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Mono-2-Ethylhexyl Phthalate Induces Oxidative Stress Responses in Human Placental Cells In Vitro

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Abstract

Di-2-ethylhexyl phthalate (DEHP) is an environmental contaminant commonly used as a plasticizer in polyvinyl chloride products. Exposure to DEHP has been linked to adverse pregnancy outcomes in humans including preterm birth, low birth-weight, and pregnancy loss. Although oxidative stress is linked to the pathology of adverse pregnancy outcomes, effects of DEHP metabolites, including the active metabolite, mono-2-ethylhexyl phthalate (MEHP), on oxidative stress responses in placental cells have not been previously evaluated. The objective of the current study is to identify MEHP-stimulated oxidative stress responses in human placental cells. We treated a human placental cell line, HTR-8/SVneo, with MEHP and then measured reactive oxygen species (ROS) generation using the dichlorofluorescein assay, oxidized thymine with mass-spectrometry, redox-sensitive gene expression with qRT-PCR, and apoptosis using a luminescence assay for caspase 3/7 activity. Treatment of HTR-8 cells with 180 μ M MEHP increased ROS generation, oxidative DNA damage, and caspase 3/7 activity, and resulted in differential expression of redox-sensitive genes. Notably, 90 and 180 μ M MEHP significantly induced mRNA expression of prostaglandin-endoperoxide synthase 2 (*PTGS2*), an enzyme important for synthesis of prostaglandins implicated in initiation of labor. The results from the present study are the first to demonstrate that MEHP stimulates oxidative stress responses in placental cells. Furthermore, the MEHP concentrations used were within an order of magnitude of the highest concentrations measured previously in human umbilical cord or maternal serum. The findings from the current study warrant future mechanistic studies of oxidative stress, apoptosis, and prostaglandins as molecular mediators of DEHP/MEHP-associated adverse pregnancy outcomes.

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Keywords

phthalates; mono-2-ethylhexyl phthalate; oxidative stress; reactive oxygen species; human placental cells; prostaglandin-endoperoxide synthase 2

Introduction

Diethylhexyl phthalate (DEHP¹) is primarily used as a plasticizer in the manufacturing of polyvinyl chloride (PVC) consumer products. Because DEHP is not covalently bound to PVC products, it migrates into various environmental media. 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 mono-2-ethylhexyl phthalate (MEHP), the active metabolite of DEHP, in 80% of urine samples analyzed (Ferguson et al., 2011). 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.

In pregnant women, higher concentrations of MEHP in urine or umbilical cord blood samples were associated with low birth weight, increased risk for preterm birth, decreased gestation length, and pregnancy loss (Latini et al., 2003; Meeker et al., 2009; Toft et al., 2011; Zhang et al., 2009). Furthermore, MEHP has been detected in placenta, amniotic fluid and umbilical cord blood of humans (Mose et al., 2007; Wittassek et al., 2009; Zhang, et al., 2009). The latter findings suggest that the gestational compartment may be a target of MEHP toxicity. Despite evidence linking DEHP exposure to adverse pregnancy outcomes, the mechanism underlying these associations is unclear.

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses that results in a series of events including damage to cellular lipids, proteins, or DNA, and ultimately, apoptosis. Oxidative stress in cells of the gestational compartment is linked to the pathology of adverse pregnancy outcomes. Specifically, placental trophoblasts from pregnancies complicated by preeclampsia, intrauterine growth restriction (IUGR), and miscarriage exhibit higher levels of oxidative stress and apoptotic markers compared to normal pregnancies (Biri et al., 2007; DiFederico et al., 1999; Heazell et al., 2011; Hempstock et al., 2003; Johnstone et al., 2011; Tomas et al., 2011). Furthermore, increased levels of urinary oxidative stress markers early in pregnancy predict preeclampsia, shortened gestation length, and low birth-weight (Peter Stein et al., 2008).

Oxidative stress and apoptosis 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, et al., 2011; Ferguson et al., 2012). In vitro MEHP treatments of neutrophils, Kupffer cells and Leydig cells generate ROS (Fan et al., 2010; Rose et al., 1999; Vetrano et al., 2010). 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 (Erkekoglu et al., 2010; Hauser et al., 2007; Kasahara et al., 2002; Richburg et al., 2000; Suna et al., 2007).

¹Abbreviations: COX-2, cyclooxygenase-2; DCF, dichlorofluorescein; DEHP, di-2-ethylhexyl phthalate; HTR-8, HTR-8/SVneo; MEHP, mono-2-ethylhexyl phthalate; oT, oxidized thymine; *PTGS2*, prostaglandin-endoperoxide synthase 2; ROS, reactive oxygen species

Although the increased presence of oxidative stress is observed in tissues from pathological pregnancies, effects of DEHP metabolites on oxidative stress responses in placental cells have not yet been explored. In the present study, we investigated the effects of MEHP treatment on oxidative stress responses in human placental cells. Specifically, we treated HTR-8/SVneo (HTR-8) cells, a human first trimester extravillous trophoblast cell line, with MEHP and assessed ROS generation, oxidative DNA damage, redox-sensitive gene expression, and apoptotic cell death. This study is the first to identify potential molecular mediators of DEHP-associated adverse pregnancy outcomes in human placental cells.

Materials and Methods

Reagents

We purchased 6-carboxy-dichlorodihydrofluorescein diacetate (carboxy-H₂DCF-DA), Hoechst 33342 dye, phosphate buffered saline (PBS), and Hank's balanced salt solution (HBSS) from Invitrogen Life Technologies (Carlsbad, CA); dimethyl sulfoxide (DMSO), deferoxamine mesylate (DFO), *tert*-butyl hydroperoxide (TBHP), and camptothecin from Sigma-Aldrich (St. Louis, MO); MEHP from Accustandard (New Haven, CT); and RPMI 1640 medium with L-glutamine without phenol red, 10,000 U/mL penicillin/10,000 µg/mL streptomycin, and fetal bovine serum (FBS) from Gibco (Grand Island, NY).

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 cell line was derived from first trimester human placental cytotrophoblasts immortalized with SV40 antigen (Graham et al., 1993). Cells between passages 71 and 84 were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a 5% CO₂ humidified atmosphere. Cells were grown to a confluence of 70–90% before treatment. From stock solutions of 362 mM MEHP in DMSO, exposure media of 11.25, 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 and solvent controls.

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 cultured for 24 h. Cells were pre-incubated with 100 µM carboxy-H₂DCF-DA in HBSS for 1 h at 37 °C. The dye solution was then removed, cultures were rinsed with HBSS, and the cells were treated with DMSO (solvent control), or with 11.25, 22.5, 45, 90, or 180 µM MEHP in replicates of 3–6 for 1 h. After washing with HBSS and adding fresh HBSS back to the cultures, 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 preliminary experiments, we determined that MEHP in 0.05% DMSO showed no effects on DCF fluorescence in cell-free HBSS buffer compared to DMSO alone.

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 carboxy-H₂DCF-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. HTR-8 cells were pretreated with DFO to chelate cellular pools of free iron, thereby limiting the availability of iron to catalyze formation of ROS (Rothman et al., 1992). Cultures were exposed to HBSS alone, DMSO (solvent control), or 180 µM MEHP for 90 min, and then counterstained with the

nucleic acid stain Hoechst 33342 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 360 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. Equivalent adjustments for brightness and contrast were applied to each image in ImageJ software (National Institutes of Health).

Oxidized thymine measurement

HTR-8 cells were seeded at a density of $3.5\text{--}4 \times 10^6$ cells in 175 cm² flasks. After 24 h of incubation, cells were treated with DMSO (solvent control), 50 μM *tert*-butyl hydroperoxide (TBHP; positive control), 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 by MALDI-TOF/TOF-MS as described elsewhere (See Supplemental Materials, Page 2 for detailed methods) (Wang et al., 2012).

Cytotoxicity and cell viability assessment

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 treated with medium alone, DMSO (solvent control), MEHP (22.5, 45, 90, or 180 μM), or 4 μM camptothecin (positive control). After 24 h exposure, we measured caspase 3/7 activity in cell lysates using the Caspase-Glo 3/7 luminescent assay (Promega; Madison, WI) following the manufacturer's recommended protocol. The MultiTox-Glo Multiplex Cytotoxicity Assay (Promega, Madison, WI) was used to quantify cytotoxicity after 24 or 48 h exposure, following the manufacturer's recommended protocol. The latter assay uses a luminescent substrate to detect cell-leaked extracellular protease activity as a measure of membrane integrity and a fluorescent substrate to detect intracellular protease activity as a measure of cell viability (Niles et al., 2007). Briefly, the fluorogenic, cell-permeable substrate glycyl-phenylalanyl-aminofluorocoumarin (GF-AFC) was added to the cell cultures. Upon entering the cell, GF-AFC is cleaved by intracellular proteases to yield a fluorescent product, aminofluorocoumarin (AFC), proportional to the number of viable cells. The intracellular proteases detected by generation of AFC are specific to viable cells. A second, luminescent, cell-impermeable substrate, alanyl-alanyl-phenylalanyl-aminoluciferin (AAF-GloTM) was used to measure membrane integrity. The AAF-GloTM substrate reacts with proteases that are released from the cell upon loss of membrane integrity to produce luminescent aminoluciferin. Because the AAF-Glo substrate is cell impermeable, it will not react with intracellular proteases of viable cells with intact membranes. Fluorescence was measured using the SpectraMax M2e Multi-Mode Microplate Reader (Molecular Devices; Sunnyvale, CA) and luminescence was measured using the Glomax Multi Plus Detection System (Promega; Madison, WI). The Caspase-Glo 3/7 and MultiTox-Glo assays were repeated in 3–4 independent experiments containing 3 replicates for each treatment.

Oxidative stress gene array and qRT-PCR validation

Because MEHP stimulated ROS generation as assessed by DCF fluorescence, we evaluated changes in gene expression in the oxidative stress response pathway using the Oxidative Stress PCR Array (SABiosciences; Valencia, CA). 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 24 h. Cells were treated with medium alone, DMSO (solvent control) or MEHP (90, or 180 μM). After 4, 8, or 24 h of exposure, RNA was extracted using the RNeasy Plus Mini Kit (Qiagen; Valencia, CA), and cDNA was synthesized using the RT² First Strand Kit (SABiosciences, Valencia, CA) following the manufacturer's recommended protocols. For the array, cDNA from the solvent control and 180 μM MEHP treatment groups was

analyzed using the Applied Biosystems 7900HT Sequence Detection System following the SABiosciences recommended protocol. Fold changes were calculated from ΔCT values (gene of interest CT value – average of all housekeeping gene CT values) using the $\Delta\Delta CT$ method. 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). With qRT-PCR, we validated the findings of the array for those genes with significant mRNA expression changes that were approximately two-fold or more with 180 μM MEHP treatment: aldehyde oxidase 1 (*AOX1*), 24-dehydrocholesterol reductase (*DHCR24*), glutaredoxin 2 (*GLRX2*), prion protein (*PRNP*), scavenger receptor class A, member 3 (*SCARA3*), thioredoxin reductase 1 (*TXNRD1*), and prostaglandin-endoperoxide synthase 2 (*PTGS2*). qRT-PCR was performed on the latter seven genes using samples from cells treated with DMSO (solvent control), 90 or 180 μM MEHP for 24 h. In addition, qRT-PCR for *PTGS2* expression was performed on RNA isolated from DMSO (solvent control), 90 μM MEHP or 180 μM MEHP treatment groups after 4 and 8 h exposures. qRT-PCR reactions were prepared with SABiosciences SYBR Green mastermix and primers, and run on a Bio-Rad CFX96 Real Time C1000 thermal cycler following the manufacturer's recommended protocols. mRNA levels of each gene of interest were normalized to β -2-microglobulin mRNA levels.

Statistical analysis

Multiple group comparisons were carried out using a one-way ANOVA or two-way ANOVA followed by Tukey's posthoc test, as appropriate, and pairwise comparisons were carried out using paired t-tests, with $p < 0.05$ as the significance cutoff. Data are expressed as the mean $\pm$ SE of 3–5 experiments.

Results

Cellular generation of reactive oxygen species

Treatment of HTR-8 cells with 45, 90 or 180 μM MEHP for 1 h resulted in significant increases in DCF fluorescence (relative fluorescence units) compared with solvent controls, indicating increased carboxy- H_2DCF -DA oxidation (Figure 1A; $p < 0.05$). When cells exposed to 180 μM MEHP were pretreated with the antioxidant iron chelator deferoxamine mesylate (DFO), MEHP-stimulated DCF fluorescence was visibly decreased, suggesting that MEHP induced Fenton-dependent production of hydroxyl radical from hydrogen peroxide (Figure 1B). Microscopy examination revealed no differences in fluorescence comparing cells from control cultures incubated in HBSS alone, HBSS with 0.05% DMSO, or HBSS with 1 mM DFO (data not shown).

Oxidized thymine (oT) formation

Figure 2 shows oxidation of thymine. Treatment with 90 μM and 180 μM MEHP for 24 h increased oT approximately 20% and 80% respectively. An increase in oT of approximately 200% was also observed with 50 μM TBHP, included as a positive control (See Figure 1 in Supplemental Material for spectrograph). These data are from three separate experiments (experiment 2 included only solvent control and 90 μM MEHP treatment groups).

Caspase 3/7 activity

MEHP treatment significantly increased caspase 3/7 activity by approximately 80% at 24 h (Figure 3A; $p < 0.05$). Treatment with 4 μM camptothecin (positive control) significantly increased caspase 3/7 activity by approximately 390 % (data not shown). We observed no

differences in caspase 3/7 activity in controls treated with medium alone compared with 0.05% DMSO (solvent control) (data not shown).

Cytotoxicity and cell viability

Treatment with 180 μ M MEHP, but not lower MEHP concentrations, significantly decreased cell viability at 48 h but not 24 h, as measured by intracellular protease activity (Figure 3B). Treatment with 4 μ M camptothecin (positive control) significantly decreased cell viability at 24 and 48 h. MEHP had no effect on cytotoxicity at either 24 h or 48 h as assessed by an extracellular protease activity assay for membrane integrity, whereas camptothecin increased cytotoxicity by approximately 200% and 250% of controls at 24 and 48 h (data not shown). We observed no differences in cytotoxicity or cell viability with medium alone compared with 0.05% DMSO (solvent control) at 24 h or 48 h.

mRNA expression

The Oxidative Stress Gene Array identified seven genes with mRNA expression significantly changed two-fold or more by 180 μ M MEHP treatment compared to solvent control (For complete mRNA array data, See Supplemental Material, Table 1). Changes in expression of the array-identified genes were then examined by qRT-PCR. Consistent with the array results, the greatest fold change of gene expression was observed by qRT-PCR for *PTGS2*, which increased 5.3, 8.4 and 6.7 fold after 4, 8, and 24 h, respectively, with exposure to 180 μ M MEHP; mRNA increased significantly after 4 and 8 h treatment with 90 μ M MEHP, also, but to a lesser extent (Figure 4A; $p < 0.05$). Moreover, MEHP induced *PTGS2* expression in a concentration-dependent manner, with mRNA increased to a greater extent with 180 μ M MEHP compared to 90 μ M MEHP at all time points (Figure 4A; $p < 0.05$). In addition, we confirmed increased expression of *GLRX2* and *TXNRD1*, and decreased expression of *DHCR24*, after 24-hr treatment with 180 μ M MEHP (Figure 4B; $p < 0.05$). Although MEHP did not significantly change mRNA expression for *SCARA3*, *PRNP*, or *AOX1* when measured with qRT-PCR, we observed the same directional changes as were observed with the expression array (Figure 4B).

Discussion

The objective of the current study is to identify MEHP-stimulated oxidative stress responses in human placental cells. Our findings demonstrate that MEHP treatment increases ROS production, oxidative DNA damage, and apoptosis, and results in differential expression of redox-sensitive genes. Notably, MEHP treatment strongly induced *PTGS2*, the gene for cyclooxygenase-2 (COX-2).

We observed significant effects with MEHP concentrations ranging from 45–180 μ M, depending on the response. The lowest in vitro concentration we used is similar to average MEHP concentrations reported by Lin et al. (Lin et al., 2008) in human umbilical cord serum (35.7 μ M) and human maternal serum (42.6 μ M), but is higher than concentrations reported by Latini et al. (Latini, et al., 2003) in their epidemiologic study of MEHP in umbilical cord serum (average of 1.8 ± 2.2 μ M, range of 0–10.6 μ M).

To our knowledge, this is the first study to document MEHP-stimulated oxidative stress responses in cells of the gestational compartment. As such, the current findings are consistent with previous reports that in vitro exposure to MEHP induces DNA damage in human lymphocytes at concentrations ranging from 100–2500 μ M (Kleinsasser et al., 2004), and stimulates ROS generation in human neutrophils, human lymphoblast cells, and mouse Leydig cells at concentrations ranging from 100–500 μ M (Rosado-Berrios et al., 2011; Vetrano, et al., 2010; Zhao et al., 2012). Studies published by Erkekoglu et al. demonstrate

ROS generation and oxidative DNA damage with MEHP concentrations as low as 3 μ M, however, the reason for detection of these effects with lower treatment concentrations is not known (Erkekoglu, et al., 2010; Erkekoglu et al., 2011). Because urinary oxidative stress markers, including urinary 8-OHdG, a marker of oxidative DNA damage, are predictive of shortened gestation length and low birth weight, our findings may have relevance to adverse pregnancy outcomes (Peter Stein, et al., 2008).

In the HTR-8 human placental cells, MEHP increased activity of the executioner caspases 3 and 7 at 24 h and decreased cell viability at 48 h, suggesting apoptotic activation and subsequent cell death or cell cycle arrest. The latter findings are consistent with previous in vitro studies evidencing apoptosis with similar or higher MEHP concentrations (196–1000 μ M) in immune cells, testicular germ cells, and Sertoli cells (Rosado-Berrios, et al., 2011; Vetrano, et al., 2010; Yao et al., 2007). Although not directly measured in this study, we suggest that the increased caspase activity observed at 24 h may be dependent on MEHP-stimulated generation of ROS in HTR-8 cells, as shown previously for MEHP in male germ cells and human lymphoblast cells (Rogers et al., 2008; Rosado-Berrios, et al., 2011). Furthermore, oxidative insult in cultured first-trimester placental trophoblasts and term chorionic membrane trophoblasts triggers apoptosis (Moll et al., 2007; Rogers, et al., 2008; Rosado-Berrios, et al., 2011; Yuan et al., 2008). Because apoptosis of first trimester placental trophoblasts and later-gestation chorionic laeve trophoblasts are features of preeclampsia and preterm birth, respectively, our finding of increased activation of caspases suggests a potential link between apoptosis and MEHP-associated adverse pregnancy outcomes. Because MEHP induced *PTGS2* expression in the HTR-8 cells and previous studies show a link between prostaglandin E2 and apoptosis (Ackerman and Murdoch, 1993; Soleymani Fard et al., 2012; Takadera et al., 2004), future studies could test the dependence of apoptosis on *PTGS2* expression in human placental cells.

Consistent with ROS generation, MEHP treatment resulted in differential expression of the redox-sensitive genes *PTGS2*, *GLRX2*, *TXNRD1*, and *DHCR24*. The increased mRNA expression of *GLRX2* and *TXNRD1* is in accord with MEHP-stimulated ROS generation because these genes code for antioxidants and oxidoreductases that confer protection from oxidative DNA damage, apoptosis, and cell death (Meyer et al., 2009). Expression of *DHCR24*, the gene for 3 β -hydroxysterol-D24 reductase, was decreased in the present study. Because *DHCR24* catalyzes the last step in cholesterol biosynthesis, reduced *DHCR24* expression could potentially interfere with synthesis of the steroid hormone progesterone with implications for maintenance of pregnancy (Kawashiro et al., 2009). Previously, MEHP was shown to inhibit progesterone production in granulosa cells (Treinen et al., 1990). Based on the observed reduction in *DHCR24* expression with MEHP treatment, future studies could investigate the effects of MEHP treatment on steroid synthesis in HTR-8 cells, including synthesis of progesterone. Differential antioxidant expression or activity is observed in gestational tissues of pregnancies complicated by preeclampsia, intrauterine growth restriction, and miscarriage (Biri, et al., 2007; Biri et al., 2006; Hoegh et al., 2010). Moreover, increased levels of GLRX and TXNRD protein are found in preeclamptic placentae compared to controls, suggesting their involvement in response of the placenta to oxidative insult (Shibata et al., 2001). Whether the gene responses observed in the present in vitro study of MEHP are relevant to human pregnancy requires additional experiments beyond the scope of the present study.

Particularly interesting is our finding that MEHP induced a robust increase in mRNA expression of *PTGS2*, which encodes for the prostaglandin biosynthetic enzyme cyclooxygenase-2 (COX-2). In HTR-8 cells and primary extravillous trophoblasts, COX-2 inhibition suppresses migration, suggesting that perturbation of COX-2 expression early in gestation may interfere with placentation (Horita et al., 2007). Additionally, COX-2 is up-

regulated in placenta and extraembryonic membranes in the third trimester of pregnancy with concomitant increased synthesis of uterotonic prostaglandins (Slater et al., 1999). *PTGS2* mRNA levels are approximately seven times higher in chorion laeve from spontaneous preterm extraembryonic membranes compared to non-laboring tissues of equivalent gestational age (Mijovic et al., 1998), an increase comparable to the 8-fold increase we observed with MEHP in the present study. The increased *PTGS2* we observed with MEHP is consistent with findings of MEHP-induced expression of *PTGS2* mRNA in murine liver cells and COX-2 protein in spermatocytes (Ledwith et al., 1997; Onorato et al., 2008). In contrast, in vivo exposure to 750 and 1500 mg/kg DEHP, MEHP's parent compound, decreased *PTGS2* mRNA and COX-2 protein in rat placenta (Xu et al., 2008). Differences between the latter in vivo study and the current study may be related to species differences, because differences in response to DEHP have been observed in studies comparing peroxisome proliferation in rat and human (Klaunig et al., 2003), or they may be related to the cell line and culture conditions used in the present study. Furthermore, although DEHP is rapidly metabolized to MEHP by gut lipases following ingestion, a percentage of DEHP remains in serum. Therefore, differences in *PTGS2* expression between our in vitro results and the latter in vivo study could also be due to differences in placental response to DEHP compared to MEHP.

Considering our observations that MEHP treatment increased ROS generation and oxidative DNA damage, we expected the oxidative stress response array to identify more genes differentially expressed with MEHP. Because the oxidative stress response array includes genes that are responsive to a wide array of reactive species, it may be that MEHP generated 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. Moreover, because the array focused on genes directly involved in response and adaptation to ROS, other redox-sensitive genes that are critical for physiological and pathological cellular functions were not evaluated. A more comprehensive gene expression analysis may identify additional redox-sensitive genes affected by MEHP treatment, including genes critical for maintenance of pregnancy.

We chose to use HTR-8 cells as a model to study the effects of MEHP on placental cells because they have a similar phenotype compared to their primary counterparts (Biondi et al., 2006; Graham, et al., 1993; Jovanovi et al., 2010). HTR-8 cells retain migratory capability and express specific placental trophoblast markers including HLA-G, cytokeratin-7, and $\alpha 5 \beta 1$ integrin up to passage number 105 (Khan et al., 2011; Nicola et al., 2005). 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 et al., 2010; Novakovic et al., 2011). For this reason, we plan to further investigate the mechanisms of toxicity of MEHP identified in the current study using primary cells of the placenta.

Our results demonstrate that MEHP induced oxidative stress responses in human placental cells; specifically, ROS production, oxidative DNA damage, apoptosis, and modification of redox-sensitive gene expression. Notably, MEHP treatment strongly induced *PTGS2*, the gene for COX-2, which has critical roles in pregnancy and parturition. We observed significant effects with MEHP concentrations ranging from 45–180 μ M, concentrations within an order of magnitude of highest MEHP concentrations measured in human maternal or umbilical cord serum (Latini, et al., 2003; Lin, et al., 2008). Our findings suggest that future studies are warranted to explore oxidative stress, apoptosis, and prostaglandin synthesis in gestational tissues as potential mediators of DEHP-associated increased risk for adverse pregnancy outcomes. Moreover, given that numerous toxicants generate cellular

oxidative stress, evaluation of adverse pregnancy outcome associations with other chemical exposures may be warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- MEHP increased reactive oxygen species, oxidative DNA damage, and caspase activity
- MEHP induced expression of PTGS2, a gene important in pregnancy and parturition
- MEHP treatment resulted in differential expression of *GLRX2*, *TXNRD1*, and *DHCR24*

A

B

Figure 1.

MEHP-induced generation of reactive oxygen species. HTR-8 cells were preloaded with carboxy-H₂DCF-DA for 1 h, then treated for 1 h (A) or 90 min (B) with DMSO (solvent controls) or MEHP. **A**) Quantification of MEHP-stimulated DCF fluorescence. Bars represent the means of 4 independent experiments (except n=2 for 11.25 μM MEHP) containing 3–6 replicates each ± SE. *p < 0.05, compared to solvent control. **B**) Fluorescence microscopy visualization of the effect of deferoxamine pretreatment (DFO) on MEHP-stimulated DCF fluorescence (n=3 experiments). The top panel shows representative

images of intracellular DCF fluorescence, and the bottom panel shows corresponding Hoechst nuclear staining.

Figure 2. MEHP effects on oxidation of the DNA base thymine. HTR-8 cells were treated with DMSO (solvent control), 90 μ M MEHP, 180 μ M MEHP, or 50 μ M TBHP for 24 h. Oxidized thymine was measured from extracted genomic DNA and normalized to total DNA bases using mass spectrometry. Three experiments are shown: only solvent control and 90 μ M MEHP treatment groups were included in experiment 2.

Figure 3. MEHP effects in HTR-8 cells on **A**) caspase 3/7 activity and **B**) cell viability. Camptothecin was included as a positive control (data not shown for A). Bars represent the means of 3–4 independent experiments containing 3 replicates each $\pm$ SE. * $p < 0.05$ compared to solvent control.

Figure 4. MEHP effects on HTR-8 cell mRNA expression of genes previously identified with an expression array. **A)** Time-course of *PTGS2* mRNA expression. **B)** MEHP concentration-dependent effects on mRNA expression of six genes after 24 h exposure. mRNA was quantified by qRT-PCR. Bars represent means $\pm$ SE (n=3 experiments). *p<0.05 comparing the treatment Δ CT values to solvent control values. #p<0.05 comparing 180 μ M MEHP to 90 μ M MEHP.