Involvement of testicular growth factors in fetal Leydig cell aggregation after exposure to phthalate in utero

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Edited by Ryuzo Yanagimachi, University of Hawaii, Honolulu, HI, and approved March 21, 2008 (received for review September 30, 2007)

Exposures to di-(2-ethylhexyl) phthalate (DEHP) have been shown to be associated with decreased adult testosterone (T) levels and increased Leydig cell numbers. As yet, little is known about DEHP effects in utero on fetal Leydig cells (FLC). The present study investigated effects of DEHP on FLC function. Pregnant Long–Evans female rats received vehicle (corn oil) or DEHP at 10, 100, or 750 mg/kg by oral gavage from gestational day (GD)2–20. At GD21, T production, FLC numbers and distribution, and testicular gene expression were examined. The percentage of FLC clusters containing 6–30 cells increased in all treatment groups, with 29 ± 2% in control vs. 37 ± 3, 35 ± 3, and 56 ± 4% in rats receiving 10, 100, and 750 mg/kg DEHP, respectively. In contrast, FLC numbers were 33% and 39% lower than control after exposures to 100 and 750 mg/kg DEHP, respectively. At these doses, mRNA levels of leukemia inhibitory factor (LIF) increased. LIF was found to induce cell aggregation in FLCs in vitro, consistent with the hypothesis that DEHP induced FLC aggregation. Testicular T levels were doubled by phthalates and halved at 750 mg/kg. The mRNA levels of IGF1, KITL, and LIF genes may contribute to the reproductive toxicity of phthalates.

Phthalates, widely used as plasticizers and solvents, are commonly found in a variety of consumer products including cosmetics, toys, medical tubing, and catheters and in the environment as an industrial waste product. Increasing public concern over lack of regulation on their use in the United States, in contrast to the European Union and 14 other countries (1), has arisen in response to reports that exposures to phthalates may be linked to abnormal reproductive development in the human male (2, 3). Epidemiological studies show statistical correlations between serum concentrations of phthalate monoesters, the primary metabolites of phthalates, and the incidence of anomalies such as cryptorchidism and shortened anogenital distance (AGD) (4, 5). Di-(2-ethylhexyl) phthalate (DEHP), the most abundant phthalate in the environment, has been shown to have adverse effects on androgen synthesis in the rodent (6).

The Agency for Toxic Substances and Disease Registry reported that, although exposure to DEHP is generally low, the exposures of preterm infants can be as high as 10–20 mg per day (7). Controversy exists over whether DEHP, at the levels found in the environment, is harmful to humans, because most studies have been conducted in rodents administered high doses. In previous studies, we showed that the administration of low-dose (10 mg/kg body weight) DEHP for 28 days during pubertal development caused elevations in testosterone (T) (8, 9). This was in contrast to doses >750 mg/kg, which significantly reduced serum and testicular T (10). Similar results were obtained in rats treated by inhalation with a dose of DEHP comparable to 1–10 mg/kg per day orally (11) and in boars exposed intramuscularly to DEHP at 50 mg/kg twice per week during puberty (12).

Fetal Leydig cells (FLCs) are a distinct population of Leydig cells that originate in the fetus. These cells secrete high levels of T that are critical for development of the penis and sex accessory glands and, along with another FLC product, insulin-like growth factor 3 (INSL3), for the scrotal descent of the testis. FLCs reach their peak numbers around birth and then gradually involute after postnatal day 7 such that few, if any, are present in the adult testis (13). The goal of the present study was to examine the effects of DEHP dose on the prenatal production of T by the FLCs. We postulated that FLCs might have distinctive responses to DEHP compared to adult Leydig cells (ALCs), given that these cells respond to different regulatory factors. For example, FLCs develop normally and secrete T in luteinizing hormone (LH) receptor knockout mice, whereas LH is required for the formation and steroidogenic function of ALCs (14). Given the essential role of fetal T production, our objective was to determine whether and how DEHP treatment in utero perturbs these cells and thus steroidogenic function in the fetus.

Results

General Reproductive Toxicology. As shown in Table 1, none of the DEHP doses affected the body weights of dams, birth rates (number of dams that delivered litters divided by the number of dams with an established pregnancy as defined by the presence of a vaginal plug), numbers of pups per dam, male-to-female sex ratio, or male pup body weight at gestational day (GD)21 in comparison to the controls. The AGD of the male pups was significantly reduced (P < 0.001) at a dose of 750 mg/kg DEHP (Table 1). Because AGD is a function of androgen action, we examined whether phthalate treatment caused a reduction in T synthesis by the fetal testis.

Testicular T Production. Intratesticular T concentrations at GD21 were measured to assess the steroidogenic function of the FLCs.

Author contributions: H.L. and R.-S.G. contributed equally to this work; R.-S.G., Q.-Q.L., and X.-K.L. designed research; H.L., R.-S.G., G.-R.C., G.-X.H., L.D., D.O.H., C.M.S., and M.P.H. performed research; R.-S.G. and X.-K.L. analyzed data; and R.-S.G., D.O.H., X.-K.L., and M.P.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0709260105/DCSupplemental.

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Relative to controls, T values were 50% higher in the 10 mg/kg DEHP exposure group but 66% lower in the 750 mg/kg group (Fig. 1A and Table 1). T values were not significantly different from controls in the 100 mg/kg group.

**Leydig Cell Distribution, Number, and Size.** FLCs are not uniformly distributed in the interstitial space of the GD21 testis but rather are found in discrete clusters (15); only 20 ± 1% were present as single cell (Table 2). The percentage of single cell decreased significantly from controls with DEHP exposures, and the average number of FLCs per cluster also was greater after DEHP exposure group but 66% lower in the 750 mg/kg group

**Effect of LIF on Leydig Cell Aggregation.** As shown in Fig. 2, LIF expression was increased in response to the 750 mg/kg DEHP dose. This dose also was associated with larger-sized FLC clusters (Fig. 1). To determine whether there might be a cause–effect relationship between increased LIF and FLC aggregation, the effects of LIF on cultured FLCs were analyzed. In response to LIF at 1 ng/ml (IC50 for LH-stimulated T production) or 10 ng/ml (concentration showing maximal inhibition) of LIF for 12 h in vitro, FLC aggregations were increased in frequency (Table 3). LIF antibody significantly antagonized the effects of LIF (Table 3).

**Testicular Gene Expression.** A panel of genetic markers was selected to assess cell type-specific function in the testis after prenatal exposure to phthalate. As shown in Fig. 2, the markers included genes encoding growth factors (Igf1, Kitl, Lif), their receptors (Igf1r, Kitl, Lhcgr, Pdgfra), cholesterol transporters (Scarb1, Star), and steroidogenic enzymes (Cyp11a1, Cyp19, Sdr5a1). In all, transcript levels of 37 testicular mRNAs were examined by real-time PCR. The gene names, symbols, and functions are listed in supporting information (SI) Table S1. Among the growth factors, Igf1 and Kitl were elevated in response to the 10 mg/kg DEHP dose, whereas Lif and Pdgfβ mRNAs were increased in response to the 750 mg/kg dose. Amh, Pdgfa, and Fgf2 expression levels were unchanged (data not shown). mRNA levels of growth factor receptors Lhcgr and

### Table 1. Reproductive parameters before and after exposure to DEHP for 19 days

| Parameters                      | DEHP, mg/kg per day |
|--------------------------------|---------------------|
|                                | 0                   | 10                  | 100                  | 750                  |
| Dams                           |                     |                     |                     |                     |
| Number of dams                 | 6                   | 6                   | 6                   | 9                    |
| Body weight before, g          | 224 ± 7             | 218 ± 3             | 222 ± 2             | 218 ± 6             |
| Body weight after, g           | 335 ± 11            | 323 ± 14            | 352 ± 6             | 320 ± 12            |
| Pups per dam                   | 11 ± 2              | 12 ± 1              | 13 ± 1              | 11 ± 3              |
| Birth rate                     | 6/6                 | 6/6                 | 6/6                 | 7/9                 |
| Pups male, %                   | 40 ± 10             | 44 ± 7              | 56 ± 7              | 57 ± 7              |
| Male pups                      |                     |                     |                     |                     |
| Number of pups                 | 30                  | 34                  | 41                  | 51                  |
| Body weight, g                 | 6.0 ± 0.1           | 5.6 ± 0.1           | 5.8 ± 0.1           | 5.6 ± 0.1           |
| AGD, mm                        | 4.5 ± 0.1           | 4.3 ± 0.1           | 4.8 ± 0.1           | 4.1 ± 0.1*          |
| Testicular T, ng/mg             | 0.89 ± 0.13         | 1.4 ± 0.19*         | 0.69 ± 0.24         | 0.29 ± 0.07*        |

Dams of Long–Evans rats were gavaged with DEHP from GD2 to GD20. Parameters were measured at GD21.

Values are mean ± SEM, n = 6 for dam's data. *P < 0.001.

*One-way ANOVA with Dunnett’s Multiple Comparison Test vs. control, significantly higher than control values.

### Table 2. Frequency distribution of cluster sizes of FLCs after in utero exposure to DEHP

| Frequency, % | DEHP, mg/kg |
|--------------|-------------|
| Cell no. per cluster | 0       | 10      | 100     | 750     |
| 1 — 5        | 20 ± 1    | 10 ± 2**| 12 ± 2**| 4 ± 1*** |
| 5 — 10       | 50 ± 2    | 51 ± 2  | 51 ± 1  | 34 ± 5  |
| 10 — 20      | 29 ± 2    | 37 ± 2* | 35 ± 3* | 56 ± 4***|
| > 20          | 1 ± 1     | 1 ± 0.4 | 1 ± 0.4 | 7 ± 2*** |
| Average       | 2.2 ± 0.2 | 3 ± 0.4 | 4 ± 0.3 | 9 ± 1*** |

Mean ± SEM, n = 6. *, P < 0.05; **, P < 0.01; ***, P < 0.001; in comparison to vehicle control (0 mg/kg).
was significantly reduced in the 750-mg/kg DEHP group. Insl3 and Hsd17b12 were reduced at high DEHP dose, but 3β-HSD were not affected. This also was true of their mRNAs. These suppression of steroidogenic function appeared to result from testosterone and circulating T levels (Fig. 1A).

T Biosynthetic Enzyme Protein Levels. T biosynthetic enzyme protein levels were also evaluated. As shown in Fig. 3, P450scc was reduced at high DEHP dose, but 3β-HSD, P450c17, and 17β-HSD were not affected. This also was true of their mRNAs. These results suggest the reduced levels of P450scc might be integrally involved in DEHP-mediated inhibition of T production at the higher DEHP doses.

Discussion

DEHP and T Production. Steroidogenic activity in FLCs peaks 1–2 days before birth on GD19 (16). The T produced at this time is critical for male secondary sexual differentiation (i.e., development of the penis and sex accessory glands) (17). FLCs also produce the hormone INSL3, which binds to the leucine-rich repeat-containing G protein-coupled receptor 8 (LRG8). INSL3 specifically binds LRG8s in the gubernaculum (18) and, together with androgen, induces scrotal descent of the testis (18, 19). Thus, interference with the development of FLCs may be a precipitating cause of cryptorchidism (20). Indeed, the incidence of cryptorchidism is significantly increased during the neonatal period after exposures to high doses of phthalates (20). Exposure to doses of phthalates at 250 mg/kg per day or higher also has been reported to be associated with increased frequencies of underdeveloped epididymides, testicular atrophy, hypospadias, and ectopic or absent testes (21).

The present study shows that low-dose exposure to DEHP (10 mg/kg) in utero resulted in increased testicular T levels, whereas a higher dose of DEHP (750 mg/kg) resulted in reduced T levels and AGD. Previous studies similarly showed that low and high doses of DEHP, when administered postnatally, had differential effects on T levels (6, 11, 12). Low-dose exposures that abnormally elevate T levels are likely to be as undesirable as the suppressive effects documented at high doses. The explanation for the differential effects of low- and high-DEHP doses is uncertain. Our and previous results suggest several plausible possibilities. With respect to the observation that testicular T levels in the 10 mg/kg group were higher than the controls, IGF-1 has been reported to stimulate T production by FLCs (22). Among the 36 genes that potentially affect FLC development and steroid production, IGF1 and KITL had significantly elevated expression levels. IGF-1 is produced by both Leydig and Sertoli cells, and c-Kit ligand (KITL) is exclusively produced by Sertoli cells. IGF-1 and KITL are required to stimulate T production by FLCs. Levels of the mRNA for IGF1 increased 5-fold after exposure to 10 mg/kg DEHP; KITL increased 50%. These increases are consistent with the increased T production seen in response to 10 mg/kg DEHP. Thus, one possibility is that increases in IGF-1 and KITL may explain the significant elevations of T in animals exposed to 10 mg/kg DEHP. It should be pointed out that elevations in T levels in response to low-dose DEHP exposures also were noted in DEHP-treated pubertal rats (9, 11, 12).

Higher doses of DEHP resulted in reduced FLC numbers, cell size, and steroidogenic enzyme activities, consistent with the

| Table 3. Aggregation of FLC after leukemia inhibitory factor treatment in vitro |
|---------------------------------|-----------------|-----------------|-----------------|
|                                | Frequency, %    |
|                                | Control         | 1 ng/ml LIF     | 10 ng/ml LIF    |
| Single cells                   |                 |                 |                 |
|                                | 96 ± 2          | 75 ± 1***       | 61 ± 3***       |
| Clusters (two or more cells)   | 5 ± 2           | 23 ± 1***       | 39 ± 3***       |
|                                |                 |                 | 90 ± 2          |

Values are mean ± SEM, n = 4–5 preparations. AB as LIF antibody (1:100 dilution). One-way ANOVA with Newman–Keuls Multiple Comparison Test. *, Significant difference compared with control at P < 0.001 in single cells or clusters.
Brief, endogenous peroxidase was blocked with 0.5% H2O2 in methanol for 30 min.

DEHP and FLC Aggregation. FLCs were aggregated rather than dispersed when doses of DEHP were increased. Previous studies similarly reported that high-dose exposures to DEHP or DBP in utero resulted in focal disruptions in the structure of the seminiferous epithelium and in abnormal aggregations of FLCs (24, 25). Stereological analyses have suggested that the increased FLC number per cluster in response to DEHP does not result from increased Leydig cell numbers (our results and ref. 26).

In our study, a dose as low as 10 mg/kg per day for 20 days (GD2–GD20) elicited Leydig cell aggregation. The physiological consequences of FLC aggregation per se are uncertain, although there is evidence that it is associated with reduced fertility (27) and increased incidence of cryptorchidism (20). It seems unlikely that the expanded clusters of FLCs observed with DEHP represent tumors. Support for this comes from observations that FLC numbers were reduced, not increased, in response to DEHP, and that expression levels of the tumor transformation gene Rcl were unchanged (data not shown).

The mechanism by which FLCs become grouped in clusters is not well understood. One possible explanation is that these cells are initially more diffusely distributed but then form aggregates under the influence of local growth factors. One such growth factor that may be active in this respect is LIF, which is primarily produced by peritubular myoid cells (28). We herein showed that LIF caused dose-dependent increases in the cluster size of FLCs in vitro. This was consistent with the known ability of LIF to stimulate aggregation in other cell types such as platelets (29) and embryonic stem cells (30).

In conclusion, these results, taken together, indicate that fetal exposures to DEHP have effects on FLC number, distribution, and most importantly, steroidogenic capacity, and suggest that abnormal expressions of IGF1, KITL and LIF genes may contribute to the reproductive toxicity of phthalates.

Materials and Methods

Animals and Treatments. Long-Evans rats were purchased from Charles River. All studies were approved by The Rockefeller University’s Animal Care and Use Committee (Protocol #040359). Adult pregnant dams were treated from GD2 to GD20 with 0 (control, n = 6), 10 (n = 6), 100 (n = 6), or 750 (n = 9) mg/kg DEHP (Sigma–Aldrich) in 1 ml/kg corn oil, administered daily by oral gavage. The body weights and AGD of male pups at GD21 were measured. The pups were killed by inhalation of CO2 on GD21.

Immunohistochemical and Histochemical Staining. Frozen testes from rats (0, 10, 100, and 750 mg/kg DEHP) were embedded in the same blocks and counterstained with Mayer’s hematoxylin. For testicular steroid levels, vanillin-hydrogen peroxide (VHP) was added to the sections to detect 3α-OH compounds (31). For testicular T analysis, the sections were incubated with 1 mg/ml bovine albumin and plated to dishes. After plating the interstitial cells, FLCs were isolated by the same procedure that was used successfully for immature Leydig cells (33). In brief, the testes from 60 GD21 male rats were removed and decapsulated. Interstitial cell nuclear antigen (LIN) was detected by the inverse of the sampling probability, and average FLC numbers per testis were calculated by the Fractionator technique, as described in ref. 9. The total number of FLCs per testis was calculated by multiplying the number of FLCs counted in a known fraction of the testis by the inverse of the sampling probability, and average FLC numbers per testis per treatment group were determined. Average cell sizes were measured by tracing the profiles of ~300 FLCs.

Stereological Analysis of FLC Number, Size, and Clusters. To enumerate FLC numbers, testicular tissues were sampled according to the manufacturers’ instructions (Invitrogen). First-strand synthesis and real-time PCR were performed as described in ref. 32. Ribosomal protein S16 (Rps16) mRNA levels were assayed in all samples as internal controls. The primers of the other 36 genes were provided in Tables S1 and S2. These genes are: growth factors including insulin growth factor 1 (Igf1), Kit ligand (Kitli), antimullerian hormone (Amhh), leukemia inhibitory factor (LIF), platelet growth factor A (Pdgfa), platelet growth factor B subunit (Pdgfb), fibroblast growth factor 2 (fgf2), and insulin-like growth factor 3 (Ins3; 3); cell proliferative genes cyclin G1 (Ccng1), cyclin D3 (Ccn3d3), c-myc related gene Rcl (Rcl1), and proliferation cell nuclear antigen (Pena); membrane receptor genes including LH receptor (Lhgr), insulin-like growth factor 1 receptor (lgfr1), platelet growth factor receptor a (Pdgfra), c-kit (Kit), anti-Mullerian hormone receptor 2 (Amhr2), and follistimulating hormone receptor (Fshr); cholesterol transport genes including cholesterol HDL receptor (Scar1), steroidogenic acute regulatory protein (Star), and peripheral benzodiazepine receptor (Pbr); nuclear receptors and transcription factors estrogen receptor a (Esr1), androgen receptor (Ar), steroidogenic factor 1 (Nrsf1at), peroxisome proliferative activated receptor a (Ppara), and peroxisome proliferative activated receptor y (Pparg); Gata 4 (Gata4), steroidogenic enzyme genes including P450scoc (Cyp17a1), P450scc (Cyp11a1), hsd1b1 (Hsd1b1), P450 17a1 (Cyp17a1), 17hSD3 (Hsd17b3), 17hSD12 (Hsd17b12), Sdr-2 (Sdr5a1), and P450 aromatase (Cyp19); and cell junction proteins including connexin 43 (Gja1) and Clusterin (Trmp2). The relative mRNA levels of targeted genes were normalized to Rps16 (internal control gene) by using the standard curve method.

Leydig Cell Steroidogenic Enzymes. P450scc and 3βHSD intensities were measured in immunohistochemically stained cryostat sections by using image analysis software. The total intensity in cells from treated animals was expressed as a percentage relative to the untreated control. For measurements of P450scc and 17βHSD3, 1 μM radiolabeled substrates (3H-progesterone) or 3H-androstenedione were added to 100 μg of protein (homogenized testis). After 120 min, products were examined by TLC separation, and radiometric scanning was performed as described.

Percoll Purification and Culture of FLCs. FLCs were isolated by the same procedure that was used successfully for immature Leydig cells (33). In brief, testes from 60 GD21 male rats were removed and decapsulated. Interstitial cells were dissociated with 0.25 mg/ml collagenase (collagenase-D, Roche Molecular Biochemicals) in medium 199 for 10 min at 34°C. The separated cells were resuspended in 55% isotonic Percoll. After centrifugation at 25,000 × g for 45 min at 4°C, the FLC fraction was collected between densities of 1.064 and 1.076 g/ml. The cells were resuspended in DMEM:F12 (1:1) supplemented with 1 mg/ml bovine albumin and plated to dishes. After plating the interstitial cells, FLCs were allowed to attach to the dishes, resulting in enrichment ~30–50% purity. FLCs (0.1–0.5 × 104) were cultured in the presence of increasing concentrations of LIF for 24 h. Neutralization of LIF was performed by using LIF receptor antibody (1:100 dilution, sc-20087, Santa Cruz).

Statistical Analysis. Values are expressed as mean ± SEM, and data were analyzed by using one-way ANOVA. At P < 0.05, Dunnett’s test was used to compare values from DEHP-treated animals to control values. For this purpose, GraphPad Prism (version 4, GraphPad Software) was used. Data for Leydig cell number per testis were tested for logtransformed before statistical analysis by ANOVA test because of their skewed distribution.

Acknowledgments. This work was supported in part by National Institute on Environmental Health Sciences Grant R01 ES10233 (to M.P.H.).
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