The influence of di-(2-ethylhexyl) phthalate on steroidogenesis by the ovarian granulosa cells of immature female rats

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Abstract

Phthalate esters are known to exert harmful effects on mammalian reproduction and fertility, but their potential adverse effects on the hormonal functions of the ovary have not yet been elucidated in detail. Here, we investigated the effects of di-(2-ethylhexyl) phthalate (DEHP) on the hypothalamic–pituitary–gonadal axis of young developing female rats, as well as on ex vivo steroidogenesis by granulosa cells (GCs) and secretion of LH by gonadotropes. Exposure of 20-day-old female rats to 500 mg DEHP by oral gavage once daily for 10 days reduced their serum levels of progesterone and estradiol, while tending to enhance levels of LH. Furthermore, primary cultures of GCs isolated from these rats exhibited an attenuated capacity to produce progesterone in response to stimulation by LH and FSH, as well as a lower degree of transport of endogenous cholesterol into mitochondria. Moreover, the ability of primary cultures of pituitary cells isolated from DEHP-treated rats to produce and secrete LH in response to GnRH was significantly enhanced. In addition, 2-ethylhexanoic acid, a metabolite of DEHP, significantly potentiated GnRH-stimulated production of LH by cultures of pituitary cells isolated from untreated 20-day-old female rats. Together, these data indicate that DEHP exerts dual effects on the pituitary–gonadal axis, stimulating the hormonal function of the pituitary and, at the same time, by inhibiting steroidogenesis by GCs.

Journal of Endocrinology (2007) 194, 603–609

Introduction

Phthalates, including the widely used plasticizer di-(2-ethylhexyl) phthalate (DEHP), are the most abundant pollutants of our general environment. These substances are components of food wraps and many medical devices (e.g. tubing, blood bags, and dialysis equipment), as well as of many cosmetic products. With time, they are leached out of these plastic products, and their volatility results in pronounced human exposure to phthalates.

Investigations on the influence of phthalates on the female reproductive system have focused primarily on fertility and teratogenicity in experimental animals and cattle (Collins et al. 1992). Little information concerning the effects of phthalates on pregnant women is presently available, although chronic occupational exposure of female factory workers to high levels of phthalates has been reported to be associated with reduced rates of pregnancy and an elevated frequency of miscarriage (Aldyreva et al. 1975). Exposure of adult rats to DEHP results in hypoestrogenic anovulatory cycles and polycystic ovaries (Davis et al. 1994a). In the intestine and liver of humans and animals, DEHP is rapidly hydrolyzed by esterases to yield mono-(2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol (Rowland 1974, Albro et al. 1983); and this hexanol is subsequently oxidized enzymatically to 2-ethylhexanoic acid (2-EHXA; Collins et al. 1992). MEHP, 2-ethylhexanol, and/or their metabolites are the immediate inducers of the majority of enzymes known to be affected by exposure to DEHP (Pollack et al. 1989).

In primary cultures of rat granulosa cells (GCs), stimulation of adenylate cyclase and progesterone synthesis by follicle-stimulating hormone (FSH) is inhibited by MEHP (Treinen et al. 1990). Moreover, this compound suppresses estradiol production by the same cells by a mechanism that is independent of the elevation in cAMP levels caused by FSH (Davis et al. 1994b). This latter phenomenon reflects direct inhibition by MEHP of transcription of the gene that encodes aromatase, the enzyme that converts androgen to estrogen (Lovekamp & Davis 2001). DEHP also suppresses the production of estradiol and ovulation in cycling rats (Davis et al. 1994a). However, in the case of immature female rats, the influence of in vivo administration of DEHP on the hypothalamic–pituitary–gonadal (HPG) axis and progesterone production by GCs isolated and cultured ex vivo has not yet been characterized. Here, we investigated the effects of DEHP on the HPG axis of immature female rats, as well as ex vivo steroidogenesis by GCs and secretion of luteinizing hormone (LH) by gonadotropes.
**Materials and Methods**

**Materials**

Dulbecco’s modified Eagle’s medium (DMEM)–Ham’s nutrient mixture F-12 (supplemented with t-glutamine and HEPES), BSA (7.5% solution), cholesterol lipids (250X), and penicillin–streptomycin (Gibco/BRL, Life Technologies); ovine FSH, ovine LH, 22R-hydroxycholesterol (22R-OHC), DEHP, EHX, and aminoglutethimide (AMG; an inhibitor of cytochrome P450scc; Sigma); MEHP (TCI Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of cytochrome P450scc; Sigma); MEHP (TCI Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Stegram Pharmaceuticals, Europe, Brussels, Belgium); trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase; Steg...
they were first incubated at 37 °C under an atmosphere containing 5% CO₂ for 24 h. Thereafter, fresh medium containing AMG (0.5 mM), an inhibitor of cytochrome P450scc, and trilostane (5 μM), an inhibitor of 3β-hydroxy-steroid dehydrogenase, was added and incubation continued for an additional 30 min, following which the medium was supplemented with LH (100 mU/ml) and incubation performed for 3 h more. Inhibition of mitochondrial cholesterol metabolism by AMG and trilostane results in accumulation of this steroid in this organelle in response to hormonal stimulation (Potts et al. 1978, Brueggemeier et al. 2005).

Subsequently, the cells were washed twice with the basic medium and then cultured in the presence of trilostane (5 μM) in AMG-free medium at 37 °C under 5% CO₂ for 2 h, after which the levels of pregnenolone and its metabolite dehydroepiandrosterone (DHEA; formed by cytochrome P450c17 via the Δ⁵ pathway) in the medium were measured. The total combined amount of these steroids produced by the cells reflects the amount of cholesterol available to the cytochrome P450scc system (Potts et al. 1978). After lysing the cells with the M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA), protein was determined using the Micro BCA Protein Assay from the same company and the total amount of these hormones produced expressed per milligram protein.

Statistical analysis

Differences in the values obtained for animals or cells subjected to different treatments were evaluated for statistical significance with Student’s t-test and one-way ANOVA, followed by the Student–Newman–Keul’s or Dunn’s test, in all cases employing the SigmaStat (v 3.00) software package (SPSS Inc., Chicago, IL, USA).

Results

Oral administration of DEHP to immature female rats decreases their serum levels of progesterone and estradiol

Administration of DEHP by oral gavage to 20-day-old female rats for 10 days resulted in two- and fivefold reductions in the concentrations of progesterone and estradiol respectively in their plasma, together with a tendency toward an elevated level of plasma LH (Fig. 1A–C). This exposure did not exert any obvious toxic effects on the animals, as reflected in their unaltered body and ovary weights (Table 1).

Treatment of immature female rats with DEHP inhibits ex vivo steroidogenesis by their GCs

In an attempt to identify the primary target through which DEHP impairs the biosynthesis of sex hormones, the capacities of GCs isolated from treated and control rats to produce progesterone under ex vivo conditions were compared. Following stimulation with FSH or LH, progesterone production by GC isolated from DEHP-treated animals was 30% lower (P<0.05) than by control cells (Fig. 2). Moreover, these cells isolated from young developing female rats, whether treated or not, were poorly responsive to gonadotropins, suggesting that the signaling machinery involved was not yet fully developed. More detailed information concerning the site in steroidogenesis inhibited by DEHP was provided using 22R-OHC, a derivative of cholesterol that diffuses readily across membranes. DEHP exerted no effect on progesterone synthesis from 22R-OHC.
suggesting that this phthalate may be disturbing the translocation of cholesterol across mitochondrial membranes.

**Treatment of immature female rats with DEHP reduces ex vivo transport of cholesterol into the mitochondria of their GCs**

Since the suppression by DEHP of steroidogenesis in GCs was abolished by 22R-OHC, we examined whether treatment with this phthalate affects the transport of cholesterol into the mitochondria of GCs. Indeed, the level of DHEA produced (which reflects the amount of cholesterol available to the mitochondrial cytochrome P450scc system) was significantly reduced (33%) by such treatment (Fig. 3). The level of pregnenolone detected was negligible, indicating that when 3β-HSD (3β-hydroxysteroid dehydrogenase) was blocked, this entire compound was converted further into DHEA by CYP17α.

**Effects of in vitro exposure to DEHP or its metabolites on LH secretion by pituitary cells treated neither with DEHP nor with vehicle**

Subsequent experiments were designed to test the hypothesis that DEHP and/or metabolites of this phthalate can directly stimulate the pituitary of developing rats to produce LH. In order to assess contribution of all these compounds into the activating effect of DEHP on these cells observed ex vivo, the effects of DEHP and its predominant metabolites, MEHP and 2-EHXA, on hormonal function of cultured pituitary cells were studied. Indeed, 2-EHXA, but neither DEHP nor MEHP, significantly enhanced (by 30%, *P*<0.05) GnRH-stimulated production of LH by cultures of pituitary cells isolated from 20-day-old female rats with no pretreatment whatsoever (Fig. 5). However, this compound had no effect on basal production of LH by the same cells (the control and 2-EHXA-exposed values were 40.8±1.0 and 46.7±2.5 ng/10⁴ cells/24 h respectively).

**Table 1** The weights of the whole body and both ovaries of 20-day-old female rats prior to and following treatment with di-(2-ethylhexyl) phthalate (DEHP) or vehicle alone for 10 days

<table>
<thead>
<tr>
<th>Group</th>
<th>Prior to treatment</th>
<th>Following treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age (days)</td>
<td>Body weight (g)</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>20</td>
<td>42.6±0.9</td>
</tr>
<tr>
<td><strong>DEHP</strong></td>
<td>45.7±1.6</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>30</td>
<td>69.5±3.7</td>
</tr>
<tr>
<td><strong>DEHP</strong></td>
<td>77.0±3.7</td>
<td>12.7±0.7</td>
</tr>
</tbody>
</table>

The rats received DEHP (500 mg/kg) or the corresponding volume of vehicle (corn oil) alone each day for 10 days (*n*=10). ND, not determined.

**Figure 3** Ex vivo transport of cholesterol into the mitochondria of granulosa cells isolated from DEHP-treated (gray bars) and control (black bars) immature female rats. Following pretreatment of the cells with aminoglutethimide (AMG; 0.5 mM) and trilostane (5 μM) for 30 min and subsequent incubation in the presence (LH) or absence (basal) of LH (100 mU/ml) for 3 h, the DHEA present in the culture medium was measured by RIA procedure. The results presented are the means±S.E.M. for three independent experiments. **P*<0.01; ***P*<0.001 when compared with the corresponding control values.
The present investigation demonstrates that the treatment of pre-pubertal 20-day-old female rats with DEHP lowers their serum levels of progesterone and estradiol and, at the same time, suppresses progesterone production by and transport of cholesterol into the mitochondria of GCs isolated from their ovaries. In addition, we found that primary cultures of pituitary cells prepared from DEHP-treated rats exhibit an enhanced capacity to secrete LH, which may explain the observed tendency for serum gonadotropin levels to increase in these treated animals. Moreover, we demonstrate here that 2-EHXA, a metabolite of DEHP, significantly enhances GnRH-stimulated production of LH by pituitary cells isolated from 20-day-old female rats that have not been treated in any way. To the best of our knowledge, this is the first example of direct modulation of the hormonal function of the pituitary by a metabolite of DEHP. Together, these results indicate that DEHP exerts a dual effect on the pituitary–ovary axis, both stimulating production of LH by the pituitary and inhibiting steroidogenesis in GCs.

Our observation that administration of DEHP to immature female rats by oral gavage reduces their serum levels of progesterone and estradiol (Fig. 1) is consistent with previous findings that serum levels of cholesterol and triglycerides are lowered by dietary administration of phthalates and/or their metabolites to rats (Moody & Reddy 1982). Two metabolites of DEHP, 2-ethylhexanol and EHXA, specifically reduce the level of cholesterol, the precursor of all steroid hormones, which may attenuate production of, for example, progesterone and estradiol. This reasoning is also consistent with the previous report that MEHP, one of the major and active metabolites of DEHP, inhibits transcription of the aromatase gene in GCs (Lovekamp & Davis 2001).

In addition, a decrease in serum levels of estradiol following exposure of cycling rats to DEHP had been observed earlier, along with lowered serum levels of progesterone at certain stages of the estrous cycle (Davis et al. 1994a). Similar inhibition of serum and total ovarian progesterone levels by dibutyl phthalate was shown in female rats during pregnancy (Gray et al. 2006). However, the present study represents for the first time that the ex vivo steroidogenic capacity of GCs isolated from rats receiving DEHP by oral administration has been examined, and we show that the responsiveness of these cells to gonadotropins is attenuated. This may result in decreased production and secretion of progesterone into the serum, which could also contribute to our present findings.

The dose of DEHP (500 mg/kg) used in this investigation was shown to effectively increase the levels of corticosterone and ACTH in plasma of 20- and 40-day-old male rats (Supornsilchai et al. 2007). As in our study, no overt toxicity was observed in these animals. The dose of DEHP used in this investigation (500 mg/kg) was lower when compared with doses commonly used by others. In similar studies on female rats, doses of DEHP as high as 1500–2000 mg/kg have been...
is capable of penetrating the placental barrier and exerting the fact that the (relatively small molecule, can do so. This idea is supported by metabolites can penetrate the blood–brain barrier remains to of ACTH (Supornsilchai and 40-day-old male rats to DEHP elevates their serum level 1982). Recently, we have also shown that oral exposure of 20- promotes development of pituitary tumors (Kluwe 1982). Hypertrophy of gonadotropes in the anterior pituitary and chronic administration of DEHP to male rats results in indirect action is supported by the observation that exposure pituitary may also be occurring. The existence of such an enhanced might probably reflect less pronounced negative effects of cholesterol transport is at least one of the deleterious effects of cholesterol might probably reflect less pronounced negative feedback by progesterone and estradiol on the hypothalamus and/or the pituitary of these developing animals. However, our data also indicate that the DEHP metabolite, 2-EHX, stimulates LH production by the pituitary directly; of course, an indirect action via attenuated negative feedback on the pituitary may also be occurring. The existence of such an indirect action is supported by the observation that exposure of pre-pubertal male rats to DEHP for 28 days stimulated their pituitary–gonadal axis, thereby resulting in elevated serum levels of LH (Akingbemi et al. 2001), as well as by the findings that chronic administration of DEHP to male rats results in hypertrophy of gonadotropes in the anterior pituitary and promotes development of pituitary tumors (Kluwe et al. 1982). Recently, we have also shown that oral exposure of 20- and 40-day-old male rats to DEHP elevates their serum level of ACTH (Supornsilchai et al. 2007).

Although the question as to whether DEHP and/or its metabolites can penetrate the blood–brain barrier remains to be resolved, it seems possible that 2-EHX, which is a relatively small molecule, can do so. This idea is supported by the fact that the (R)-enantiomer of sodium 2-ethylhexanoate is capable of penetrating the placental barrier and exerting severe teratogenic effects in mice (Collins et al. 1992), as well as by our own data revealing that 2-EHX directly enhances the sensitivity of the gonadotropes to GnRH.

In conclusion, oral administration of DEHP to immature female rats reduces their plasma levels of progesterone and estradiol and enhances the responsiveness of their pituitary cells to GnRH. Such alterations in the HPG axis, caused by exposure to phthalates from various sources, may have deleterious effects on the health of the developing organism.

Acknowledgements

This work was supported financially by the EU 6th Framework Programme ‘Food Quality and Safety’, project PIONEER (contract no. 513991), the CASCADE Network of Excellence (EU contract no. FOOD-CT-2004-506319), the Swedish Research Council (project 8282), the Frimurare Barnhuset Foundation in Stockholm, the Swedish Children’s Cancer Fund, the Swedish Environmental Protection Agency, HRH Crown Princess Lovisa’s Society for Pediatric Health Care, the Society for Child Care, the Wera Ekström Foundation for Pediatric Research, and grants from the Karolinska Institute, Stockholm, Sweden. The authors would like to thank Dr C. Carlsson-Skwirrut for her help in the iodination of LH and Josephine Sundborger for her help with the animals. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 21 June 2007
Accepted 26 June 2007
Made available online as an Accepted Preprint 27 June 2007