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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 μM 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 μM 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.

Introduction

Diethylhexyl phthalate (DEHP) 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

Abbreviations: 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.

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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, 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).

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-H_2DCF-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% CO_2 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-H_2DCF-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-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. 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 $3.5–4\times10^6$ cells in 175 cm^2 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-Glo™) was used to measure membrane integrity. The AAF-Glo 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