Gender-specific adverse effects of mono-ethylhexyl phthalate on steroidogenesis in immature granulosa cells and rat Leydig cell progenitors in vitro

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INTRODUCTION

An increased incidence of disorders of sex development (DSD), such as hypospadias and cryptorchidism as well as testicular cancer and poor semen quality during the last decades in humans have been attributed to the influence of endocrine disruptors (ED). These compounds constitute a heterogeneous group of xenobiotics of anthropogenic origin which can mimic natural hormones, inhibit the action of hormones, or alter their biosynthesis and/or metabolism (Lottrup et al., 2006).

Phthalates, including the widely used plasticizer di-(2-ethylhexyl) phthalate (DEHP), are components of many plastic products, such as food wraps, toys, and some medical devices (e.g., tubings, bags for intravenous infusions, blood transfusion, and dialysis equipment; Thomas and Thomas, 1984). DEHP is loosely held with the plastic polymer and can easily be released into the environment (Koch et al., 2006). High concentrations of phthalate metabolites were detected in the urine of women of childbearing age (Blount et al., 2000), a finding that can be associated with extensive use of personal care products (e.g., skin creams and nail polish) containing phthalates. Children on dialysis are suggested to be one of the most vulnerable groups for phthalates exposure, because they may receive high levels of phthalates from dialysis tubing (Loff et al., 2000; Brock et al., 2002). Further, in this context, children and women of childbearing age were demonstrated to have higher urinary levels of phthalates than other groups (Blount et al., 2000). Moreover, maternal phthalate exposure during pregnancy has been associated with decreased anogenital distance (AGD) in male infants (Swan et al., 2005), a finding suggested to be due to attenuation of androgen production by the fetal testis.

Di-(2-ethylhexyl) phthalate, one of the phthalates most widely distributed in the environment, causes reproductive toxicity that is attributable to the action of its primary metabolite, mono-(2-ethylhexyl) phthalate (MEHP). Here, we have investigated the effects of MEHP on steroidogenesis by primary cultures of rat Leydig cell progenitors and immature granulosa cells. This phthalate stimulated basal steroidogenesis and steroidogenic acute regulatory protein (StAR) expression in both types of steroidogenic cells. However, when MEHP was incubated with (Bu)2CAMP, steroid production was increased in granulosa cells and suppressed in Leydig cell progenitors, a process associated with up-regulation of StAR expression. Our data suggest that MEHP exerts gender-specific adverse effects on the hormonal function of the developing gonads. This may be involved in the development of pathological conditions including disorders of prenatal sex development that may attenuate future reproductive health.

Keywords: Leydig cell progenitors, immature granulosa cells, MEHP, steroidogenesis, StAR

Experiments on animal models have demonstrated that DEHP causes reproductive toxicity in both prenatal and postnatal stages of development. This effect was attributed to its primary metabolite, mono-(2-ethylhexyl) phthalate (MEHP; Sjoberg et al., 1986; Dostal et al., 1988; Gray et al., 2001). This mono-phthalate impairs the function of steroid-producing gonadal cells, such as Leydig and granulosa cells in both in vivo and in vitro experimental paradigms. MEHP was observed to inhibit the stimulation of testosterone production in primary cultures of rat Leydig cells by luteinizing hormone (LH; Jones et al., 1993) and the activation of steroidogenesis by human chorionic gonadotropin (hCG) in the MA-10 Leydig cell line (Dees et al., 2001). We have recently demonstrated that MEHP decreased hCG-activated steroidogenic acute regulatory protein (StAR) expression, a process that was associated with reduced transport of cholesterol into mitochondria and attenuated androgen production in immature and adult Leydig cells (Svechnikov et al., 2008). Similarly, MEHP was reported to attenuate progesterone production by follicle stimulating hormone (FSH)-stimulated primary cultures of rat granulosa cells in vitro (Tirenien et al., 1990) and chronic administration of DEHP to rats inhibited sex hormone production in immature female rats in vivo and ex vivo (Svechnikova et al., 2007). In addition, MEHP was demonstrated to suppress estradiol production by granulosa cells through direct inhibition of transcription of the aromatase (CYP19) gene (Lovekamp and Davis, 2001).

However, the mechanism(s) underlying the action of MEHP on developing Leydig and granulosa cells at their early stages of differentiation has not yet been investigated. Thus, the aim of the present study was to examine the effects of MEHP on steroidogenesis in progenitor Leydig cells and immature granulosa cells isolated from 20-day-old male and female rats.
**MATERIALS AND METHODS**

**MATERIALS**

Dulbecco’s modified Eagle’s medium (DMEM) – Ham’s nutrient mixture F-12 (supplemented with L-glutamine and HEPES), bovine serum albumin (BSA; 7.5% solution), cholesterol lipid concentrate (250 ×), and penicillin-streptomycin were bought from Gibco/BRL, Life Technologies (Paisley, Scotland); BSA (fraction V), percoll, HEPES, collagenase type I, (Bu)2cAMP, ovine FSH, (1 mM) or 22R-OHC (10 μM) and medium containing cholesterol lipid concentrate (diluted 1:250; intended to mimic the cholesterol content of the follicular fluid in vivo).

**ANIMALS**

The testes and ovaries from 20-day-old Sprague-Dawley rats (B&K Laboratories, Sollentuna, Sweden) were used for the isolation of immature granulosa cells and Leydig cell progenitors. These animals had access to a standard laboratory chow (Lantmännen, Kinmast, Sweden) and water ad libitum. The rats were anesthetized by phenobarbital before the testes and ovaries were removed. These experiments were approved by the Northern Stockholm Animal Ethics Committee (registration no. N319/08).

**ISOLATION AND PRIMARY CULTURE OF GRANULOSA CELLS**

Granulosa cells were isolated from the ovaries of untreated immature (20-day-old) female Sprague-Dawley rats (B&K Laboratories, Sollentuna, Sweden) as described previously (Svechnikov et al., 2007). In brief, after trimming away surrounding tissue, the ovaries were placed in 3 ml DMEM:F-12 (1:1) medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 0.5% BSA and thereafter punctured with a 25-gauge needle. The resulting cell suspension was then filtered through a 70-μM cell strainer, followed by collection of cells by centrifugation at 200 × g for 7 min. After washing once with the same medium the cells were resuspended in a corresponding volume of fresh DMEM:F-12 (1:1) medium containing cholesterol lipid concentrate (diluted 1:250; intended to mimic the cholesterol content of the follicular fluid in vivo).

For culturing, 100 μl of a suspension containing 1 × 10⁵ cells/ml was plated into each well of a 96-well Falcon plate and cultured for 2 h and thereafter incubated with or without MEHP (100 and 250 μM) for 24 h at 37°C in an atmosphere of air and 5% CO₂. Then, culture medium was replaced by fresh medium containing MEHP at the same concentrations and an optimal concentration of FSH (50 mU/ml) and/or (Bu)₂cAMP (1 mM) for another 24 h.

**STEROID ASSAYS**

Media from the cultures performed as described above were stored at −20°C prior to analysis of testosterone, progesterone and 5α-androstane-3α, 17β-diol (3α-Adiol). The sum of the levels of testosterone and 3α-Adiol, the predominant steroids synthesized by Leydig cell precursors, was used as an indicator of the total capacity of these immature steroidogenic cells to produce androgens in culture. Testosterone and progesterone were quantified employing the Coat-a-Count RIA kit (Diagnostic Products Corp., Los Angeles, CA, USA), in accordance with the manufacturer’s instructions; while the levels of 3α-Adiol were determined by RIA using specific antisera (Cosmo Bio Co. Ltd., Tokyo, Japan). For this purpose, 5α-[9,11,13H (N)] androstane-3α,17β-diol (specific activity, 40 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA, USA). Intra-assay and inter-assay coefficients for testosterone and progesterone RIA were 5.0, 3.5% and 6.4, 3.9%, respectively. The same parameters for 3α-Adiol RIA were 10 and 6.9%. The sensitivity of the RIAs was 0.04, 0.02, and 0.08 ng/ml for testosterone, progesterone and 5α-androstane-3α, 17β-diol, respectively.

**WESTERN BLOT ANALYSIS**

The influence of MEHP on basal and (Bu)₂cAMP-stimulated SF-1 and StAR expression was analyzed by PAGE/western blotting as described earlier (Svechnikov et al., 2008). For this purpose, both types of steroidogenic cells were washed twice with PBS and then lysed and sonicated in a buffer containing 62.5 mM Tris–Cl (pH 6.8), 2% SDS, 50 mM dithiothreitol and 10% glycerol. Subsequently, the fraction thus solubilized was collected by removal of debris by centrifugation at 10,000 × g for 6 min and the solubilized proteins (50 μg from each sample) resolved by electrophoresis on 10% SDS/polyacrylamide gels and thereafter transferred electrophoretically to Hybond-P polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK), using 25 mM Tris–Cl, 185 mM glycine, pH 8.3, containing 20% methanol. These membranes were routinely stained with 5% Ponceau
We observed that MEHP enhanced FSH-stimulated progesterone production by Leydig cell progenitors and markedly (three-fold over control, p < 0.05) stimulated testosterone and its metabolite production by cultured immature granulosa cells. The most effective concentration of MEHP (250 μM) significantly (12-fold over untreated control, p < 0.05) stimulated testosterone and its metabolite production by Leydig cell progenitors and markedly (three-fold over control, p < 0.05) enhanced progesterone secretion by immature granulosa cells.

S in order to confirm equal transfer of the proteins. Only membranes with equal levels of proteins from all lanes were processed further.

Following this transfer, each membrane was incubated in blocking buffer (TBS-0.1% Tween (TBST) containing 5% non-fat dry milk) for 1 h, followed by three washes with TBST (3 × 10 min). Mouse monoclonal anti-SF-1 antibody (Perseus Proteomics, Tokyo, Japan) and polyclonal antisera directed against StAR (kindly provided by Dr. Stocco, Texas Tech University, USA; Clark et al., 1994) were used for incubation at a dilution of 1:500 and 1:3000, respectively in TBS containing 5% non-fat dry milk overnight at 4°C. Antibody against tubulin (mouse monoclonal IgG, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and actin (rabbit polyclonal IgG, Sigma-Aldrich, St. Louise, MO, USA) were used as an internal standard. After washing, the membranes were then incubated with secondary donkey anti-rabbit or sheep anti-mouse antibodies conjugated with horseradish peroxidase (Amersham Pharmacia Biotech) and the bands detected by incubation with ECL Plus™ western blotting agent (Amersham Pharmacia Biotech) and subsequent exposure to Hyperfilm ECL (Amersham Pharmacia Biotech). Finally, the ECL Hyperfilms were scanned with an HP Scan Jet 5100C and HP Precision scan software (Hewlett-Packard Sverige AB, Kista, Sweden) and the extent of antibody binding quantitated employing the NIH Image 1.57 software.

STATISTICAL ANALYSIS
The differences between various values were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by the Dunnett t-test, using the SigmaStat (v 3.00) package (SPSS, Inc, Chicago, IL, USA). p < 0.05 was considered to be statistically significant.

RESULTS
MEHP STIMULATES BASAL STEROIDOGENESIS IN IMMATURE GRANULOSA AND LEYDIG CELLS
As depicted in Figure 1 both concentrations of MEHP (100 and 250 μM) activated steroid production by cultured immature granulosa cells and Leydig cell progenitors. The most effective concentration of MEHP (250 μM) significantly (12-fold over untreated control, p < 0.05) stimulated testosterone and its metabolite production by Leydig cell progenitors and markedly (three-fold over control, p < 0.05) enhanced progesterone secretion by immature granulosa cells.

OPPOSITE EFFECT OF MEHP ON (Bu)2cAMP-ACTIVATED STEROIDOGENESIS IN GRANULOSA CELLS AND LEYDIG CELL PROGENITORS
We further investigated whether MEHP can influence steroidogenesis in the steroidogenic cells stimulated by (Bu)2cAMP beyond LH/FSH receptor. Similarly to FSH, MEHP significantly and dose-dependently promoted (Bu)2cAMP-stimulated progesterone production by immature granulosa cells (Figure 3A), while the responsiveness of Leydig cell progenitors to the stimulator was significantly (by 44%, p < 0.05) attenuated by the highest (250 μM) concentration of MEHP (Figure 3B). In contrast, MEHP exerted no significant effect on androgen synthesis from 22R-OHC, a derivative of cholesterol that diffuses readily across membranes (22R-OHC treatment, 153.7 ± 1.2 vs 139 ± 7.4 ng/10⁵ cells/24 h with 22R-OHC + MEHP treatment; p = 0.12, n = 4), suggesting that MEHP may disturb the translocation of cholesterol across mitochondrial membranes in Leydig cell progenitors.

OPPOSITE EFFECT OF MEHP ON STEROIDOGENESIS BY CULTURED GRANULOSA AND LEYDIG CELL PROGENITORS IN RESPONSE TO FSH AND hCG
We observed that MEHP enhanced FSH-stimulated progesterone production by granulosa cells, where the highest concentration of the phthalate (250 μM) increased FSH-activated steroid production by two-fold compared to FSH alone (p < 0.05; Figure 2A). In contrast, the same concentration of MEHP significantly (by 48%, p < 0.05) suppressed hCG-activated steroidogenesis in Leydig cell progenitors (Figure 2B).

OPPOSITE EFFECT OF MEHP ON STEROIDOGENESIS IN GRANULOSA CELLS AND LEYDIG CELL PROGENITORS ACTIVATED BY (Bu)2cAMP
We further tested the hypothesis that MEHP can differentially affect the expression of StAR (which is responsible for the translocation of cholesterol from the outer to the inner mitochondrial membranes) by cultured immature granulosa cells and Leydig cell progenitors. MEHP significantly (by 48%, p < 0.05) suppressed StAR protein expression in immature granulosa cells treated with 100 μM MEHP (Figure 4A). Similarly, MEHP decreased expression of StAR in Leydig cell progenitors treated with 100 μM MEHP (Figure 4B).
FIGURE 2 | Opposite effect of MEHP on FSH and hCG-stimulated steroidogenesis in immature granulosa cells (A) and Leydig cell progenitors (B). The steroidogenic cells were cultured without or with MEHP at different concentrations for 24 h and then incubated with hCG (10 ng/ml) and FSH (50 mU/ml) in the absence or presence of the same concentrations of MEHP for additional 24 h. Testosterone (T) and 3α-Adiol and progesterone in the culture media were then measured by RIA. The mean values ± SEM for four independent steroidogenic cell preparations are presented. *p < 0.05 compared to treatment with FSH and hCG.

FIGURE 3 | Opposite effect of MEHP on (Bu)2cAMP-stimulated steroidogenesis in immature granulosa cells (A) and Leydig cell progenitors (B). The steroidogenic cells were cultured without or with MEHP at different concentrations for 24 h and then incubated with (Bu)2cAMP (1 mM) in the absence or presence of the same concentrations of MEHP for an additional 24 h. Testosterone (T), 3α-Adiol and progesterone in the culture media from the steroidogenic cells were then measured by RIA. The mean values ± SEM for four independent steroidogenic cell preparations are presented. *p < 0.05, **p < 0.01 compared to treatment with (Bu)2cAMP.

DISCUSSION

Here we demonstrate for the first time that MEHP exerts gender-specific adverse effects on steroidogenesis by immature granulosa cells and Leydig cell progenitors isolated from 20-day-old rats. This mono-phthalate significantly stimulated basal steroidogenesis in both types of steroidogenic cells, while gonadotropin (FSH and hCG) and (Bu)2cAMP-activated steroid production was differently affected by the phthalate in male and female cells. We observed that MEHP caused an additive effect on the stimulator activated progesterone biosynthesis by granulosa cells but attenuated significantly hCG and (Bu)2cAMP-evoked steroidogenesis in Leydig cell progenitors. The observation that MEHP had no effect on steroidogenesis stimulated by 22R-OHC, a form of cholesterol that readily penetrates mitochondrial membranes, indicates that MEHP may act by disturbing cholesterol trafficking across mitochondrial membranes and influence STAR expression in Leydig cell progenitors. Indeed, further analysis of STAR expression demonstrated that MEHP enhanced (Bu)2cAMP-activated STAR expression in both male and female steroidogenic cells, an event accompanied by activation of progesterone production in granulosa cells and inhibition of steroidogenesis in Leydig cell progenitors.
In addition, we also found that cAMP-PKA signaling activated by (Bu)₂cAMP up-regulated SF-1 expression in both types of the cells, a cellular event that was suppressed by MEHP in Leydig but not in granulosa cells.

Our present findings are in line with those of a recent study showing that MEHP stimulates basal steroid synthesis in KK-1 granulosa tumor cells and in mouse Leydig tumor cells (MLTC-1) but suppresses hCG-induced progesterone production in MLTC-1. The observed stimulatory effect of MEHP was STAR independent and associated with an up-regulation of the enzymes involved in cholesterol mobilization, suggesting that MEHP increases the amount of cholesterol available for steroidogenesis (Gunnarsson et al., 2008). In contrast, we found that MEHP significantly stimulated STAR expression in both types of gonadal cells, a process associated with the activation of steroidogenesis. This difference in the mechanisms responsible for the activation of steroidogenesis by MEHP in the steroidogenic cells may be explained by different experimental models used in our study and that by Gunnarsson et al. (2008). In our study we employed primary cultures of native immature steroidogenic cells, while Gunnarsson et al. (2008) exploited steroidogenic tumor cells which may have an aberrant steroidogenic machinery and signal transduction pathway(s). Moreover, our findings agree well with another recent study demonstrating that exposure to MEHP increased STAR gene expression in the rat testis (Lahousse et al., 2006).

We also observed that (Bu)₂cAMP-activated expression of STAR was significantly up-regulated by MEHP in both types of steroidogenic cells. These events were associated with a decrease in androgen production by Leydig cell progenitors and activation of steroidogenesis in granulosa cells. This finding let us to suggest that molecular events activated by MEHP in the (Bu)₂cAMP-stimulated steroidogenic cells and triggered STAR gene expression were similar in both types of cells but post-translational modifications of STAR which activate its function were suppressed in Leydig cell progenitors. It is well-known that STAR needs to be phosphorylated to reach maximal activity, a process dependent on the action of the PKA pathway (Arakane et al., 1997). Therefore, attenuation of STAR protein phosphorylation but not its expression in Leydig
cell progenitors co-treated with MEHP and (Bu)_2cAMP may result in accumulation of functionally inactive StAR with low capacity to deliver cholesterol into the mitochondria. In contrast, StAR function and steroidogenesis were activated in granulosa cells co-treated with MEHP and (Bu)_2cAMP, indicating that this phthalate did not counteract post-translational modifications induced by cAMP-PKA dependent signaling pathway in the female cells. In addition, we have recently demonstrated that MEHP decreased hCG-activated StAR protein expression, a process that was associated with reduced transport of cholesterol into mitochondria and attenuated androgen production in immature and adult Leydig cells (Svechnikov et al., 2008).

Our data have also shown that the activation of StAR expression by cAMP-PKA signaling was associated with up-regulation of SF-1 expression in both types of steroidogenic cells, a finding that is in line with previous report (Sandhoff et al., 1998). However, in contrast to StAR, co-treatment with (Bu)_2cAMP and MEHP attenuated SF-1 expression in Leydig cells and had no significant effect on the transcription factor level in granulosa cells. This observed disagreement between StAR and SF-1 expression can be explained by a complex regulation of STAR expression in steroidogenic cells that includes dynamic and effective interaction between several transcription factors (e.g., SF-1, CREB, GATA, cJun) and coactivators (Stocco and Clark, 1996; Stocco, 2001), where SF-1 may not play a primary role. This suggestion is supported by one recent study showing that attenuation of the expression of SF-1-regulated genes (e.g., StAR, P450scc, P450c17) in the fetal testis of rats that underwent di (n-butyl) phthalate (DBP) exposure in utero was not associated with changes in SF-1 mRNA and protein levels, suggesting that this downregulation was indirect and perhaps involved cofactor starvation (Plummer et al., 2007). Moreover, (Bu)_2cAMP and cigitazone stimulated STAR expression in immortalized KK1 mouse granulosa cells and MA-10 mouse Leydig tumor cells was linked to activation of PPARγ and up-regulation of cjun expression but not SF-1 (Kowalewski et al., 2009). We also observed that basal and MEHP-treated granulosa cells did not express detectable amount of SF-1. This finding may be due to weak steroidogenic activity of immature granulosa cells compare to Leydig cells, which in our experiments reached 10- to 20-fold differences, suggesting that SF-1 is not a major player in the regulation of basal granulosa cell steroidogenesis.

The concentrations of MEHP (100 and 250 μM) employed in the present in vitro investigations are relevant to in vivo situations in humans. Human exposure to DEHP can be as high as 4.3 mg/kg/day for adults (Lovekamp-Swan and Davis, 2003) and 10–20 mg/kg/day for infants exposed to neonatal transfusion or parenteral nutrition (Loff et al., 2000; Calafat et al., 2004), resulting in 150 and 400 μM concentrations in the blood, respectively.

In our present study we have used primary cultures of steroidogenic cells isolated from 20-day-old rats to explore the effects of MEHP. This age represents prepubertal stage of human development. Phthalates are widely used as plasticizers in the production of plastics and children are thought to be more susceptible for harmful action of these anthropogenic chemicals than adults. Recent studies reported the negative correlation between AGD and maternal (urinary) levels of phthalates in male infants (Swan et al., 2005; Swan, 2008). Moreover, these studies also demonstrated that AGD correlated to penile volume and the incidence of cryptorchidism (Swan et al., 2005). Additionally, phthalates present in breast milk are proposed to alter the function of the hypothalamic–pituitary–gonadal axis in male offspring (Main et al., 2006). However, it should be noted that negative effect of MEHP on testicular steroidogenesis in human fetal testis is not yet proven. One in vitro study reported that this phthalate had no effect on basal and LH-stimulated steroidogenesis in first-trimester human fetal testis explant (Lambrot et al., 2009).

Alteration in steroidogenesis during development may significantly affect the reproductive health of children. Pubertal development is closely regulated by sex hormones in both sexes and activation or suppression of steroidogenesis may induce hormonal disturbances resulting in pathological conditions. For example, increased production of progesterone by MEHP stimulated granulosa cells may induce their advanced luteinization, a process that may lead to dysregulation of the estrous cycle and anovulatory cycles in rats (Davis et al., 1994). Further, in this context, a recent study demonstrated that exposure of prepubertal rats to low environmentally relevant DEHP levels for 28 days increased Leydig cell capacity to produce testosterone that was associated with enhanced plasma levels of LH (Akingbemi et al., 2001), indicating potential for phthalates to modulate the pituitary–testicular axis in rodents. Similarly, we have recently reported that the ability of primary cultures of pituitary cells isolated from DEHP-treated prepubertal female rats to produce LH in response to GnRH was significantly enhanced (Svechnikova et al., 2007).

Taken together, our current data indicate that MEHP affects gonadal steroidogenesis in a gender-specific manner. This phthalate stimulated basal steroidogenesis and StAR expression in both male and female steroidogenic cells. However, when MEHP was incubated with (Bu)_2cAMP, steroid production was increased in granulosa cells and suppressed in Leydig cell progenitors, a process associated with up-regulation of StAR expression. Based on these results it may be hypothesized that males and females react differentially to the endocrine disrupting actions of phthalates, with boys being more susceptible than girls. This may be related to the clinical observation that DDS are more prevalent in boys than girls.

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