MONO-2-ETHYLHEXYL PHTHALATE STIMULATES PARTURITION-RELATED RESPONSES IN HUMAN GESTATIONAL TISSUES AND CELLS

By

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To my mother, Bonnie J. Tetz, and my father, C. David Tetz

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TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Diethyhexyl phthalate (DEHP) is an environmental pollutant used universally as a plasticizer in polyvinyl consumer products. Exposure to DEHP increases risk of adverse pregnancy outcomes in humans, including decreased gestation length, preterm birth, low birth weight, and early pregnancy loss. Moreover, mono-ethylhexyl phthalate (MEHP), the active metabolite of DEHP increases oxidative stress and inflammatory responses in vitro. Because oxidative stress and inflammatory responses are linked to the pathogenesis of preterm birth, we investigated MEHP stimulated oxidative stress and inflammatory responses in human gestational cells and tissues as mechanisms by which MEHP exposure may contribute to preterm birth. To identify whether MEHP exposure induces oxidative stress responses in the gestational compartment, we treated human placental cells (HTR-8/SVneo) with MEHP and measured reactive oxygen species (ROS) generation using the dichlorofluorescein (DCF) assay, oxidized thymine (oT) with mass-spectrometry, redox-sensitive gene expression with qRT-PCR, and activation of caspase 3/7 using a luminescence assay. We found that MEHP increased ROS generation, oxidative DNA damage, and apoptosis, and modified redox-sensitive gene expression. Notably, MEHP significantly induced

mRNA expression of prostaglandin-endoperoxide synthase 2 (PTGS2), the gene for COX-2, an enzyme important for prostaglandin synthesis. To assess whether MEHP may stimulate inflammatory responses in the gestational compartment, we treated human primary placental macrophages, primary decidual macrophages, gestational membrane explants, and HTR-8 cells with MEHP and measured prostaglandin and cytokine release using enzyme-linked immunosorbent assays (ELISA) and PTGS2 mRNA expression using qRT-PCR. Our results demonstrate that MEHP treatment significantly increased total prostaglandin, PGF2α, and PGE2 release in human primary placental macrophage Hofbauer cells, and induced PTGS2 mRNA expression in the HTR-8 human trophoblast cell line. MEHP treatment showed no effect on pro-inflammatory cytokine release. The results from the present study are consistent with the hypothesis that MEHP stimulates oxidative stress and prostaglandin synthesis in gestational tissues and cells. The findings from the current study warrant future epidemiological studies of oxidative stress and prostaglandin synthesis as mechanisms by which MEHP may contribute to preterm birth and other adverse pregnancy outcomes.

CHAPTER I

Introduction

DEHP is an abundant environmental pollutant

Di-2-ethylhexyl phthalate (DEHP) (Figure 1.1A), a phthalic acid ester, is a ubiquitous environmental pollutant used primarily in plastic products to impart flexibility. DEHP can be found in a wide variety of consumer products ranging from medical tubing and blood storage bags to plastic food containers, shower curtains, children's toys, clothing, automobile and furniture upholstery. Because DEHP is not covalently bound to plastic, it is particularly susceptible to leeching into the environment. Consequently, DEHP has been found in at least 733 of the 1,613 Environmental Protection Agency National Priorities List sites and in 2005, the EPA listed DEHP as an ATSDR priority chemical. The ubiquitous nature of DEHP has resulted in wide-spread exposure in the US population. A recent study conducted by the National Health and Nutrition Examination Survey (NHANES) found measureable levels of monoethylhexyl phthalate (MEHP) (Figure 1.1B), the active monoester metabolite of DEHP, in nearly every urine sample analyzed. Because DEHP is rapidly metabolized to its active monoester metabolites and excreted in the urine, the latter finding suggests that exposure to DEHP is essentially a daily occurrence.

DEHP is a reproductive toxicant

Exposure to DEHP is associated with a number of negative reproductive health outcomes in both humans and animals, including abnormal Leydig cell aggregation, disruption of steroidogenesis and cholesterol uptake in rat testes (Ge, Chen et al. 2007), and decreased anogenital distance—an index of prenatal androgen status-- in human male infants (Marsee, Woodruff et al. 2006). In female rats, DEHP exposure reduces serum levels of progesterone and estradiol, and suppresses ovulation (Davis, Maronpot et al. 1994). DEHP is associated with adverse pregnancy outcomes in humans including decreased gestation length, preterm birth, low birth weight, and early pregnancy loss (Latini, De Felice et al. 2003). Furthermore, measureable levels of MEHP are found in human cord blood, amniotic fluid, and placenta (Mose, Mortensen et al. 2007; Wittassek, Angerer et al. 2009; Lin, Ku et al. 2011). Though the latter studies suggest that the gestational compartment may be a target of MEHP toxicity, further studies are needed to elucidate the mechanisms underlying adverse pregnancy outcomes.

Preterm birth is a significant health problem

Preterm birth, defined as live birth occurring before 37 completed weeks of gestation, affects 21 million infants worldwide and half a million, or 1 in 8, infants per year in the U.S. Furthermore, preterm birth accounts for nearly one-third of all neonatal mortalities (Callaghan, MacDorman et al. 2006). Among preterm infants that survive, many develop long-term health complications later in life,

including blindness, deafness, cerebral palsy, and low IQ. Consequently, medical costs due to preterm birth represent a significant economic burden, costing the U.S. nearly $26 billion per year. The Center for Disease Control noted research into maternal, child, and infant health - including preterm birth - as an objective under the Healthy People 2020 initiative. Intrauterine infection and preeclampsia are the leading known causes of preterm birth, but only represent 25 and 15 percent of total preterm births, respectively (Goldenberg and Rouse 1998; Romero, Chaiworapongsa et al. 2003). In fact, greater than half of preterm births are attributed to unknown causes (Hamilton, Martin et al. 2005). A recent report from The Institute of Medicine highlighted the importance of further research into factors contributing to preterm birth, including environmental factors such as pollutants. The coincidental rise of environmental pollutants, such as phthalates gives further impetus to study the relationship between exposure to xenobiotics and adverse pregnancy outcomes such untimely parturition.

The placenta and gestational membranes play a role in initiation of parturition

The gestational membrane tissues, consisting of amniotic, chorionic and decidual tissue, extend from the placenta to surround the fetus and provide a barrier between the maternal and fetal compartments (Figure 1.2). The amnion layer of the gestational membranes includes a thick collagen layer that imparts strength and flexibility to the membranes. The proximity of the gestational membranes to the myometrium allows for transfer of secreted factors between these tissues. Trophoblast cells provide a barrier between fetal and maternal

blood flow, and contribute structural integrity to the placenta and gestational membranes (Figure 1.2). In addition to providing structural integrity, the trophoblasts produce protein and steroid hormones throughout gestation. Placental and gestational membrane chorionic trophoblasts also synthesize and secrete inflammatory mediators throughout gestation, including prostaglandins and pro-inflammatory cytokines. Other resident cells of the gestational membranes and placenta, including decidual macrophages, placental Hofbauer macrophages (Figure 1.2), amnion fibroblasts, and amnion epithelial cells, contribute to synthesis and secretion of prostaglandins and pro-inflammatory cytokines as well.

Measurable levels of MEHP are found in the gestational compartment

Following ingestion, DEHP is absorbed by the intestinal epithelium and rapidly hydrolyzed by lipases to MEHP (Rusyn, Peters et al. 2006). Consequently, blood is the primary vehicle for exposure to MEHP as distal organs, including the placenta and gestational membranes, are exposed primarily to MEHP through circulating blood. Measureable concentrations of MEHP have been found in maternal blood, cord blood, placenta, and amniotic fluid with the highest concentrations measured in maternal and cord blood. Specifically, median cord blood concentrations ranging from 1.8 to 35 µM, and median maternal blood concentrations ranging from 42.6 µM have been detected in Italian and Chinese cohorts, respectively (Latini, Del Vecchio et al. 2006; Lin, Zheng et al. 2008). At term, between 450 and 650 ml of blood per minute circulates through the placenta, therefore, circulating MEHP represents a significant source of exposure

for the gestational compartment (Edman, Toofanian et al. 1981).   The

concentrations of MEHP used in the present study (10-180 µM) are

physiologically relevant and within an order of magnitude of MEHP

concentrations measured in human maternal and cord blood.

Pro-inflammatory cytokines and prostaglandins mediate normal and preterm parturition

Both preterm and normal human parturition are associated with increased

pro-inflammatory cytokine and prostaglandin synthesis and release from cells of

the gestational compartment. Specifically, increased amniotic fluid

concentrations of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α and the

prostaglandins PGE2 and PGF2α are associated with preterm birth (Romero,

Manogue et al. 1989; Hillier, Witkin et al. 1993; Carroll, Abbas et al. 1995; Lee,

Park et al. 2009; Menon, Fortunato et al. 2011). This activation of the

inflammatory response is central to the pathogenesis of intrauterine infection, the

leading known cause of preterm birth. Elevated pro-inflammatory cytokines and

prostaglandins are observed in the amniotic fluid of preterm birth cases with

intrauterine infections (Keelan, Blumenstein et al. 2003). Furthermore, exposure

to the pro-inflammatory stimulus lipopolysaccharide (LPS) increases pro-

inflammatory cytokine and prostaglandin release from gestational tissues and

induces preterm birth in mice (Miller and Loch-Caruso 2010; Phillippe, Diamond

et al. 2011). Preceding labor, the amnion, chorion, and decidua of the

gestational membranes, in addition to resident immune cells of the placenta,

participate in production of pro-inflammatory cytokines and prostaglandins. Pro-

inflammatory cytokines are secreted proteins that act through autocrine and paracrine mechanisms to initiate inflammatory events, including recruitment of circulating leukocytes to sites of infection. Prostaglandins represent a class of lipid mediators that have pleiotropic physiological functions in nearly every tissue. These functions range from vasodilation/vasoconstriction to platelet function and cytoprotection (Miller 2006). During pregnancy, pro-inflammatory cytokines stimulate bioactive prostaglandin synthesis through induction of the prostaglandin catabolic enzyme COX-2 (Hansen, Keelan et al. 1999). COX-2 synthesizes prostaglandin E2 (PGE2) and prostaglandin F2α (PGF2α), which bind to excitatory receptors in the uterus to initiate myometrial contractions (Olson 2003). Inhibition of prostaglandin synthesis through COX-2 inhibition delays pregnancy and prevents preterm labor in rodents, and in vivo or in vitro exposure to bioactive prostaglandins stimulates myometrial contractions (Wikland, Lindblom et al. 1984; Lee, Kim et al. 2003; Fischer, Hutchinson et al. 2008).   In addition to their role in prostaglandin synthesis, pro-inflammatory cytokines also stimulate expression and activation of matrix metalloproteinases and activation of apoptosis, contributing to gestational membrane weakening and cervical ripening (Vadillo-Ortega and Estrada-Gutierrez 2005). In summary, pro-inflammatory cytokine release from gestational tissues stimulates prostaglandin synthesis, apoptosis, and activation of matrix-metalloproteinases to promote membrane rupture, uterine contractions, cervical ripening, and ultimately, parturition (Figure 1.3).

DEHP exposure is associated with inflammation and induction of inflammatory mediators

In humans, urinary metabolites of DEHP are associated with increases in urinary markers of inflammation, including C-reactive protein and absolute neutrophil count (Ferguson, Loch-Caruso et al. 2011; Meeker and Ferguson 2011).Consistent with an observed increase in inflammatory markers in humans exposed to DEHP, airway inflammation and allergic responses have also been observed in DEHP-exposed humans and animals (Deutschle T 2008, Kolarik B).

Exposure to DEHP or its metabolite MEHP stimulates release and synthesis of inflammatory mediators that are known to be associated with preterm labor. MEHP treatment of primary neonatal neutrophils, rat alveolar macrophages, murine monocyte-macrophages, human lung epithelial cells, and mouse Leydig cells increases pro-inflammatory cytokine release (Jepsen, Abildtrup et al. 2004; Rakkestad, Holme et al. 2010; Vetrano, Laskin et al. 2010). Furthermore, MEHP treatment increases release of the bioactive prostaglandin PGF2α in cultured bovine ovarian and endometrial cells, and increases expression of the prostaglandin catabolizing enzyme COX-2 in spermatocytes and rat and human mast cells (Ledwith, Pauley et al. 1997; Onorato, Brown et al. 2008; Oh, Lim et al. 2010; Wang, Shang et al. 2010; Oh and Lim 2011).

Of direct relevance to the present work is the finding that DEHP exposure (750 or 1500 mg/kg/day oral gavage) decreases COX-2 expression and total prostaglandin concentration in rat placental tissue (Xu, Agrawal et al. 2008). The

findings from the latter study are contrary to studies in other species and organ systems demonstrating increased COX-2 and prostaglandins with DEHP or MEHP exposure. For example, MEHP treatment increases PGE2 release from cultured bovine ovarian and endometrial cells, and increases COX-2 expression in spermatocytes and rat and human mast cells (Ledwith, Pauley et al. 1997; Onorato, Brown et al. 2008; Oh, Lim et al. 2010; Wang, Shang et al. 2010; Oh and Lim 2011). Differences could be due to species or organ system differences in prostaglandin pathway responses to MEHP. Furthermore, the doses of DEHP used in the latter study exceeded the 95th percentile of estimated adult human intake of 21 µg/kg/day by approximately four orders of magnitude (Koch, Preuss et al. 2006). Further studies testing the effects of physiologically relevant concentrations of MEHP on prostaglandin synthesis and pro-inflammatory cytokine release in human gestational tissues and cells would provide additional insight into the role of MEHP in stimulation of inflammatory responses during pregnancy.

Oxidative stress is associated with preterm and normal term parturition

Oxidative stress is characterized by an excess of damaging reactive oxygen species (ROS) resulting from an imbalance between oxidant species generation and cellular antioxidant capacity. Excess ROS can result in damage to macromolecules, including lipid peroxidation, oxidative DNA adduct formation, and protein oxidation. Exposure to pollutants that induce oxidative stress, including air pollution, cigarette smoke, and organochlorine pesticides increases risk for preterm birth (Cnattingius 2004; Malmqvist, Rignell-Hydbom et al. 2011;

Chang, Reich et al. 2012). Specifically, exposure to organochlorine pesticide residues during pregnancy is associated with elevated cord blood concentrations of malondialdehyde and decreased cord blood concentrations of reduced glutathione, indicative of lipid peroxidation and antioxidant depletion (Pathak, Suke et al. 2010).

Oxidative stress in the cells and tissues of the gestational compartment is linked to preterm labor and normal human parturition. Normal human parturition corresponds with increased protein oxidation and lipid peroxidation and decreased antioxidants in myometrial tissues (Khan, Matharoo-Ball et al. 2010). Similarly, preterm labor is associated with oxidative stress markers including the lipid peroxidation products F2-isoprostane and malondialdehyde, and decreases in the antioxidant enzyme glutathione peroxidase (Fainaru, Almog et al. 2002; Mocatta, Winterbourn et al. 2004). Furthermore, increased levels of the urinary oxidative stress markers 8-isoprostane and 8-hydroxydeoxyguanosine (8-OHdG) in early or mid-gestation are predictive of preeclampsia and decreased gestation length (Peter Stein, Scholl et al. 2008; Hsieh, Chen et al. 2012). Although the latter observations suggest that oxidative stress events correspond with preterm and normal term parturition, the function of these events in initiation of parturition is not fully understood.

Evidence suggests that reactive oxygen species generation in cells of the gestational compartment may activate cellular pathways relevant to preterm birth including NF-κB, pro-inflammatory cytokine response, and apoptosis. Specifically, antioxidant pretreatment prevents LPS-stimulated release of pro-

inflammatory cytokines and NF-kB activation in gestational membrane explant cultures, and prevents LPS-induced preterm labor in mice (Buhimschi, Buhimschi et al. 2003; Lappas, Permezel et al. 2003). In addition, oxidative insult in cultured placental and chorionic membrane trophoblasts initiates apoptosis (Rosado-Berrios, Velez et al. 2011). The latter studies provide compelling evidence to suggest that parturition relevant pathways, including apoptosis, pro-inflammatory cytokine release, and lipid mediator synthesis may be mediated through oxidative stress. Furthermore, these studies suggest that oxidative stress may have more than an associative relationship to parturition events and that, in fact, oxidative insult may be capable of initiating these events.

DEHP exposure induces oxidative stress responses

Both apoptosis and oxidative stress are mechanisms common to the toxicity of many environmental pollutants, including MEHP. In humans, urinary MEHP or its oxidized metabolites are correlated with urinary markers of oxidative stress including decreased bilirubin and increased gamma glutamyltransferase (GGT), malondialdehyde (MDA), and 8-hydroxydeoxyguanosine (8-OHdG) (Hong, Park et al. 2009; Ferguson, Loch-Caruso et al. 2011; Ferguson, Loch-Caruso et al. 2011). In vitro, MEHP treatment induces reactive oxygen species generation in neutrophils and cells of the liver and testes. (Rose, Rivera et al. 1999; Erkekoglu, Rachidi et al. 2010; Fan, Traore et al. 2010; Vetrano, Laskin et al. 2010; Erkekoğlu, Rachidi et al. 2011; Rosado-Berrios, Vélez et al. 2011). Furthermore, MEHP toxicity in germ cells and Leydig cells of the testes is linked to decreases in antioxidant levels, increased oxidative DNA damage, and induction of

apoptosis (Richburg, Nañez et al. 2000; Kasahara, Sato et al. 2002; Hauser, Meeker et al. 2007; Suna, Yamaguchi et al. 2007; Erkekoglu, Rachidi et al. 2010). Despite the evidence linking MEHP-induced oxidative stress to disease outcomes in the liver, immune system, and male reproductive system, little attention has been given to the role of oxidative stress in adverse female reproductive outcomes.

Research Objectives

Based on the evidence that MEHP induces oxidative stress and inflammatory mediator synthesis in other organ systems, and because MEHP exposure is associated with adverse pregnancy outcomes in humans, *we hypothesize that MEHP activates cellular responses relevant to preterm birth in human gestational tissues and cells.* The specific aims of this project are to: 1) Examine the effects of MEHP treatment on reactive oxygen species generation, oxidative DNA damage, antioxidant gene expression, and apoptotic cell death in human placental trophoblast cells; and 2) Examine the effects of in vitro MEHP treatment on prostaglandin synthesis and pro-inflammatory cytokine release in primary human placental and decidual macrophages, human placental trophoblast cells, and gestational membrane explants.
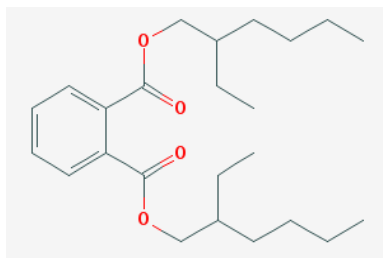
Figure 1.1A. Structure of diethylhexyl phthalate (DEHP).
Image taken from http://pubchem.ncbi.nlm.nih.gov.
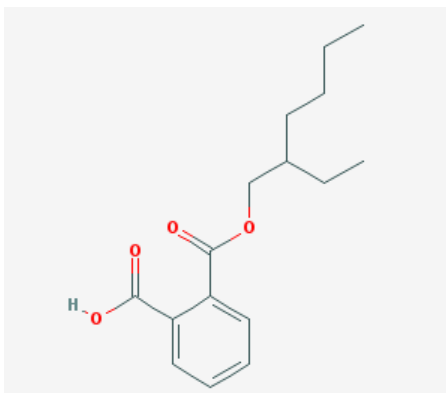
Figure 1.1B. Structure of monoethylhexyl phthalate (MEHP).
Image taken from http://pubchem.ncbi.nlm.nih.gov.

Gestational membranes

amnion
chorion
decidua
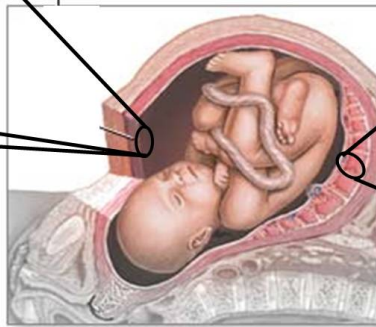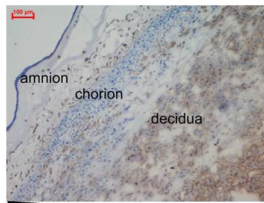
Placenta – Chorionic villi

trophoblast

Hofbauer cell
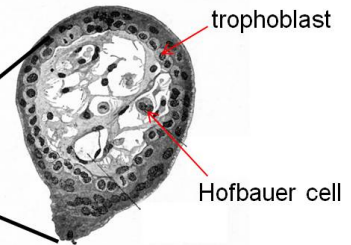
Figure 1.2. Anatomy of the pregnant uterus.
Diagram adapted from A.D.A.M Interactive Anatomy 2009. Gestational membrane histology section from Thiex 2008. Chorionic villi image adapted from Miller 1921.
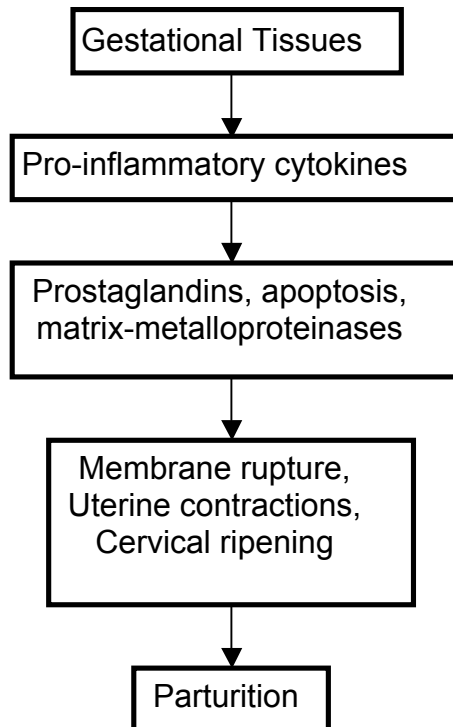
Figure 1.3. Model of parturition initiation. Pro-inflammatory cytokine release from gestational tissues stimulates prostaglandin synthesis, apoptosis, and activation of matrix-metalloproteases to promote membrane rupture, uterine contractions, cervical ripening, and ultimately, parturition.

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CHAPTER II

Mono-2-ethylhexyl phthalate-induced oxidative stress
in human placental cells

Abstract

Di-2-ethylhexyl phthalate (DEHP) is an environmental contaminant used as a plasticizer in polyvinyl chloride products. Mono-ethylhexyl phthalate (MEHP), the active metabolite of DEHP, increases reactive oxygen species production and decreases antioxidants in liver, kidney and testicular cells. To investigate whether placenta is a potential target of MEHP, we evaluated MEHP effects on reactive oxygen species generation, oxidative DNA adduct formation, apoptosis and expression of redox-sensitive genes in the human extravillous trophoblast cell line HTR-8/SVneo (HTR-8). HTR-8 cells were treated with MEHP concentrations ranging from 10-180 µM. Solvent controls were exposed to dimethyl sulfoxide (DMSO; 0.05% v/v). Stimulation of oxidant species generation was assessed by formation of the fluorescent product 2,7′-dichlorofluorescein (DCF) in HTR-8 cells loaded with 5-(and-6)-carboxy-2′7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) and exposed for 1 h to MEHP. Treatment with 45, 90, or 180 µM MEHP for 1 h significantly increased oxidant species after 1 h of exposure. In other experiments, HTR-8 cells were loaded with H2DCF-DA, pretreated for 1 h with or

without the iron chelator deferoxamine mesylate (DFO; 1 mM), and then exposed for 1 h to MEHP. Pretreatment with DFO reduced MEHP-induced DCF fluorescence. To measure oxidized thymine adduct formation, cells were treated 24 h with MEHP. DNA then was extracted, and concentrations of oxidized thymine were quantified using mass-spectrometry. Treatment with 180 μM MEHP increased oxidized thymine adduct formation two-fold compared with solvent controls. To assess apoptosis, caspase 3/7 activation was measured using a luminescence-based assay following 24-h exposure to MEHP. A 1.5-fold increase in caspase 3/7 activation was observed with 180 μM MEHP treatment ($p \leq 0.05$). RNA expression of 84 redox-sensitive genes was measured in cells exposed to MEHP for 4, 8 or 24 h using a qRT-PCR array (n=3 experiments). Using qqRT-PCR, we validated the findings of the array for those genes with significant mRNA expression changes that were approximately two–fold or more with 180 μM MEHP treatment: AOX1, DHCR24, GLRX2, PRNP, SCARA3, TXNRD1, and PTGS2.   Treatment with 180 μM MEHP significantly increased mRNA expression of PTGS2, GLRX2, and TXNRD1 and significantly decreased mRNA expression of DHCR24. These results show that MEHP induces an oxidative stress response in human placental cells.

Introduction

Diethylhexyl phthalate (DEHP) is primarily used as a plasticizer in the manufacturing of polyvinyl chloride (PVC) and is found in a wide variety of consumer products including food packaging, intravenous medical tubing, clothing, furniture and automobile upholstery, and children's toys. DEHP is not covalently bound to PVC products and as a result leaches into the environment where it collects in dust particles or in food contained in PVC plastic. DEHP is a pervasive environmental contaminant, present in 733 out of 1613 Environmental Protection Agency (EPA) National Priority List sites. Exposure to DEHP is widespread and frequent in the US population. Data collected from the National Health and Nutrition Examination Survey (NHANES) datasets from 1999 to 2006 show measurable levels of monoethylhexyl phthalate (MEHP), the active metabolite of DEHP, in 98% of urine samples analyzed. Because DEHP is rapidly metabolized to its active monoester metabolites and excreted in the urine, the latter finding suggests that human exposure to DEHP is a widespread and daily occurrence.

Exposure to DEHP is linked to adverse pregnancy outcomes. In humans, higher concentrations of MEHP in urine or cord blood samples of pregnant women are associated with low birth weight, increased risk for preterm birth, decreased gestation length, and pregnancy loss (Latini, De Felice et al. 2003; Lin, Zheng et al. 2008; Meeker, Hu et al. 2009; Toft, Jonsson et al. 2011). Furthermore, MEHP has been detected in placenta, amniotic fluid and cord blood of humans (Mose, Mortensen et al. 2007; Wittassek, Angerer et al. 2009; Lin, Ku

et al. 2011). The latter two findings suggest that the gestational compartment may be a target of MEHP toxicity. Despite the evidence linking DEHP exposure to adverse pregnancy outcomes, the mechanism underlying these associations is unclear.

Oxidative stress, defined as an imbalance between the production of damaging reactive oxygen species (ROS) and cellular antioxidant defenses, and apoptosis in cells of the gestational compartment are closely linked to the pathology of adverse pregnancy outcomes, as evidenced by increased markers of apoptosis and oxidative stress in pathological pregnancies. For example, placental trophoblasts from pregnancies complicated by preeclampsia, intrauterine growth restriction, and miscarriage express higher levels of oxidative stress and apoptotic markers compared to normal controls (DiFederico, Genbacev et al. 1999; Hempstock, Jauniaux et al. 2003; Tomas, Prusac et al. 2011). Furthermore, increased levels of urinary oxidative stress markers early in pregnancy predict preeclampsia and shortened gestation length (Peter Stein, Scholl et al. 2008; Bazavilvaso-Rodríguez, Hernández-Valencia et al. 2011; Miranda Guisado, Vallejo-Vaz et al. 2011).

Apoptosis and oxidative stress are mechanisms common to the toxicity of many environmental pollutants, including MEHP. In humans, urinary MEHP or its oxidized metabolites are associated with urinary markers of oxidative stress (Ferguson, Loch-Caruso et al. 2011; Ferguson, Loch-Caruso et al. 2011). In vitro MEHP treatment of neutrophils, Kupfer cells and Leydig cells generates (ROS) (Rose, Rivera et al. 1999; Erkekoglu, Rachidi et al. 2010; Fan, Traore et al. 2010;

Vetrano, Laskin et al. 2010; Erkekoğlu, Rachidi et al. 2011; Rosado-Berrios, Vélez et al. 2011). Furthermore, MEHP toxicity in germ cells or Leydig cells of the testes is linked to decreased levels of GSH and ascorbic acid, decreased thioredoxin reductase expression, decreased glutathione peroxidase activity, increased DNA damage, and induction of apoptosis (Richburg, Nañez et al. 2000; Kasahara, Sato et al. 2002; Hauser, Meeker et al. 2007; Suna, Yamaguchi et al. 2007; Erkekoglu, Rachidi et al. 2010). In human first trimester trophoblast cells, in vitro DEHP treatment increases apoptosis and expression of the pro-apoptotic gene BAX2 (Wang, Shang et al. 2010). The biological relevance of the latter study is challenged, however, by the fact that DEHP is rapidly metabolized to MEHP by lipases in the gut or lungs after ingestion or inhalation, respectively, converting most DEHP to MEHP before distribution to distal organs of the body, including the placenta (Frederiksen, Skakkebaek et al. 2007).

In the present study, we hypothesize that MEHP induces oxidative stress responses and subsequent apoptotic cell death in cells of the gestational compartment. We examined the effects of MEHP treatment on ROS generation, oxidative DNA damage, redox-sensitive gene expression, and apoptotic cell death in HTR-8/SVneo (HTR-8) cells, a human first trimester extravillous trophoblast cell line.

Materials and Methods

Reagents. We purchased carboxy-dichlorodihydrofluorescein diacetate (H_2DCF-DA), Hoechst dye, phosphate buffered saline (PBS), and Hank's balanced salt solution (HBSS) from Invitrogen Life Technologies; dimethyl sulfoxide (DMSO), deferoxamine mesylate, *tert*-butyl hydroperoxide (TBHP), and camptothecin from Sigma-Aldrich (St. Louis, MO, USA); and MEHP from Accustandard (New Haven, CT).

Cell culture and treatment. The HTR-8 cells were a gift from Dr. Charles Graham (Queens University, Ontario, Canada). The HTR-8 cells were isolated from first trimester human placenta and immortalized with SV40 antigen (Graham, Hawley et al. 1993). Similar to their primary counterparts, HTR-8 cells express human chorionic gonadropin, stain positive for the epithelial marker cytokeratin-7, and retain migratory capabilities in culture (Graham, Hawley et al. 1993). Cells between passages 71 and 84 were cultured in RPMI 1640 medium with L-glutamine without phenol red (Gibco; Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin (Gibco) at 37°C in a 5% CO_2 humidified atmosphere. For most experiments, cells were grown to a confluence of 70-80% before treatment. For the dichlorofluorescein (DCF) plate-based assay, cells were grown to a confluence of 80-90% before treatment. From stock solutions of 392 µM MEHP in DMSO, exposure media solutions of 10, 22.5, 45, 90, or 180 µM MEHP were made immediately prior to initiating the experiment. The DMSO concentration was 0.05% for all exposure groups.

ROS measurement. Stimulation of ROS generation was assessed spectrofluorometrically using the dichlorofluorescein (DCF) assay. The HTR-8 cells were seeded at a density of 30,000 cells per well in a 96-well black, clear bottom plate and incubated at 37°C in a 5% CO_2 atmosphere for 24 h. Cells were then pre-incubated with 100 µM H_2DCF-DA in HBSS for 60 min at 37 °C. The dye solution was then removed, the cultures were rinsed with HBSS, and the cells were then treated with dimethyl sulfoxide (DMSO; 0.05% v/v; solvent control), or with 10, 22.5, 45, 90, or 180 µM MEHP in replicates of 6 for 60 min. Treatment with the prototypical chemical oxidant *tert*-butyl hydroperoxide (TBHP;100 µM) served as a positive control. After washing with HBSS and then adding fresh HBSS back to the cells, fluorescence was measured from the bottom of the culture plate with the Molecular Devices SpectraMax Gemini M2e at an excitation wavelength of 492 nm and emission wavelength of 522 nm. In previous experiments, we determined that MEHP in 0.05% DMSO showed no effects on DCF fluorescence in cell-free HBSS buffer compared to DMSO alone (Appendix A, Figure A1.)

Inhibition of DCF fluorescence was assayed by fluorescence microscopy. The HTR-8 cells were seeded at a density of 400,000 cells per well in a 6-well plate and cultured for 24 h before incubation with 100 µM H_2DCF-DA in HBSS for 1 h. After removal of the dye solution and rinsing with HBSS, cultures were incubated for an additional 1 h with 1 mM deferoxamine mesylate (DFO) as an antioxidant treatment. Previously, we had determined that 1mM deferoxamine was not cytotoxic to HTR-8 cells, using the Promega Cyquant viability assay

(data not shown). Cultures were exposed to HBSS alone, 0.05% DMSO (solvent control), or 180 µM MEHP for 90 min, and then counterstained with the nucleic acid stain Hoechst for 5 min. Using an EVOS digital inverted fluorescence microscope, intracellular DCF fluorescence was visualized at 470 nm excitation and 525 nm emission, and Hoechst stain was visualized at 357 nm excitation and 447 nm emission. Five images per treatment were taken, one image in each of the four quadrants and one in the center of the well.

Oxidized thymine measurement. HTR-8 cells were seeded at a density of 3.5-4 x 10^6 cells in 175 cm^2 flasks. After 24 h of incubation, cells were treated with DMSO (solvent control), 50 µM TBHP, 90 µM MEHP, or 180 µM MEHP for 24 h. Genomic DNA was extracted using the Qiagen Blood and Cell Culture DNA Midi Kit following the manufacturer's protocol. Oxidized thymine (oT) was measured in collaboration with the Giese laboratory at Northeastern University as described in detail elsewhere by the following sequence of steps: (1) DNA was digested to nucleotides with nuclease P1 and phosphodiesterase; (2) the enzymes were removed by ultrafiltration; (3) the nucleotides were separated by reversed-phase HPLC and fractions eluting between dCMP and TMP were collected; (4) the nucleotides in the collected fraction were converted to phosphorimidazolides with a pair of isotopologue (d_o, d_4) benzoylhistamine (BH) reagents in the presence of a water-soluble carbodiimide; (5) cation exchange filtration was conducted to remove residual reagents; (6) capillary reversed-phase HPLC was conducted by spotting onto a MALDI target followed by matrix addition containing an internal standard (BH-labeled 1,N^6-etheno-dAMP) ; and (7) BH-labeled-oT and internal

29

standard were measures by MALDI-TOF-MS with structural confirmation by MALDI-TOF/TOF-MS (Wang, Fisher et al. 2012).

Caspase 3/7 activity assay. We measured caspase 3/7 activity in cell lysates using the Caspase-Glo 3/7 luminescent assay (Promega) following the manufacturer's recommended protocol. The HTR-8 cells were seeded at a density of 10,000 cells per well in a 96-well white, clear-bottom plate 24 h prior to treatment. Cells were then treated with medium alone, DMSO (solvent control), MEHP (22.5, 45, 100, or 180 µM), or 4 µM camptothecin (positive control) for 4, 8, or 24 h. We observed no significant differences between medium alone and solvent control (DMSO) cultures (data not shown).

Cytotoxicity and viability assay. The MultiTox-Glo Multiplex Cytotoxicity Assay (Promega, Madison, WI, USA) was used to quantify cytotoxicity using a luminescent substrate for extracellular, dead-cell protease, and to quantify viability using a fluorescent substrate for intracellular proteases, following the manufacturer's recommended protocol. The HTR-8 cells were seeded at 10,000 cells/well in a 96-well, white, clear-bottomed plate 24 h prior to treatment. Cells were treated with medium alone, DMSO (solvent control), MEHP (22.5, 45, 90, or 180 µM), or 4 µM camptothecin (positive control) for 24 or 48 h. Fluorescence was measured using the SpectraMax M2 Multi-Mode Microplate Reader (Molecular Devices) and luminescence was measured using the Glomax Multi Plus Detection System (Promega).

Oxidative stress and antioxidant response gene array and qRT-PCR

validation. Because MEHP stimulated reactive ROS generation as assessed by

DCF fluorescence, we evaluated changes in gene expression in the oxidative

stress response pathway using the Oxidative Stress and Antioxidant PCR Array

from SABiosciences. The HTR-8 cells were seeded at a density of 400,000 cells

per well in a 6-well cell culture plate and allowed to adhere for one day. Cells

were treated with medium alone, DMSO (solvent control), MEHP (90, or 180 µM)

or 50 µM TBHP. After 4, 8, or 24 h of exposure, RNA was extracted using the

RNeasy Plus Mini Kit (Qiagen), and cDNA was synthesized from 1 µg of total

RNA using the RT^2 First Strand Kit (SABiosciences) following the manufacturers'

recommended protocols. For the array, the cDNA from solvent control and 180

µM MEHP treatments was analyzed using the ABI 7900HT Sequence Detection

System following the SABiosciences recommended protocol. Fold changes were

calculated using the ΔΔCT method, normalizing each sample to the average CT

of all housekeeping gene CT values. Mean ΔCT values were compared between

groups using paired t-tests from the Limma package of Bioconductor (Smyth

Gordon 2004). The resulting p-values were adjusted for multiplicity using the

Benjamini and Hochberg false discovery rate method (Benjamini and Hochberg

1995). Using qRT-PCR, we validated the findings of the array for those genes

with significant mRNA expression changes that were approximately two–fold or

more with 180 µM MEHP treatment: AOX1, DHCR24, GLRX2, PRNP, SCARA,

TXNRD1, and PTGS2. qRT-PCR was performed on these seven genes using

samples from cells treated with medium alone, DMSO (solvent control), 90 or

31

180 μM MEHP, or 50 μM TBHP for 24 h. Using the SABiosciences RT^2 First

Strand Kit, cDNA was made from 1 μg of mRNA. PCR reactions were run with

the same SABiosciences primers used in the Oxidative Stress and Antioxidant

PCR Array and SYBR green mastermix (SABiosciences), with 2-5 μL of

template. qRT- PCR reactions were run on the Bio-Rad CFX96 Real Time C1000

thermal cycler following the manufacturer's recommended protocol.

Statistical analysis. Multiple group comparisons were carried out using a

paired one-way ANOVA or two-way ANOVA for two-factor analyses and pairwise

comparisons were carried out using paired t-tests, with $p < 0.05$ as the

significance cutoff. Data are expressed as the mean ± SD of between 3 and 5

experiments.

Results

Cellular generation of reactive chemical species. Treatment of HTR-8 cells

with 45, 90 or 180 μM MEHP for 1 h resulted in a significant increase in relative

fluorescence units over the solvent control, indicating increased H_2DCF-DA

oxidation (Figure 2.1). Interestingly, this increase was larger than that observed

with the prototypical chemical oxidant, TBHP (100 μM). When cells were

pretreated with the iron chelator, deferoxamine mesylate, DCF fluorescence was

visibly decreased in cells treated with 180 μM MEHP, suggesting a Fenton-

dependent production of hydroxyl radical from hydrogen peroxide with MEHP

treatment (Figure 2.2). Using microscopy, we observed no differences in

32

fluorescence when cells were incubated in HBSS alone or HBSS with 0.05% DMSO (data not shown).

Oxidized thymine (oT) formation. Figure 2.3A shows oxidation of thymine in three separate experiments. Treatment with MEHP for 24 h increased oT approximately 20% with 90 µM and approximately 80% with 180 µM MEHP, on average. An increase in oT of approximately 200% was also observed with 50 µM TBHP treatment. To obtain these results, DNA from the samples was digested to nucleotides and the appropriate HPLC fraction was subjected to labeling with a mass tag prior to analysis by mass spectrometry. Samples were paired, where one sample (after HPLC collection) was labeled with a nonisotopic (d_0) tag, whereas the other sample was labeled with a corresponding isotopic (d_4) tag, prior to combining the samples to assess the relative amounts of oT. At the same time, the absolute amounts were determined based on the internal standard (BH-labeled 1,N^6-etheno-dAMP). In these experiments, the tags were also reversed. Representative data is shown in Figure 2.3B.

Caspase 3/7 activity. MEHP treatment significantly increased caspase 3/7 activation by approximately 80% at 24 h (Figure 2.4; p=0.01). This increase was modest, though, in comparison to the positive control (4 µM camptothecin). We observed no significant treatment effects at 4 h or 8 h, and no differences in caspase 3/7 activity in controls treated with medium alone compared with 0.05% DMSO (solvent control) at 4, 8, or 24 h (data not shown).

Cytotoxicity and cell viability. MEHP treatment had no effect on cytotoxicity, at either 24 h or 48 h, as assessed by an extracellular protease activity assay for membrane integrity (data not shown). We observed no differences in cytotoxicity or viability with medium alone compared with 0.05% DMSO at 24 h or 48 h. Treatment with 180 μM MEHP, but not lower MEHP concentrations, significantly decreased cell viability at 48 h but not 24 h, as measured by intracellular protease activity (Figure 2.5; p<0.001).

mRNA expression. We identified seven genes with the Oxidative Stress and Antioxidant Response Gene Array whose mRNA expression significantly changed two-fold or more with 180 µM MEHP treatment compared to solvent control (Table 2.1; adjusted p value<0.05). The results of the array showed that MEHP treatment significantly increased expression of PTGS2 at 4, 8, and 24 h, and GLRX2, PRNP, TXNRD1, and AOX1 at 24 h; whereas expression significantly decreased for SCARA3 and DHCR24 at 24 h. With qRT-PCR analysis of RNA extracts from samples treated for 24 h, we confirmed MEHP-stimulated changes in gene expression for four of these genes: increased expression of PTGS2, GLRX2, TXNRD1 and decreased expression of DHCR24 (p-value=0.001, 0.01, 0.048, and 0.002, respectively) (Figure 2.6 A, B, C, and D, respectively). Although the expression changes observed with MEHP treatment were not significant for SCARA, PRNP, or AOX1 (Figure 2.6E, F, and G, respectively), we observed the same directional changes in expression as were observed with the qRT-PCR array. Cells exposed to 180 µM MEHP had similar magnitude changes in gene expression as were observed with 50 µM TBHP;

however, these groups were not compared by statistical analysis due to low sample size in the TBHP treatment group. Likewise, statistical comparisons were not performed between the solvent (DMSO) control group and the control group exposed to medium alone because we had an n of 2 for the medium alone treatment group.

Discussion

The goal of the current study was to investigate oxidative stress as a cellular mechanism by which MEHP exposure may contribute to adverse pregnancy outcomes. Our findings demonstrate that MEHP induces oxidative stress responses in human placental cells, specifically, ROS production, oxidative DNA damage, and modification of redox-sensitive gene expression. The direction and magnitude of redox-sensitive gene expression changes observed with MEHP treatment were comparable to those observed with tert-butyl hydroperoxide (TBHP) treatment, suggesting that MEHP acts similarly to a prototypical chemical oxidant to modify antioxidant and redox sensitive gene expression in the HTR-8 cells. Notably, MEHP treatment strongly induced PTGS2, the gene for COX-2, suggesting that MEHP may stimulate synthesis of prostaglandins necessary for stimulation of myometrial contractions and ultimately, parturition.

This is the first study to document MEHP-stimulated oxidative stress responses in cells of the gestational compartment, consistent with previous

reports that MEHP stimulates ROS production and oxidative DNA damage are observed in other cell types. In humans, urinary levels of MEHP are correlated with increased DNA damage in sperm. In vitro, MEHP treatment induces DNA damage in human liver carcinoma cells at concentrations ranging from 25 to 100 μM, and human lymphocytes at concentrations ranging from 100-2500 μM (Yang, Zhou et al. ; Kleinsasser, Harreus et al. 2004; Hauser, Meeker et al. 2007). In addition, in vitro MEHP treatment stimulates ROS generation in human prostate adenocarcimona cells at a concentration of 3 μM and human neutrophils, human lymphoblast cells, and mouse Leydig cells at concentrations ranging from 100-500 μM (Zhao, Ao et al. ; Erkekoglu, Rachidi et al. 2010; Erkekoglu, Rachidi et al. 2010; Rosado-Berrios, Vélez et al. 2011).

Previous studies demonstrating that increased urinary 8-OHdG is predictive of shortened gestation length and low birth weight highlight the relevance of our findings to the etiology of adverse pregnancy outcomes. The intrauterine environment is maintained at relatively low-oxygen concentrations in order to support proper development of the placenta. As a consequence, early trophoblast cells tend to express lower levels of antioxidants (Davis and Auten 2010). This suggests that first trimester trophoblasts may be more susceptible to oxidative stress from chemicals that increase the generation of ROS compared to third trimester trophoblast cells.

In the HTR-8 cells, MEHP increased activity of the executioner caspases 3 and 7 at 24h and decreased cell viability at 48h, suggesting apoptotic activation and subsequent cell death. This finding is supported by in vitro studies

36

evidencing apoptosis with MEHP concentrations ranging from 196-1000 µM in immune cells, testicular germ cells, Sertoli cells, and mouse embryonic stem cells (Yao, Lin et al. 2007; Lim, Kim et al. 2009; Vetrano, Laskin et al. 2010; Rosado-Berrios, Vélez et al. 2011). Interestingly, MEHP-induced apoptosis is dependent on ROS in male germ cells and TK6 human lymphoblast cells and, similarly, oxidative insult in cultured first-trimester placental trophoblasts and term chorionic membrane trophoblasts triggers apoptosis (Moll, Jones et al. 2007; Rogers, Ouellet et al. 2008; Yuan, Ohyama et al. 2008; Rosado-Berrios, Velez et al. 2011). ROS and oxidative DNA damage, both of which were increased with MEHP in the HTR-8 cells, can activate caspases directly by interaction with redox-sensitive cysteines on caspases or by p53 induction, respectively (Circu and Aw 2010). Although not directly measured in this study, we suggest that the increased caspase activity observed at 24 h may be dependent on the MEHP-stimulated generation of ROS in HTR-8 cells. Because apoptosis of first trimester placental trophoblasts and later-gestation chorionic laeve trophoblasts is linked to preeclampsia and preterm birth, and our results show increased activation of caspases, we suggest that apoptosis may be implicated in MEHP-induced adverse pregnancy outcomes. We observed decreased cell viability with 180 µM MEHP at 48 h without any measurable cytotoxic effects. A possible explanation for this may be that extracellular proteases released into cell medium do not remain viable for longer treatment periods and therefore may not be the most suitable indicator of cytotoxicity at later time points.

Differential antioxidant expression is observed in gestational tissues of pregnancies complicated by preeclampsia, IUGR, and miscarriage (Sahlin, Wang et al. 2000; Biri, Kavutcu et al. 2006; Hoegh, Borup et al. 2010). Our gene expression array results demonstrate that MEHP treatment resulted in a robust induction of the dual peroxidase and oxidase PTGS2, modest changes in antioxidant genes GLRX2, TXNRD1, DHCR24, PRNP, and SCARA3, and modest changes in the phase 1 metabolizing enzyme gene AOX1. TXNRD1, PRNP, and AOX1 each contain an antioxidant response element in their promoter regions (Varela-Nallar, Toledo et al. 2006; Suvorova, Lucas et al. 2009). The latter results suggest that Nrf2 may be activated as a protective response to ROS generated with MEHP treatment. GLRX2 and TXNRD1, both classic antioxidants and oxidoreductases, confer protection from oxidative DNA damage, apoptosis, and cell death (Meyer, Buchanan et al. 2009). Increased levels of GLRX and TXNRD protein are found in preeclamptic placentae compared to controls, suggesting involvement of GLRX and TXNRD proteins in protection of the placenta from oxidative insult (Shibata, Ejima et al. 2001). Increases in GLRX2 and TXNRD1 gene expression observed in this study may serve to protect the cell from increased ROS generated by MEHP. AOX1 is the gene for aldehyde oxidase 1, a phase 1 metabolizing enzyme that transfers electrons to molecular oxygen, resulting in formation of radicals. Because of the possibility of radical formation with aldehyde oxidase activity, we suggest that the observed decrease in AOX1 with MEHP treatment may be a protective cellular response. SCARA3, DHCR24, and PRNP have radical scavenging and cyto-

protective functions; however, the function of these genes in the gestational compartment is unknown (Han, Tokino et al. 1998; Kawashiro, Fukata et al. 2009). The decrease observed in this study in DHCR24, the gene for 3β-hydroxysterol-D24 reductase, which catalyzes the last step in cholesterol biosynthesis converting desmosterol to cholesterol, suggests that MEHP may interfere with steroid synthesis, which has implications for sustaining progesterone levels necessary for maintenance of pregnancy (Kawashiro, Fukata et al. 2009). The parallel changes in the magnitude and direction of gene expression between 180 µM MEHP and TBHP treatment for all genes measured is not surprising considering that TBHP works through the Fenton reaction to generate hydroxyl radical from hydrogen peroxide and our results from the DCF assay suggest that MEHP generates hydroxyl radical through the Fenton reaction also.

Considering our observations that MEHP treatment increases both ROS generation and oxidative DNA damage, we expected to see more genes differentially expressed with MEHP. Because the antioxidant and oxidative stress response array includes genes that are responsive to a wide array of reactive species, it may be that MEHP treatment is generating only a subset of these reactive species. Furthermore, because gene expression is often transient and we measured expression at only three time points, some MEHP-induced changes in expression may have been missed. Future experiments could test additional time points and validate these gene expression changes with protein expression data.

Particularly interesting is our finding that MEHP induced a robust increase in expression of PTGS2, the gene for cyclooxygenase-2 (COX-2) enzyme, an enzyme necessary for the initiation of parturition. COX-2, the translated enzyme product of PTGS2, catalyzes synthesis of bioactive prostaglandins, including PGE2 and PGF2α, which bind to excitatory receptors and stimulate myometrial contractions, a hallmark of labor induction. In chorion laeve, PTGS2 mRNA levels are approximately seven times higher in tissues from spontaneous preterm labor compared to non-laboring tissues of equivalent gestational age, an increase comparable to the 8-fold increase that we observed with 180 μM MEHP compared to the solvent control in the present study (Mijovic, Zakar et al. 1998). In HTR-8 cells and primary extravillous trophoblast cells, COX-2 inhibition suppresses migration, suggesting that perturbation of COX-2 expression early in gestation may interfere with placentation (Horita, Kuroda et al. 2007). The observed increase in PTGS2 levels with MEHP treatment is corroborated by studies showing increased PTGS2 mRNA expression with MEHP treatment in murine liver cells and increased COX-2 protein expression with MEHP treatment in spermatocytes and rat and human mast cells (Ledwith, Pauley et al. 1997; Onorato, Brown et al. 2008; Oh, Lim et al. 2010; Oh and Lim 2011). In contrast, an in vivo study found that 750 and 1500 mg/kg DEHP exposure decreased PTGS2 mRNA and COX-2 protein expression in rat placenta. Differences between the current study and previously published studies may be related to species differences, because differences in response to DEHP have been observed in studies comparing peroxisome proliferation in rat and human

hepatocytes (Xu, Agrawal et al. 2008). Furthermore, the doses of DEHP used in

the rat study were four orders of magnitude above the 95th percentile of relevant

human intake estimates (Koch, Preuss et al. 2006). Further in vivo studies

testing exposure doses that are closer to physiologically relevant concentrations

are needed. In addition, it is possibly that MEHP may exert differential effects in

the in vivo context compared to in vitro.

We chose to use HTR-8 cells as a model to study the effects of MEHP on the

gestational compartment because these cells exhibit normal chromosome

numbers as compared to other immortalized cell lines from the gestational

compartment and they have a similar phenotype compared to their primary

counterparts (Graham, Hawley et al. 1993; Nicola, Timoshenko et al. 2005;

Biondi, Ferretti et al. 2006; Nicola, Chirpac et al. 2008; Jovanović and Vićovac

2009; Jovanović, Stefanoska et al. 2010). It is important to note, however, that

these cells may have a different gene expression and gene methylation profile

compared to primary extravillous trophoblast cells (Bilban, Tauber et al. 2010;

Novakovic, Gordon et al. 2011). For this reason, we plan to further investigate

the mechanisms of toxicity of MEHP which were identified in the current study in

primary cells of the gestational compartment.

In the present study, we have identified oxidative stress as a mechanism by

which MEHP may influence pregnancy outcomes using an immortalized human

first trimester trophoblast cell line as a model. In the future, we intend to study

MEHP-induced oxidative stress in primary human gestational cells and tissues

from late term pregnancies, as well as in an in vivo rodent model, with the goal of

identifying mechanisms that may explain the association between MEHP

exposure and preterm birth. Furthermore, based on our finding of increased

PTGS2 gene expression with MEHP treatment, we are interested in studying the

effects of MEHP treatment on prostaglandin synthesis pathways, specifically,

PGE2 and PGF2α, in primary human gestational tissues and cells. The findings

from the current study warrant future epidemiological studies of oxidative stress,

apoptosis, and prostaglandin synthesis as potential cellular mechanisms by

which MEHP exposure, and possibly other environmental contaminants, may

contribute to increased risk for adverse pregnancy outcomes.

		4h		8h		24h	
Gene	Protein product	Fold Change	Adj. p-value	Fold Change	Adj. p-value	Fold Change	Adj. p-value
PTGS2	Cyclooxygenase 2	**5.06**	0.01	**9.93**	0.0004	**8.68**	0.0004
GLRX2	Glutaredoxin 2	1.11	0.95	1.40	0.10	**2.24**	0.0001
PRNP	Prion protein	1.16	0.95	1.47	0.23	**2.60**	0.001
TXNRD1	Thioredoxin reductase 1	1.03	0.96	1.06	0.90	**1.95**	0.001
SCARA3	Scavenger receptor class A, member 3	0.89	0.95	0.82	0.84	**0.44**	0.003
DHCR24	24-dehydrocholesterol reductase	0.92	0.95	0.80	0.90	**0.34**	0.01
AOX1	Aldehyde oxidase 1	0.82	0.95	1.10	0.90	**1.97**	0.01

Table 2.1. Oxidative stress and antioxidant response gene expression array results. HTR-8 cells were treated with DMSO (0.05% v/v; solvent control) or 180 µM MEHP for 4, 8, or 24 h. The Oxidative Stress and Antioxidant Response qRT-PCR Array was performed as described in the "Materials and Methods" section. Results are shown for those genes with a significant change of approximately two-fold or more compared to solvent controls at 4, 8, or 24 h. Statistically significant changes of two-fold or more are highlighted in bold (adjusted p-value < 0.05).
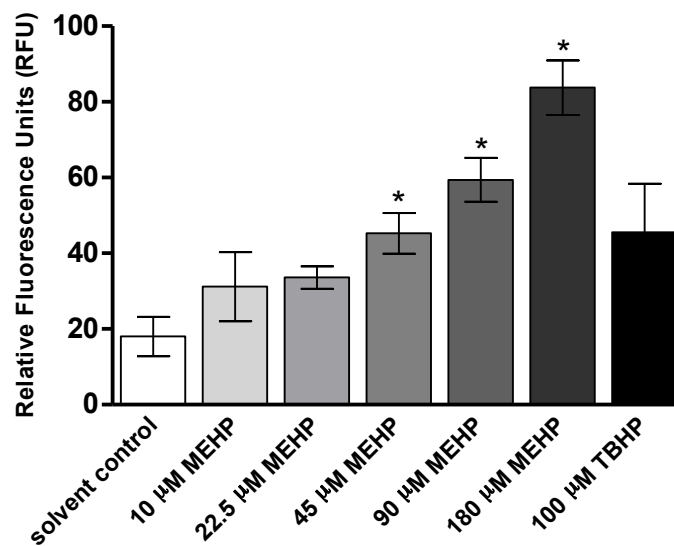
Figure 2.1. MEHP-stimulated generation of reactive oxygen species (ROS). MEHP treatment increased ROS generation in HTR-8 cells in a concentration-dependent manner as measured by the DCF assay. HTR-8 cells were pre-loaded with $H_2DCF-DA$ for 1 h, then treated with DMSO, (0.05% v/v; solvent control) 10, 22.5, 45, 90, or 180 μM MEHP (*n* = 3-5 experiments) or 100 μM TBHP (n=2 experiments) for 1 h as described in the "Materials and Methods" section. Bars represent means ± SE. *p < 0.05, compared to solvent control.
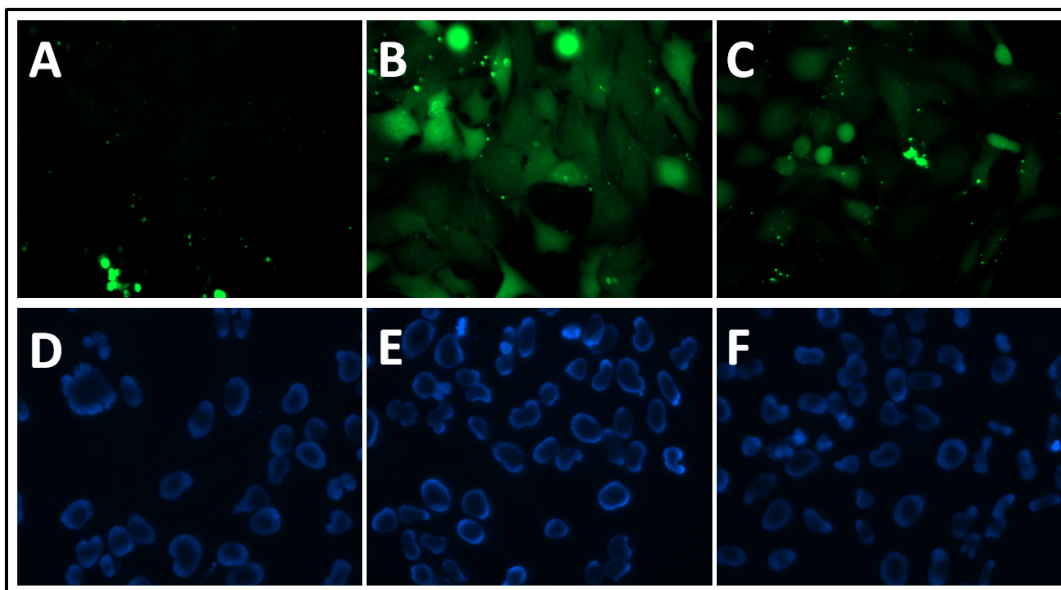
Figure 2.2. Effect of pretreatment with the antioxidant deferoxamine (DFO) on MEHP-stimulated reactive oxygen species (ROS) generation. HTR-8 cells were pre-loaded with H_2DCF-DA for 1 h. After removal of H_2DCF-DA, cells were treated with 1 mM DFO for 1 h, and then treated with DMSO (0.05% v/v; solvent control) or 180 µM MEHP as described in the "Materials and Methods" section (n=3 experiments). The top panels show representative images of intracellular DCF fluorescence of A) solvent control, B) 180 µM MEHP, and C) 180 µM MEHP + 1 mM DFO. The bottom panel shows corresponding Hoescht nuclear staining of D) solvent control, E) 180 µM MEHP, and F) 180 µM MEHP + 1 mM DFO.
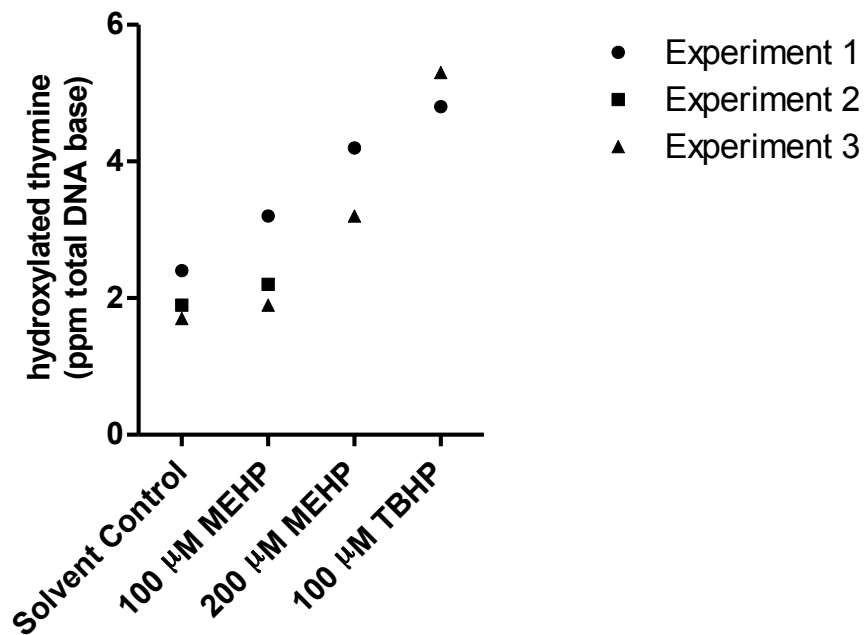
Figure 2.3A. MEHP treatment effects on oxidation of the DNA base thymine. HTR-8 cells were treated with solvent control, 90 µM MEHP, 180 µM MEHP, or 50 µM TBHP for 24 h. Levels of oxidized thymine were measured from extracted genomic DNA and normalized to ppm total DNA base using mass spectrometry, as described in the "Materials and Methods" section. All treatments were tested in experiment 1 (circle) and experiment 3 (triangle), and only solvent control and 90 µM MEHP were tested in experiment 2 (square).
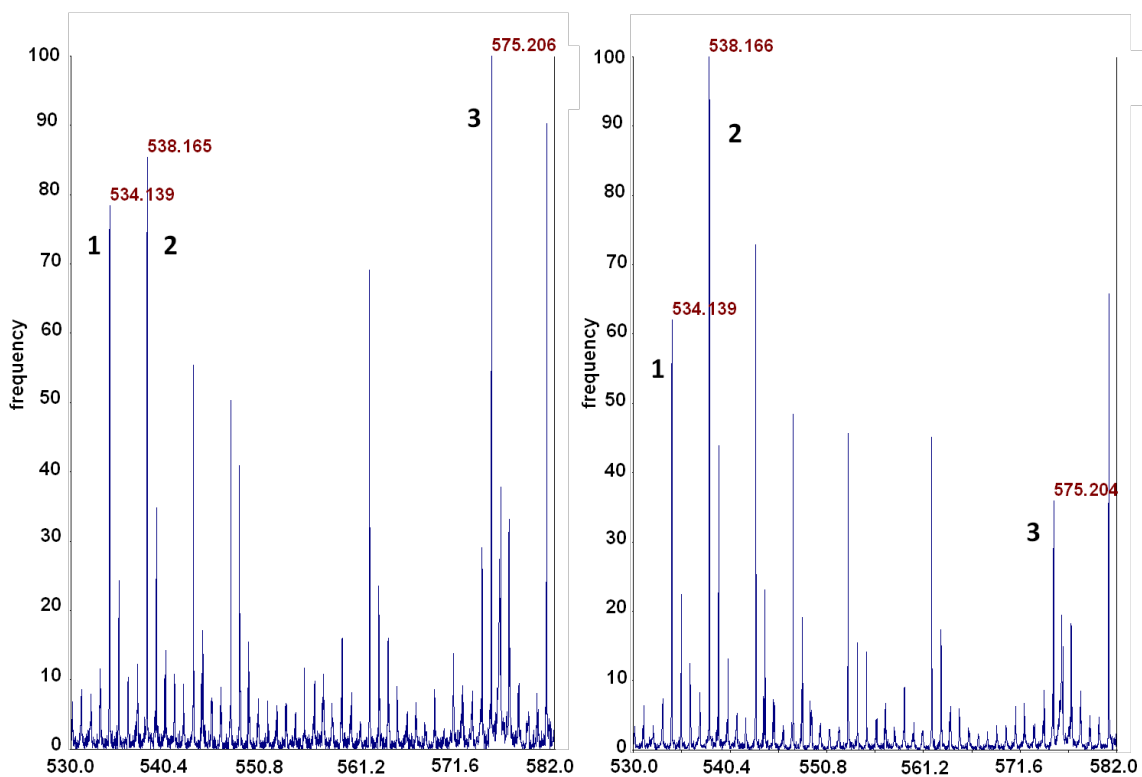
Figure 2.3B. Detection of oxidized thymine (as a mass-tagged deoxynucleotide) by MALDI-TOF-MS. Peaks 1 and 2: oxidized thymine labeled with d_0 and d_4 mass tags, respectively. Peak 3: mass tagged N^2-ethyl dGMP spiked (25 fmol) into each MALD1 spot as an internal standard. A: peak 1 (1.7 ppm of oxidized thymine in DNA nucleobases) represents the DMSO sample and peak 2 (1.9 ppm) represents the 90 µM MEHP sample. B: peak 1 (3.7 ppm) represents the180 µM MEHP sample and peak 2 (6.0 ppm) represents the 50 µM TBHP sample.
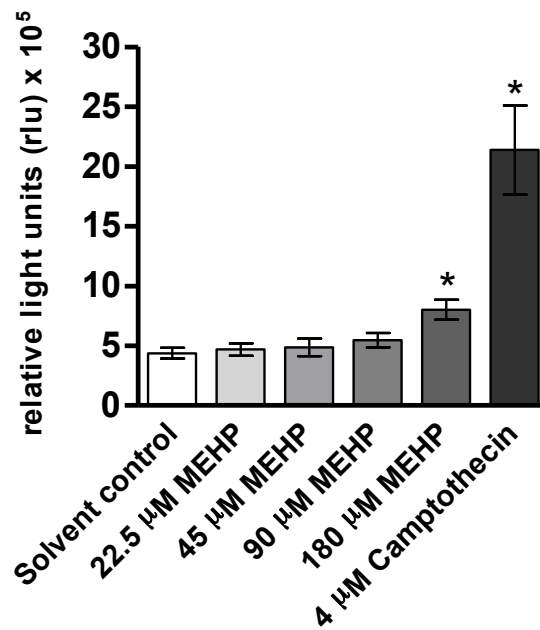
Figure 2.4. MEHP treatment effects on caspase 3/7 activity. HTR-8 cells were treated with DMSO (0.05% v/v; solvent control), MEHP (22.5, 45, 90, or 180 μM), or 4 μM camptothecin (positive control) for 24 h, then caspase 3/7 activity was assessed as described in the "Materials and Methods" section (n = 3). Bars represent means ± SE. *$p<0.05$ compared to solvent control.

Figure 2.5. Effects of MEHP on HTR-8 cell viability. Cells were treated with DMSO (0.05% v/v; solvent control), MEHP (22.5, 45, 90, or 180 µM), or 4 µM camptothecin (positive control) for 24 h or 48 h, then the cultures were assessed for cell viability using a commercial intracellular protease activity assay as described in the "Materials and Methods" section. Bars represent means ± SE. *$p<0.05$ compared to solvent control.

A
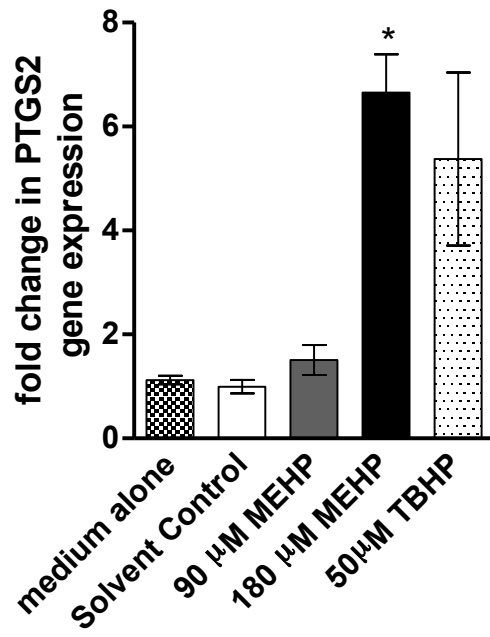
B
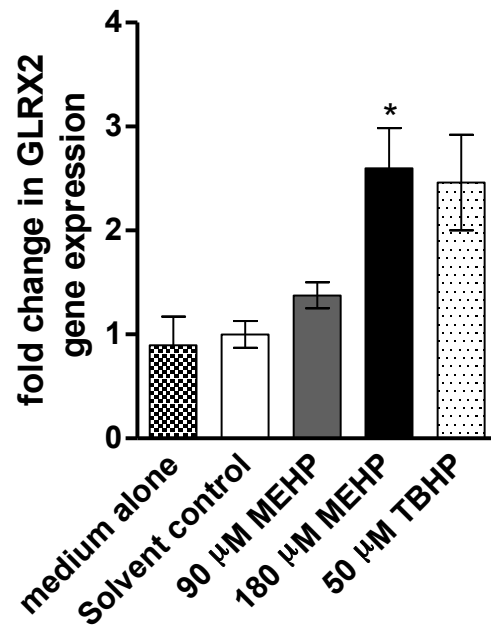
C
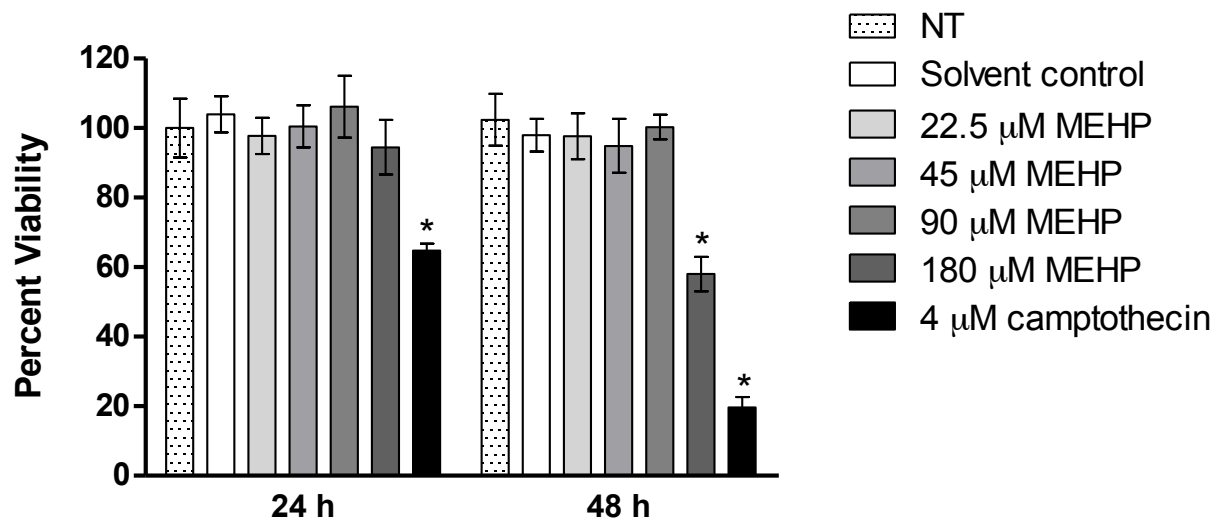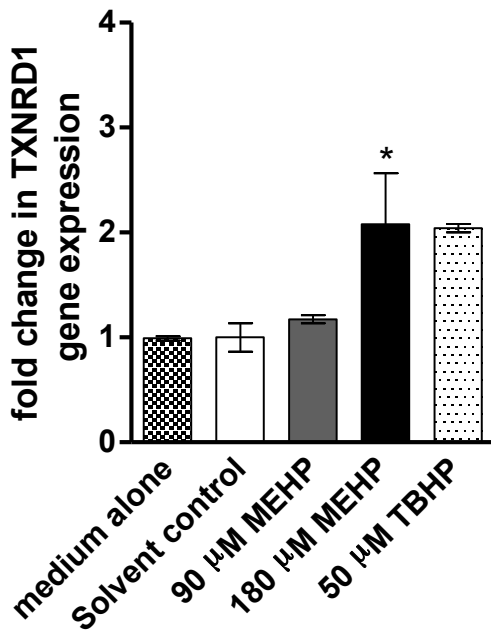
D
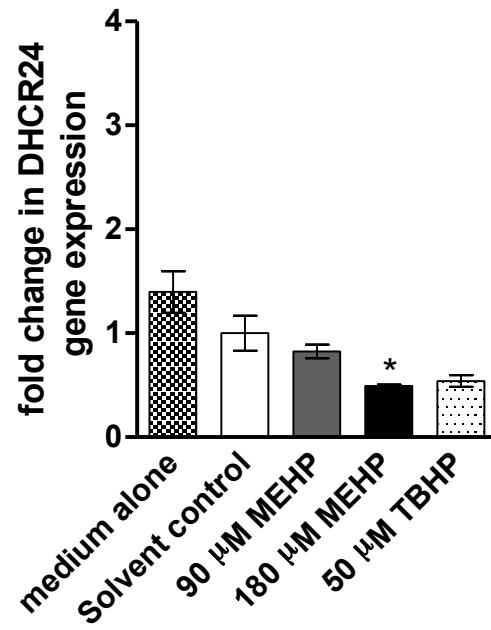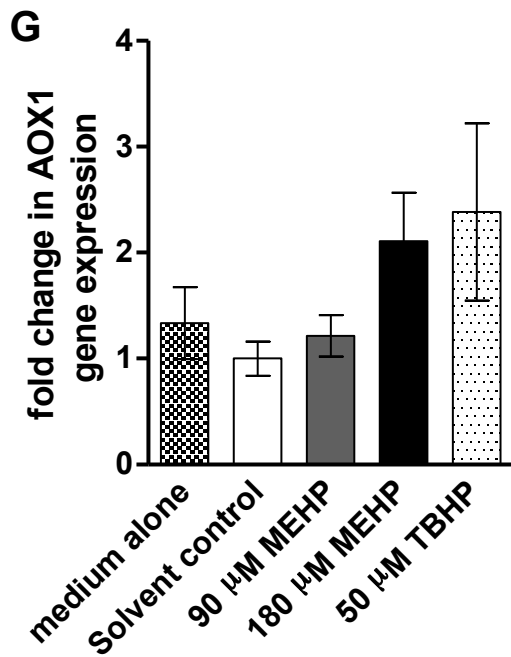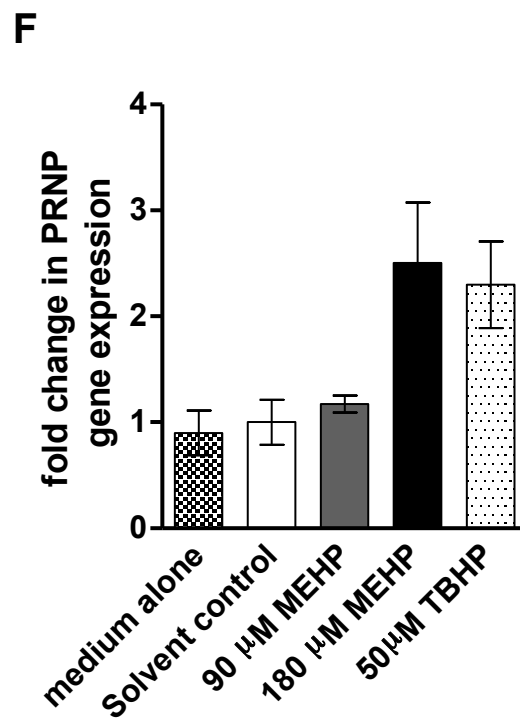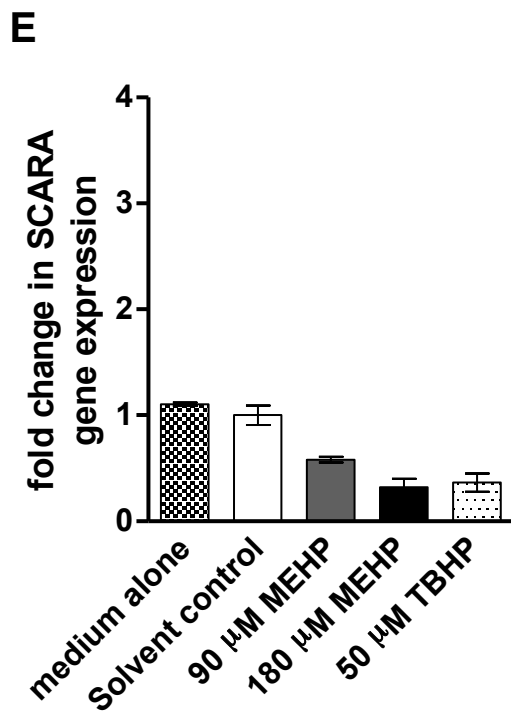
Figure 2.6. qRT-PCR validation of gene array results (from Table 1), showing MEHP treatment effects on mRNA expression: A) PTGS2; B) GLRX2; C)TXNRD1; D) DHCR24; E) SCARA3; F) PRNP; G) AOX1. Cells were treated for 24 h with medium alone (n=2), DMSO (0.05% v/v; solvent control), MEHP (90 µM or 180 µM), or TBHP (50 µM) (n=3). qRT-PCR reactions were run as described in "Materials and Methods" section. Bars represent means ± SE. *p<0.05 comparing treatments to solvent control.

APPENDIX A

MEHP effects on DCF fluorescence in cell-free buffer solutions

Materials and Methods

Measurement of MEHP-stimulated fluorescence with the $H_2DCF-DA$ assay.
To assess the effects of MEHP on DCF fluorescence in cell-free buffer, 45, 90,
and 180 µM MEHP solutions were prepared in either HBSS buffer or to RPMI
medium with or without 10% fetal bovine serum (FBS). $H_2DCF-DA$ reagent was
mixed with each treatment or solvent control (0.05% DMSO) in HBSS or RPMI to
a final concentration of 10 µM $H_2DCF-DA$ and 200 µl aliquots were added to a
96-well plate in replicates of six. Fluorescence readings were taken every 10 min
for 1 h using a plate spectrofluorometer.

Results

MEHP effects on DCF fluorescence. Exposure to MEHP for 1 h had no effect
on DCF fluorescence in HBSS containing $H_2DCF-DA$ or RPMI medium
containing $H_2DCF-DA$ in the absence of serum (Figure A.1, HBSS buffer, RPMI
buffer). However, 45, 90, and 180 µM MEHP concentrations significantly

increased DCF fluorescence when cell-free RPMI medium containing 10% serum and H$_2$DCF-DA was used as the buffer (Figure A.1; p<0.0001).

Conclusion

The results from the present study suggest that the DCF assay is appropriate for studying MEHP affects on ROS production when serum-free conditions are applied.
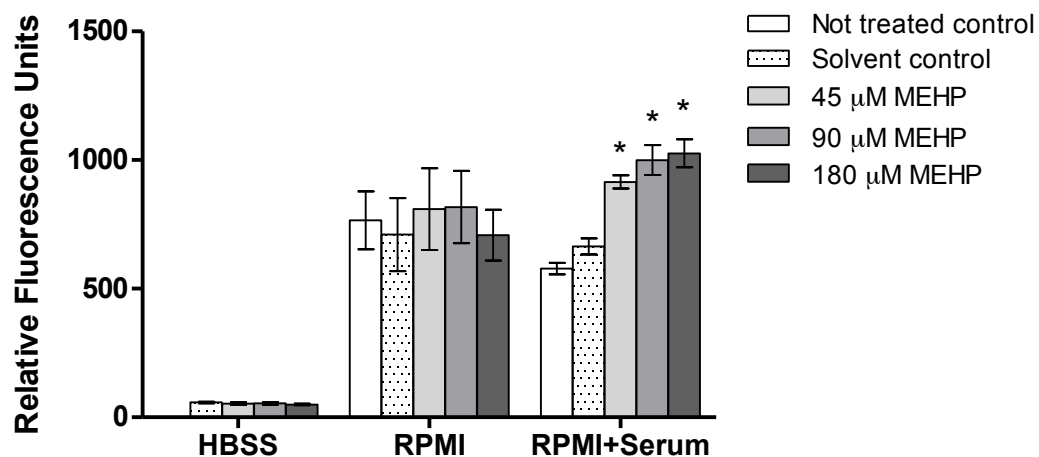
Figure A.1. MEHP effects on DCF fluorescence in HBSS and RPMI medium solutions. MEHP effects on DCF fluorescence were determined after a1-h incubation in cell-free solutions containing 10 µM $DCFH_2$-DA in HBSS or in RPMI medium with or without serum. The bars represent the means of 3 independent experiments containing 6 replicates each (n = 3). There were significant increases in DCF fluorescence with MEHP treatments compared to solvent control when RPMI + serum was used as the buffer, but not when RPMI (without serum) or HBSS was used as the buffer. Asterisks indicate statistically significant increases compared with not treated and solvent controls (p<0.0001).

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CHAPTER III

MEHP stimulates release of prostaglandins but not inflammatory cytokines in human gestational cells and tissues

Abstract

Di-2-ethylhexyl phthalate (DEHP) is a ubiquitous environmental pollutant used in a wide variety of consumer polyvinyl chloride (PVC) products. Monoethylhexyl phthalate (MEHP), the active monoester metabolite of DEHP, increases release of inflammatory mediators, including prostaglandins and inflammatory cytokines in various tissues and cell types.   To investigate the effects of MEHP on inflammatory responses in the gestational compartment, we assessed MEHP-stimulated prostaglandin and inflammatory cytokine responses in gestational membrane explants, placental macrophage Hofbauer cells, decidual macrophage cells, and human placental cells (HTR-8/SVneo). With IRB approval, term gestational tissues were obtained from healthy non-laboring term pregnancies delivered by cesarean section and first trimester decidual macrophages were obtained from women undergoing surgical abortion. Gestational cells and tissues were treated for 4, 8, or 24 h with MEHP concentrations ranging from 10-180 µM. PTGS2 mRNA expression of was measured in HTR-8 cells with qRT-PCR. Treatment with 180 µM MEHP for 4, 8, or 24 h, or 90 µM MEHP for 4 or 8 h

significantly induced PTGS2 mRNA expression. Release of total prostaglandin, PGE2, PGF2α, IL-6, IL-8, IL-1β, TNF-α, IL-10, and TGF-β in cell culture medium was assessed using enzyme-linked immunosorbant assays (ELISA). We found that 90 or 180 μM MEHP treatment significantly increased release of total prostaglandins in placental macrophage Hofbauer cells, decidual macrophages, and gestational membrane explants. Treatment with 90 or 180 μM MEHP also increased PGF2α, and PGE2 in human primary placental macrophage Hofbauer cells. MEHP treatment suppressed IL-10 levels in decidual macrophage cells but had no effects on inflammatory cytokine release in other in vitro models. These data suggest that MEHP may contribute to initiation of preterm labor through increased bioactive prostaglandin synthesis in the gestational compartment.

Introduction

Diethylhexyl phthalate (DEHP) is a ubiquitous environmental contaminant used widely as a plasticizer in polyvinyl chloride (PVC) consumer products. Because DEHP is not covalently bound to PVC, it is released into the environment where it collects in dust particles or in material contained in PVC plastic. Consequently, human exposure to DEHP is widespread in the general population. A recent study conducted by NHANES found measureable levels of monoethylhexyl phthalate (MEHP), the active metabolite of DEHP, in 98% of urine samples analyzed. Because DEHP is rapidly converted to MEHP, which is excreted within hours of exposure, the NHANES findings suggest that exposure to DEHP is a widespread and daily occurrence.

Exposure to DEHP is associated with adverse pregnancy outcomes in humans. Specifically, phthalate exposure was associated with decreased gestation length in studies of women living in New York City and Italy (Latini, De Felice et al. 2003; Adibi, Hauser et al. 2009). Likewise, phthalate urinary metabolites were associated with preterm birth in a Mexico City cohort. Conversely, a negative association between urinary DEHP metabolites and preterm birth was found in another New York City cohort (Wolff, Engel et al. 2008).

Measureable levels of MEHP are found in human cord blood, placenta, and amniotic fluid, suggesting that tissues of the gestational compartment may be targets of MEHP toxicity (Mose, Mortensen et al. 2007; Wittassek, Angerer et

al. 2009; Lin, Ku et al. 2011). The placenta and extraplacental membranes of the gestational compartment are significant sources of signaling molecules, including inflammatory mediators, which are important for parturition. Specifically, the placenta and extraplacental membranes produce and release pro-inflammatory cytokines and prostaglandins which together activate parturition events. Pro-inflammatory cytokines stimulate synthesis of the bio-active prostaglandins PGE2 and PGF2α in gestational tissues through induction of prostaglandin synthesis enzymes, including cyclooxygenase-2 (COX-2) (Hansen, Keelan et al. 1999). Bioactive prostaglandins PGE2 and PGF2α released from gestational tissues stimulate myometrial contractions (Olson 2003).   Pro-inflammatory cytokines tumor-necrosis factor alpha (TNF-α) and interleukin-8 (IL-8) also promote cervical dilation and membrane rupture through activation of apoptosis and induction of matrix metalloproteinase (MMP) expression, respectively (Vadillo-Ortega and Estrada-Gutierrez 2005). Furthermore, increased amniotic fluid levels of pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α and prostaglandins PGE2 and PGF2α are evident in preterm labor (Romero, Manogue et al. 1989; Hillier, Witkin et al. 1993; Carroll, Abbas et al. 1995; Lee, Park et al. 2009; Yoneda, Shiozaki et al. 2011). Hence, pro-inflammatory cytokine signaling and prostaglandin synthesis pathways work in concert to initiate myometrial contractions, cervical ripening, and membrane rupture, leading to initiation of human parturition and preterm labor.

Recent reports suggest that exposure to DEHP or its metabolite MEHP may result in activation of inflammatory responses. In humans, urinary MEHP or

66

its oxidized metabolites are associated with urinary markers of inflammation (Ferguson, Loch-Caruso et al. 2011). In addition, MEHP treatment increases pro-inflammatory cytokine release in primary neonatal neutrophils, rat alveolar macrophages, murine monocyte-macrophages, and human lung epithelial cells, suggesting activation of inflammatory responses with MEHP (Jepsen, Abildtrup et al. 2004; Rakkestad, Holme et al. 2010; Vetrano, Laskin et al. 2010). Similarly, MEHP treatment increases prostaglandin release from cultured bovine ovarian and endometrial cells, and increases COX-2 expression in spermatocytes and rat and human mast cells (Ledwith, Pauley et al. 1997; Onorato, Brown et al. 2008; Oh, Lim et al. 2010; Wang, Shang et al. 2010; Oh and Lim 2011). Previous data collected from our laboratory show that MEHP treatment strongly induces expression of PTGS2, the gene for COX-2, in human placental cells. The latter finding contrasts with a report that placental COX-2 expression and prostaglandin production are decreased in pregnant rats exposed to DEHP compared to unexposed animals (Xu, Agrawal et al. 2008).

In the present study, we utilize a variety of in vitro model systems to examine the effects of MEHP treatment on prostaglandin synthesis and pro-inflammatory cytokine release in cells and tissues of the gestational compartment. Specifically, we assess MEHP-stimulated responses in first trimester placental cells (HTR-8/SVneo), primary decidual and placental macrophages, and gestational membrane explants.

Materials and Methods

Reagents. We purchased dimethyl sulfoxide (DMSO), deferoxamine mesylate, *tert*-butyl hydroperoxide (TBHP), and camptothecin from Sigma-Aldrich (St. Louis, MO, USA); FBS and charcoal-stripped FBS from HyClone Laboratories (Waltham, MA); RPMI 1640, Dulbecco's Modified Eagle Medium (DMEM), penicillin/streptomycin solution, and DPBS from Life Technologies-Invitrogen (Carlsbad, CA); recombinant IL-1β from R&D Systems (Minneapolis, MN); and MEHP from Accustandard (New Haven, CT).

Human subjects. This study was reviewed and approved by the Institutional Review Board (IRB) at the University of Michigan. In compliance with the IRB, the investigators did not collect any personal identifiable information, the investigators had no direct interaction with the patient, and the tissues would otherwise have been discarded.

Third trimester gestational tissue acquisition. Extraplacental gestational membranes and placental tissue were collected from women undergoing normal, medically indicated cesarean section delivery at the University of Michigan Women's Hospital Birth Center between 37 and 39 weeks of gestation. Exclusion criteria included the following: preeclampsia, diabetes, multifetal pregnancy, collagen vascular disease, cervical cerclage, immune-compromised conditions, bacterial vaginosis or clinical chorioamnionitis (as noted in the chart or suspected by attending physician), prescription of antibiotics in the past two weeks (with the exception of routine, pre-operative antibiotics), cigarette

68

smoking, third trimester bleeding, major maternal medical conditions (e.g.,

chronic renal disease, sarcoidosis, hepatitis, HIV), or any condition requiring the

tissues to undergo pathological examination.

Immediately following delivery, extraplacental membranes were excised

from the placental disk, maintaining a 3-mm margin from the chorionic plate, and

submerged in warm isotonic Dulbecco's phosphate buffered saline (DPBS). For

placental macrophage isolations, a 5 g sample of tissue was excised from the

chorionic villous stalk of the placenta and placed in warm DPBS. Samples were

then transported to the laboratory for further processing.

First trimester decidual tissue acquisition. First trimester decidual tissues

were collected from women age 18-44 undergoing surgical abortion at Planned

Parenthood of Mid-Michigan. Exclusion criteria included subjects receiving

immunosuppressive medications, subjects with acute reproductive tract infection,

and subjects with chronic medical conditions of the skin, heart, lung, liver,

gastrointestinal tract, endocrine, reproductive, hematological, neurological,

musculoskeletal, or immune systems. Tissues requiring pathological

examination, as determined by the physician, were excluded from this study.

Immediately following the abortion procedure, decidual tissue was separated

from the gestational sac and fetal tissues by the physician and then placed in

isotonic DPBS by the researchers.

Placental and decidual macrophage isolation. Placental and decidual

macrophage isolations were performed as described previously (Thelen, Hao et

al. 2010). Before mincing, placental tissues were washed 3 times with DPBS to remove blood. The purity of decidual and placental isolates was between 92-94%, determined with flow cytometry as cells positive for $CD14^+$. Cells were seeded at a density of 200,000-400,000 cells per well in a polystyrene, 24-well culture plate in RPMI with antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and 10% charcoal dextran FBS, and then incubated for 24 h in a humidified atmosphere at 37°C and 5% CO_2.

Extraplacental gestational membrane tissue culture and treatment. Under aseptic conditions, tissues were washed 3 times in warm DPBS to remove blood and 12-mm punches of full-thickness tissue were made using a biopsy punch. Punches were then placed, one punch per well, in a 12-well polystyrene culture plate containing 1 mL per well of warm DMEM supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin. Following 4 h of culture in a humidified atmosphere at 37°C and 5% CO_2, medium was removed and replaced with fresh medium, and the punches were incubated for an additional 24 h. The medium was replaced and tissue was allowed to incubate for 1 h before treatment. Tissue punches were treated in triplicate with medium alone, 0.05% dimethyl sulfoxide (DMSO; solvent control), or 22.5, 45, 90, or 180 µM MEHP, or 100 ng/mL LPS for 24 h.

Placental and decidual macrophage culture and treatment.   Primary macrophage cells were treated with medium alone, 0.05% DMSO (solvent control), 90 µM MEHP or 180 µM MEHP in triplicate in RPMI 1640 medium with L-glutamine without phenol red supplemented with 10% fetal bovine serum and

100 U/mL penicillin and 100 µg/mL streptomycin for 8 or 24 h in a humidified atmosphere at 37°C and 5% CO_2.

HTR-8/SVneo cell culture and treatment. The HTR-8/SVneo (HTR-8) cells were a gift from Dr. Charles Graham (Queens University, Ontario, Canada). The HTR-8 cells were isolated from first trimester human placenta and immortalized with SV40 antigen (Graham, Hawley et al. 1993). Similar to their primary counterparts, HTR-8 cells express human chorionic gonadropin, stain positive for the epithelial marker cytokeratin-7, and retain migratory capabilities in culture (Graham, Hawley et al. 1993). Cells were cultured in RPMI 1640 medium with L-glutamine without phenol red, supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a 5% CO_2 humidified atmosphere. Cells were grown to approximately 70-80% confluence before treatment. For cytokine and prostaglandin measurements, cells were seeded at 100,000 cells per well in a 24-well culture plate. Following a 24 h incubation, cells were treated with 0.05% DMSO (solvent control), 10, 22.5, 45, 90 or 180 µM MEHP, or 100 ng/mL LPS (positive control) for 24 h. The 24-h time point was selected based on a preliminary time-course experiment in which we determined that 24 h gave optimal cytokine responses. For qRT-PCR, cells were seeded 400,000 cells per well in a 6-well culture plate. Following a 24-h incubation, cells were treated with medium alone, 0.05% DMSO (solvent control), 90 or 180 µM MEHP for 4, 8, or 24 h.

Prostaglandin quantification. Because MEHP increased expression of PTGS2 mRNA in our previous study (Chapter II), we measured secreted prostaglandin

71

concentrations in HTR-8 cells, primary placental and decidual macrophages, and gestational membrane punches.   Total prostaglandin release was measured in gestational tissue and primary macrophage cell medium using the Prostaglandin Screening EIA kit (Cayman Chemical, Ann Arbor, MI). Prostaglandin E2 (PGE2) concentrations in primary macrophage and HTR-8 cell medium and prostaglandin F2α (PGF2α) concentrations in primary macrophage medium were quantified with enzyme immunoassay following the manufacturer's recommended protocol (EIA; Cayman Chemical, Ann Arbor, MI, USA).

Cytokine quantification. Interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), transforming growth factor beta (TGF-β) and interleukin-10 (IL-10) concentrations were quantified in cell and tissue medium by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's recommended protocol (Duosets, R&D systems, Minneapolis, MN) in the Immunology Core Facility at the University of Michigan Cancer Center.

qRT-PCR for PTGS2 mRNA expression.   After 4, 8, or 24 h of exposure, RNA was extracted from HTR-8 cells using the RNeasy Plus Mini Kit (Qiagen), and cDNA was synthesized from 1 µg of total RNA using the RT^2 First Strand Kit (SABiosciences) following the manufacturers' recommended protocols. Using the SABiosciences RT^2 First Strand Kit, cDNA was made from 1 µg of mRNA. PCR reactions were run with the SYBR Green Mastermix (SABiosciences), with 5 µL of template. RT- PCR reactions were run on the Bio-Rad CFX96 Real Time C1000 thermal cycler following the manufacturer's recommended protocol.

Results

***PTGS2 mRNA expression, prostaglandin, and cytokine response in HTR-8
cells.*** Treatment of HTR-8 cells with 180 μM MEHP for 4, 8, or 24 h increased
PTGS2 expression 5.3 (±0.6), 8.4 (±2.0), and 6.7 (±0.7) fold, respectively (Figure
3.1). Treatment with 90 μM MEHP for 4 or 8 h resulted in fold increases in
PTGS2 expression of 2.2 (±0.2) and 3.3 (±0.7), respectively. PGE2
concentrations in the medium were below the limit of detection of the assay for all
treatments assayed (data not shown). Treatment with MEHP had no effect on IL-
6 or IL-8 release whereas 100 ng/mL LPS, included as a positive control,
stimulated both IL-6 and IL-8 release (Figure 3.2). IL-10 and TNF-α
concentrations were near or below the limit of detection for all treatments
assayed (data not shown).

Prostaglandin release in placental macrophage cultures. Treatment of
primary placental macrophages with 90 or 180 μM MEHP for 8 or 24 h increased
total prostaglandin release (Figure 3.3), PGF2α release (Figure 3.4), and PGE2
release (Figure 3.5) into the culture medium. Treatment of primary placental
macrophages from subject 1, 2, or 3 with MEHP significantly increased
prostaglandin release by 34.5, 11.9, and 4.5 fold, respectively; PGF2α release by
3.2, 1.3, and 1.4 fold, respectively; and PGE2 release by 2.2 and 1.9 fold,
respectively. Solvent control cultures (0.05% DMSO v/v) showed no significant
changes of total prostaglandin, PGF2α, or PGE2 release compared to cultures
exposed to medium alone (data not shown).

Prostaglandin and cytokine release in decidual macrophage cultures.

Consistent with the response in placental macrophages, treatment of primary decidual macrophages with 90 μM MEHP for 24 h significantly increased total prostaglandins release by 1.7 fold (Figure 3.6). Treatment with MEHP for 8 h significantly decreased release of IL-10 into the culture medium (Figure 3.7A) but had no effect on IL-6, IL-8, TNF-α, or TGF-β release after 8 or 24 h of treatment (Figure 3.7 B, C, D, and E, respectively). Treatment with 10 ng/mL IL-1β (positive control) for 8 or 24 h significantly increased release of all cytokines measured (Figure 3.7).

Prostaglandin and cytokine release in gestational membrane explant cultures. Treatment of gestational membrane punches with 180 μM MEHP for 24 h significantly increased release of total prostaglandins by 2.5 fold (Figure 3.8 A). However, within-tissue variability was high for most of the subjects (Figure 3.8. B, C, D, and E). MEHP had no significant effects on release of any of the cytokines measured (Figure 3.9). However, treatment of gestational membrane punches for 24 h with 100 ng/mL LPS, included as a positive control, stimulated IL-6, IL-8, TNF-α, and IL-10 (Figure 3.9 A, B, C, and D, respectively). IL-6 and IL-8 release in gestational tissues was highly variable both between subjects and within subjects (within subject data not shown). The solvent control (0.05% DMSO) showed no effects on prostaglandin release compared to medium alone. In earlier experiments, we found that 0.05% DMSO showed no effects on IL-6, IL-8, or IL-1β release (Miller 2009).

Discussion

The objective of the current study was to investigate inflammatory responses in various gestational tissues and cells following MEHP treatment in vitro. Our results demonstrate that MEHP treatment significantly increased release of total prostaglandins, PGF2α, and PGE2 in human primary placental macrophage Hofbauer cells, and induced PTGS2 mRNA expression in the HTR-8 human trophoblast cell line. Furthermore, our data suggest that MEHP suppresses levels of the anti-inflammatory cytokine IL-10 in decidual macrophage cells, though these results require replication using cells from additional subjects. These data suggest that MEHP may contribute to initiation of preterm labor through increased bioactive prostaglandin synthesis in the gestational compartment.

Bioactive prostaglandin synthesis is a critical event for the initiation of human parturition, mediating such events as myometrial contractions and cervical ripening. Inhibition of prostaglandin synthesis with COX-2 inhibitors delays pregnancy and prevents preterm labor in rodents, and in vitro exposure to bioactive prostaglandins stimulates myometrial contractions and labor (Wikland, Lindblom et al. 1984; Lee, Kim et al. 2003; Fischer, Hutchinson et al. 2008). In humans, increases in the bioactive prostaglandins PGE2 and PGF2 in tissues and fluids of the gestational compartment precede and correspond with preterm and term labor (Mazor, Wiznitzer et al. 1990; Lee, Romero et al. 2008). Though the placenta is considered the primary producer of prostaglandins in the gestational compartment, only one published study exists that has measured

prostaglandin release in placental macrophage Hofbauer cells, which reside in the lumen of the chorionic villi of the placenta (Wetzka, Clark et al. 1997). Novel to the present study is the finding that MEHP significantly increases release of bioactive prostaglandins PGE2 and PGF2α and total prostaglandins in human primary term placental macrophage Hofbauer cells at physiologically relevant concentrations. Also novel is our finding that MEHP stimulated release of total prostaglandins in term gestational membrane explants and first trimester primary decidual macrophage cells. These results are corroborated by the findings from a previous study where MEHP treatment increased release of PGF2α in bovine ovarian and endometrial cells (Wang, Shang et al. 2010). However, our results are inconsistent with the findings from a previous study where DEHP exposure (750 – 1500 mg/kg/day oral gavage) resulted in decreases in total placental prostaglandins in pregnant rats (Xu, Agrawal et al. 2008). The differences between the latter study and the present study prostaglandin responses to MEHP could be explained by species differences in prostaglandin synthesis pathway responses or differences between in vivo and in vitro systems. Furthermore, the doses of DEHP used in the latter study were at least four orders of magnitude greater than the 95th percentile of estimated adult human intake of 21 μg/kg/day (Koch, Preuss et al. 2006).

Interestingly, the concentration and magnitude of increase of PGE2 and PGF2α release observed with MEHP treatment in placental macrophages did not fully explain the increase in total prostaglandin levels observed with MEHP treatment. In addition to PGE2 and PGF2α, the prostaglandin screening EIA

recognizes arachadonic acid-derived COX-dependent thromboxane and prostacyclin as well as prostaglandins synthesized by COX conversion of eicosapentaenoic acid and dihomo-*gamma*-linolenic acid. Additionally, the prostaglandin screening EIA recognizes COX-independent isoprostanes, 8-iso prostaglandin F2α (8-iso PGF2α) and 8-iso prostaglandin E2 (8-iso PGE2), that are synthesized from reactive oxygen species (ROS) oxidation of arachadonic acid. Based on our finding that MEHP strongly induces PTGS2 expression in HTR-8 cells, future experiments in placental macrophages could include measurements of PTGS2 mRNA expression, COX-2 protein expression, and release of COX-2 dependent thromboxane and prostacyclin. In addition, based on our findings from Chapter II that MEHP treatment induces oxidative stress responses, we intend to measure release of isoprostanes following MEHP treatment of placental macrophages. Increased urinary isoprostanes early in gestation are predictive of preterm labor and preeclampsia; however, the function of isoprostanes in the parturition processes is not known (Peter Stein, Scholl et al. 2008). Previous studies demonstrate, though, that isoprostanes are bioactive and interact with excitatory receptors, similar to PGE2 and PGF2 (Hoffman, Moore et al. 1997; Friel, Sexton et al. 2006). Similarly, we plan to further investigate PGE2, PGF2α, and isoprostane release in the gestational membrane explant model to identify specific prostaglandin increases that may explain the observed increase in total prostaglandins.

Pro-inflammatory cytokines are important mediators of both preterm and normal human parturition. A widely discussed model for labor initiation describes

labor as an inflammatory event, with pro-inflammatory cytokine induction. Pro-inflammatory cytokines mediate prostaglandin synthesis and release, matrix metalloproteinase activation and expression, and recruitment of inflammatory cells, leading to labor events including membrane rupture, cervical ripening, and uterine contractions (Kamel 2010). In our gestational tissue and cell models, MEHP stimulated prostaglandin synthesis without effects on pro-inflammatory cytokine release. The latter finding suggests that MEHP may work through a non-traditional, pro-inflammatory cytokine independent pathway to initiate prostaglandin synthesis. Previous studies from other laboratories demonstrate that antioxidant treatment prevents COX-2 induction and PGE2 synthesis and release (Mögel, Baumann et al. 2011; Temma-Asano, Tskitishvili et al. 2011). In addition, results from Chapter II of this thesis demonstrate that MEHP induces ROS generation in gestational cells. Based on the latter two findings, we propose that MEHP may stimulate prostaglandin synthesis through ROS generation. Alternatively, PPARs, for which MEHP is a ligand, can initiate prostaglandin synthesis by interacting with the PTGS2 promoter to induce PTGS2 gene expression (Kusu, Oishi et al. 2008). However, the effects of PPARs on PTGS2 gene expression are cell-type specific and in some cases PPAR activation can result in suppression of PTGS2 expression (Kang, Mbonye et al. 2007; Scoditti, Massaro et al. 2010). Further research, beyond the scope of the present study, is needed to elucidate these early events in the up regulation of prostaglandin synthesis.

Although we observed no changes in pro-inflammatory cytokine release with MEHP in any of our in vitro models, we did observe a significant suppression of IL-10 release in first-trimester decidual macrophages with MEHP treatment. This novel finding suggests that exposure to MEHP during the first trimester of pregnancy may have effects on the ability of macrophages to maintain sufficient IL-10 levels necessary for protection from innate immune activation and fetal tissue rejection (Nagamatsu and Schust 2010). IL-10 deficiency or polymorphism is associated with preeclampsia, miscarriage, and preterm birth, and DEHP exposure has recently been associated with early pregnancy loss and preterm birth in humans (Hennessy, Pilmore et al. 1999; Kaur 2011; Ruiz, Jallo et al. 2012). Due to difficulty in obtaining first-trimester tissues, we only have data from one subject demonstrating MEHP suppression of IL-10. Further experiments in isolates from additional subjects are necessary to determine whether or not these effects are reproducible. Furthermore, because IL-10 is important for suppression of innate immune responses and women with IL-10 polymorphisms are at greater risk of preterm birth, future studies could investigate the effects of MEHP treatment on term decidual macrophage IL-10 secretion.

The findings from this study suggest a possible mechanism whereby MEHP exposure during pregnancy could stimulate myometrial contractions and ultimately preterm labor through induction of bio-active prostaglandin synthesis. Based on our findings, future studies investigating the associations between

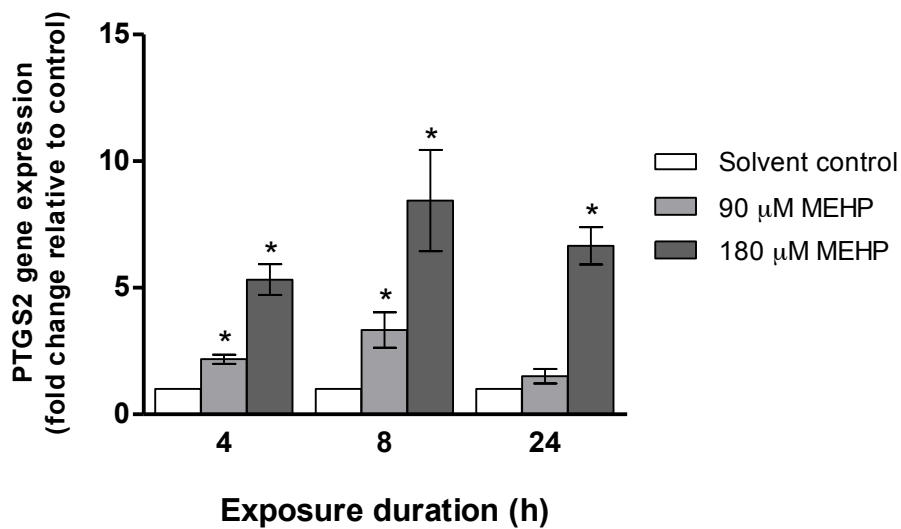PGE2 and PGF2α urinary metabolite levels, MEHP exposure, and preterm birth in human populations are warranted.

Figure 3.1. MEHP treatment effects on mRNA expression of PTGS2 in HTR-8 cells. Cells were treated for 4, 8, or 24 h with medium alone, DMSO (0.05% v/v; solvent control), 90 µM MEHP or 180 µM MEHP, and then qRT-PCR reactions were run as described in the "Materials and Methods" section. Solvent control cultures showed no significant changes of PTGS2 expression compared to cultures exposed to medium alone (data not shown). Bars represent means ± SEs (n = 3). *p<0.05, compared to solvent controls.
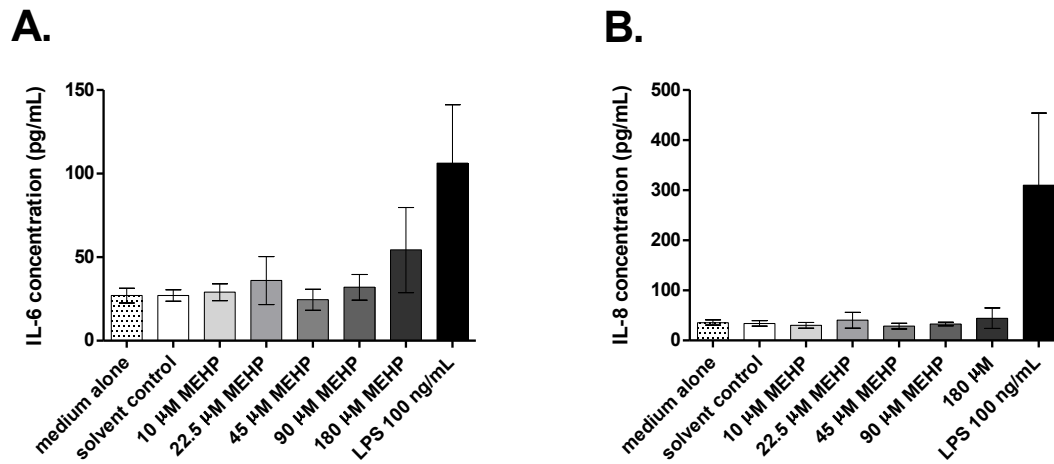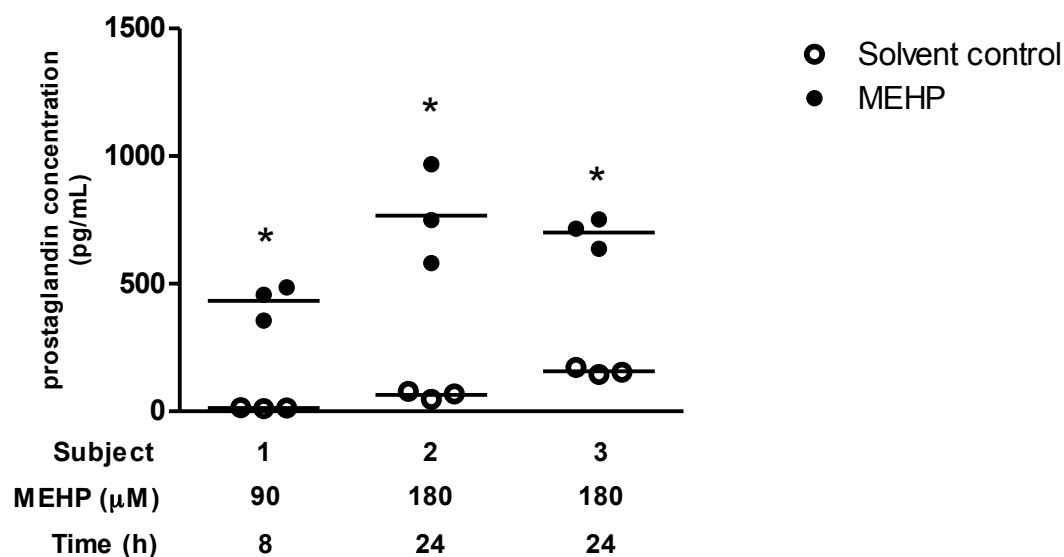
Figure 3.2. MEHP treatment effects on cytokine release from HTR-8 cells into culture medium. Cells were treated for 24 h with medium alone, DMSO (0.05% v/v; solvent control), or 10, 22.5, 45, 90 or 180 μM MEHP. A) IL-6 release in HTR-8 cells. B) IL-8 release in HTR-8 cells. IL-6 and IL-8 ELISAs were performed as described in "Materials and Methods" section. Solvent control cultures (0.05% DMSO v/v) showed no significant changes of cytokine release compared to cultures exposed to medium alone (data not shown). Bars represent means ± SEs (n = 3-4 experiments). *p<0.05, compared to solvent controls.
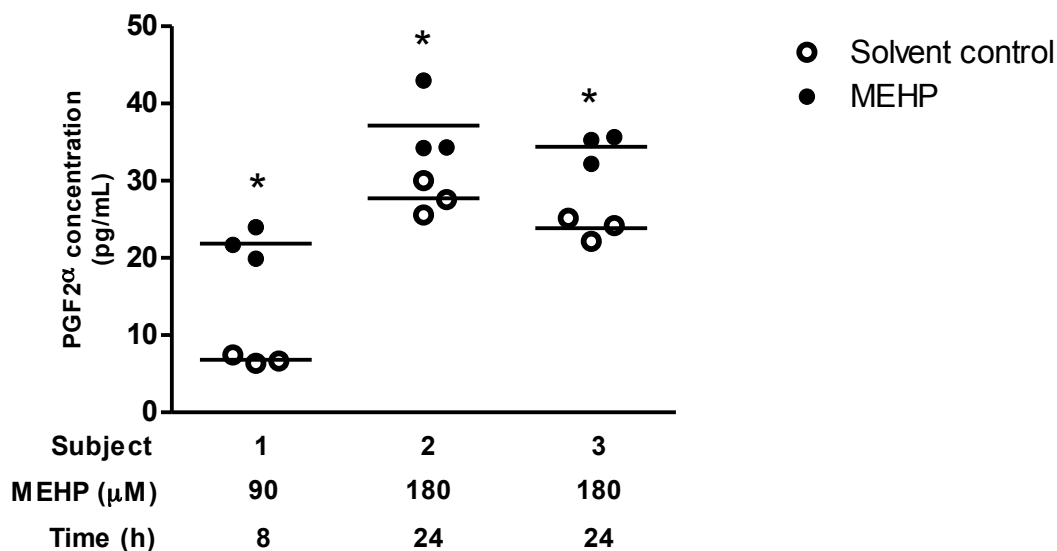
Figure 3.3. MEHP treatment effects on total prostaglandin release from primary placental macrophages into cell culture medium. Data are shown from three subjects. Cells were treated for 8 h (Subject 2) or 24 h (Subjects 1 and 3) with medium alone, DMSO (0.05% v/v; solvent control), 90 μM MEHP or 180 μM MEHP. The prostaglandin screening EIA was performed as described in the "Materials and Methods". Data points represent individual wells for each subject: open circles are solvent control values and filled circle are from cultures exposure to MEHP (n = 3 wells per treatment). Horizontal lines indicate mean values for each treatment within each subject. *p<0.05, compared to solvent controls.

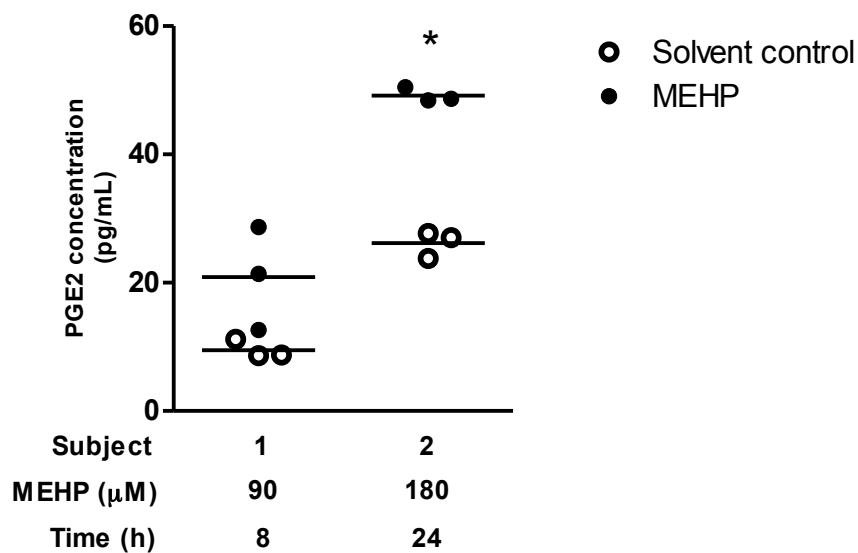**Figure 3.4**. MEHP treatment effects on PGF2α release from primary placental macrophages into cell culture medium. Data are shown from three subjects. Cells were treated for 8 h (Subject 2) or 24 h (Subject 1 and 3) with medium alone, DMSO (0.05% v/v; solvent control), 90 µM MEHP or 180 µM MEHP. The PGF2α EIA was performed as described in the "Materials and Methods" section. Data points represent individual wells for each subject: open circles are solvent control values and filled circle are from cultures exposure to MEHP (n = 3 wells per treatment). Horizontal lines indicate mean values for each treatment within each subject. *$p<0.05$, compared to solvent controls.

Figure 3.5. MEHP treatment effects on PGE2 release from primary placental macrophages into cell culture medium. Data are shown from two subjects. Cells were treated for 8 h (Subject 2) or 24 h (Subject 1) with medium alone, DMSO (0.05% v/v; solvent control), 90 or 180 µM MEHP. The PGE2 EIA was performed as described in the "Materials and Methods" section. Data points represent individual wells for each subject: open circles are solvent control values and filled circle are from cultures exposure to MEHP (n = 3 wells per treatment). Horizontal lines indicate mean values for each treatment within each subject. *$p<0.05$, compared to solvent controls.
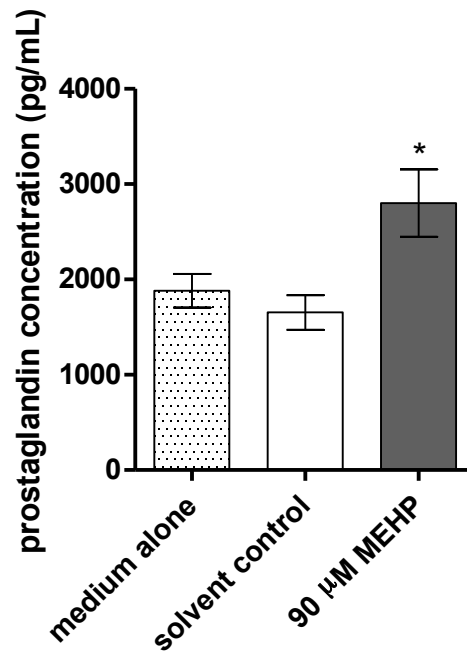
Figure 3.6. MEHP treatment effects on total prostaglandin release from primary decidual macrophage cell medium. Cells isolated from one subject were treated for 24 h with medium alone, DMSO (0.05% v/v; solvent control), or 90 µM MEHP. The prostaglandin screening EIA was performed as described in "Materials and Methods" section (means ± SE, n = 3 wells per treatment). Solvent control cultures (0.05% DMSO v/v) showed no significant changes of total prostaglandin release compared to medium alone.   *$p < 0.05$, comparing treatments to solvent control.
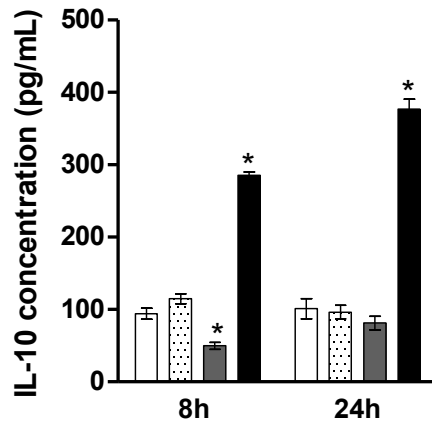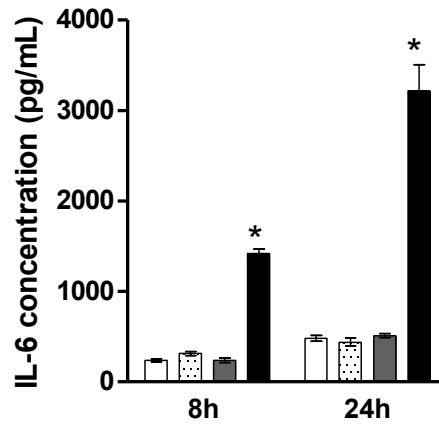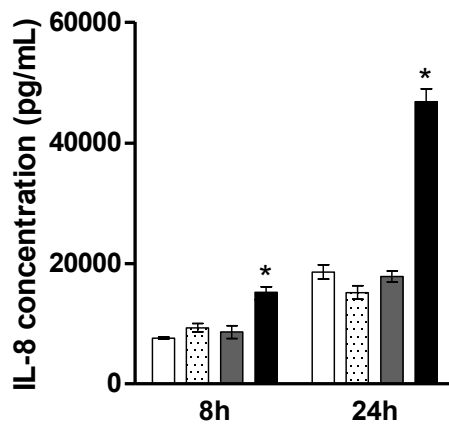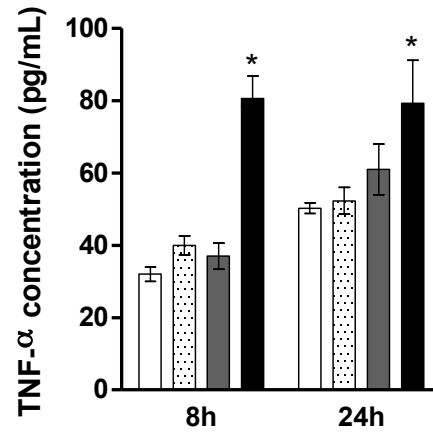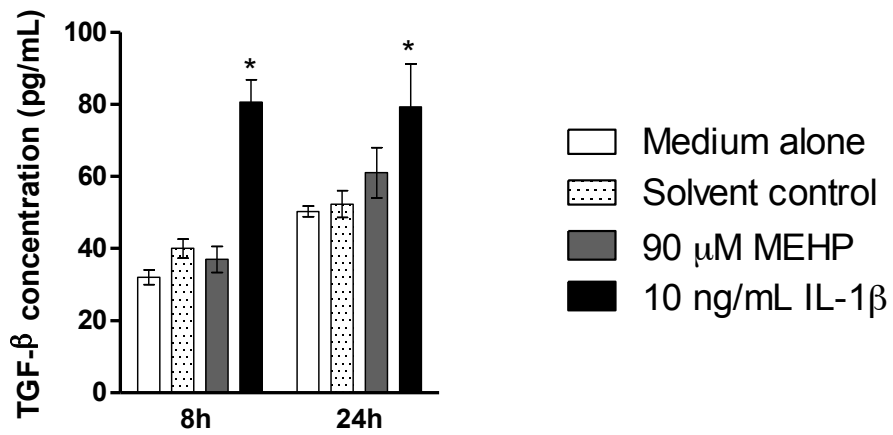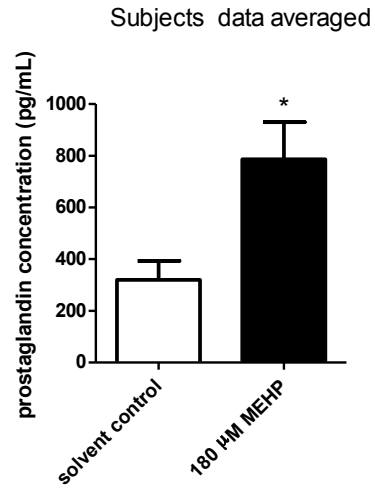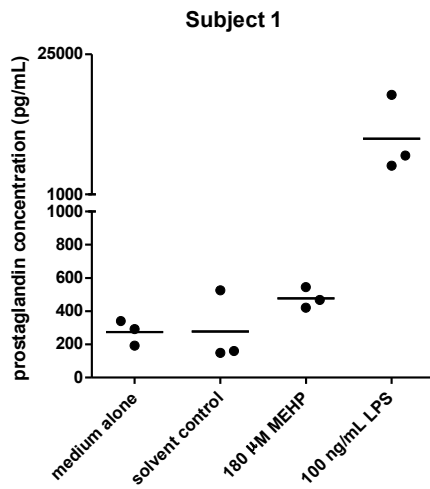
Figure 3.7. MEHP treatment effects on cytokine release from decidual macrophages into cell culture medium: A) IL-10; B) IL-6; C) IL-8; D) TNF-α; and E) TGF-β. Cells were treated for 8 h or 24 h with medium alone, DMSO (0.05% v/v; solvent control), 90 μM MEHP or IL-1β (positive control), and then cytokine concentrations in the culture medium were determined by ELISA as described in the "Materials and Methods" section. Data shown are from macrophages collected from one subject. *$p < 0.05$, comparing treatments to solvent control.
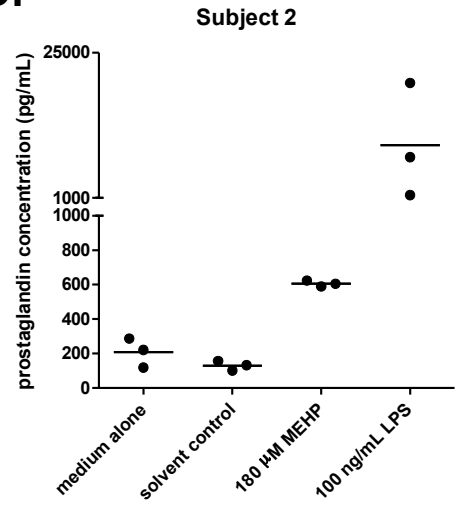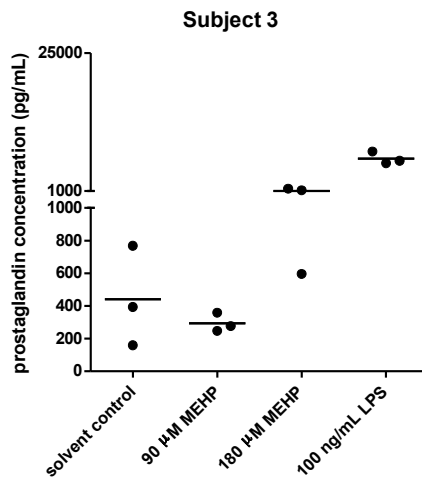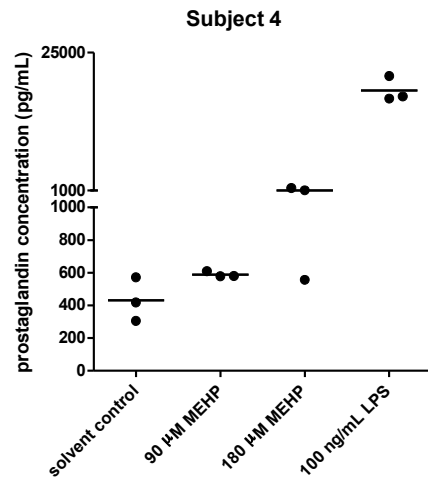
Figure 3.8. MEHP treatment effects on total prostaglandin release in gestational membrane explant medium. Figure 4A shows the subject averages for DMSO (0.05% v/v; solvent control), and 180 µM MEHP. Figures B- E show the individual replicate punch measurements for each subject (n=3 punches per subject). Cells were treated for 24 h with medium alone, DMSO (0.05% v/v; solvent control), 180 µM MEHP, or 100 ng/mL LPS. The prostaglandin screening EIA was performed as described in the "Materials and Methods" section. (means ± SE, n = 4 subjects. Figures B-E show data points representing 3 individual wells for each subject (n = 3 wells per treatment). Horizontal lines indicate mean values for each treatment within each subject. Solvent control cultures (0.05% DMSO v/v) showed no significant changes of prostaglandin release compared to medium alone (n=2 subjects). *$p < 0.05$, comparing treatments to solvent control.
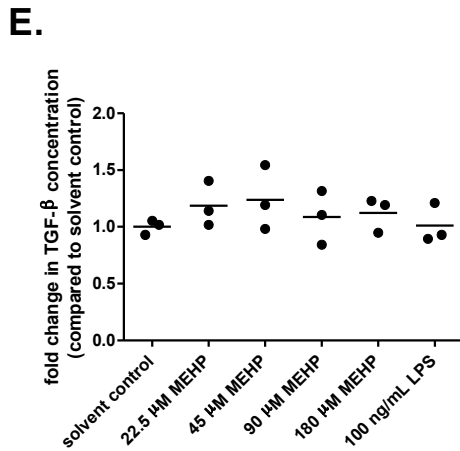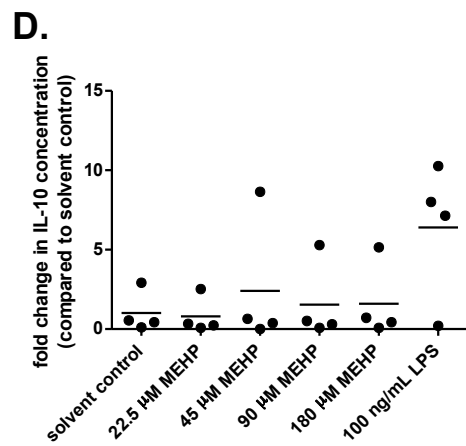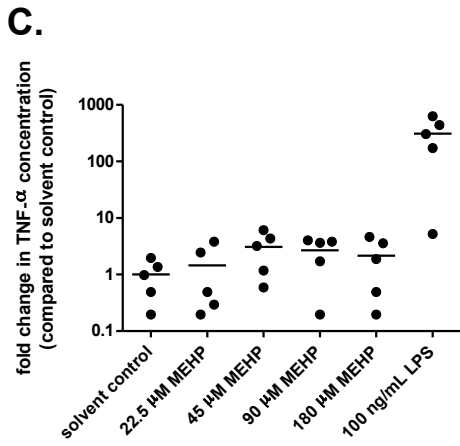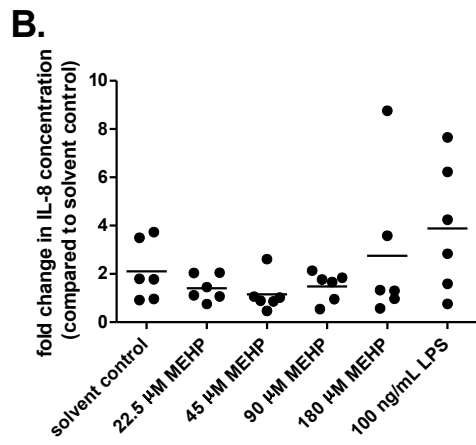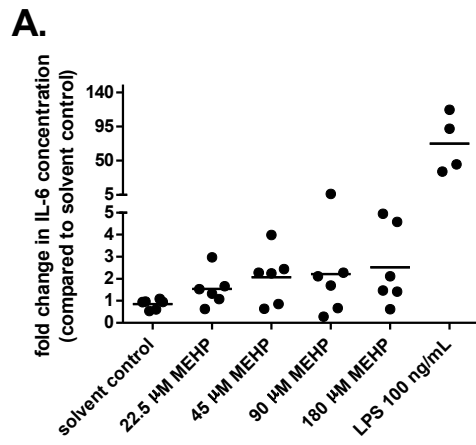
Figure 3.9. MEHP treatment effects on cytokine release from gestational membrane explants into cell culture medium: A) IL-6; B) IL-8; C) TNF-α; D); E) IL-10; and F)TGF-β. Punches were treated for 24 h with medium alone, DMSO (0.05% v/v; solvent control), 22.5, 45, 90 or 180 µM MEHP, or 100 ng/mL LPS and. Cytokine ELISAs were performed as described in the "Materials and Methods" section. Each black circle represents the mean of three wells from one subject. *p<0.05, comparing treatments to solvent control.

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CHAPTER IV:

Discussion

The present study is the first to identify cellular mechanisms by which MEHP may contribute to adverse pregnancy outcomes in human in vitro gestational cell and tissue models. Our results demonstrate that MEHP induces oxidative stress responses ranging from generation of hydroxyl radical, increased DNA damage, and modification of redox sensitive gene expression to activation of apoptotic proteases caspase 3/7. In addition, MEHP treatment stimulated prostaglandin synthesis, evidenced by increases in total prostaglandin release from immortalized human placental cells, decidual macrophages, placental macrophage Hofbauer cells, and gestational membrane explants. Moreover, MEHP increased release from placental macrophage Hofbauer cells of PGE2 and PGF2α, bioactive prostaglandins that promote uterine contractions.

Though the importance of prostaglandin synthesis in the initiation of parturition is clear, the role of oxidative stress responses in labor is not well understood. Association studies suggest a link between oxidative stress endpoints and preterm birth; however, few studies examine generation of ROS as a mediator of parturition pathways (Al-Gubory, Fowler et al. 2010; Pathak, Suke et al. 2010; Menon, Fortunato et al. 2011; Clerici, Slavescu et al. 2012).

98

Some insight can be gained from N-acetyl cysteine antioxidant pretreatment studies demonstrating that ROS species generation is necessary for LPS-induced preterm birth in mice and necessary for LPS-stimulated pro-inflammatory cytokine and prostaglandin release in gestational membrane explant cultures (Buhimschi, Buhimschi et al. 2003; Lappas, Permezel et al. 2003) . Similarly, oxidative insult can induce apoptosis in placental and gestational membrane chorionic trophoblast cells (Moll, Jones et al. 2007; Rogers, Ouellet et al. 2008; Yuan, Ohyama et al. 2008). Results from our laboratory suggest that treatment with the prototypical chemical oxidant, TBHP stimulates parturition-related pathways, including prostaglandin synthesis, apoptosis, and signal transduction in human placental cells, suggesting that ROS signaling may be important for initiation of these events (Cassandra Korte, personal communication). Furthermore, anti-oxidant pretreatment prevents MEHP-induced apoptosis in male germ cells and TK lymphoblasts and prevents DEHP-induced antral follicle injury in mice, suggesting that toxicologic and pathologic outcomes of MEHP treatment can be mediated through ROS generation (Rosado-Berrios, Velez et al. 2011; Wang, Craig et al. 2012).

Although not directly tested, the data in this dissertation are consistent with the following proposed model: that ROS generation stimulates prostaglandin synthesis, apoptosis, redox-sensitive gene expression and oxidative DNA damage in gestational tissues and cells, promoting the events of parturition (Figure 4.1). PTGS2 gene expression is controlled by a number of transcription factors that can be activated by ROS species , including AP-1, SP-1, and NF-κB

(Kang, Mbonye et al. 2007). These transcription factors can be activated by ROS through oxidation of redox-sensitive residues, such as cysteines existing within the protein itself, or through activation or modification by ROS of inhibitory proteins or upstream regulatory factors. For example, intracellular ROS generation resulting from an oxidative stimulus can lead to dissociation and degradation of the NF-κB inhibitory protein IκB, resulting in migration of the NF-κB subunit to the nucleus where it can initiate transcription (Allen and Tresini 2000). Similarly, ROS can inactivate phosphatases through direct oxidation of cysteine residues, resulting in increased kinase activity (Ma 2010); this signal can then be propagated to downstream kinase-dependent transcription factors, such as AP-1, NF-κB, and SP-1 (Allen and Tresini 2000; Ma 2010). Additionally, executioner caspases, including caspase 3/7 can be directly activated by ROS through redox sensitive cysteines, or indirectly through p53 activation following oxidative damage to DNA (Circu and Aw 2010).

Our finding that MEHP treatment increases ROS generation in human placental cells is corroborated by results from other studies demonstrating ROS generation with MEHP in cells and tissues from other organ systems. Despite the evidence linking MEHP exposure to increases in ROS generation, the mechanism underlying this occurrence is unknown. Because MEHP can activate the nuclear receptor PPAR through direct binding, some authors postulate that MEHP may increase hydrogen peroxide generation through peroxisome proliferation. This latter hypothesis is challenged, however, by studies demonstrating that MEHP-induced ROS generation is independent of PPARα

(Rusyn, Kadiiska et al. 2001; Kamijo, Hora et al. 2007). MEHP can also affect mitochondrial permeability, resulting in leakage of superoxide anion into the cytoplasm; however, it is unknown how MEHP affects mitochondrial permeability (Rosado-Berrios, Vélez et al. 2011). Additionally, cytochrome P450 metabolism of MEHP to its oxidized metabolites may generate superoxide and thereby represent another possible mechanism of MEHP-induced ROS generation. Lastly, because some phthalates can act as iron chelators, some phthalates, including MEHP, may increase Fenton-dependent hydroxyl radical production through facilitation of iron transport into and within the cell (Chang and Zylstra 1999). Further work is need to elucidate the mechanism of MEHP induced ROS generation.

With the exception of total prostaglandin release and cytokine analysis in whole tissue explants, the majority of this work was performed in cell culture models. The benefits of using cell culture models to study mechanisms of toxicity include ease of studying cell signaling mechanisms and measurement of ROS generation using cell-permeable, ROS-sensitive probes. Furthermore, as evidenced by the work in this proposal, primary cell isolates exhibit less within-subject variability in cytokine and prostaglandin responses as compared to gestational tissue explants. We recognize, however, that important cell-cell and tissue-tissue interactions may be missed when using cell culture models. Therefore, future studies should include measurements of oxidative stress responses with MEHP in whole tissue and in vivo exposure scenarios. Recent development of a photostable, tissue-permeable hydrocyanine ROS dye provides

possibilities for studying ROS generation both in vivo and in whole tissue culture (Kim, Choi et al. 2011; Selvam, Kundu et al. 2011). Furthermore, measurement of oxidative stress endpoints, including oxidized thymine measurements, caspase activity, and redox-sensitive gene expression can be applied to in vivo and whole tissue explant MEHP exposure scenarios.

The mechanisms of MEHP-induced toxicity identified in the present study can inform future molecular epidemiology studies to further strengthen the link between MEHP exposure and adverse pregnancy outcomes, including preterm birth. To strengthen the hypothesis that MEHP targets the gestational compartment, further studies could correlate urinary or gestational tissue MEHP concentrations with measurements of oxidative stress markers, including 8-hydroxy-2-deoxyguanosine (8-OHdG), malondialdehyde (MDA) and antioxidant protein expression, in addition to prostaglandin pathway factors including COX-2 protein and PTGS2 mRNA. Furthermore, circulating or urinary measures of 8-OHdG, MDA, antioxidants, and prostaglandin metabolites can also be correlated with MEHP exposure and preterm birth (Minuz, Covi et al. 1988).

In the current study, we were interested in effects of MEHP on preterm birth related responses relevant to the third trimester of gestation. Due to the limited availability of immortalized human gestational cells with normal chromosome number and phenotypes similar to primary cells or term primary macrophage cells, we chose to work with the HTR-8 cells and primary decidual macrophages, which originate from first trimester placenta, to study mechanisms relevant to parturition. We recognize, however, that the mechanisms studied in

this dissertation may be relevant to first-trimester adverse pregnancy outcomes as well, including preeclampsia and early pregnancy loss. As described in Chapter I, preeclampsia is linked to oxidative stress and apoptosis. In addition, PGE2 promotes migration of first trimester trophoblast cells in culture, a process important for proper vascularization of the placenta (Nicola, Lala et al. 2008). Furthermore, as described in Chapter II, IL-10 release by decidual macrophages helps to prevent maternal tissue rejection of the fetal allograft, a pathology linked to early pregnancy loss (Nagamatsu and Schust 2010). Future studies could elaborate on apoptosis, oxidative stress, and prostaglandins as mechanisms of MEHP-induced, first-trimester adverse pregnancy outcomes.

The concentrations of MEHP tested in the present study are comparable to concentrations of MEHP tested for ROS generation in previous studies in other cells types. Furthermore, the concentrations at which MEHP induced ROS production are comparable to the higher range of MEHP concentrations found in the gestational compartment. Specifically, the concentrations used in the current study were within an order of magnitude of reported human umbilical cord blood concentrations that range from 0.01 µM to 35 µM (Latini, De Felice et al. 2003; Lin, Zheng et al. 2008; Lin, Wang et al. 2011). Concentrations of MEHP in placental tissue range from 0 to 14.96 ug/kg (n=5 subjects) (Mose, Mortensen et al. 2007). Further studies with larger samples sizes are needed to more accurately assess placental exposure.

In summary, the findings from this study suggest a possible mechanism whereby MEHP exposure during pregnancy could contribute to preterm birth

through induction of bio-active prostaglandin synthesis, apoptosis, and oxidative stress. Based on these findings, future studies investigating the associations between the latter endpoints, MEHP exposure, and preterm birth in human populations are warranted.   Furthermore, prostaglandin synthesis, oxidative stress and apoptosis may represent mechanisms by which other environmental pollutants could contribute to preterm birth. Therefore, this work warrants future studies investigating the effects of other environmental pollutants on prostaglandin synthesis, oxidative stress, and apoptosis in human gestational tissues and cells.
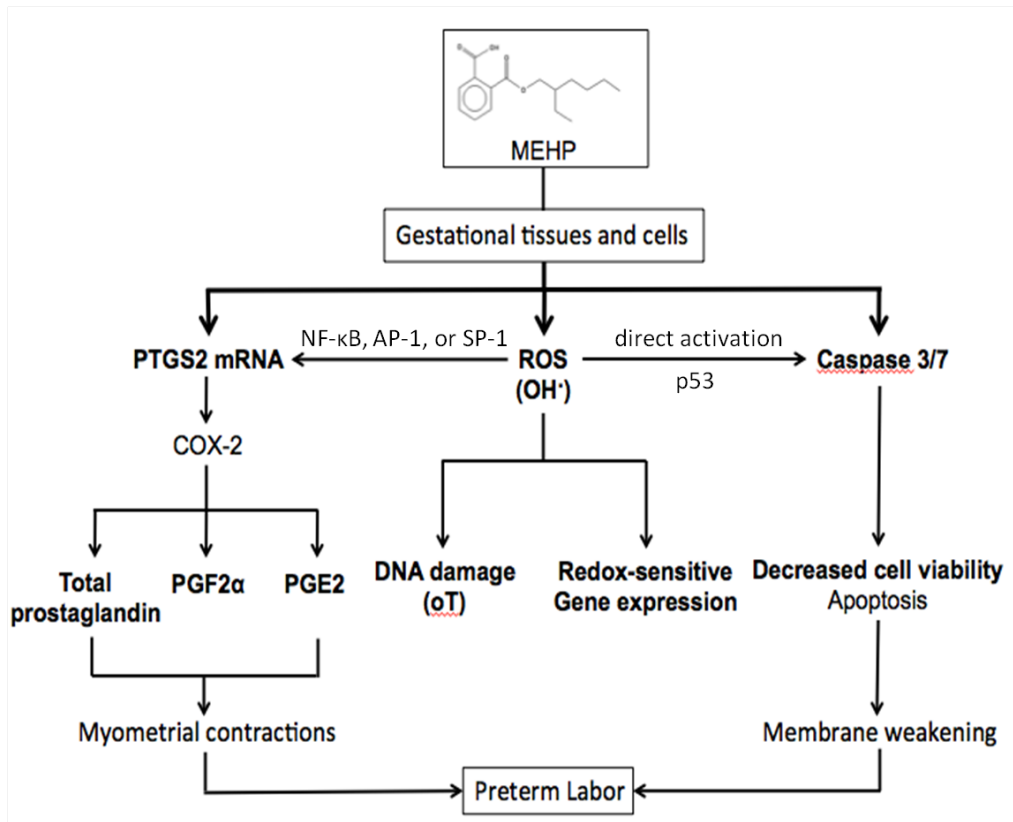
Figure 4.1. Conceptual model of MEHP effects in gestational tissues and cells. Reactive oxygen species (ROS) generation stimulates prostaglandin synthesis, apoptosis, redox-sensitive gene expression and oxidative DNA damage in gestational tissues and cells, promoting the events of parturition. Endpoints that were directly tested in this dissertation are highlighted in bold.

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