Cindrova-Davies et al. 2007b), and over 10 fold in gestational membranes (Mijovic et al. 1998). Indomethacin, a non-selective inhibitor (Johnson et al. 1995) of PTGS activity (Vane 1998), significantly decreased TBHP-stimulated $PGE_2$ release, supporting dependence of $PGE_2$ release on PTGS activity. We found no significant changes in mRNA expression of PTGES (also known as mPGES-1; Appendix A, Figure A.1B), an inducible, predominant prostaglandin E synthase isoform (Park et al. 2006). The latter finding fails to support a role for increased PTGES enzymes in TBHP-stimulated $PGE_2$, suggesting that the increase is regulated upstream of PTGES. Because there is an associated induction of PTGS2 but not PTGS1 mRNA (Appendix A, Figure A.1A), we posit that TBHP-stimulated $PGE_2$ release is due, at least in part, to PTGS2 enzymatic activity. Indeed, in mouse embryonic stem cells, $H_2O_2$-stimulated $PGE_2$ is mediated by PTGS2 activity (Lee et al. 2009).

We found a significant decrease in mRNA expression of the prostaglandin catabolic enzyme HPGD. In addition to increased PTGS activity, diminished $PGE_2$ catabolic activity (Tai et al. 2006) could contribute to the increased $PGE_2$ concentrations observed following treatment with TBHP. During pregnancy, HPGD expression in the chorion of the extraplacental membranes remains elevated until parturition (Challis et al. 2002; Challis 2000), then decreases during parturition (Germain et al. 1994; Johnson et al. 2004; Sangha et al. 1994). Thus, decreases in HPGD protein expression in the extraplacental membranes may contribute to increased prostaglandin concentrations observed during labor (Challis 1999). Results from the present study are the first to

suggest that ROS may be involved in the regulated decrease in expression of HPGD observed during labor.

TBHP is a model oxidant chemical popular for experiments with a variety of cells and tissues. TBHP produces oxygen-containing peroxyl and alkoxyl radicals in a reaction catalyzed by iron (Van der Zee et al. 1989). It stimulates responses sensitive to alterations in the cellular redox state, including depletion of reduced glutathione (Buc-Calderon et al. 1991; Eklow et al. 1984; Lam et al. 2011; Martín et al. 2001), lipid peroxidation (Buc-Calderon et al. 1991; Eaddy et al. 2012; Haidara et al. 2002; Kanupriya et al. 2007; Martín et al. 2001; Yajima et al. 1995), and apoptosis (Azizi et al. 2011; Haidara et al. 2002; Kalia and Bansal 2008; Kanupriya et al. 2007). DPPD and BHA, which inhibited TBHP-stimulated $PGE_2$ in the present study, are lipophilic, inhibit lipid peroxidation (Clark et al. 1985; Sogabe et al. 1996; Verhagen et al. 1991), and prevent oxidative membrane damage (Catalá 2009) in some cells. Because TBHP stimulated both $PGE_2$ release and PTGS2 mRNA expression, one mechanism by which BHA and DPPD may be blocking $PGE_2$ production is by directly scavenging TBHP-derived ROS that drive PTGS2 expression. Along with PTGS activity, prostaglandin production is also limited by release of arachidonic acid from the cell membrane (Balsinde et al. 1999). Thus, another point of possible regulation for $PGE_2$ production is via lipid peroxidation stimulated release of arachidonic acid. Indeed, lipid peroxidation is known to free arachidonate from cell membranes by stimulating phospholipase $A_2$ ($PLA_2$) activity in murine fibroblasts (van Rossum et al. 2004), macrophages (Martinez and Moreno 1996), and mesangial cells (Han et al. 2003) treated with $H_2O_2$ or superoxide. One study in human lung fibroblasts suggests that TBHP-stimulated prostaglandin release involves

regulation at two points in the prostaglandin synthetic pathway: release of arachidonic acid from the cell membrane and conversion of arachidonic acid to final prostaglandin products (Taylor et al. 1983). More studies are needed to determine whether TBHP stimulates $PGE_2$ from HTR-8/SVneo cells in a similar two-step manner. An additional point of regulation at the cell membrane level may explain why we observed significant increases in $PGE_2$ production with 25 μM TBHP, yet PTGS2 mRNA expression was not significantly increased with the same exposure.

MAPKs are speculated to participate in pathways associated with parturition. Increased p38 MAPK phosphorylation is observed in labored cervical stroma (Wang and Stjernholm 2007), amnion, chorion trophoblasts (Lappas et al. 2011), decidual stroma, and myometrium (Takanami-Ohnishi et al. 2001). Here we found that TBHP-stimulated $PGE_2$ release and PTGS2 mRNA expression were inhibited by PD169316, a pharmacologic inhibitor of p38 MAPK. Adding further weight to the potential involvement of p38 MAPK in TBHP-stimulated prostaglandin synthesis, TBHP stimulated phosphorylation of p38 MAPK, indicating activation of p38 MAPK (Roux and Blenis 2004). Consistent with this study, placental explants exposed to hypoxia reperfusion injury have increased p38 MAPK phosphorylation and PTGS2 protein expression, which are inhibited by PD169316 (Cindrova-Davies et al. 2007a). Taken together, these findings suggest that p38 MAPK may be a key parturition signaling protein whose activity can be stimulated by ROS-producing chemicals. However, future studies are needed to examine the mechanistic signaling targets in the pathway linking TBHP-stimulated lipid peroxidation, ROS, and p38 MAPK activity. One potential

molecular target includes the MAPK regulatory protein phosphatases whose activity is inhibited upon cysteine oxidation within their catalytic domains (Salmeen 2005).

We recognize that there are limitations to this study. First, the levels of $PGE_2$ detected in this study are rather low, perhaps because of the low prostaglandin synthetic potential of HTR-8/SVneo cells (Horita et al. 2007; Nicola et al. 2005). In addition, because the HTR-8/SVneo cell line used in this study is an immortalized cell line derived from first trimester extravillous trophoblasts, targets identified in these experiments should be validated in late gestation primary cells and tissues that may be more relevant to premature birth. Moreover, recent publications suggest the epigenetic (Novakovic et al. 2011) and transcriptomic (Bilban et al. 2010) profiles of HTR-8/SVneo cells differ from primary extravillous trophoblasts. Nevertheless, HTR-8/SVneo cells have proven to be a useful cell culture model for the study of trophoblast physiology (Hannan et al. 2010). Furthermore, HTR-8/SVneo cells are phenotypically similar to primary cells. Specifically, primary extravillous trophoblast responses to interleukin (IL)-6 (Jovanović and Vićovac 2009), IL-8 (Jovanović et al. 2010), and $PGE_2$ (Nicola et al. 2005) are similar to that of HTR-8/SVneo cells.

Moreover, these results may have implications for adverse pregnancy outcomes due to early events of pregnancy. In one study with the HTR-8/SVneo cells, $PGE_2$ inhibited trophoblast invasion and migration (Biondi et al. 2006), whereas others have shown stimulatory effects (Horita et al. 2007; Nicola et al. 2005; Nicola et al. 2008a; Nicola et al. 2008b). Dysregulation of extravillous trophoblast migration and, as a consequence, incomplete spiral artery transformation, have been implicated in the pathogenesis of preeclampsia (Merviel et al. 2004; Wang et al. 2009).

In sum, we found that exposure of a human trophoblast-derived cell line to the ROS-producing chemical TBHP induced synthesis of prostaglandins, mediators of particular relevance to the initiation of parturition. As such, stimulation of prostaglandin production may then increase risk for preterm birth by initiating myometrial contractions. Environmental chemical exposure has been linked to preterm birth in epidemiology studies (Dietrich 1991; Jelliffe-Pawlowski et al. 2006; Meeker et al. 2009; Pathak et al. 2009; Pathak et al. 2010; Torres-Sánchez et al. 1999). Such chemicals, including phthalates (Ferguson et al. 2011; Kasahara et al. 2002; Seo et al. 2004; Tetz et al. 2013), lead (Ercal et al. 2001; Jomova and Valko 2011; Liu et al. 2010), and organochlorine pesticides (Ahmed et al. 2008; Banerjee et al. 2001; Pathak et al. 2010; Siddharth et al. 2012), have been shown to stimulate production of ROS. Therefore, the present study identifies a potential mechanistic explanation for some reported associations between environmental chemicals and preterm birth that warrants further research.

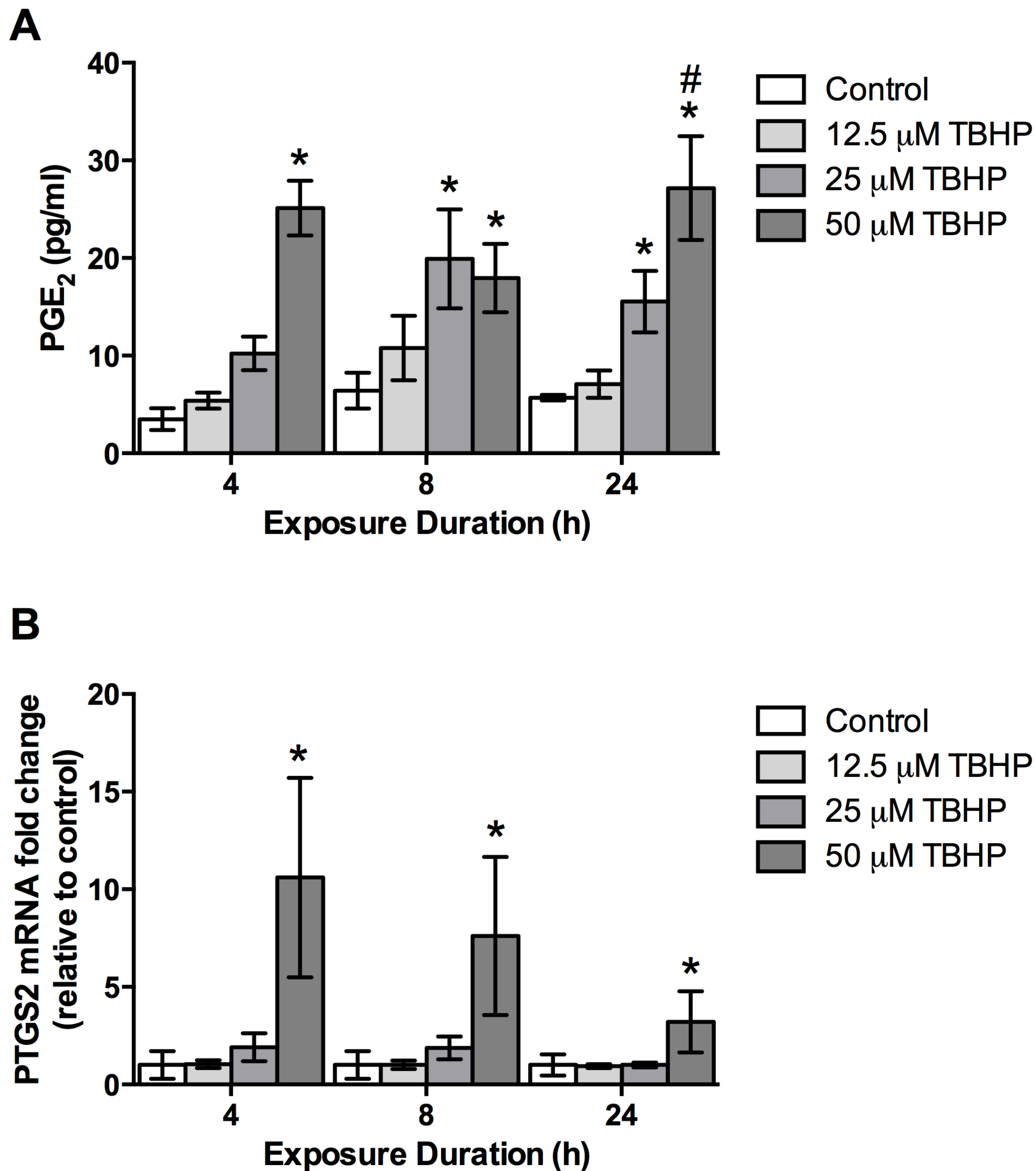



**Figure 3.1. Treatment effects of TBHP on $PGE_2$ release and PTGS2 mRNA expression.** HTR-8/SVneo cells were treated with 0, 12.5, 25, or 50 μM TBHP for 4, 8, or 24 h. A) PGE2 concentrations in culture medium. B) Cellular PTGS2 mRNA expression. Data are represented as mean ± SEM of 3-4 experiments. Fold changes in PTGS2 mRNA expression were calculated using the ΔΔCt method (Yuan et al. 2006). To calculate fold changes in mRNA expression, the Ct values for each sample were normalized to its own housekeeping gene control, and then normalized its respective time-matched control. *, Significantly different compared to time-matched controls ($p<0.05$). #, Significantly different compared to time-matched 25 μM TBHP treated cells $p<0.05$.

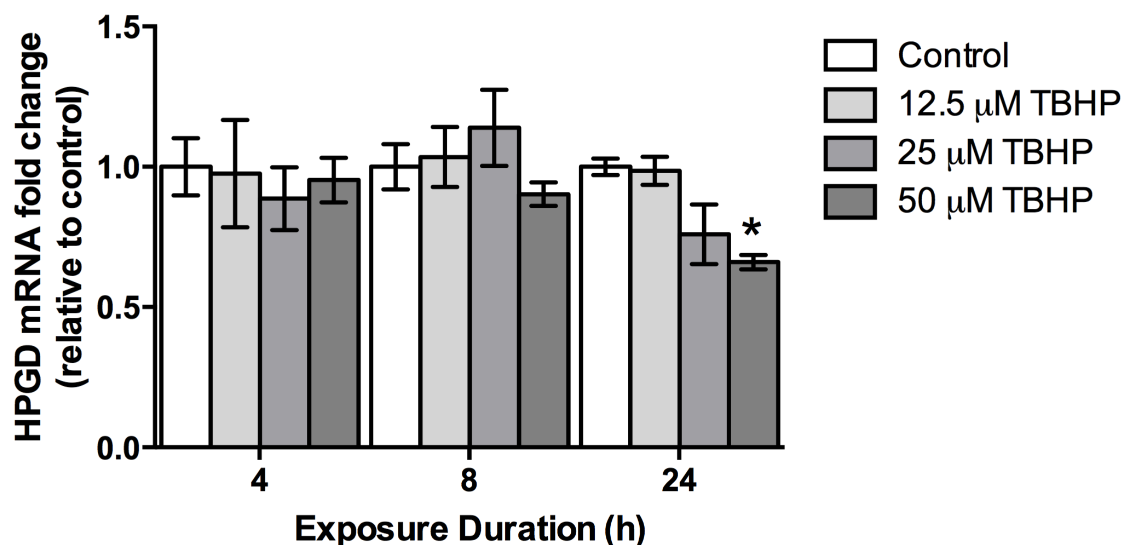
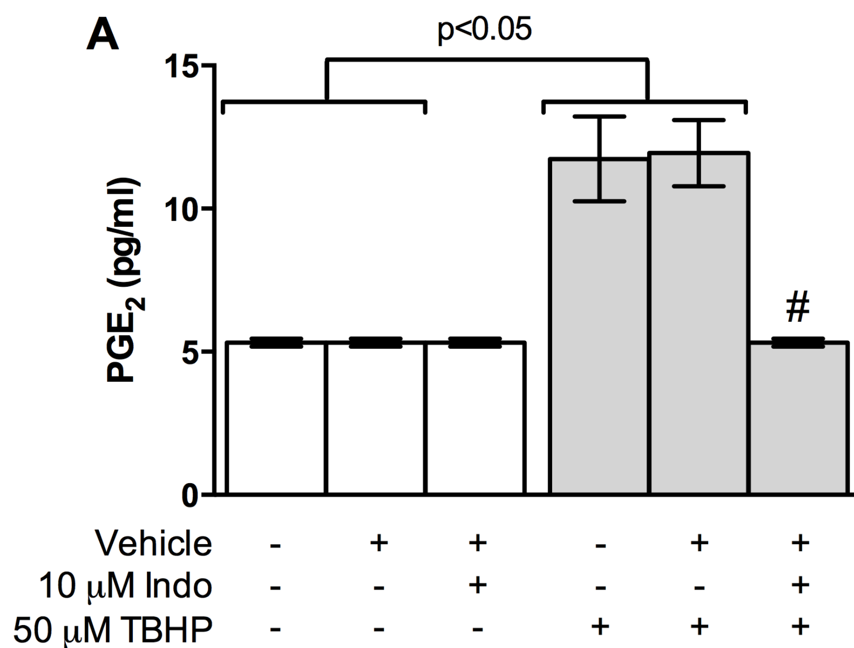



**Figure 3.2. Effect of TBHP on prostaglandin metabolic enzyme mRNA expression.** Changes in HPGD were measured in HTR-8/SVneo cells following treatment with 12.5, 25, or 50 μM TBHP for 4, 8, or 24 h. Fold changes in HPGD mRNA expression were calculated using the ΔΔCt method (Yuan et al. 2006). Values represent mean fold changes ± SEM for 3 experiments. *, Significantly different $p<0.05$ compared with time-matched control ($p<0.05$).



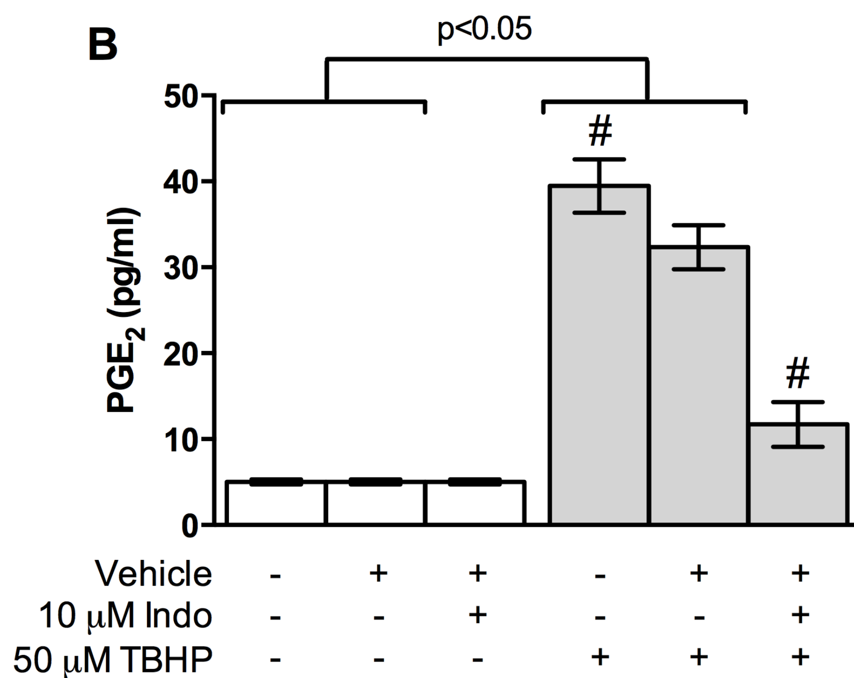




**Figure 3.3. Effect of indomethacin (Indo) on TBHP stimulated $PGE_2$ release.** HTR-8/SVneo cells were cotreated with 50 µM TBHP (filled bars) or without TBHP (open bars), and vehicle (0.1% DMSO), or the PTGS inhibitor indomethacin (10 µM) for A) 4 h or B) 24 h. Bars represent mean ± SEM $PGE_2$ concentration in culture medium for 3 experiments. #, Signficantly different compared to vehicle + TBHP control ($p<0.05$).

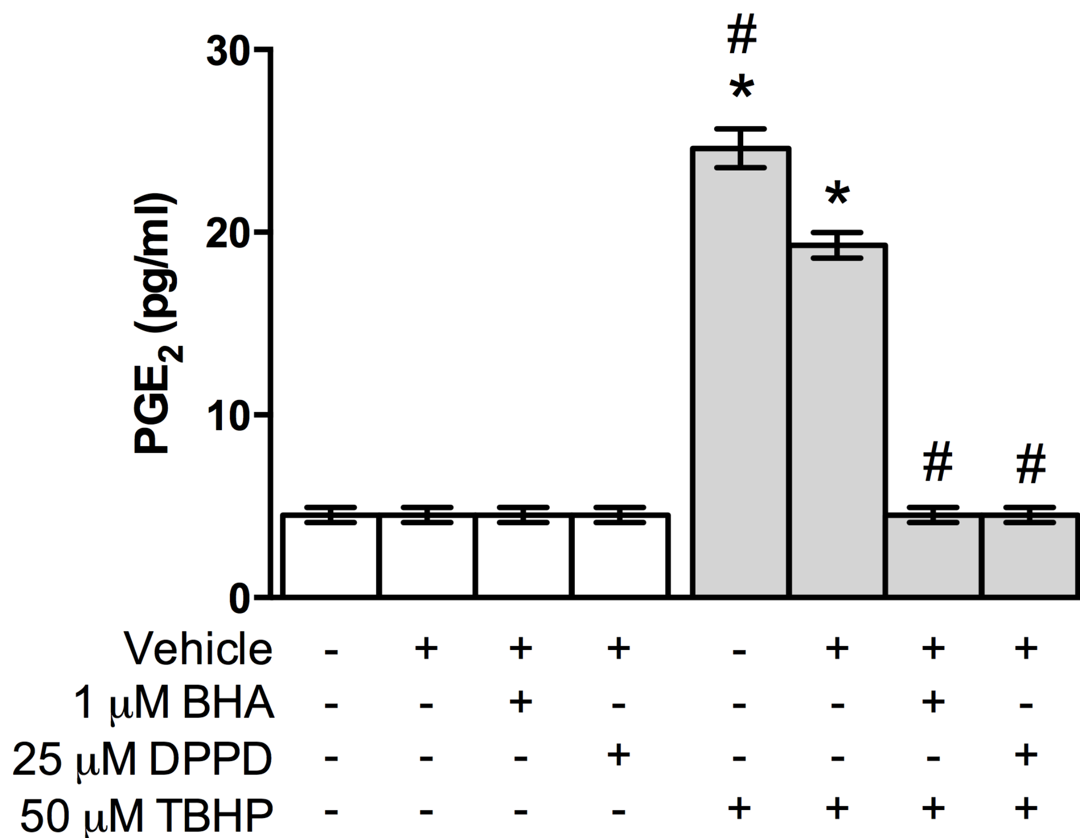
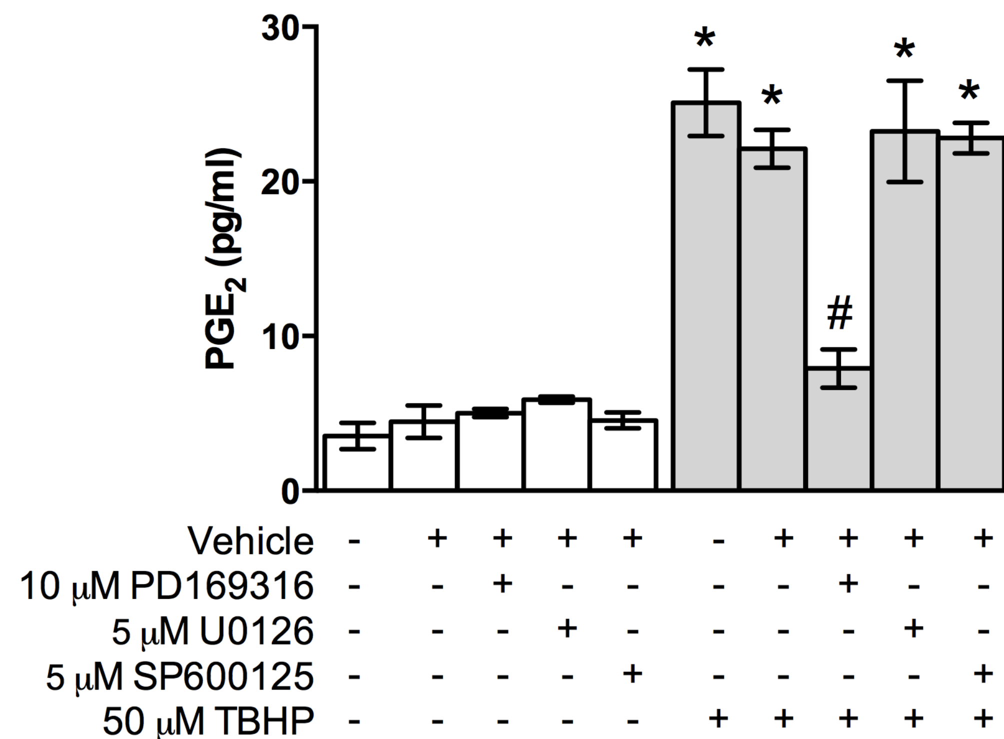



**Figure 3.4. Effect of antioxidants on TBHP-stimulated $PGE_2$.** HTR-8/SVneo cells were cotreated with 50 μM TBHP (gray bars), vehicle (0.1% DMSO), 1 μM butylated hydroxyanisole (BHA), or 25 μM diphenyl phenylenediamine (DPPD). Data represent mean ± SEM $PGE_2$ concentration in culture medium for 3 experiments. *, Significantly different compared with cotreatment matched control ($p<0.05$). #, Significantly different compared to vehicle + TBHP control ($p<0.05$).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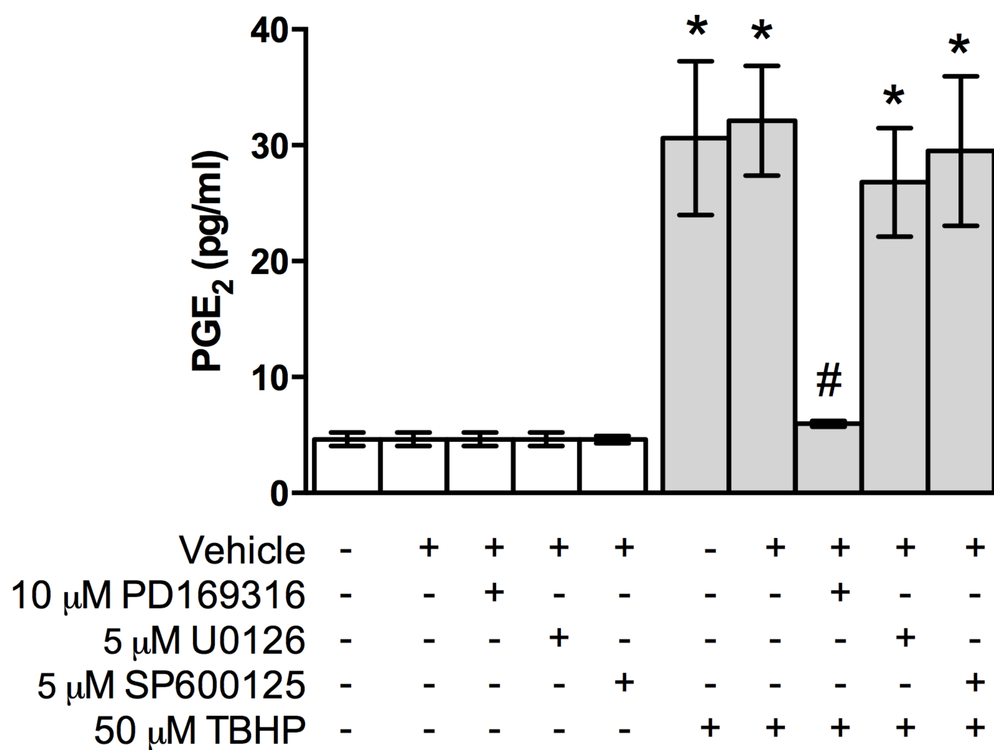
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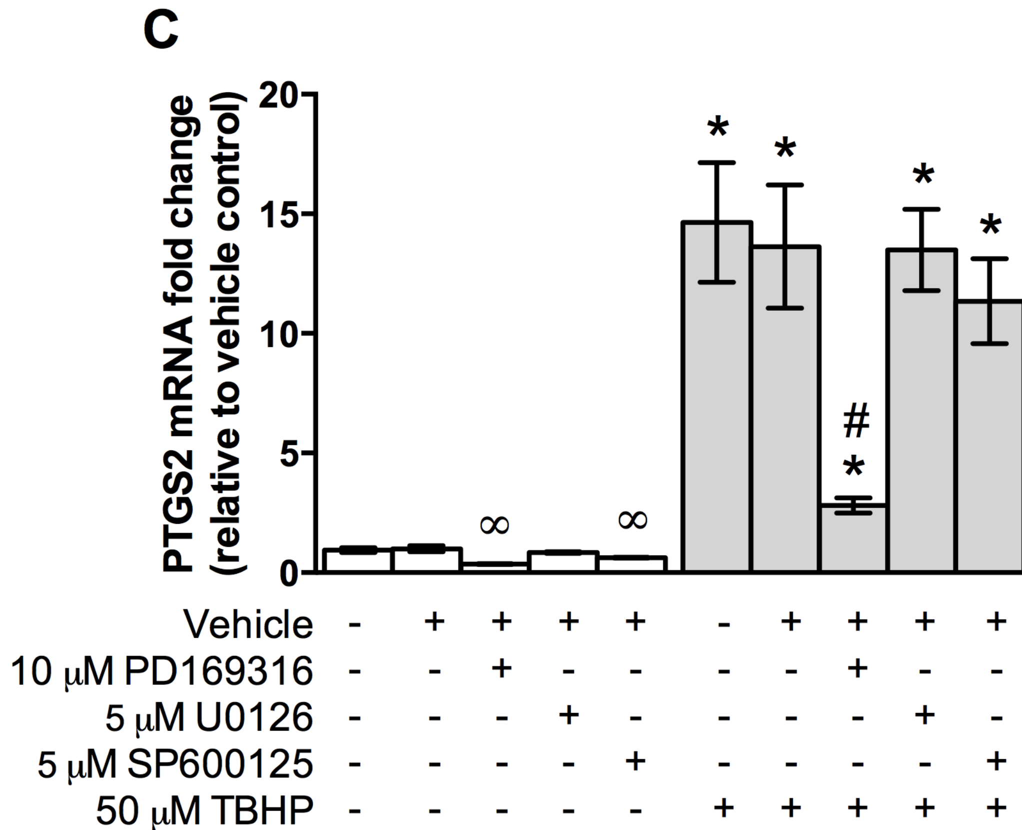


**Figure 3.5. Effect of MAPK inhibitors on TBHP-stimulated $PGE_2$ release and PTGS2 mRNA expression.** HTR-8/SVneo cells were pretreated with vehicle (0.4% DMSO), 10 µM PD169316, 5 µM U0126, or 5 µM SP600125 for 1 h. Pretreatments were removed and cells were then treated with 50 µM TBHP (filled bars) or received no further treatment for 4 or 24 h. $PGE_2$ concentrations in culture medium at A) 4 h and B) 24 h. C) Fold changes in PTGS2 mRNA after 4 h treatment with TBHP. Values represent mean ± SEM for 3 experiments. *, Significantly different compared to pretreatment matched control ($p<0.05$). #, Significantly different compared to vehicle + TBHP control ($p<0.05$). ∞, Significantly different compared with vehicle alone ($p<0.05$).

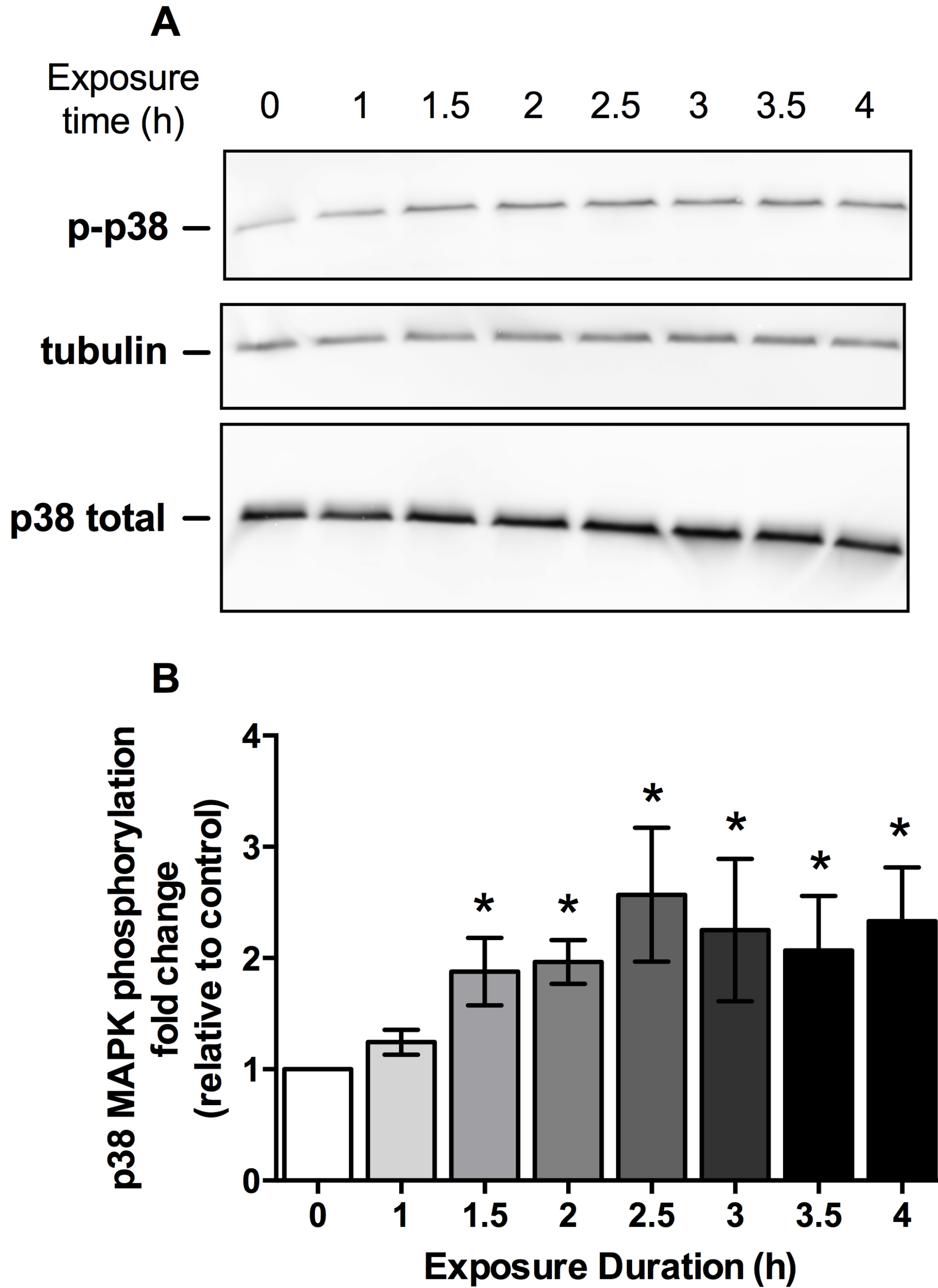


**Figure 3.6. Effect of TBHP on p38 MAPK phosphorylation.** Phosphorylation of p38 MAPK was assessed by western blot following treatment of HTR-8/SVneo cells with 50 μM TBHP for 1, 1.5, 2, 2.5, 3, 3.5, or 4 h. A) Representative image of western blotting for phospho-p38 MAPK, total p38 MAPK, and β-tubulin loading control. B) Fold changes in p38 MAPK phosphorylation relative to 0 h time point (i.e., the untreated control). Densitometry data for p38 MAPK phosphorylation were normalized to the β-tubulin loading control. Data represent mean ± SEM for 3 experiments. *, Significantly different compared with 0 h time point ($p<0.05$)

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# CHAPTER 4

## *TERT*-BUTYL HYDROPEROXIDE STIMULATES RELEASE OF THE CYTOKINE INTERLEUKIN-6 FROM THE HUMAN PLACENTAL CELL LINE HTR-8/SVNEO

### ABSTRACT

As a leading cause of infant morbidity and mortality, preterm birth is a problem that warrants further study. Damage due to reactive oxygen species (ROS) has also been associated with preterm birth. While the link between inflammatory pathways and initiation of parturition is well described, the effect of ROS on stimulated cytokine responses in human gestational tissues is unclear. As such, this study examines the effect of TBHP, a prototypical pro-oxidant chemical, on the cytokine interleukin-6 (IL-6) and nuclear factor-κB (NF-κB) in the human placental trophoblast-derived cell line HTR-8/SVneo. Treatment with 50 μM TBHP at 24 h induced significant increases in IL-6 release and NF-κB reporter activity ($p<0.05$). Stimulated IL-6 was blocked by the antioxidants butylated hydroxyanisole (BHA), diphenyl phenylenediamine (DPPD), and deferoxamine. ($p<0.05$). We also assessed dependence of responses on the redox sensitive mitogen activated protein kinases (MAPKs). Inhibition of p38 MAPK ablated the TBHP-stimulated the IL-6, but not NF-κB, response. Results from this study suggest that pro-oxidant chemicals may stimulate inflammatory responses from human trophoblasts.

# INTRODUCTION

Preterm birth is a significant public health problem associated with increased risk for mortality (Callaghan et al. 2006) and persistent morbidity (Saigal and Doyle). Inflammatory pathway activation is a key feature of both term and preterm labor (Christiaens et al. 2008). Cytokines are secreted messenger proteins that mediate inflammatory events and stimulate downstream pathways that drive labor (Christiaens et al. 2008). For instance, interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α are cytokines that induce uterotonic prostaglandin production from gestational tissues, including the placenta (Pomini et al. 1999) and extraplacental membranes (Kent et al. 1993a; Kent et al. 1993b). Cytokines also stimulate myometrial contractions via induction of prostaglandins (Sadowsky et al. 2000) and early labor in vivo (Romero et al. 1991; Sadowsky et al. 2006). Consequently, the identification of cellular mechanisms that lead to production of cytokines within the gestational compartment is critical to our understanding of the pathogenesis of preterm birth.

Although the link between stimulation of inflammation and bacterial infection within the gestational compartment is well defined (Romero et al. 2007), preterm birth also occurs in the absence of detectable infection (Muglia and Katz 2010). Inflammation at the maternal-fetal interface may, therefore, have alternative etiologies, such as through reactive oxygen species (ROS)-initiated signaling (Pathak et al. 2010). Indeed, markers for oxidative DNA, lipid and protein damage, lipid peroxidation have been associated with shortened gestation length (Stein et al. 2008) and preterm birth (Pathak et al. 2010).

Reactive oxygen species can act as signal transduction second messengers (Nathan 2003; Paulsen and Carroll 2009; Reth 2002). Reactive oxygen species mediate

inflammation and the production of proinflammatory cytokines in a variety of disease phenotypes including, diabetes, neurodenerative disease, and chronic obstructive pulmonary disease, among others (Chiurchiù and Maccarrone 2011; Hald and Lotharius 2005; Rahman and Adcock 2006). However, there are limited studies to suggest such pathways exist in human gestational cells (Buhimschi et al. 2003; Cindrova-Davies et al. 2007; Lappas et al. 2003). This study examines the effect of the chemical oxidant *tert*-butyl hydroperoxide (TBHP) on release of the parturition-associated cytokine IL-6 in a human placental trophoblast cell line, HTR-8/SVneo. Specifically, we utilized TBHP to demonstrate ROS-mediated IL-6 release and the role of immunomodulatory signal transducers, MAPK and NF-κB, on this response.

## MATERIALS AND METHODS

### Materials

RPMI 1640 medium supplemented with L-glutamine and 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, and Opti-MEM I Reduced Serum Medium supplemented with L-glutamine and hydroxyethyl piperazineethanesulfonic acid (HEPES) and without phenol red were purchased from Gibco Life Technologies (Carlsbad, CA). Deferoxamine mesylate salt (DFO), 70% *tert*-butyl hydroperoxide (TBHP), dimethyl sulfoxide (DMSO), N,N'-diphenyl-p-phenylenediamine (DPPD), butylated hydroxyanisole (BHA), and indomethacin were purchased from Sigma-Aldrich (St. Louis, MO). PD169316 was purchased from EMD Millipore (Billerica, MA), and U0126 and SP600125 were purchased from Enzo Life Sciences (Farmingdale, NY). Lipopolysaccharide (LPS,

*Salmonella typhimurium*) was from List Biological Laboratories (Campbell, CA). Dual Luciferase luminescent assays were purchased from Promega (Madison, WI). NF-κB Cignal reporter assay kit and Attractene transfection reagent were purchased from Qiagen (Germantown, MD).

**Cell culture and treatments**

HTR-8/SVneo cells were kindly provided as a gift from Dr. Charles H. Graham (Queen's University, Kingston, Ontario, Canada). The HTR-8/SVneo cells are human extravillous trophoblast cells derived from first trimester placentae and immortalized using the SV40 T antigen (Graham et al. 1993). These cells share many phenotypic similarities to primary extravillous trophoblasts (Jovanović and Vićovac 2009; Jovanović et al. 2010; Nicola et al. 2005). HTR-8/SVneo cells between passages 73-87 were maintained at 37°C in a humidified incubator with 5% $CO_2$ in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin for 2-3 days until disaggregated by trypsinization. For time-course IL-6 experiments, 500,000 cells/well were seeded in 6-well plates. For antioxidant and MAPK inhibitor experiments, HTR-8/SVneo cells were seeded at a density of 100,000 cells/well in 24-well plates. For transfection assays, cells were seeded at density of 20,000 cells/well in 96-well white, clear-bottom plates. HTR-8/SVneo cultures were allowed to reach about 70-80% confluence 24 h after subculture before initiating an experiment. All treatments were performed in triplicate.

Cells were treated with *tert*-butyl hydroperoxide as a model ROS-producing chemical that readily passes across the plasma membrane to enter cells (Ahmed-Choudhury et al. 1998). Intracellularly, TBHP produces *tert*-butyl alkoxyl and *tert*-butyl

peroxyl radicals in a series of iron-catalyzed reactions similar to the Fenton and Haber-Weiss reactions (Huang et al. 2005). For time-course experiments, culture medium was aspirated and cells were treated with 12.5, 25, or 50 μM TBHP (dissolved directly into culture medium), or medium alone. For experiments with treatments that preceded TBHP exposure, medium was aspirated and cells were pretreated with 1 mM DFO (dissolved directly into medium), 0.1% DMSO (solvent control for MAPK inhibitors), 10 μM PD169316, 5 μM U0126, 5 μM SP600125, or medium alone (untreated control). One hour later, medium was aspirated, and cells were treated with 50 μM TBHP or medium alone for 24 h. For experiments that included indomethacin or antioxidants, cells were coexposed to 50 μM TBHP and 0.1% DMSO (solvent control), 10 μM indomethacin, 1 μM BHA, or 25 μM DPPD for 24 h. Additional cultures of HTR-8/SVneo cells were exposed to 100 ng/mL LPS, included as a positive control for cytokine release (Svinarich et al. 1996) and NF-κB reporter activity (Dai et al. 2011).

**Analysis of IL-6 release**

TBHP-stimulated IL-6 release into culture medium was determined by ELISA. Culture medium was collected, clarified by centrifugation, aliquoted, and stored at -80°C until analysis. Samples were analyzed in duplicate for IL-6 using a human specific ELISA kit (R&D systems Duoset) by the University of Michigan Cancer Center Immunology Core, according to manufacturer's instructions. 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.

**Transfections and reporter assays**

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 CMV promoter. Suspensions of HTR-8/SVneo cells were seeded into 96-well plates containing transfection reagent (Qiagen) complexed with negative control, positive control, or NF-κB reporter constructs. Cells were allowed to adhere and transfect for 6 h followed by an 18 h recovery period at 37°C in a humidified incubator with 5% $CO_2$. Cells were then treated in triplicate with TBHP for 24 h, medium was aspirated, and cells were lysed in Passive Lysis Buffer (Promega). Dual luciferase assays (Promega) were performed according to manufacturer's instructions. NF-κB firefly luciferase activity was normalized to luciferase activity of *Renilla*, included as an internal control for cell number and transfection efficiency. Data are presented as the fold change in luciferase activity normalized to the control. Cells were maintained in OptiMEM I + 1% NEAA + 3% FBS throughout the duration of transfection experiments.

**Statistics**

Data were compared by repeated-measures one-way or two-way analysis of variance (ANOVA) with Tukey post-tests using SigmaPlot version 11.0 (Systat Sofware).

## RESULTS

**Effect of TBHP on IL-6 release from HTR-8/SVneo cells**

Exposure to 50 μM TBHP for 24 h stimulated a significant 2.3 fold increase in IL-6 release from HTR-8/SVneo cells compared to untreated control (Figure 4.1; p=0.005). Exposure to TBHP at earlier time points (4 and 8 h) and with lower

concentrations (12.5 and 25 μM TBHP) failed to significantly increase IL-6 release. LPS (100 ng/mL), included as a positive control, stimulated significant increases ($p<0.001$) in IL-6 release of about 3 fold compared to the untreated control at each of the time points assessed. Based on these results, we used treatment with 50 μM TBHP for 24 h in subsequent experiments to explore the mechanism of ROS-stimulated IL-6 release.

**Antioxidant exposure effects on TBHP-stimulated IL-6 release**

Because TBHP stimulates formation of free radicals via an iron-catalyzed reaction (Davies 1989; Van der Zee et al. 1989), we used the iron-chelating agent DFO to test the hypothesis that TBHP stimulates IL-6 release by an iron-dependent mechanism. As observed in Figure 4.2A, treatment with 50 μM TBHP for 24 h significantly increased IL-6 release compared with the non-pretreated group ($p<0.001$). Notably, pretreatment with 1 mM DFO for 1 h prevented TBHP-stimulated IL-6 release ($p<0.001$). Exposure to 1 mM DFO alone had no significant effect on IL-6 release compared with untreated control.

Because BHA and DPPD inhibited the effects of TBHP in our previous experiments (see Chapter 3), we used the antioxidants BHA and DPPD to assess dependency of IL-6 release on TBHP-induced ROS. HTR-8/SVneo cells were co-exposed to 50 μM TBHP, 1 μM BHA, 25 μM DPPD, antioxidant vehicle (0.1% DMSO; vehicle controls), or medium alone (untreated controls). Cotreatment with BHA significantly suppressed TBHP-stimulated IL-6 release compared with the vehicle control ($52.3 \pm 3.7$ pg/ml vs. $81.0 \pm 10.6$ pg/ml, respectively; Figure 4.2B; $p<0.001$). Similarly, cotreatment with DPPD quelled stimulated IL-6 release ($51.6 \pm 4.1$ pg/ml) compared with the TBHP + vehicle control ($p<0.001$). However, the BHA and DPPD treatments were

not able to completely block IL-6 release, and TBHP still stimulated significant increases of 2.3 and 2.2 fold, respectively, compared with vehicle controls ($p<0.001$). Treatment with antioxidants without TBHP had no significant effect on IL-6 release.

**Effect of indomethacin on TBHP-stimulated IL-6 release**

Previously, we found that TBHP increased $PGE_2$ release within 4 h exposure (see Chapter 3). Because $PGE_2$ release preceded IL-6 release temporally, we hypothesized dependency of IL-6 release on $PGE_2$. We pretreated with PTGS inhibitor indomethacin to test this hypothesis. Indomethacin failed to block TBHP-stimulated IL-6 release in HTR-8/SVneo cells, with similar levels of IL-6 observed in cultures co-exposed to 50 μM TBHP and 10 μM indomethacin compared with TBHP + vehicle (0.1% DMSO) or TBHP alone (Figure 4.3; $p<0.05$).

**Effect of MAPK inhibitors on TBHP-stimulated IL-6 release**

We used inhibitors of specific MAPK pathways to examine the dependency of TBHP-stimulated IL-6 release on MAPK activity. Pretreatment for 1 h with 10 μM PD169316, a p38 MAPK inhibitor, blocked TBHP-stimulated IL-6 release (Figure 4.4; compare vehicle + PD169316 + TBHP group with vehicle + TBHP group; $p<0.001$). However, a 1 h pretreatment with ERK1/2 or JNK inhibitors (5 μM U0126 and 5 μM SP600125, respectively), did not significantly alter TBHP-stimulated IL-6 release (IL-6 concentrations remained increased compared with their respective control; $p<0.05$). These data suggest that TBHP-stimulated IL-6 release is mediated by p38 MAPK activity and fails to support a role for the ERK 1/2 or JNK pathways.

**NF-κB reporter gene activity**

To determine whether exposure to TBHP stimulates NF-κB activity, HTR-8/SVneo cells were transfected with NF-κB reporter gene and treated with different concentrations of TBHP for 24 h. Exposure to 50 μM TBHP resulted in 6.7 fold increased reporter activity compared to the untreated control (Figure 4.5A; p=0.002), a level similar to the response elicited by 100 ng/ml LPS (7.1 fold; p=0.001), included as a positive control. We also examined whether NF-κB activity depended on p38 MAPK by pretreating with 10 μM PD169316 for 1 h. Inhibition of p38 MAPK failed to inhibit TBHP-stimulated NF-κB reporter gene activity (Figure 4.5B).

## DISCUSSION

The role of ROS in stimulation of parturition-associated pathways in cells of human gestation has been scarcely studied. The purpose of the present study was to examine the effect of a prototypical pro-oxidant chemical, TBHP, on IL-6 production in a human gestational cell model. Increased IL-6 is consistently observed in the amniotic fluid and cervicovagnial fluid of women who deliver preterm (Wei et al. 2010).

Here, we found that TBHP stimulated a significant 2.3 fold increase in IL-6 production from HTR-8/SVneo cells, a human placental trophoblast cell line. This magnitude of IL-6 change is similar to the 2 to 3 fold changes that occur with term labor in amniotic fluid and extraplacental membrane tissue (Laham et al. 1996). Several studies suggest that oxidative insult stimulates increases in parturition-associated cytokines. Of relevance to this study, term placental explants exposed to superoxide or hypoxia-reoxygenation injury produce cytokines TNFα (Coughlan et al. 2004; Hung et al. 2004), IL-1β (Cindrova-Davies et al. 2007), and IL-1α, but not IL-6 (Malek et al. 2001). Differences in the milleu of cytokines released from placental explants compared to the

results found here may be due to the nature of the oxidant used or differences in experimental models. This is the first study to identify IL-6 release in response to oxidative insult in human gestational cells.

The prototype chemical pro-oxidant used in this study, TBHP, undergoes an iron-catalyzed reaction to produce peroxyl and alkoxyl radicals (Van der Zee et al. 1989). In rat hepatocytes, the iron chelator DFO decreased ROS produced as a result of exposure to TBHP, thereby inhibiting arachidonic acid release from the cell membrane (Martín et al. 2001) and mitochondrial permeability transition (Nieminen et al. 1997). Similar cytoprotective roles for DFO have been reported with TBHP in other cell types (Kruidering et al. 1997; Sogabe et al. 1996; Yajima et al. 1995). Although we did not directly measure free radical production by TBHP here, we found that DFO pretreatment inhibited TBHP-stimulated IL-6 release from HTR-8/SVneo cells. Similarly, cotreatment with the antioxidants DPPD or BHA (Clark et al. 1985; Sogabe et al. 1996; Verhagen et al. 1991) decreased IL-6 release from HTR-8/SVneo cells, although inhibition was incomplete compared with DFO pretreatment. Taken together our data suggest TBHP-stimulated IL-6 release is due to ROS formation. Because DPPD and BHA are effective inhibitors of lipid peroxidation (Clark et al. 1985; Sogabe et al. 1996; Verhagen et al. 1991), our data suggest dependence, at least in part, on lipid peroxidation. A more thorough examination of the nature of the TBHP-stimulated IL-6 response, however, is warranted to elucidate the relative contributions of lipid peroxidation-dependent and -independent mechanisms that contribute to cytokine signaling.

Previously, we showed that TBHP stimulated $PGE_2$ release from HTR-8/SVneo cells and that the $PGE_2$ release was inhibited by cotreatment with the PTGS inhibitor

indomethacin (see Chapter 3). In the present study, we evaluated the contribution of $PGE_2$ to TBHP-stimulated IL-6 because $PGE_2$ stimulates release of IL-6 in human chondrocytes (Wang et al. 2010) and increases cytokines IL-8, IL-10, and monocyte chemotactic peptide-1 (MCP-1) in perfused human placentae (Denison et al. 1998). Additionally, HTR-8/SVneo cells produce EP receptors 1, 2, 3, and 4 (Nicola et al. 2005), enabling them to respond to prostaglandins. However, TBHP-stimulated IL-6 release was not affected by cotreatment with indomethacin. Our data, therefore, fail to support a role for $PGE_2$ in the TBHP-stimulated IL-6 response and indicate that the stimulated IL-6 release is dependent on a different mechanism.

MAPK signaling pathways are critical for the release of proinflammatory cytokines (Zhang and Dong 2005). Here, we found that TBHP-stimulated IL-6 release was blocked using the p38 MAPK inhibitor PD169316. Consistent with our findings, inhibition of p38 MAPK blocked early release of TNF-α and IL-1β from human placental explants exposed to hypoxia-reoxygenation injury (Cindrova-Davies et al. 2007). Dependence of IL-6 release on TBHP-stimulated p38 activity is consistent with the known sensitivity of p38 MAPK to the cellular redox environment (McCubrey et al. 2006). In contrast, our data do not support a role for ERK 1/2 or JNK in TBHP-stimulated IL-6 release from the HTR-8/SVneo cells, although ERK 1/2, JNK, and p38 are activated in the murine uterus during parturition (Takanami-Ohnishi et al. 2001) and phosphorylation of key kinases in these MAPK pathways is increased in uterine cervical stroma of women after delivery at term (Wang and Stjernholm 2007). The concentrations of ERK1/2 inhibitor (5 μM U0126) and JNK (5 μM SP600125) used in the present study were reported to inhibit TNFα-induced activation of respective MAPK pathways in HTR-

8/SVneo cells (Renaud et al. 2009), yet we failed to observe significant decreases in TBHP-stimulated IL-6. Because of the limitations of pharmacologic inhibitors, future studies could use a genetic approach to deplete MAPK pathways, and thereby further assess involvement of these pathways in TBHP-mediated signaling.

NF-κB is a transcription factor critical to the regulation of inflammatory pathway gene expression (Vallabhapurapu and Karin 2009). Important to parturition, NF-κB mediates gestational cell PTGS2 and cytokine expression in response to stimulation with LPS and cytokines (Ackerman et al. 2008; Keelan et al. 2009; Yan et al. 2002). Increased NF-κB binding activity and nuclear translocation is found in gestational tissues during term and preterm labor (Chapman et al. 2004; Vora et al. 2010). In addition, pharmacologic inhibition of NF-κB delays onset of labor in a mouse model of premature labor (Condon et al. 2004). The latter reports are consistent with the 6.7-fold increase in reporter activity found in HTR-8/SVneo cells exposed to 50 μM TBHP for 24 h. Although we found associated increases in IL-6, we did not assess a causal relationship between stimulated NF-κB activity and IL-6. Exposure of intestinal epithelial cells to IL-6 stimulates NF-κB-mediated increases in adhesion molecule expression (Wang et al. 2003). On the other hand, NF-κB transactivation induces IL-6 synthesis following LPS, interferon-γ (IFNγ), and TNFα treatment in a human macrophage cell line (Libermann and Baltimore 1990). Therefore, further experiments are needed to determine interdependence of IL-6 on NF-κB or vice versa.

Our data failed to support a role for p38 MAPK in induction of NF-κB reporter activity. For the NF-κB reporter experiments, we used 10 μM PD169316, the same concentration that was effective in inhibiting the TBHP-stimulated IL-6 release. NF-κB-

dependent transcription of cytokines in LPS-stimulated neutrophils is ablated when a different p38 MAPK inhibitor, SB203580, was added to cultures, suggesting p38 MAPK may directly or indirectly activate NF-κB (Nick et al. 1999). Other studies suggest that p38 MAPK activity and NF-κB cooperate in the nucleus to stimulate transcriptional activity (Bergmann et al. 1998; Read et al. 1997; Saccani et al. 2002). As such, we expected that the p38 MAPK inhibitor PD169316 would abrogate TBHP-stimulated NF-κB activity. Inconsistencies in the results of these studies might be due to differences in physiology of cell types or the stimuli used to initiate p38 MAPK and NF-κB activity. An alternative mechanism responsible for stimulation of NF-κB activity might include direct TBHP-stimulated redox-mediated phosphorylation of the NF-κB regulating chaperone, inhibitor of κB (IκB), thereby promoting nuclear shuttling (Morgan and Liu 2011; Oliveira-Marques et al. 2009). Additionally, TBHP has been shown to induce NF-κB activity, purportedly through its lipid peroxidation activity (Bowie et al. 1997). Further studies are needed to elucidate mechanisms by which TBHP contributes to stimulation NF-κB.

A potential limitation of our study is the use of a first trimester extravillous trophoblast cell line, HTR-8/SVneo. Though the HTR-8/SVneo cells have similar phenotypes as primary extravillous trophoblast cells (Biondi et al. 2006; Jovanović and Vićovac 2009; Jovanović et al. 2010; Nicola et al. 2005; Nicola et al. 2008) and are genetically stable (Drennan et al. 2010), a recent study has shown that the epigenetic map (Novakovic et al. 2011) of these cells differs from primary cells. Perhaps more importantly, given that this cell line is derived from placenta in the first trimester, these cells may respond differently than gestational cells isolated during periods more relevant

to preterm birth. Nevertheless, HTR-8/SVneo cells respond by producing the proinflammatory cytokine IL-6, similar to cytokines produced by term placental explants exposed to ischemia-reoxygenation (Cindrova-Davies et al. 2007). Primary cell cultures and tissue cultures from human placentae later in pregnancy may provide more directly relevant models for preterm parturition. These experiments, however, are beyond the scope of the present investigation.

As an extravillous trophoblast cell line derived from first trimester human placenta, the HTR-8/SVneo cell results may have relevance to early events of pregnancy. Importantly, dysfunctional extravillous trophoblast migration and subsequent impaired spiral artery transformation has been linked to adverse pregnancy outcomes including preeclampsia (Merviel et al. 2004; Wang et al. 2009) and preterm birth (Kim et al. 2002; Kim et al. 2003). However, the role of IL-6 in placentation is unclear. Treatment of extravillous trophoblasts with IL-6 induces trophoblast migration (Jovanović and Vićovac 2009), suggesting an important function for IL-6 in physiologic placental development. However, treatment of extravillous trophoblast cells (Anton et al. 2012; Kim et al. 2005) with LPS, which stimulated IL-6 release, also resulted in decreased trophoblast migration. Therefore the potential relevance of the TBHP-stimulated IL-6 release for trophoblast migration is unclear.

Limited studies suggest that environmental exposures activate pathways related to parturition. For example, cigarette smokers are more likely to deliver preterm (Cnattingius 2004) and have increased systemic biomarkers for oxidative damage and inflammation (Yanbaeva et al. 2007). Similarly, increased organochlorine pesticide exposures are associated with increased damage markers and increased rates of preterm

delivery (Pathak et al. 2010). The present study provides a potential mechanistic link that explains the associations between environmental exposures and preterm birth. This study is the first, to our knowledge, to use a chemical oxidant on a human trophoblast-derived cell line to assess parturition-related end points, namely cytokine release.

In summary, we have shown that exposure to the chemical oxidant TBHP stimulated release of the parturition-associated cytokine IL-6 in a human placental trophoblast-derived cell line. Importantly, induction of this cytokine plays an important role in the induction of pathways involving prostaglandins, matrix metalloproteinases, and other parturition-mediating cytokines. As such, ROS may be an important mediator in the initiation of these events. These findings are novel in that IL-6, which has been consistently linked to preterm birth (Wei et al. 2010), has not been previously identified as regulated by ROS in human cells of gestation. Furthermore, we identified p38 MAPK as critical to the TBHP-stimulated IL-6 response, suggesting p38 may be an important signaling node in trophoblast cell responses to oxidative insult. Because many environmental chemicals associated with preterm birth also increase ROS, these findings suggest a mechanism by which exposure to environmental contaminants may contribute to increased risk for preterm birth.

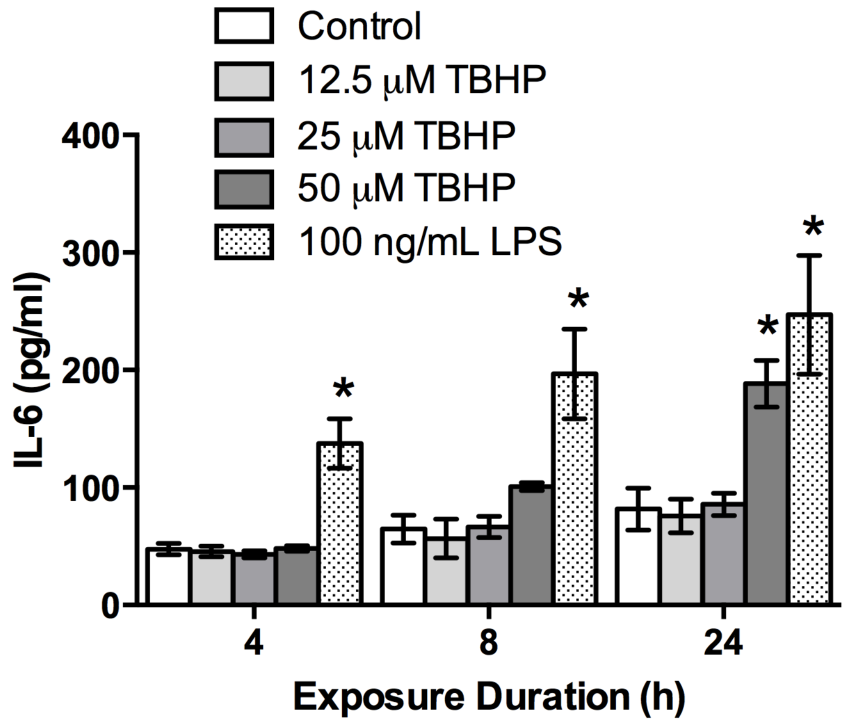


**Figure 4.1. TBHP-stimulated IL-6 release.** HTR-8/SVneo cells were exposed to 0, 12.5, 25, or 50 μM TBHP for 4, 8, or 24 h. LPS (100 ng/ml) was included as a positive control. IL-6 release into culture medium was measured using ELISA. Data are presented as mean ± SEM from 3 experiments. *, Significantly different compared with the time-matched control ($p<0.05$).

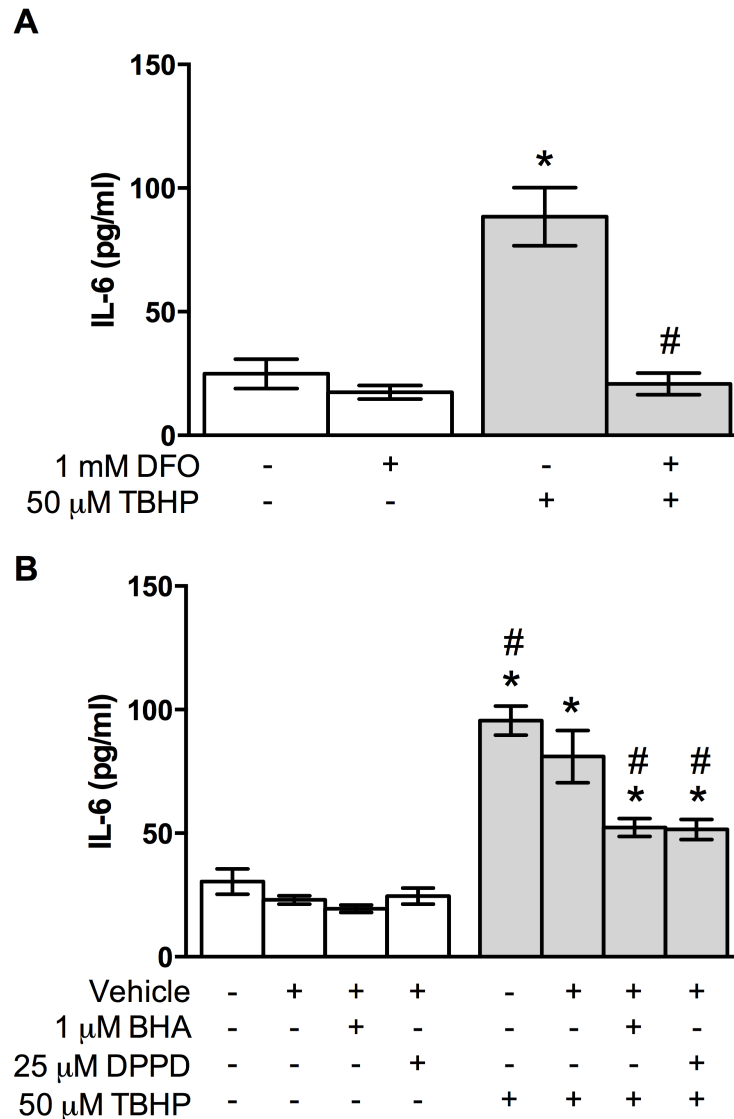


**Figure 4.2. Effect of antioxidant treatment on TBHP-stimulated IL-6 release.** A) HTR-8/SVneo cells were pretreated with 1mM DFO for 1 h, the DFO-containing medium was removed, and then cells were treated with 50 μM TBHP or fresh medium alone for 24 h. Data are presented as mean ± SEM of 4 experiments. *, Significantly different compared with the untreated controls ($p<0.001$). #, Significantly different compared with the samples treated with TBHP alone ($p<0.001$). B) Cells were cotreated with 50 μM TBHP and 1 μM BHA, 25 μM DPPD, or vehicle (0.1% DMSO; control) for 24 h. Filled bars represent treatment with TBHP and unfilled bars represent cells not treated with TBHP. Data are presented as mean ± SEM ± SEM of 3 experiments. *, Significantly different compared with co-treatment matched control ($p<0.001$). #, Significantly different compared to vehicle + TBHP ($p<0.05$).

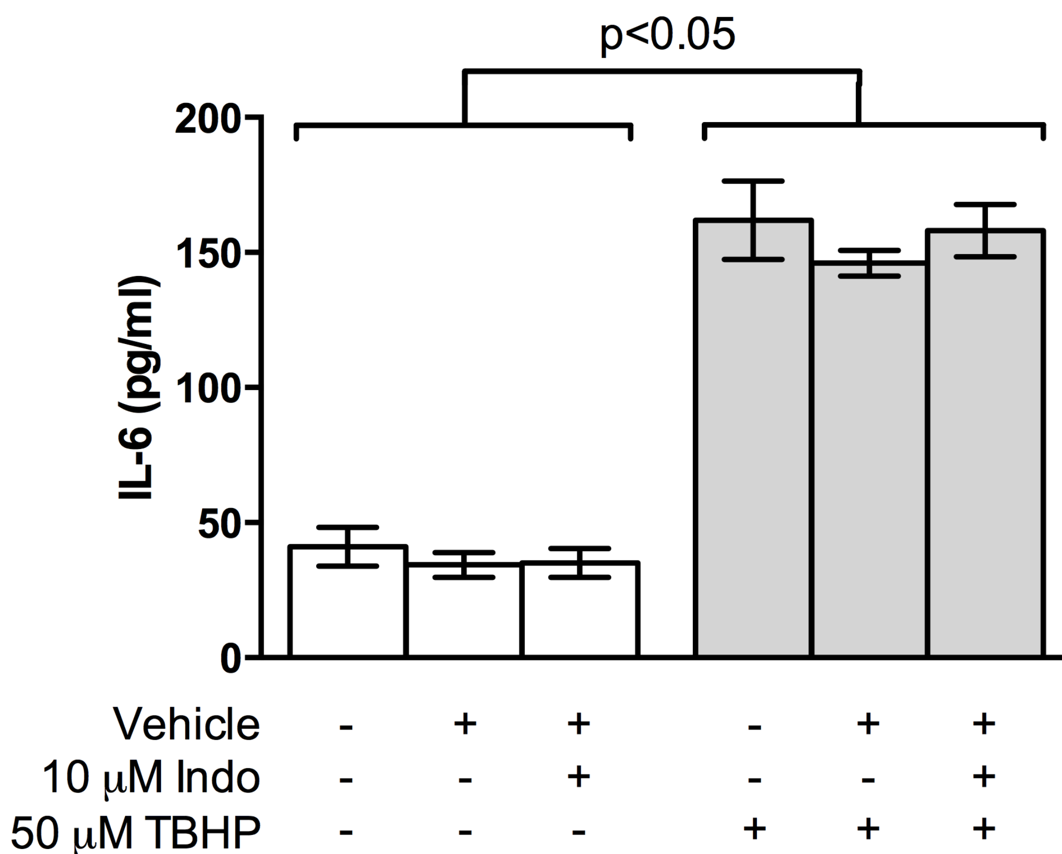


**Figure 4.3. Effect of the PTGS inhibitor indomethacin (Indo) on TBHP-stimulated IL-6 release.** HTR-8/SVneo cells were treated with 50 μM TBHP in the presence or absence of 10 μM indomethacin for 24 h. Cultures exposed to 0.1% DMSO were vehicle controls for indomethacin. Filled bars represent treatment with TBHP and unfilled bars represent cells not treated with TBHP. Bars represent mean ± SEM IL-6 concentration in culture medium for 3 experiments.

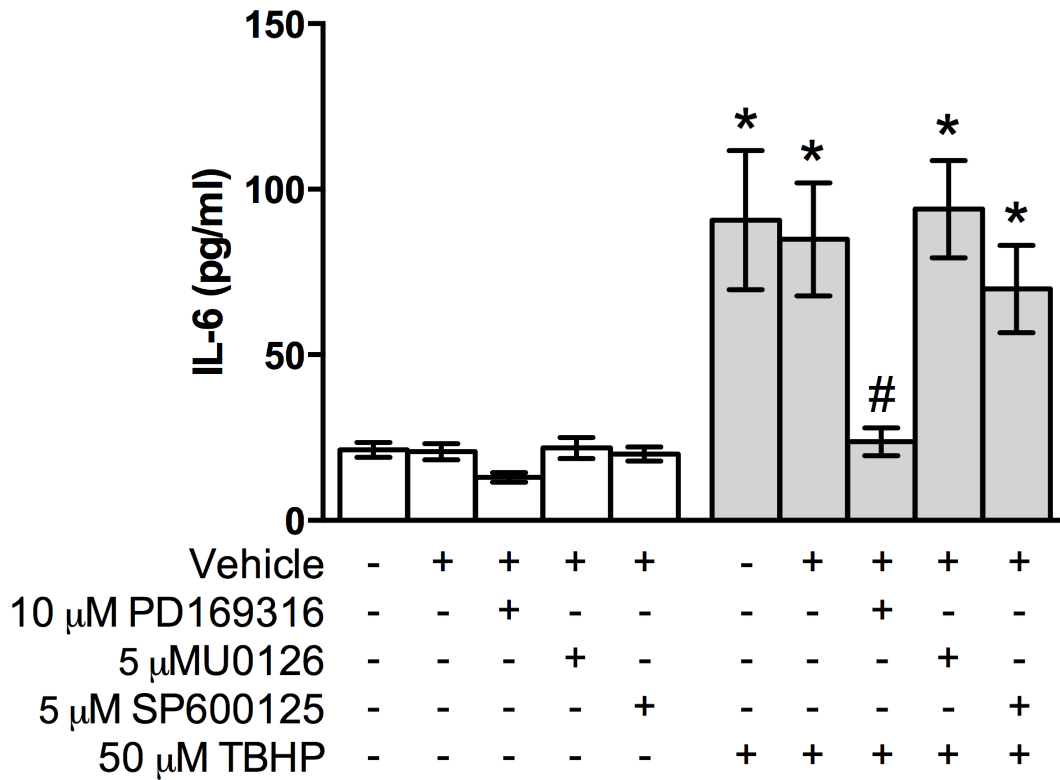


**Figure 4.4. Effect of MAPK inhibitors on TBHP-stimulated IL-6 release.** HTR-8/SVneo cells were pretreated with vehicle (0.1% DMSO; control), 10 μM PD169316 (p38 MAPK inhibitor), 5 μM U0126 (ERK 1/2 inhibitor), 5 μM SP600125 (JNK inhibitor) or medium alone for 1 h, and then exposed to 50 μM TBHP for 24 h. IL-6 release into culture medium was assessed by ELISA. Filled bars represent treatment with TBHP and unfilled bars represent cells not treated with TBHP. Data are presented as mean ± SEM of 3 experiments, performed in triplicate. *, Significantly different compared with the control that received the same pretreatment ($p<0.05$). #, Significantly different compared with the vehicle + TBHP group ($p<0.001$).

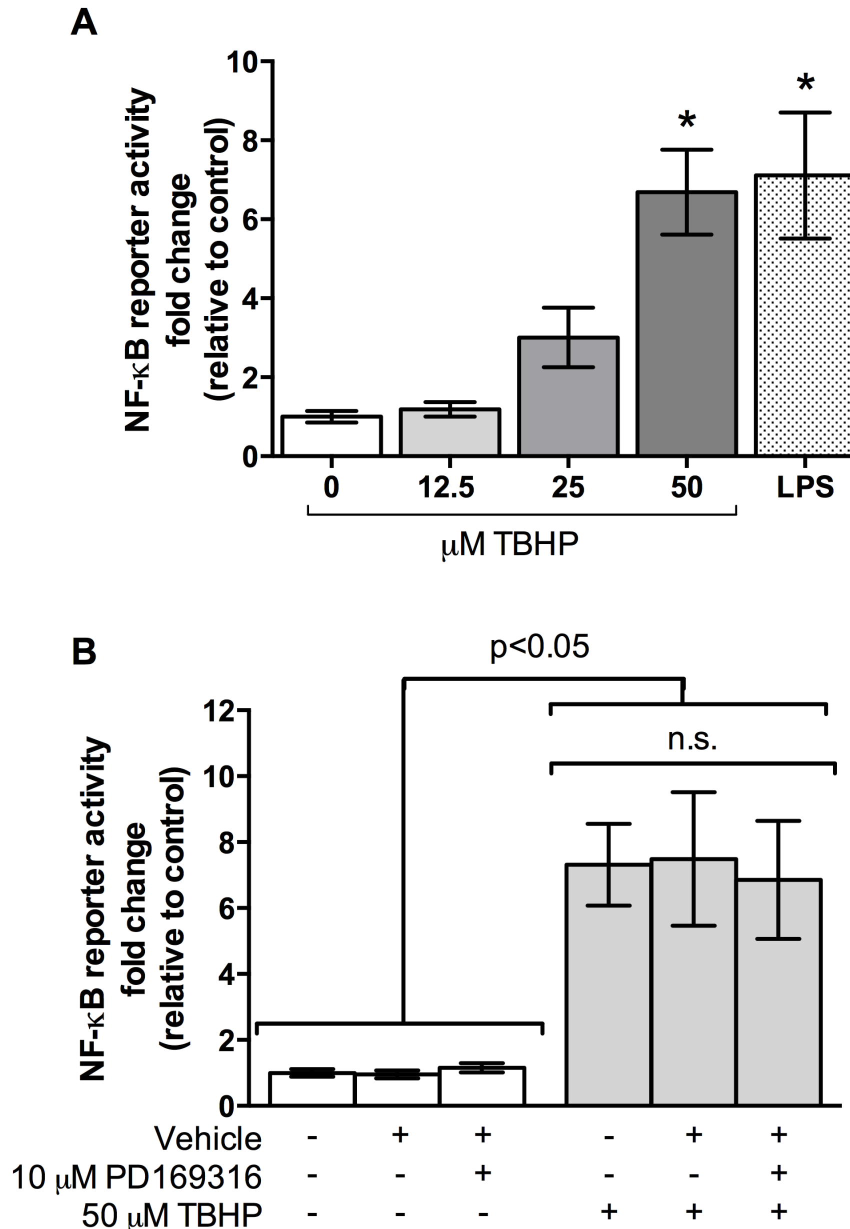


**Figure 4.5. Effect of TBHP and p38 MAPK inhibitor on NF-κB reporter activity.** HTR-8/SVneo cells were transfected with NF-κB reporter gene constructs and allowed an 18 h recovery time. A) After transfection, cells were treated with 0, 12.5, 25, or 50 μM TBHP for 24 h. LPS (100 ng/ml) was included as a positive control. B) Cells were pretreated with 10 μM PD169316 (p38 MAPK inhibitor) or vehicle (0.4% DMSO) for 1 h, pretreatments were removed, and cells were then treated with 50 μM TBHP for 24 h. Dual luciferase assays were performed to assess NF-κB activation. Data are presented as fold change (mean ± SEM) over the untreated controls from 3-4 experiments. Relative light units (RLU) measured from *Firefly* luciferase activity were divided by RLU from the internal control, *Renilla* luciferase, then fold changes were calculated relative to the non-treated samples (control) in A or vehicle control in B. *, Significantly different compared with the untreated controls ($p<0.05$).

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# CHAPTER 5

# DISCUSSION

This thesis explores mechanisms by which cellular reactive oxygen species (ROS) may stimulate parturition-associated pathways within placental cells. Whereas roles of prostaglandins, cytokines, and apoptosis in parturition have been described, the effect of ROS on these parturition pathways is unclear. Several studies support ROS involvement in parturition pathways (Buhimschi et al. 2003; Lappas et al. 2003; Matsumoto et al. 2013), and evidence of cellular damage due to ROS has been associated with shortened gestation length (Comporti et al. 2004; Mocatta et al. 2004; Pathak et al. 2010; Stein et al. 2008) and labor at term (Mocatta et al. 2004; Mongelli et al. 1997; Rogers et al. 1998; Yaacobi et al. 1999).

We demonstrated that *tert*-butyl hydroperoxide (TBHP), a prototypical chemical oxidant, stimulated release of prostaglandin (PG) $E_2$, the cytokine interleukin (IL)-6, and caspase activity in the human placental cell line HTR-8/SVneo. This is the first study to identify stimulation of IL-6 from a human gestational tissue in response to an oxidative insult. In addition, whereas Cindrova-Davies et al. (2007) identified prostaglandin endoperoxide synthase (PTGS)-2, an enzymatic constituent within the prostaglandin synthetic process, as a target induced by hypoxia-reoxygenation injury, this study is the first to identify $PGE_2$ as a potential prostaglandin mediator produced by placental tissues in response to a chemical that produces ROS. This study is also consistent with models of placental hypoxia-reoxygenation, in observing stimulation of apoptosis-related pathways

as a response to oxidative injury (Cindrova-Davies et al. 2007a; Hung et al. 2002). A potential caveat to earlier models that employed hypoxia-reoxygenation to generate ROS is that the quantitation of ROS and overall physiologic relevance may be difficult to decipher. In contrast, this study used a precise amount of ROS-producing chemical in each experiment, facilitating comparisons between our results and studies using other ROS. We observed that TBHP generated parturition-related responses while also generating modest changes in the cellular redox potential, suggesting that these endpoints can be stimulated even in the absence of overt oxidative damage.

Research presented in this thesis identifies p38 MAPK as a critical mediator in the induction of prostaglandin and cytokine release. This finding is in accordance with in vitro explant studies with human extraplacental membrane and human placenta (Lappas et al. 2007; Lappas et al. 2011; Wang and Stjernholm 2007) and known responsiveness of p38 MAPK to cellular redox regulation (McCubrey et al. 2006). Taken together, these studies suggest p38 MAPK may represent a viable target with therapeutic potential for women who are at risk for preterm birth. Another potential therapy may include supplementation with antioxidants. However, a recent meta-analysis suggests that antioxidant vitamins C and E have no significant effect on preterm birth as an outcome in randomized control trials (Dror and Allen 2012), suggesting that antioxidant therapy alone may be insufficient as an intervention for general populations.

Based on our results, we propose the model shown in Figure 5.1. In this model, TBHP promotes formation of ROS within the placental cell, supported by findings that TBHP-stimulated parturition-related endpoints were inhibited by the antioxidants butylated hydroxanisole (BHA) and diphenyl phenylenediamine (DPPD). Inhibition of

downstream responses by the iron chelator deferoxamine further suggests that TBHP forms iron-dependent alkoxyl and peroxyl radicals, as previously described (Van der Zee et al. 1989). Resultant ROS may then stimulate phosphorylation, and thereby activation, of p38 MAPK, as supported by TBHP-stimulated p38 phosphorylation in the placental cells. Because the p38 MAPK inhibitor PD169316 blocked the prostaglandin synthetic responses, 38 MAPK kinase activity may be driving PTGS2 transcription and subsequent $PGE_2$ production. The PTGS2 gene contains consensus sequences of a number of transcription factors, including activator protein (AP)-1, whose activation is kinase dependent (Chun and Surh 2004).

Because antioxidants BHA, DPPD, deferoxamine and the p38 MAPK inhibitor PD169316 all blocked TBHP-stimulated IL-6 release, we propose that ROS-dependent p38 MAPK also drives placental cell production of IL-6 (Figure 5.1). Temporally, $PGE_2$ production preceded that of IL-6 suggesting a potential stimulatory effect of $PGE_2$ on IL-6. Although $PGE_2$ can stimulate cytokine release from human placental tissue (Denison et al. 1998), our data did not support this finding. This may be because the levels of $PGE_2$ produced by HTR-8/SVneo cells were relatively low, and thus did not meet the threshold to have an effect. Alternatively, this could be because these pathways are simply not linked in this situation. On the other hand, the effects of ROS may outweigh any effect $PGE_2$ may have in stimulating IL-6 or apoptosis, this inhibiting $PGE_2$ production would appear to have no effect.

Treatment with TBHP also increased NF-κB reporter transactivation at the same time point it stimulated IL-6, suggesting a possible causal link between ROS activation of NF-κB and increased IL-6 release (Figure 5.1). Indeed, like PTGS2, the gene for IL-6

contains NF-κB consensus sequences (Libermann and Baltimore 1990). Similar to MAPK activation, stimulation of NF-κB activity may occur by redox-mediated phosphorylation of the NF-κB regulating chaperone, inhibitor of κB (IκB) (Morgan and Liu 2011; Oliveira-Marques et al. 2009). Although MAPK signaling can stimulate NF-κB transactivation (Nick et al. 1999), our data did not support this mechanism.

We suggest that ROS drives caspase-mediated apoptosis (Figure 5.1). Although we did not measure apoptosis per se, TBHP-stimulated caspase activation was inhibited by deferoxamine. One possible mechanistic explanation for this finding is that TBHP causes HTR-8/SVneo DNA damage (Tetz et al. 2013) and may thusly induce subsequent p53-dependent activation of apoptosis pathways (Wyllie 2010). Although previous reports suggest roles for $PGE_2$ and caspase activation in apoptosis (Ackerman and Murdoch 1993; Fard et al. 2012; Takadera et al. 2004), and p38 MAPK (Cindrova-Davies et al. 2007a; Grethe et al. 2004), our results did not support these mechanisms of apoptosis activation in the placental cells. In addition, it is worth noting that caspase activity has been reported in quiescent myometrial cells, or, in other words, living, non-contracting myometrium (Jeyasuria et al. 2009). Therefore, we are cautious in suggesting the only interpretation to increased caspase activity is apoptotic cell death. However, we did find a concomitant decrease in cell viability with caspase activation.

The TBHP-stimulated redox responses we observed were relatively modest in magnitude. Nonetheless, the apoptosis, prostaglandin, and cytokine responses were inhibited by antioxidants, suggesting that TBHP-induced effects are ROS dependent. Treatment of HTR-8/SVneo cells with TBHP stimulated increases in cellular oxidized glutathione (GSSG) (Figure 5.1) without significant changes in reduced glutathione

(GSH). We speculate this may be because peroxides like TBHP (Toussaint et al. 1993) can also inhibit glutathione peroxidase, the enzyme responsible for detoxifying TBHP. Exposure to TBHP also induced modest reporter activity at the antioxidant response element (ARE). This suggests that TBHP may oxidize residues on kelch-like ECH-associated protein 1 (KEAP1), freeing nuclear factor (erythroid-derived 2)-like 2 (Nrf2) to drive transcription at ARE (Motohashi and Yamamoto 2004). Once unobstructed, Nrf2 then drives antioxidant gene expression, including thioredoxin reductase 1 (TXNRD1) (Hintze et al. 2003) and glutaredoxin 2 (GLRX2) (Gallogly et al. 2009), as observed in the present study. Taken together, these data may suggest that ROS can activate cellular pathways involving prostaglandins, cytokines, and even cell death in the absence of major changes in the cell's redox status. These results are not unheard of; in human keratinocytes treated with epidermal growth factor (EGF), redox-sensitive cytosolic thioredoxin can be oxidized while the glutathione redox potential remains unchanged (Halvey et al. 2005). As such, we speculate that environmental chemicals, which may produce a relatively low concentration of ROS, may still alter parturition-promoting pathways resulting in an increased risk for prematurity in women. Further studies are needed to characterize additional redox-related changes induced by TBHP and identify exact molecular targets linking TBHP, ROS, and parturition-associated endpoints.

We recognize that there are limitations to this study. We are cautious in interpreting results from cell lines, because the process of immortalization itself and subsequent passaging for extended periods can lead to genetic drift that may not adequately model phenotypes (Kaur and Dufour 2012). We also recognize that HTR-8/SVneo cells are derived from first trimester, and may not be phenotypically similar to

cells that contribute to preterm parturition. As such, repeating these experiments in primary cells from later in gestation is logical next step for this research. However, as the HTR-8/SVneo cells are derived from first trimester placenta, results presented in this thesis may have relevance to early events of pregnancy and its subsequent adverse outcomes. Prostaglandins (Biondi et al. 2006; Horita et al. 2007; Nicola et al. 2005; Nicola et al. 2008a; Nicola et al. 2008b) and cytokines (Jovanović and Vićovac 2009) are both modulators of placental development, dysregulation of which has been implicated in preeclampsia (Merviel et al. 2004; Wang et al. 2009)

This study provides a potential mechanistic explanation by which environmental pollutant chemicals may increase risk for preterm birth. That is, many environmental chemicals that have been associated with shortened gestation length (Jelliffe-Pawlowski et al. 2006; Meeker et al. 2009; Pathak et al. 2009) have also been shown to stimulate ROS or oxidative stress (Ercal et al. 2001; Pathak et al. 2010; Tetz et al. 2013). Toxicant-derived ROS within the gestational compartment may then be responsible for driving parturition-associated pathways involving prostaglandins, cytokines, and apoptosis. Therefore, experimental studies seeking to examine the link between toxicant exposure and early birth in animal models, may choose to examine toxicant effects on ROS and parturition-promoting pathways in gestational tissues. Furthermore, by providing a potential mechanistic link between exposure and outcome, we now have a hypothesis to test in further human-based work. Future epidemiologic studies examining the link between toxicant exposure and preterm birth, may also want to include markers for damage due to ROS including lipid peroxidation or DNA damage in tissue, blood, urine, or amniotic fluid to add a mechanistic component to their studies. In addition, studies

involving antioxidant administration in pregnant women may choose to examine environmental chemical exposure status as a potential modifier of risk of adverse outcomes.

Bacterial infection is recognized as a leading cause of preterm labor. In addition to direct signaling to produce proinflammatory cytokines (Akira and Takeda 2004), toll-like receptor (TLR) activation by bacterial products induces NADPH oxidase activity. This enzyme is responsible for making ROS that not only kill bacteria (Laroux et al. 2005), but may also contribute to cell signaling that promotes parturition. Similarly, ROS may be contributing to the progression of normal parturition. Contractions of the myometrium during labor can compress blood vessels and lead to intermittent uterine blood supplies (Janbu and Neshetm 1987), lending potential for hypoxia-reoxygenation injury during labor (Cindrova-Davies et al. 2007b). Resultant ROS may then further contribute to labor in a feed-forward manner. Once labor is started, ROS are produced in response to hypoxia-reoxygenation, which can then further stimulate prostaglandin and cytokine responses.

In summary, we have identified a potential contributing mechanism for the induction normal and preterm labor. Whether produced as a result of environmental chemical exposure, bacterial infection, or hypoxia-reoxygenation injury, this thesis shows that ROS can activate cytokines, prostaglandins, and apoptosis within the gestational compartment. Each of these endpoints are critical components to the progression of labor. Therefore, further studies examining the causal link between ROS and increased risk for early labor are warranted. As a leading cause of infant morbidity and mortality, preterm birth is a vexing public health problem whose etiology is not fully understood. By

identifying a potential signaling node in the pathophysiology of this event, we have found a potential site to modify risk for disease.

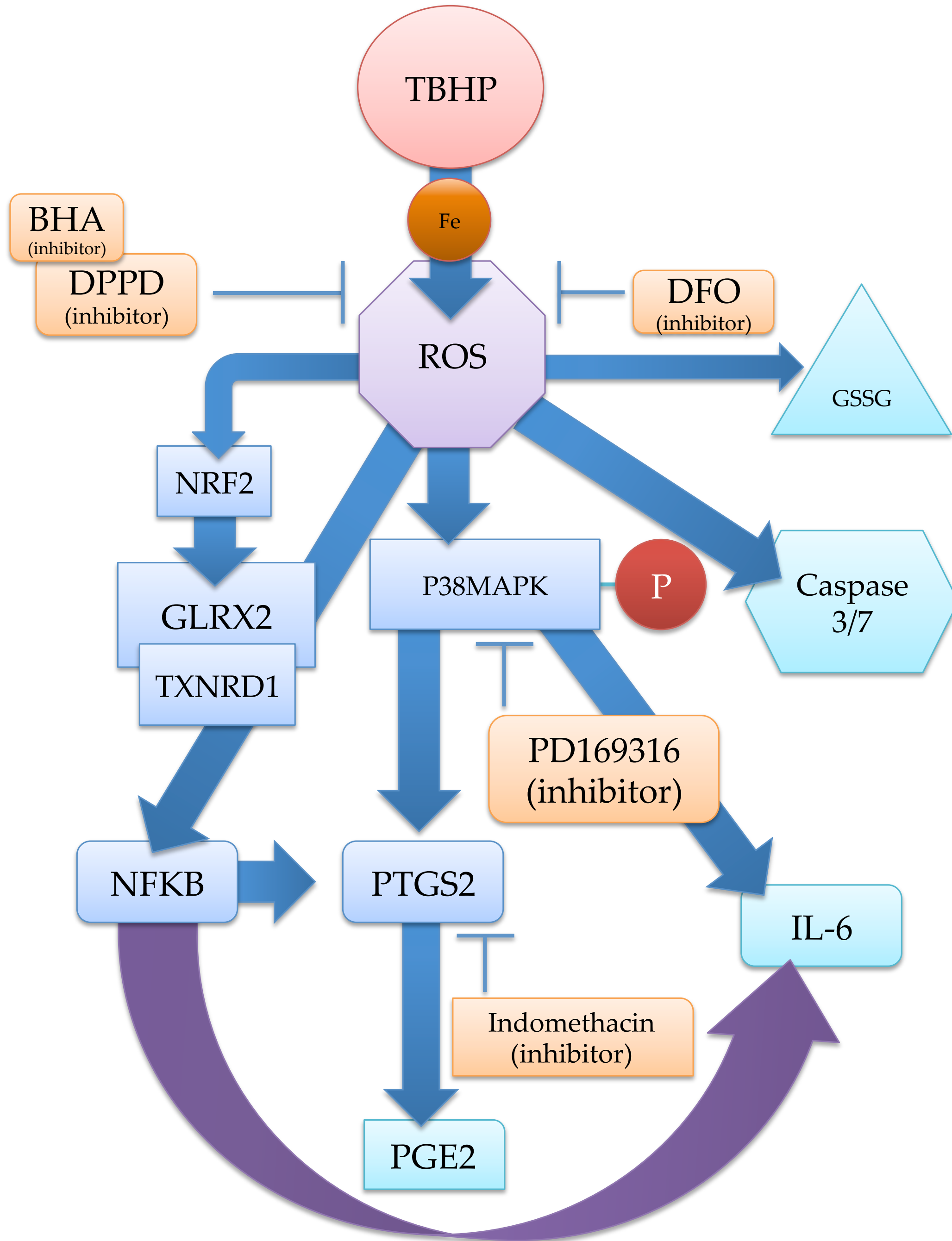


**Figure 5.1. Conceptual model of TBHP effects in placental trophoblast cells.** TBHP generates ROS in an iron-dependent manner. Resultant ROS drive the phosphorylation, and thereby activation, of p38 MAPK. Activity of p38 MAPK stimulates IL-6 release, PTGS2 expression, and $PGE_2$ synthesis ROS also induce NF-κB transactivation, which may be responsible for IL-6 release. Caspase activity, and subsequent apoptosis, are also stimulated by TBHP-generated ROS. Finally, ROS may also be responsible for modest changes in GSSG, Nrf2 transactivation, and subsequent expression of the antioxidant genes GLRX2 and TXNRD1. Image generated in collaboration with Lucas J. Korte.

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## APPENDIX A

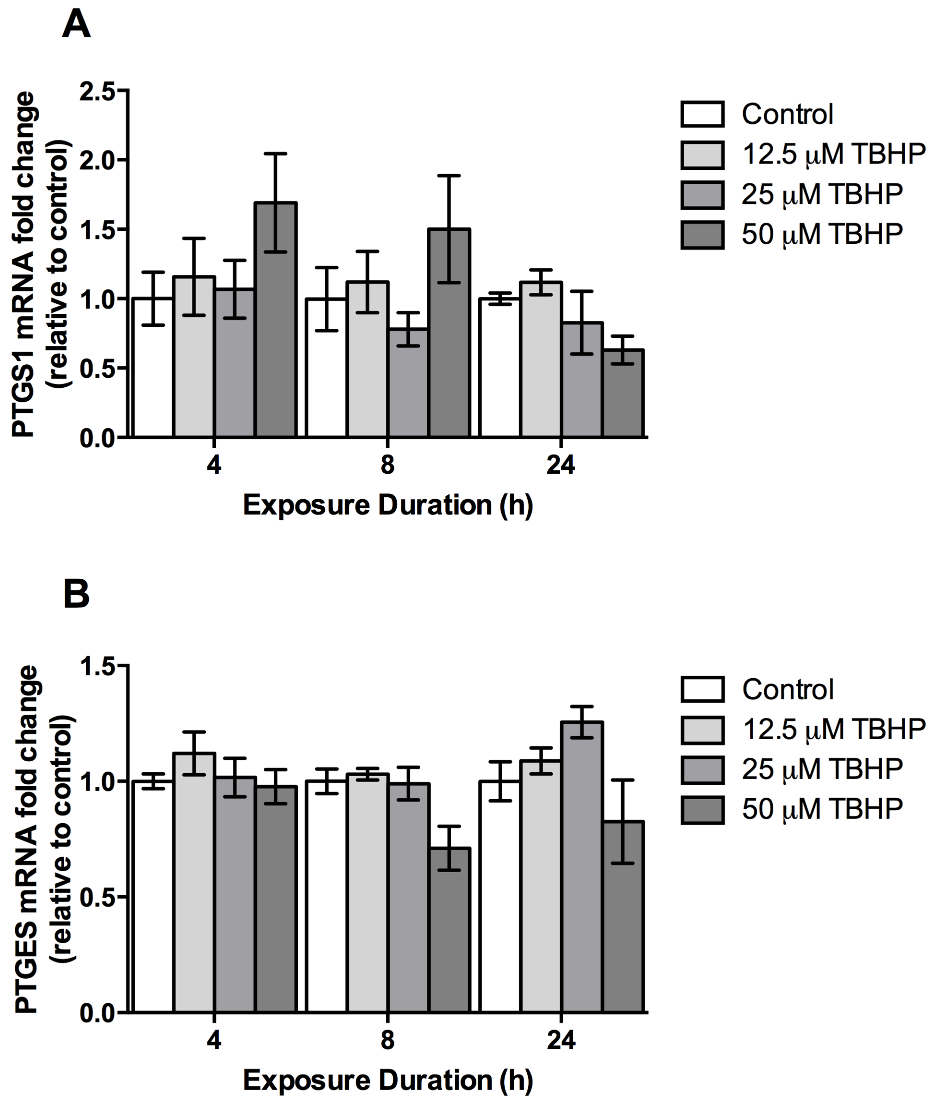



**Figure A.1. Effect of TBHP on prostaglandin synthetic enzyme expression.** Changes in mRNA expression of A) PTGS1 and B) PTGES following treatment of HTR-8/SVneo cells with 12.5, 25, or 50 μM TBHP for 4, 8, or 24 h. Values represent mean fold changes ± SEM for 3-4 experiments. Changes in the gene expression were not statistically significant.

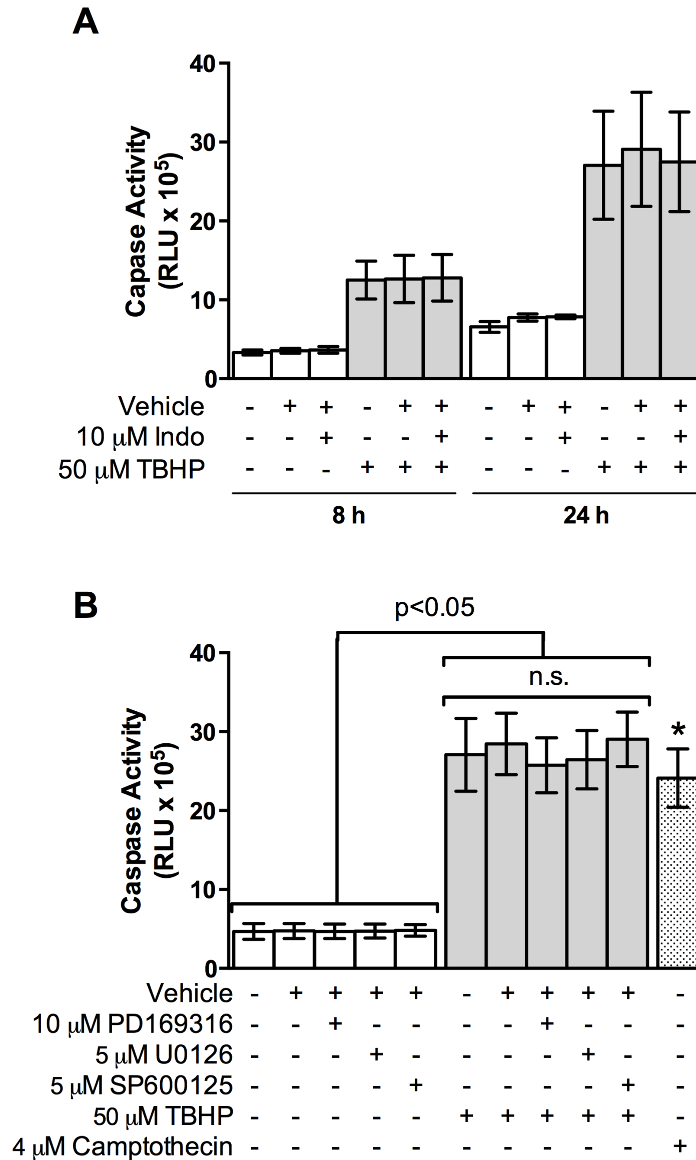
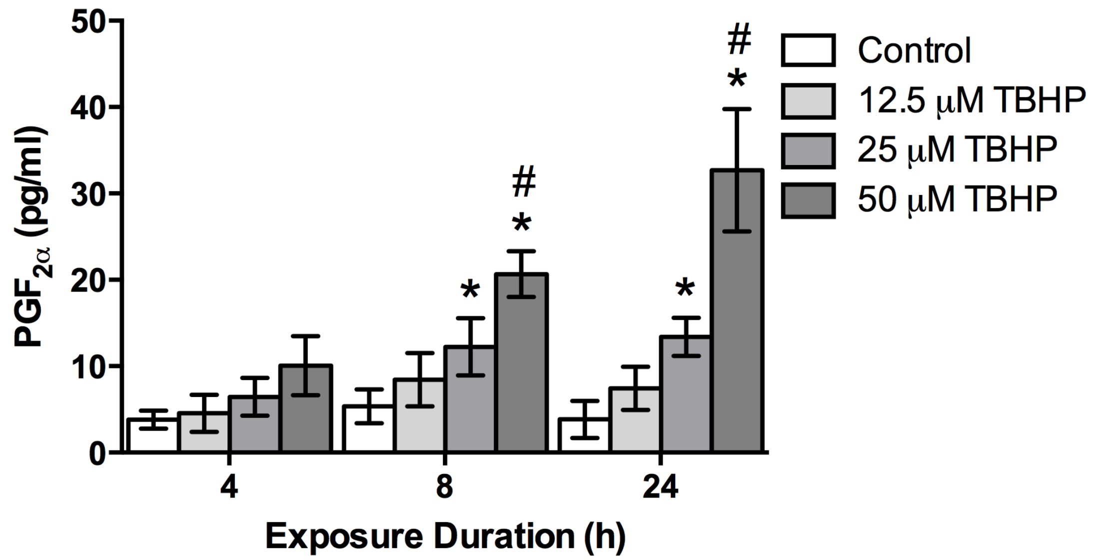



**Figure A.2. Effects of inhibitors of prostaglandin synthesis and MAPKs on TBHP-stimulated caspase 3/7 activity.** A) HTR-8/SVneo cells were treated with 50 μM TBHP in the presence or absence of 10 μM indomethacin for 8 or 24 h. Cultures exposed to 0.1% DMSO were vehicle controls for indomethacin. Filled bars represent treatment with TBHP and unfilled bars represent cells not treated with TBHP. Bars represent mean ± SEM for 2 experiments. We did not observe an inhibitory effect when cotreating with indomethacin. B) HTR-8/SVneo cells were pretreated with vehicle (0.1% DMSO; control), 10 μM PD169316 (p38 MAPK inhibitor), 5 μM U0126 (ERK 1/2 inhibitor), 5 μM SP600125 (JNK inhibitor) or medium alone for 1 h, and then exposed to 50 μM TBHP for 24 h. We did not observe a significant effect of MAPK inhibitors on TBHP-stimulated caspase 3/7 activity. Filled bars represent treatment with TBHP and unfilled bars represent cells not treated with TBHP. Data are presented as mean ± SEM of 3 experiments. *, Significantly different compared with vehicle-treated control ($p<0.05$).


A
PGF2α (pg/ml)
50
40
30
20
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0
4
8
24
Exposure Duration (h)
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12.5 μM TBHP
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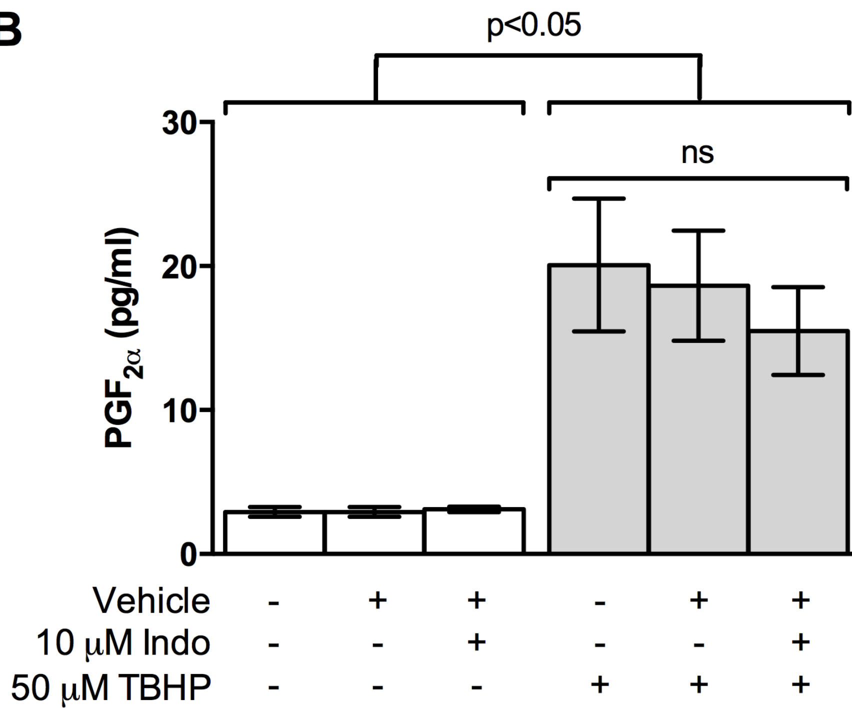
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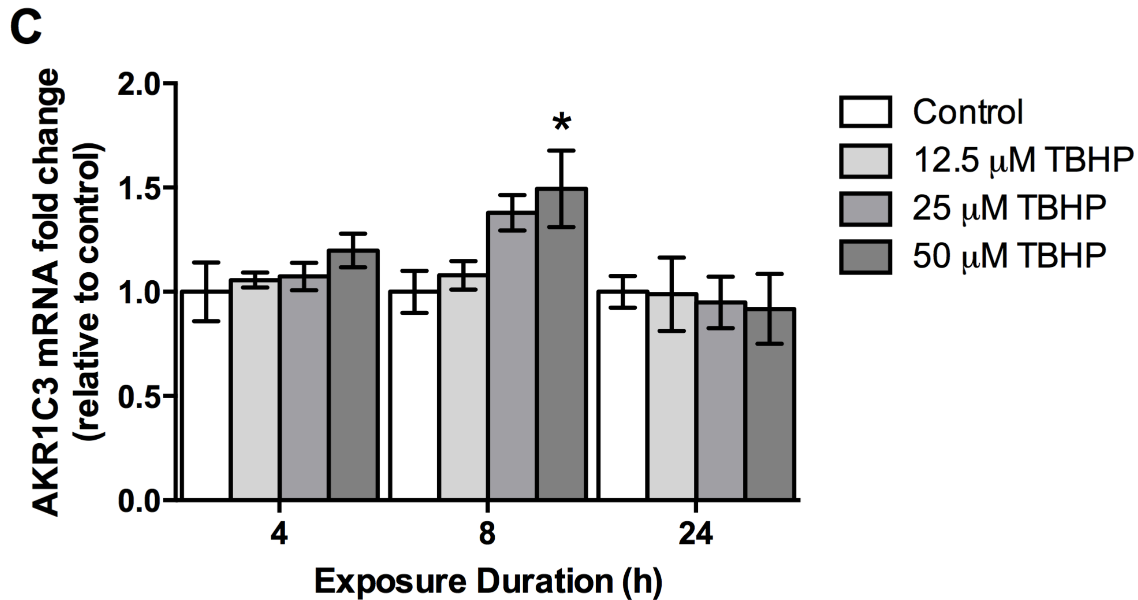


**Figure A.3. Effect of TBHP on $PGF_{2\alpha}$ and $PGF_{2\alpha}$ synthetic enzyme expression.** A) Time course and concentration response for TBHP on $PGF_{2\alpha}$ release into culture medium. HTR-8/SVneo cells were treated with 0, 12.5, 25, or 50 μM TBHP for 4, 8, or 24 h. We did not observe any significant changes in $PGF_{2\alpha}$ release with cells exposed to TBHP for 4 h or with exposure to 12.5 μM TBHP at any time point. However, 25 μM TBHP stimulated significant increases of 2.3 and 3.5 fold over time-matched controls at 8 and 24 h ($p<0.05$). Treatment with 50 μM TBHP stimulated increases of 3.9 and 8.5 fold over controls at the same respective time points ($p<0.05$). Futhermore, the stimulated $PGF_{2\alpha}$ response was concentration-dependent, increases with 50 μM TBHP exposure were also statistically different from cells treated with 25 μM TBHP ($p<0.05$). B) Effect of indomethacin (Indo) on TBHP stimulated $PGF_{2\alpha}$ release. To assess dependency of $PGF_{2\alpha}$ release on PTGS activity, we cotreated HTR-8/SVneo cells with 50 μM TBHP (shown in gray) and vehicle (0.1% DMSO) or 10 μM indomethacin. All of the TBHP treated cells were significantly increased over their cotreatment matched controls ($p<0.05$). Among the TBHP treated cells, there was no statistically significant difference between the indomethacin and vehicle co-exposed. C) Effect of TBHP on prostaglandin F synthase enzyme, AKR1C3. HTR-8/SVneo cells were treated with 12.5, 25, or 50 μM TBHP for 4, 8, or 24 h. TBHP did not stimulate any significant changes in AKR1C3 mRNA at 4 or 24 h. At 8 h, 50 μM TBHP stimulated a significant 1.5 fold increase in expression ($p<0.05$). Values represent mean ± SEM for 3-6 experiments. *, Statisically significant compared to time-matched controls ($p<0.05$). #, Statistically significant compared to time-matched 25 μM TBHP treated cells ($p<0.05$).