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Di(2-ethylhexyl) phthalate inhibits antral follicle growth, induces atresia, and inhibits steroid hormone production in cultured mouse antral follicles

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ABSTRACT

Di(2-ethylhexyl) phthalate (DEHP) is a ubiquitous environmental toxicant found in consumer products that causes ovarian toxicity. Antral follicles are the functional ovarian units and must undergo growth, survival from atresia, and proper regulation of steroidogenesis to ovulate and produce hormones. Previous studies have determined that DEHP inhibits antral follicle growth and decreases estradiol levels in vitro; however, the mechanism by which DEHP elicits these effects is unknown. The present study tested the hypothesis that DEHP directly alters regulators of the cell cycle, apoptosis, and steroidogenesis to inhibit antral follicle functionality. Antral follicles from adult CD-1 mice were cultured with vehicle control or DEHP (1–100 μg/ml) for 24–96 h to establish the temporal effects of DEHP on the follicle. Following 24–96 h of culture, antral follicles were subjected to gene expression analysis, and media were subjected to measurements of hormone levels. DEHP increased the mRNA levels of cyclin D2, cyclin dependent kinase 4, cyclin E1, cyclin A2, and cyclin B1 and decreased the levels of cyclin-dependent kinase inhibitor 1A prior to growth inhibition. Additionally, DEHP increased the mRNA levels of BCL2-associated agonist of cell death, BCL2-associated X protein, BCL2-related ovarian killer protein, B-cell leukemia/lymphoma 2, and Bcl2-like 10, leading to an increase in atresia. Further, DEHP decreased the levels of progesterone, androstenedione, and testosterone prior to the decrease in estradiol levels, with decreased mRNA levels of side-chain cleavage, 17α-hydroxylase-17,20-desmolase, 17β-hydroxysteroid dehydrogenase, and aromatase. Collectively, DEHP directly alters antral follicle functionality by inhibiting growth, inducing atresia, and inhibiting steroidogenesis.

Introduction

Di(2-ethylhexyl) phthalate (DEHP) is the most commonly used phthalate ester, and it is predominantly used in the manufacturing of a wide range of polyvinyl chloride consumer, medical, and building products to impart flexibility (Heudorf et al., 2007; Anon., 2002). Because of its incorporation in numerous commonly used consumer products, DEHP is produced in vast quantities. Domestic production of dioctyl phthalates, a subgroup of phthalate esters in which DEHP is classified, exceeds 300 million pounds annually (Anon., 2002). Humans are exposed to DEHP on a daily basis via oral ingestion, inhalation, and dermal contact (Heudorf et al., 2007). This is because DEHP is non-covalently bound to the plastic, allowing the chemical to frequently leach out into the environment and in the products that humans consume on a daily basis (Heudorf et al., 2007). In fact, it is estimated that the range of daily human exposure to DEHP is between 3 and 30 μg/kg/day (Doull et al., 1999; Kavlock et al., 2002). Continuous, daily exposure to DEHP is a major concern because DEHP and its metabolite mono(2-ethylhexyl) phthalate (MEHP) have been identified in human blood samples (Kato et al., 2004; Hogberg et al., 2008), urine samples (Heudorf et al., 2007; Kato et al., 2004; Hogberg et al., 2008; Silva et al., 2004a; Becker et al., 2004; Marsee et al., 2006), amniotic fluid samples (Huang et al., 2009; Silva et al., 2004b; Wittassek et al., 2009), cord blood samples from newborns (Latini et al., 2003; Lin et al., 2008), breast milk samples (Hogberg et al., 2008), and in ovarian follicular fluid samples tested (Krotz et al., 2012), indicating the ability of these chemicals to reach the ovary.

Important for public health, DEHP is a known endocrine disrupting chemical and reproductive toxicant (Heudorf et al., 2007; Anon., 2002). In women, chronic occupational exposure to phthalates is associated with an increased risk of miscarriage and a decreased rate of pregnancy (Heudorf et al., 2007). In laboratory animals, DEHP causes pregnancy complications, including reduced implantations, increased

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resorptions, and decreased fetal weights of offspring (Lovekamp-Swan and Davis, 2003; Kaul et al., 1982).

The mechanisms by which DEHP disrupts these endocrine and reproductive events remain unknown, but interestingly, antral follicles from the ovary are critical regulators of these processes. Follicles are the functional units of the ovary, and the antral follicle is the most mature follicle type. Antral follicles are the major sources of sex steroid hormone production in the female and are the only follicle type capable of ovulation (Hirshfield, 1991). Normal antral follicle function requires follicle growth, survival from atresia, and appropriate regulation of steroidogenesis (Hirshfield, 1991).

Antral follicle growth is predominantly regulated by proliferation of granulosa cells and theca cells, which are somatic cells located in the follicle (Hirshfield, 1991). Proliferation of these cells, like most mammalian cells, is regulated by cyclins, cyclin dependent kinases, and cyclin dependent kinase inhibitors (Grana and Reddy, 1995; Robker and Richards, 1998; Sicinski et al., 1996). Although some antral follicles are rescued from atresia and ovulate in a female's reproductive lifespan, the vast majority of follicles (99%) are lost via atretic demise (Hirshfield, 1988, 1991). The regulation of follicular atresia involves a balance of pro- and anti-apoptotic factors that signal through the B cell leukemia/lymphoma 2 (BCL2) signaling pathway promoting caspase-induced apoptosis (Ratts et al., 1995; Flaws et al., 2001; Perez et al., 1999; Greenfeld et al., 2007; Hsu and Hsueh, 2000; Green and Reed, 1998). Antral follicle production of sex steroid hormones, a process termed ovarian steroidogenesis, is also essential for reproductive and non-reproductive health (Bagur and Mautalen, 1992; Cooper and Sandler, 1998; Everson et al., 1995; Hu et al., 1999; Armamento-Villareal et al., 1992; Christiansen, 1993; Mosca, 1998; Bush et al., 1987; Dennerstein et al., 1999; Woods et al., 2002; Krege et al., 1998; Couse and Korach, 1998; Couse et al., 2003, 2005; Britt and Findlay, 2002; Findlay et al., 2001; V.L. Davis et al., 1994; Fauser et al., 2011). Steroidogenesis is the enzymatic conversion of cholesterol to 17β-estradiol and other necessary sex steroid hormones.

Interestingly, recent work also suggests that DEHP targets the ovary and adversely affects antral follicle functionality. Specifically, in vivo studies using oral exposure to DEHP have shown that DEHP decreases serum estradiol levels, decreases aromatase levels, decreases antral follicle size, causes anovulation, and disrupts estrous cyclicity (Davis et al., 1994a; Hirosawa et al., 2006; Ma et al., 2011; Liu et al., 2014; Hannon et al., 2014). Further, studies using ovarian granulosa cell cultures and minced ovary cultures show that DEHP also decreases estradiol and aromatase levels (Laskey and Berman, 1993; Svechnikova et al., 2007). Our group has also reported that DEHP inhibits antral follicle growth and decreases estradiol and aromatase levels in a whole antral follicle culture system following 96 h of culture (Gupta et al., 2010). However, few studies have investigated the effects of DEHP on the precursor hormones and enzymes upstream of estradiol and aromatase. Further, the direct effect of DEHP on antral follicle atresia is unclear. Additionally, the mechanisms by which DEHP disrupts antral follicle growth, health, and steroidogenesis remain unknown.

The present study was designed to conduct a time-course study using an antral follicle culture system to investigate the initial and time specific DEHP-induced defects in antral follicle growth, atresia, and steroidogenesis upstream of the previously reported effects on estradiol. Specifically, we tested the hypothesis that DEHP directly alters regulators of the cell cycle, apoptosis, and steroidogenic pathway to inhibit antral follicle growth, induce atresia, and inhibit steroid production. To test this hypothesis, antral follicles were cultured with vehicle or DEHP for 24, 48, 72, and 96 h. Following culture, antral follicles were collected for histological evaluation of atresia and the measurements of mRNA levels of regulators of the cell cycle (*Ccnd2*, *Cdk4*, *Ccne1*, *Ccna2*, *Ccnb1*, and *Cdkn1a*), apoptosis (*Bad*, *Bax*, *Bok*, *Bcl2*, *Bcl2l10*, *Casp8*, and *Casp3*), and the enzymes responsible for generating estradiol (*Star*, *Cyp11a1*, *Hsd3b1*, *Cyp17a1*, *Hsd17b1*, and *Cyp19a1*). Further, media were collected for the measurements of antral follicle produced progesterone, DHEA, androstenedione, testosterone, estrone, and estradiol.

Materials and methods

Chemicals. DEHP (99% purity) was purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of DEHP were prepared using dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) as the vehicle in various concentrations (1.33, 13.3, and 133 mg/ml). This allowed for an equal volume of each stock to be added to the culture wells to control for vehicle concentration. Final concentrations of DEHP in culture were 1, 10, and 100 μg/ml, which is approximately equivalent to 2.77, 27.7, and 277 μM respectively.

The concentrations of DEHP were chosen based their ability to cause inhibition of antral follicle growth, decreased estradiol production from antral follicles, and decreased antral follicle *Cyp19a1* expression (Gupta et al., 2010; Wang et al., 2012). These concentrations of DEHP also have been determined to be clinically relevant in reproductive and non-reproductive cell and tissue cultures (Gillum et al., 2009; Lenie and Smitz, 2009; Mlynarcikova et al., 2009). Additionally, the selected concentrations of DEHP are environmentally relevant. Plasma concentrations of DEHP in healthy women have been reported to be 0.18 μg/ml, and peritoneal fluid concentrations of DEHP in these women were reported to be 0.46 μg/ml, which are close to the lowest concentration in this study (Cobellis et al., 2003). The no-observed-adverse-effect level (NOAEL) for DEHP is 5.8 mg/kg/day which equates to 14.3 μM (Anon., 2002). In addition, patients undergoing consistent medical care have markedly higher levels of DEHP than healthy people due to the extensive use of DEHP in medical care products (Pak et al., 2007; Kamrin, 2009). In intensive neonatal care units, patients receiving blood transfusions have plasma levels of DEHP at 11.1 μg/ml (Sjoberg et al., 1985). Further, the lowest-observed-adverse-effect level (LOAEL) of DEHP is 140 mg/kg/day, which equates to 344.6 μM (Anon., 2002). Each of the selected concentrations of DEHP falls below the LOAEL concentration.

Animals. Cycling, adult CD-1 female mice (34–37 days of age) were obtained from Charles River Laboratories (Wilmington, MA). The mice were housed in groups of 4 in the College of Veterinary Medicine Animal Facility at the University of Illinois at Urbana–Champaign and were allowed to acclimate to the facility prior to experimentation. The mice were housed in a controlled animal room environment (temperature at 22 ± 1 °C and 12-hour light–dark cycles) and were provided food and water ad libitum. The Institutional Animal Use and Care Committee at the University of Illinois at Urbana–Champaign approved all procedures involving animal care, euthanasia, and tissue collection.

In vitro antral follicle culture. Unprimed, female CD-1 mice were euthanized and their ovaries were aseptically removed for antral follicle isolation. Antral follicles were isolated from the ovary based on relative size (250–400 μm) and were cleaned of interstitial tissue using watchmaker's forceps (Gupta et al., 2006; Miller et al., 2005). At least 2–3 mice were used in each experiment, in which we could obtain approximately 20–30 antral follicles per mouse. Each treatment group contained 10–16 follicles. Isolated antral follicles were randomly and individually plated in wells of a 96-well culture plate containing unsupplemented α-minimal essential medium (α-MEM, Life Technologies, Grand Island, NY) prior to treatment.

Treatment groups included DMSO (vehicle control) and DEHP (1, 10, and 100 μg/ml) and were prepared in supplemented α-MEM. Supplemented α-MEM contained 1% ITS (10 ng/ml insulin, 5.5 ng/ml transferrin, 5.5 ng/ml selenium, Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO), 100 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO), 5 IU/ml human recombinant follicle-stimulating hormone (FSH; Dr. A. F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical