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Effects of Neonatal Exposure to 4-*Tert*-Octylphenol, Diethylstilbestrol, and Flutamide on Steroidogenesis in Infantile Rat Testis

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Exposure of neonatal testis, populated by fetal-type Leydig cells, to endocrine-active compounds may have far-reaching consequences. Our aim was to resolve the sensitivity of testosterone synthesis of infant rat (Sprague-Dawley) testis to diethylstilbestrol (DES; 0.1–1.0 mg/kg), 4-*tert*-octylphenol (OP; 10–100 mg/kg), and Flutamide (FLU; 2.0–25 mg/kg) given by daily sc injections from birth to postnatal day 4. Testes and serum were collected on day 14 when body and testis weight, testicular histology, circulating testosterone, LH and FSH levels, and steroidogenic acute regulatory protein (StAR) and 3β-hydroxy-steroid-dehydrogenase (3β-HSD) protein levels were determined. DES at each dose and FLU at 25 mg/kg dose reduced testis weight and the diameter of seminiferous cords. FLU caused some Leydig cell hyperplasia. Plasma testosterone was reduced in all DES animals, LH elevated in DES 0.5 mg/kg and FLU 25 mg/kg animals, and FSH reduced in the DES 1.0 mg/kg group. Basal testicular *ex vivo* progesterone and human chorionic gonadotropin (hCG)–stimulated testosterone production were decreased in DES animals. Despite a decrease in hCG-induced cyclic adenosine-3′,5′-monophosphate (cAMP) production, intratesticular testosterone was increased in the FLU 10 and 25 mg/kg groups. OP 100 mg/kg elevated hCG-induced progesterone production only. No changes were seen in 3β-HSD protein levels in any treatment group. StAR levels were reduced in DES animals. The results indicate the sensitivity of postnatal fetal-type Leydig cells to endocrine-active compounds. Suppression of StAR expression level was an early sign of the DES-induced steroidogenic lesion. FLU-induced changes suggest the importance of androgen receptor–mediated regulation of testosterone synthesis in the postnatal rat testis. Octylphenol appeared less effective in bringing about acute steroidogenic changes.

Key Words: **diethylstilbestrol; octylphenol; Flutamide; testosterone; rat; testis; endocrine disruption.**

Testicular androgen synthesis in developing male mammals is regulated in a time-dependent manner. Abnormal androgen production profile combined with altered androgen-estrogen ratio has a great potency to demasculinize androgen-dependent differentiation, growth, and function of male reproductive organs and central nervous system (Casto *et al.*, 2003; Rivas *et al.*, 2002; Sharpe *et al.*, 2003). Events causing demasculinization can be traced back to changes in gonadal sex steroid synthesis (Majdic *et al.*, 1996, 1997; Saunders *et al.*, 1997) and in hypothalamic-pituitary-gonad axis (Goyal *et al.*, 2003; Sweeney *et al.*, 2000), and may involve altered expression pattern of androgen and estrogen receptors in androgen-dependent target tissues (Goyal *et al.*, 2003; Gray *et al.*, 1999; McKinnell *et al.*, 2001; Mylchreest *et al.*, 1999; Nishikawa *et al.*, 1999; Sohoni and Sumpter, 1998; Williams *et al.*, 2001). Androgen receptors in fetal and postnatal rat testis are expressed in nonsteroidogenic interstitial cells and Leydig cells and in postnatal Sertoli cells (Shan *et al.*, 1997; Williams *et al.*, 2001; You and Sar, 1998). Estrogen receptor α is found in fetal-type Leydig cells (Fisher *et al.*, 1997; Saunders *et al.*, 1997; Williams *et al.*, 2001) and type β in Leydig and Sertoli cells and gonocytes (Fisher *et al.*, 1997; Saunders *et al.*, 1998; van Pelt *et al.*, 1999; Williams *et al.*, 2001). Therefore, a number of cell types in developing rat testis are potential targets of steroid hormone receptor–mediated endocrine disruption.

Several man-made chemicals have a potency to alter testicular and pituitary functions via steroid receptor–dependent and –independent pathways. Many of the known demasculinizing compounds possess binding affinity to estrogen receptors and are referred to as xenoestrogens. This group includes, for instance, alkylphenols, bisphenol A, and phytoestrogens. So far, only a few chemicals, such as herbicides linuron, procymidone, *p,p′*-dichlorodiphenyldichloroethylene (Gray *et al.*, 1999), and viclozolin metabolites, have been identified as androgen receptor antagonists, but the number of chemicals identified as antiandrogens acting via androgen receptor is increasing (Korner *et al.*, 2004; O'Connor *et al.*, 2002; Vinggaard *et al.*, 2005).

For the present study, three differently endocrine-active compounds, diethylstilbestrol (DES), Flutamide (FLU), and octylphenol, were selected to analyze their potency to acutely interfere with the growth and steroid hormone synthesis of

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infant rat testis. DES, a synthetic nonsteroidal estrogen introduced neonatally, has a high affinity to estrogen receptors, causes a variety of reproductive tract anomalies, and alters androgen status by modifying biosynthesis of sex steroids (Majdic *et al.*, 1996, 1997) and the expression pattern of androgen and estrogen receptors (Goyal *et al.*, 2003; McKinnell *et al.*, 2001; Williams *et al.*, 2001). FLU, a nonsteroidal antiandrogen drug, effectively prevents androgen action at receptor level and is used for the treatment of prostate cancer and acne, and as a model compound for developmentally induced demasculinization (Anahara *et al.*, 2004; Miyata *et al.*, 2002; O'Connor *et al.*, 2002). In Leydig cells, FLU may inhibit androgen receptor–dependent autoregulation of testosterone synthesis (Houk *et al.*, 2004; Ruiz de Galarreta *et al.*, 1983). Octylphenol (4-*tert*-octylphenol, OP), a constituent of alkylphenol polyetoxylates, is used as a surfactant in detergents, paints, and herbicides and may accumulate in sewage sludge (White *et al.*, 1994). In the fish, avian and mammalian species, OP has been shown to interfere with the development of reproductive system (Blake and Boockfor, 1997; Sweeney *et al.*, 2000; White *et al.*, 1994). OP binds to estrogen receptors, but compared to DES, the binding affinity is 5000- to 10,000-fold weaker (Gutendorf and Westendorf, 2001). In developing rat testis, OP may cause a prolonged suppression of testosterone synthesis (Yoshida *et al.*, 2001).

The aim of the present study was to evaluate the sensitivity of fetal-type Leydig cells to neonatally induced endocrine disruption by measuring circulating testosterone and gonadotropin levels, testicular testosterone and progesterone production, and steroidogenic acute regulatory protein (StAR) and 3β-hydroxy-steroid-dehydrogenase (3β-HSD) type I protein expression levels in 14-day-old infant male rats. In the rat, fetal-type Leydig cells are the major androgen-producing cell population in infancy and become gradually replaced by adult-type Leydig cells from day 14 postpartum (Ariyaratne and Mendis-Handagama, 2000; Mendis-Handagama *et al.*, 1998; Tapanainen *et al.*, 1984). Compared to adult-type Leydig cells, the physiology of fetal-type Leydig cells differs in several aspects, including high sensitivity to LH/human chorionic gonadotropin (hCG), the fast recovery of LH receptors, and the lack of LH-induced steroidogenic desensitization until the age of 15–20 days (Huhtaniemi *et al.*, 1984). In neonatal and infant male mammals, circulating gonadotropin and androgen levels are relatively low (Döhler and Wuttke, 1974) but sufficient to support the prepubertal masculinization and growth of reproductive system. If elevated, like in the male mice overexpressing hCG (Rulli *et al.*, 2003), infertility and severe alterations in the urogenital tract may occur. In rats, high postnatal androgen levels may cause premature onset of puberty which negatively impinges on androgen status and spermatogenesis in adulthood (Chandrasekharam *et al.*, 2003; Duckett *et al.*, 1997; Pomerantz, 1984). Therefore, it is important to clarify the sensitivity of steroidogenesis in infantile testis to endocrine-active chemicals.

TABLE 1
Body and Testis Weights of 14-Day-Old Male Rat Infants Treated Neonatally with Vehicle (CTRL), DES, OP, or FLU

Treatment (mg/kg)	*n*	Body weight (g)	Testis weight (mg)
CTRL	30	29.04 ± 2.81	43.48 ± 5.65
DES 0.1	12	24.80 ± 2.02	24.92 ± 3.07***
DES 0.5	18	25.89 ± 2.26	25.91 ± 2.69***
DES 1.0	12	25.32 ± 3.07	25.89 ± 2.08***
OP 10	11	28.00 ± 3.01	43.10 ± 5.08
OP 50	10	27.70 ± 2.38	43.40 ± 3.21
OP 100	20	27.54 ± 2.69	42.15 ± 6.90
FLU 2	22	30.70 ± 1.39	42.26 ± 6.69
FLU 10	25	31.77 ± 2.02	44.39 ± 2.85
FLU 25	30	28.04 ± 2.22	34.90 ± 6.20*

Note. Values are means ± SDs. *$p < 0.05$, ***$p < 0.001$, in comparison with vehicle-treated controls. n = number of animals.

MATERIALS AND METHODS

Animals. Pregnant Sprague-Dawley female rats (The Animal Center of Turku University, Turku, Finland) were housed individually under a 12 h light/12 h dark cycle in plastic cages with woodchips as the floor covering. The temperature of the breeding room was set at 22 ± 2°C and humidity at 55 ± 10%. The rats were fed *ad libitum* with standard laboratory animal food (Commercial RM3(E) SQC, Special Diet Services, Witham, England) and tap water. The day of birth was designated as postnatal day (PND) 0. Pups grew with the dams until the day of analysis. All animal experiments were approved by the Turku University Committee on the Ethics of Animal Experimentation.

Chemicals and treatments. DES, OP, and FLU were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). DES and OP were dissolved in 33% dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) in water giving 50 μl/kg as the final daily volume of 33% DMSO in the animals. FLU was dissolved in corn oil. Daily sc exposure doses were for DES 0.1, 0.5, and 1.0 mg/kg body weight, OP 10, 50, and 100 mg/kg, and FLU 2, 10, and 25 mg/kg. For each dam, four male pups were randomly allocated from different litters. In each group, one male served as a control treated with the vehicle only and the three were treated with the serial doses of one of the compounds. The first neonatal injection was given 6 h after the birth and then daily until the PND 4, i.e., five doses altogether. In the preliminary experiments, no differences were found in steroid hormone levels of oil-treated and DMSO-treated control animals. Total number of control and treated male pups is given in Table 1.

Hormone assays. Hormone measurements were carried out by time-resolved fluoroimmunoassays (DELFIA, Dissociation-Enhanced Lanthanide Fluoroimmuno-assay; PerkinElmer Life and Analytical Sciences Ltd., Wallac, Turku, Finland) as described earlier (Haavisto *et al.*, 2001, 2003). Testosterone was measured in ether-extracted plasma samples, testis homogenates of freshly isolated testes and testes cultured for 3 h, and in culture media. Progesterone was assayed from culture media. LH and FSH levels were measured in plasma samples. For plasma, the animals were anesthesized with carbon dioxide and killed by neck dislocation on PND 14. Blood was collected by heart puncture into heparinized syringes, kept on ice (> 1 h), and centrifuged at 1000 g at +4°C for 5 min. Plasma was stored at −85°C until analysis. Testicular testosterone was measured from the lysis supernatant after incubation for 30 min at 4°C in the lysis buffer (25mM Tris-HCl, 120mM NaCl, 0.5% NP-40, 4mM NaF, 100μM Na_3VO_4, 10 μg/ml aprotinin, 1mM phenylmethylsulphonyl fluoride (PMSF), and 10 μg/ml leupeptin). For the measurement of hCG-stimulated testosterone production, the testes were excised and one of them was incubated in the absence (control) and one in the presence of 50 ng/ml of recombinant

hCG (4700 IU/mg, Akzo Nobel, N.V. Organon, Netherlands) for 3 h. Incubation was carried out in Dulbecco's modified Eagle's medium/HAM's F-12 supplemented with 15mM HEPES, 2 mg/l pyridoxine, 1.2 g/l $NaHCO_3$ (Sigma-Aldrich, Irvine, United Kingdom), 0.365 g/l L-glutamine (Life Technologies, Gibco BRL, Paisley, Scotland), 0.1% bovine serum albumin (BSA) (Sigma Chemical Co.), and 0.1 g/l gentamycin (Biological Industries, BetHaEmek, Israel).

***Testis culture* ex vivo.** Testes, aseptically excised from PND 14 rats, were decapsulated and transferred into 4-well plastic culture dishes (NUNC, Roskilde, Denmark), one testis in a well containing 1 ml of Dulbecco's modified Eagle's medium/HAM's F-12 with the supplements described. After the incubation for 3 h at 37°C under 5% CO_2 in air, culture media were collected and stored at −20°C for hormone measurements.

Futamide* ex vivo *cAMP assay. For the analysis of *ex vivo* pattern of testicular testosterone production in FLU-treated pups, basal and hCG-stimulated cAMP production was measured from the testis of control and FLU-treated (25 mg/kg) animals. The highest dose of FLU was selected for the assay due to its potency to induce significant increase in plasma LH levels and testosterone production *in vitro*. For a 3-h tissue incubation of single testis in the supplemented Dulbecco's modified Eagle's medium/HAM's F-12, 250μM 3-isobutyl-1-methylxanthine (Sigma) was added to the culture medium as a phosphodiesterase inhibitor. hCG was added at concentrations of 0, 10, 50, or 100 ng/ml. After incubation, media (1 ml) and testes were taken into Eppendorf tubes containing 110 μl of 4.4.M perchloric acid and then processed as described earlier (El-Gehani *et al.*, 1998; Haavisto *et al.*, 2001, 2003). cAMP levels were measured by protein-binding assay (Nordstedt and Fredholm, 1990). Briefly, the samples and standards (0–320nM) were incubated for 2.5 h at 4°C with protein extract from bovine adrenal cortex containing cAMP-binding protein and [^{3}H]-cAMP (specific activity 54 Ci/mmol, Amersham, Buckinghamshire, UK) diluted with distilled water to get approximately 15,000 cpm per well. The bound and free cAMPs were separated in Millipore MultiScreen filtration plates. The wells were washed three times with 200 μl of 50mM Tris-HCl, pH 7.4. After drying (+37°C, 1 h), 50 μl of SuperMix scintillation fluid (PerkinElmer Wallac Scintillation Products) was added to the well. Radioactivity was counted using a MicroBeta scintillation counter (Wallac, Turku, Finland). Standards were prepared in the range of 0–320nM. The intra- and interassay coefficients of variation were 5 and 10%, respectively.

Western blot analysis. Testes lysate was centrifuged at 14,000 rpm for 25 min at +4°C, and protein concentration in the supernatant was determined. Protein samples (20–30 μg) were dissolved in 7.5 μl of 2x Laemmli sample buffer and set to 15 μl with buffer C (25% glycerol, 0.42 M NaCl, 1.5mM $MgCl_2$, 0.2 mM EDTA, 20 mM HEPES, 0.5 mM DTT, and 0.5 mM PMSF). After electrophoresis in 12.5% sodium dodecyl sulfate–polyacrylamide gel (Mini Protean II system, Bio-Rad laboratories, Hercules, CA), proteins were transferred onto a nitrocellulose membrane (Hybond, Amersham). After incubation in blocking buffer (10 mM Tris-HCl, pH 8.0, 0.3% Tween 20, and 3% nonfat milk powder) for 1.5 h at room temperature, rabbit anti-human StAR antibodies (courtesy of Dr. J. F. Strauss III, Center for Research on Reproduction and Women's Health, University of Pennsylvania, University of Pennsylvania, PA) or rabbit anti-mouse recombinant 3β-HSD type 1 antibodies (courtesy of Dr. A. Payne, Stanford University Medical Center) were added on separate strips at dilutions 1:2000 and 1:5000 in 1% BSA-PBS, respectively. Washed (10mM Tris-HCl, pH 8.0, 0.3% Tween 20) membranes were incubated with horseradish peroxidase–linked anti-rabbit IgG (Amersham Science) diluted 1:5000 for StAR and 1:20,000 for 3β-HSD type 1. Antibody binding was visualized on X-ray film (Kodak, X-OMAT, Rochester, NY) with an ECL Western Blot Detection Kit (Amersham). Reprobing with mouse anti-β-actin antibody (Sigma; 1:20 000) was done on the washed strips. Antibody binding was visualized by peroxidase-linked anti-mouse IgG (Amersham Pharmacia Biotech; 1:50 000) labeling. The densitometric data obtained by Chemi Imager 4400 program (version 5.5, Alpha Innotech Corporation, San Leandro, CA) was normalized for actin signal. For each experiment, Western blot analysis was repeated at least for three times.

Microscopy. For histological analysis and for the measurement of the seminiferous cord diameter, the testes were dissected from pups on PND 14, one testis from four animals in each group. Tunica albuginea was punched with thin needles, and the testes were prefixed in 5% glutaraldehyde in 0.16 mol/l s-collidine-HCl buffer (pH 7.4) for 1 h at room temperature. Thereafter, the testis was cut into four horizontal slices with a razor blade and returned to the fixative for 24–48 h. After washing in s-collidine buffer, the tissues were postfixed in potassium ferrocyanide-osmium fixative. For toluidine blue staining and light microcopic analysis under Leica DMRXA light microscope, five dehydrated 1-mm^3 samples from every horizontal testis slice were embedded in Epon and cut into 1-μm-thick semithin sections. From each 1-mm^3 sample, a series of three semithin sections were cut at 250-μm intervals yielding in 240 sections from one testis. From each section, the diameter of 12 seminiferous cords was measured.

Statistical analysis. The results were analyzed by one-way ANOVA followed by Dunnett's post hoc two-sided *t*-test or independent-samples *t*-test using the SPSS for Windows 9.0 program. Values for animal body weights and testis weights were expressed as mean ± SD and all the other values as mean ± SEM. A probability p less than 0.05 was chosen as the limit of statistical significance.

RESULTS

Body and Testis Weight

Body and testis weight data of 14-day-old male rat infants are shown in Table 1. Body weight was not affected by any of the treatments, but the testis weight was reduced approximately by 40% at every dose of DES ($p < 0.001$). The highest dose of FLU (25 mg/kg/day) suppressed testis weight by approximately 15% ($p < 0.05$). OP had no effect on testis weight.

Testis Histology and the Diameter of Seminiferous Cords

Testicular histology was analyzed in 14-day-old males neonatally exposed to 0.5 mg DES/kg, 100 mg OP/kg, and 25 mg FLU/kg. The average seminiferous cord diameter in the control testes was 85.51 ± 1.9 μm (Fig. 1). In the DES group, it was reduced by 17.1% ($p < 0.001$) and by 7.6% ($p < 0.01$) in the FLU-treated offspring. A nonsignificant ($p < 0.069$) reduction in the cord diameter was recorded in the animals treated with OP. Leydig cell hyperplasia/hypertrophy was seen in one out of four testes of FLU-treated males. Large Leydig cell islets shown in Figure 2, however, occurred only sporadically and comprised only 2–5% of the total number of Leydig cell islets. Compared to controls, the testes of DES- and OP-treated males showed no overt histological changes in Leydig cells and seminiferous cords.

Plasma Testosterone, LH, and FSH

In males exposed to DES (0.1, 0.5, or 1.0 mg/kg/day), plasma testosterone (Fig. 3A) was suppressed by 82–91% ($p < 0.05$–0.01), but the levels were not significantly altered in the groups treated with OP and FLU. A twofold increase in LH (Fig. 3B) was seen in the animals treated with 0.1 and 1.0 mg DES/kg, but the change was not statistically significant due to

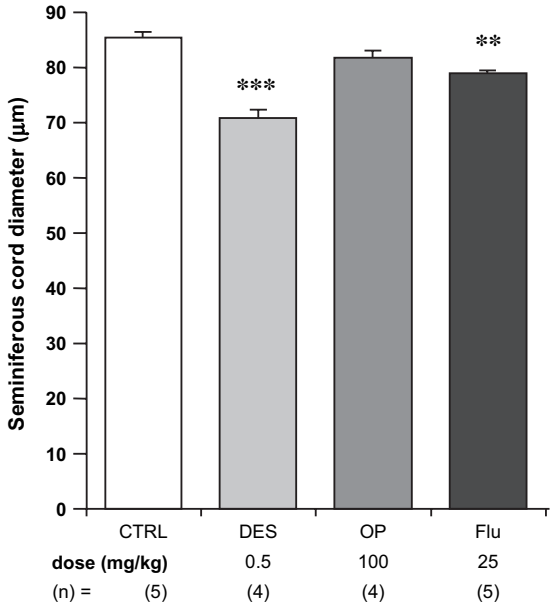

FIG. 1. Diameter of seminiferous cords in the testes of 14-day-old male rats treated neonatally with vehicle (CTRL), DES, OP, or FLU. Values are means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, in comparison with respective value for vehicle-treated controls. n = number of animals.

a relatively high interindividual variation. However, a 2.8-fold increase in LH was significant ($p < 0.05$) in males exposed to 0.5 mg DES/kg. While OP treatment did not affect plasma LH levels, a dose-dependent increase was seen in the FLU-treated animals. A threefold increase at 25 mg FLU/kg dose was statistically significant ($p < 0.01$). Plasma FSH levels (Fig. 3C) were reduced by 46% ($p < 0.01$) in the 1.0 mg/kg DES group, but no effect was seen in the animals treated with OP or FLU.

Testosterone and Progesterone Production Ex vivo

Basal testosterone secretion during a 3-h *ex vivo* culture of PND 14 testes was not significantly affected by any of the treatments (Fig. 4A). In control testes, basal testosterone secretion was stimulated by 2.3-fold in the presence of hCG. In the DES group, stimulation rate was 1.8- to 1.6-fold corresponding to approximately 40% decrease ($p < 0.05$) from the control levels. Testes of FLU-treated rats (25 mg/kg/day) showed a 5.2-fold increase ($p < 0.05$) in the hCG-stimulated secretion of testosterone. None of the changes in the OP-treated animals were statistically significant.

As for intratesticular testosterone (Fig. 4B), the profile of basal and hCG-stimulated testosterone levels confirmed the pattern observed for secreted testosterone. In the absence of hCG, none of the compounds caused statistically significant changes in intratesticular testosterone levels. In the DES groups, the doses 0.1 and 0.5 mg/kg/day decreased hCG-stimulated testosterone content by 58.5% ($p < 0.05$) and the dose 1.0 mg/kg/day by 68.5% ($p < 0.01$). Testosterone concentration in the OP group remained at the control level throughout. In the FLU-treated animals, the highest dose (25 mg/kg/day) caused a significant ($p < 0.05$) increase in hCG-induced testosterone production exceeding 1.7-fold the level measured from the stimulated control testes. The elevation from the control basal level was 5.4-fold.

Progesterone secretion (Fig. 4C) of the control testes was not altered after hCG stimulation. In the testes of DES-exposed males, however, basal secretion was reduced in a dose-independent manner by 34–37% ($p < 0.05$–0.01). After hCG stimulation, progesterone levels in the culture media of the DES group remained slightly under the control levels. Basal progesterone secretion was not affected by OP. However, OP 100 mg/kg/day significantly ($p < 0.05$) elevated hCG-stimulated progesterone levels. In the FLU-exposed animals, progesterone remained at control level throughout.

Effects of FLU on Acute Intratesticular Testosterone and cAMP Production

In order to assess whether the stimulatory effect of FLU observed in testicular testosterone production *ex vivo* could be acutely seen in the freshly isolated testes, testosterone was measured on PND 14 from the groups exposed to 2, 10, or 25 mg FLU/kg/day (Fig. 5). In the 10-mg/kg/day exposure group,

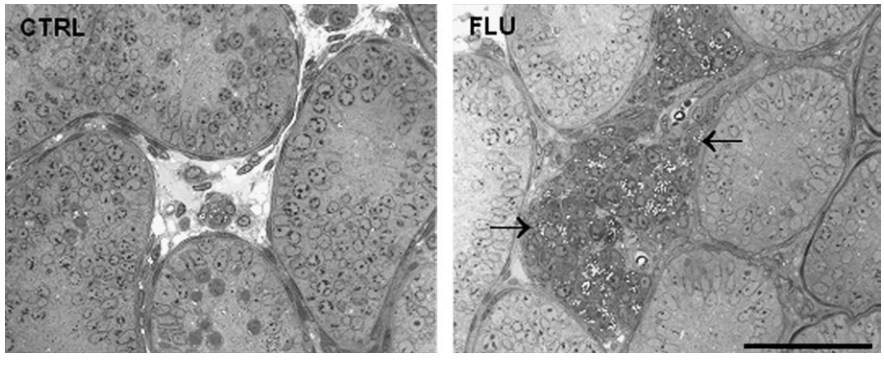

FIG. 2. Toluidine blue–stained testis sections of 14-day old male rats treated neonatally with vehicle (CTRL) or FLU 25 mg/kg/day. The latter shows a profound Leydig cell hyperplasia/hypertrophy (arrows). Bar, 50 µm.

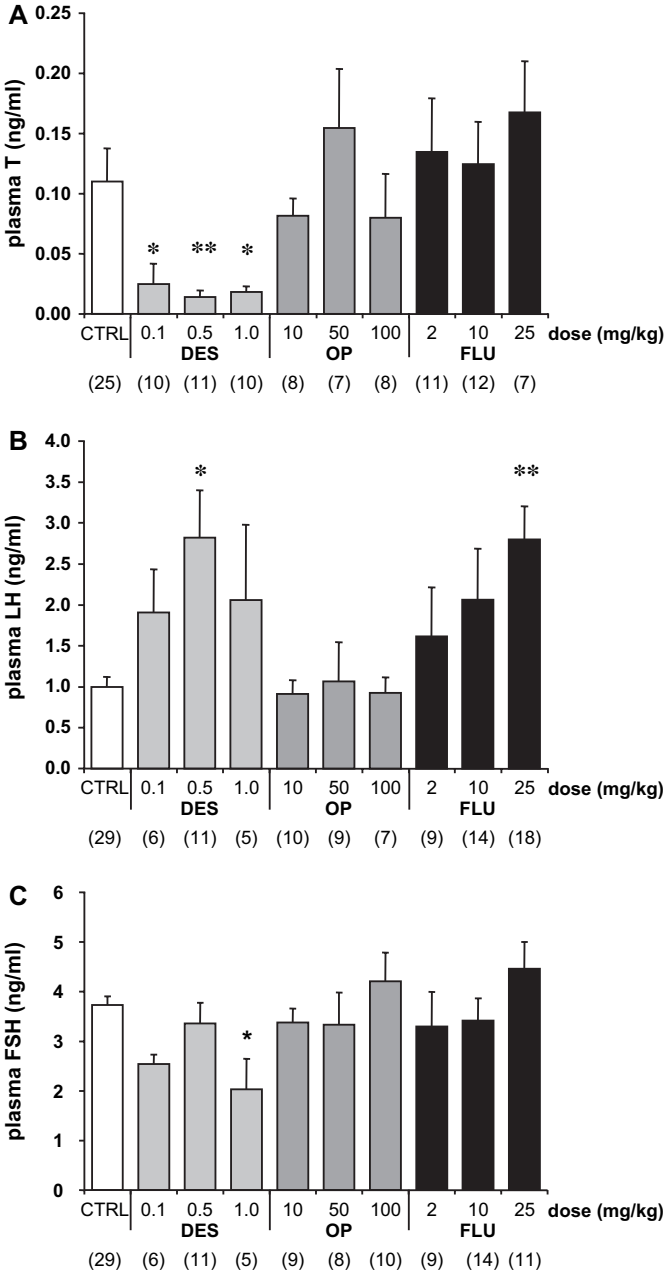

FIG. 3. Plasma testosterone (A), LH (B), and FSH (C) concentrations in 14-day-old male rats treated neonatally with vehicle (CTRL), DES, OP, or FLU. Values are means ± SEM. *$p < 0.05$, **$p < 0.01$ compared with vehicle-treated controls. Number of animals is given in parentheses.

a significant ($p < 0.001$) 1.8-fold increase and in the 25-mg/kg/day group a 2.2-fold increase ($p < 0.001$) in intratesticular testosterone content were recorded. In the cAMP assay carried out after a 3-h *ex vivo* culture of the testes neonatally exposed to 25 mg FLU/kg/day, basal cAMP production did not differ from the controls (Fig. 6). However, in the presence of hCG (10 or 50 ng/ml), a significant ($p < 0.01$–0.001) decrease in the cAMP levels was observed in the FLU-exposed group.

Expression of StAR and 3β-HSD 1 Proteins

In the Western blot analysis of the tissue homogenates from freshly isolated PND 14 rat testis, a major band at 30 kDa was recognized with anti-StAR antibody (Fig. 7A). In DES-treated groups, testicular StAR protein expression was suppressed by 41–44% ($p < 0.05$) from controls (Fig. 7B). OP did not alter the pattern of StAR protein expression. In the FLU group, StAR values remained at or slightly above the control level. For 3β-HSD type 1 protein expression, no significant changes were found in any of the exposure groups (Figs. 7A and 7C).

DISCUSSION

The results indicate the sensitivity of infantile rat testis to neonatally introduced estrogens and antiandrogens. Given that postnatal steroidogenic profile based on the activity of fetal Leydig cells (Ariyaratne and Mendis-Handagama, 2000) is of a pivotal importance for the prepubertal and pubertal sexual maturation, the observed changes may be early events in the chain that leads to the manifestation of reproductive lesions described in adult progeny exposed neonatally to endocrine-active compounds. The most striking, albeit opposing, steroidogenic effects were obtained in the male infant rats exposed to DES and FLU. That the highest concentration of octylphenol, a weak estrogenic compound, increased hCG-stimulated progesterone production is in line with the steroidogenic changes seen in the prenatal rat testis exposed to octylphenol *in vitro* (Haavisto *et al.*, 2003). Otherwise, the effects of octylphenol on postnatal testis seem to remain less severe. The observed decrease in testis weight in male infants treated with DES and FLU is a characteristic feature of demasculinization and interference of androgen-dependent growth (Goyal *et al.*, 2003; Miyata *et al.*, 2002). The decrease in tissue weight, however, does not solely explain steroidogenic effects observed.

In the DES-exposed infants, the observed steep suppression of plasma testosterone levels is in line with the reported results in 18-day-old male rats submitted for a longer postnatal exposure period (Williams *et al.*, 2001). From the present study, it appears that steroidogenic changes are induced relatively fast and after a short neonatal exposure period. Strongly altered steroidogenic profile in infancy apparently is involved in the origin of reproductive anomalies described in neonatally estrogenized adult male rats, including the reduction in pubertal testis size, Sertoli cell numbers, and lumen formation of the seminiferous epithelium, retarded spermatogenesis, and abnormal development of rat testis (Atanassova *et al.*, 1999, 2000; Goyal *et al.*, 2003; Rivas *et al.*, 2002) and expression pattern of androgen and estrogen receptors (Tena-Sempere *et al.*, 2000). Described morphologic changes due to estrogenization and altered estrogen-androgen balance may include reduced testicular cell proliferation, changes in cell differentiation program, increased rate of apoptosis (Williams *et al.*,

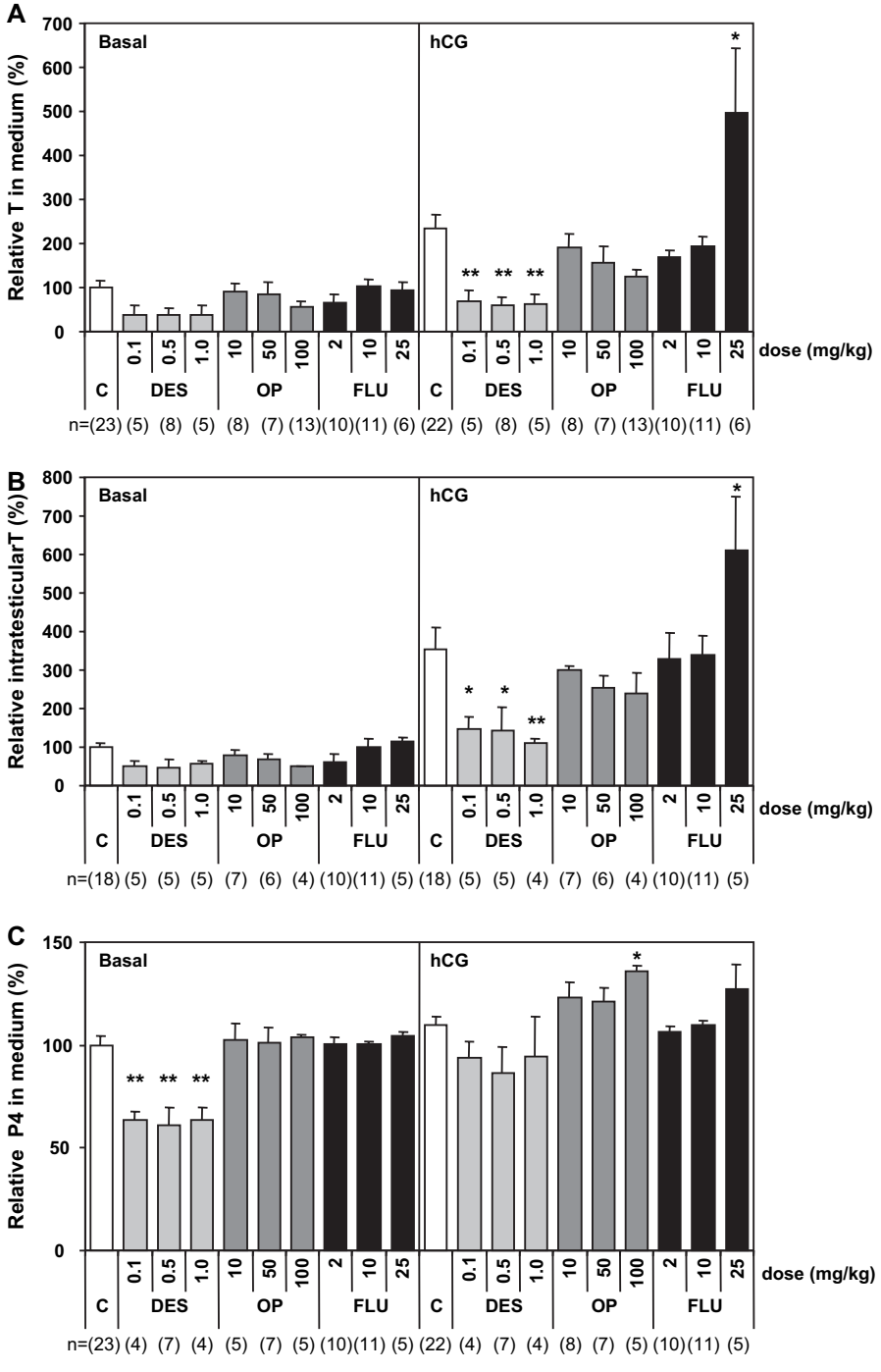

FIG. 4. (A) Basal and hCG-stimulated (50 ng/ml) secretion of testosterone (T), (B) level of intratesticular testosterone, and (C) secreted progesterone (P4) after a 3-h *ex vivo* culture of neonatally exposed infant rat testes. Bold numbers in the bottom panel indicate neonatal exposure concentrations (mg/kg) of DES, OP, and FLU. Numbers in parentheses, the number of animals. The mean of the basal control level is given as 100%. Values are means ± SEM. $*p < 0.05$, $**p < 0.01$ compared with vehicle-treated controls (C).

2001), and downregulation of androgen receptors (Fritz *et al.*, 2003; McKinnell *et al.*, 2001; Williams *et al.*, 2001).

In fetal-type Leydig cells, inhibition of androgen synthesis by DES and other estrogens seems to be mediated via estrogen receptor alpha (Delbès *et al.*, 2005). By binding to estrogen receptor, DES may inhibit steroidogenic pathway at multiple steps (Fielden *et al.*, 2002; Majdic *et al.*, 1996, 1997). According to the observed response to hCG stimulation and our previous results in the *in utero* DES-exposed testes (Haavisto *et al.*, 2003), steroidogenic lesion apparently does

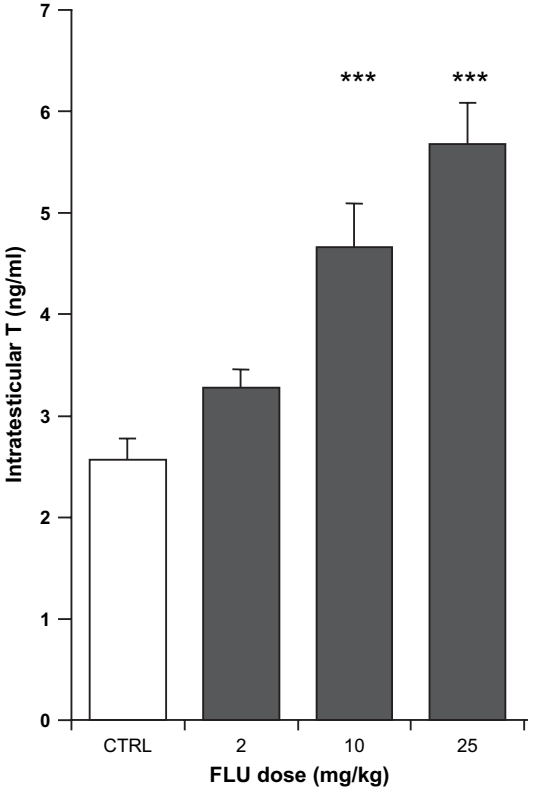

FIG. 5. Dose-response values of basal intratesticular testosterone levels measured acutely from freshly isolated 14-day-old rat testes of neonatally FLU-exposed (FLU) animals. CTRL, vehicle-treated controls. Values are means ± SEM for eight individual samples. ***$p < 0.001$ compared with controls.

not involve LH receptor–mediated signaling. Also LH secretion from the infant pituitary seems to be resistant to DES-induced defects. Instead, as indicated in the present and earlier studies in developing testis (Guyot *et al.*, 2004), StAR protein

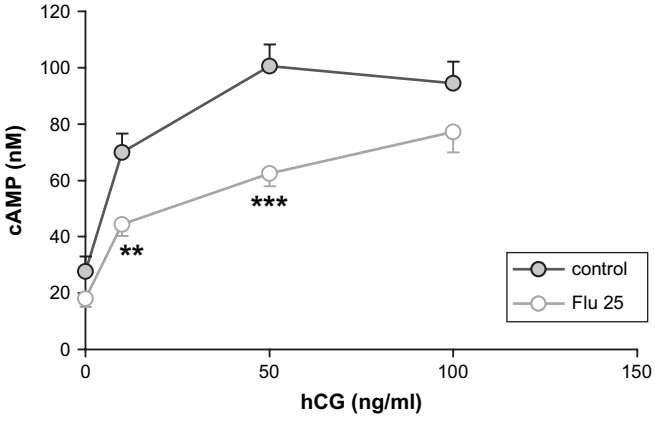

FIG. 6. cAMP levels measured *ex vivo* from the testes of male rats treated neonatally with vehicle (CTRL) or 25 mg/kg of FLU. Testes from 14-day-old male rats were incubated in the presence of hCG (10, 50, 100 ng/ml) for 3 h. Values are means ± SEM for six individual samples. **$p < 0.01$, ***$p < 0.001$ compared with vehicle-treated controls.

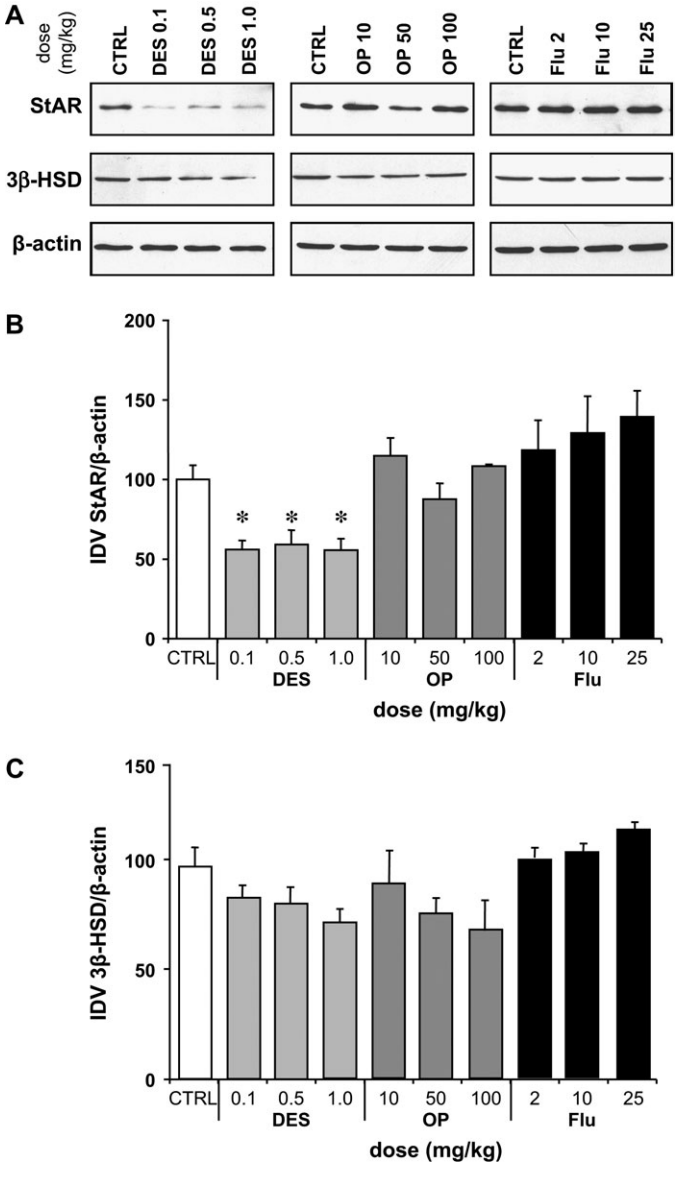

FIG. 7. (A) Western blot analyses of StAR and 3β-HSD/Δ^5-Δ^4 isomerase type 1 protein expression levels in the testes of 14-day-old male rats treated neonatally with vehicle (CTRL), DES, OP, or FLU. Anti-β-actin labeling served as an internal protein loading control. Quantitated (IDV = integrated density value) values for StAR (B) and 3β-HSD (C). The mean of control density is set as 100%. Values are means ± SEM. Each experiment was repeated three times at least. *$p < 0.05$ compared with vehicle-treated controls.

synthesis seems to be among the most sensitive targets of the adverse effects of DES. Significant decrease in StAR protein levels in Western blot analysis suggests that the steroidogenic changes in the testis of DES-treated offspring can not only be explained by the reduced testis weight. Also in Leydig MA-10 cells, estrogens strongly inhibit StAR expression (Houk *et al.*, 2004). Conversely, in estrogen receptor alpha knockout neonate male mice, StAR expression is upregulated (Delbès *et al.*, 2005). Therefore, that the testes of DES-exposed male

offspring do not react positively to elevated plasma LH levels and only marginally to hCG stimulation could be due to the upregulation estrogen receptors and estrogen receptor–mediated enzymatic inhibition of androgen synthesis, the cascade shown to occur in Leydig cells (Tena-Sempere *et al.*, 2000; Tsai-Morris *et al.*, 1986). 3β-HSD enzyme converting pregnenolone to progesterone remained unchanged at protein level, suggesting that the production of the enzyme is not a potential target of DES action, as not in the prenatal rat testes either (Saunders *et al.*, 1997). Studies on fetal mouse (Delbès *et al.*, 2005; Guyot *et al.*, 2004) and rat (Majdic *et al.*, 1996; Saunders *et al.*, 1997) testis propose 17-α hydroxylase C17,20 lyase (P450c17) as a target of DES action. Inhibited transcription of P450c17 apparently is a key factor causing steroidogenic lesion in gonadotropin-induced adult rat Leydig cells (Lu *et al.*, 1991). As for steroidogenic factor 1, a transcription factor known to regulate the expression of P450 enzyme genes, the effects of DES in developing testes have remained controversial (Guyot *et al.*, 2004; Saunders *et al.*, 1997).

Similar to DES used at the dose of 1.0 mg/kg (present study), treatment of newborn male rats with estradiol benzoate significantly reduces serum testosterone and FSH levels in 15-day-old and older rats (Tena-Sempere *et al.*, 2000). As a regulator of Sertoli cells, reduced FSH levels could adversely affect the function and proliferation of Sertoli cells, testis size, and sperm production capacity as reported in estrogenized males (Atanassova *et al.*, 2000; Sharpe, 1998). Under prevailing low aromatase activity and low enstradiol synthesis in the infant rat testis (George and Ojeda, 1987; Pomerantz, 1980), the altered FSH level apparently has no significant effect on intratesticular estradiol levels. Due to a low affinity to steroid hormone carrier proteins (Milligan *et al.*, 1998), DES alone has a great potency to cause the physiological effects indicated also in the present study.

Confirming the androgen dependence of testis growth, FLU (25 mg/kg) reduced testis growth, even though not as effectively as DES. At higher doses (100 mg/kg), in utero-introduced FLU has been shown to interfere with body growth and masculinization of male sex organs (Mylchreest *et al.*, 1999). The results from the perinatal exposure studies at 10–100 mg FLU/kg (Miyata *et al.*, 2002), in turn, have confirmed the irreversibility of FLU-induced reproductive anomalies. Circulating testosterone levels in the present pups were not significantly elevated but this apparently would occur at higher perinatal exposure doses (Miyata *et al.*, 2002). In the present study, intratesticular testosterone levels and circulating LH, however, were increased. In line with the above findings, in 15-day screening assay in adult rats, exposure to FLU (5-100 mg/kg/day) significantly elevates testosterone, estradiol, and dihydrotestosterone levels and causes hypergonadotropism (O'Connor *et al.*, 2002). The changes reported exclude LH-mediated regulation as a negative target of FLU action. As we showed *ex vivo*, hCG-induced intratesticular and secreted testosterone levels were significantly elevated in 14-day-old rat testis. The *ex vivo*-induced increase was not seen in progesterone production, which apparently is due to the rapid turn over of the hormone.

Under normal physiological conditions, androgens are known to reduce testosterone biosynthesis in prepubertal and adult Leydig cells and Leydig cell lines in an autoregulative way, apparently by the receptor-mediated inhibition of StAR expression (Houk *et al.*, 2004; Ruiz de Galarreta *et al.*, 1983). Androgen-dependent regulation of Leydig cell function seems to become functional postnatally (O'Shaughnessy *et al.*, 2002) and, therefore, fetal testicular testosterone production is not markedly affected by FLU (Mylchreest *et al.*, 2002). The present observation of the increased *ex vivo* testosterone production in the presence of relatively high StAR protein levels in the testes of FLU-treated infant rats is in line with the observation of Houk *et al.* (2004): in mouse Leydig cell cultures, the inhibition of testosterone binding to androgen receptors prevents the normal downregulation of StAR. Inhibition of transcriptional activity or the expression of androgen receptors (McKinnell *et al.*, 2001; Mylchreest *et al.*, 2002; Ohsako *et al.*, 2003) seems to have a great impact on Leydig cell steroidogenesis.

In order to further resolve the mechanisms of FLU action in postnatal rat Leydig cell population, hCG-stimulated cAMP production was measured from the testis of control and FLU-treated (25 mg/kg) pups. Despite hCG-induced stimulation of testosterone production, testicular *ex vivo* cAMP levels were lower in FLU-treated than in control animals. The mechanism behind the phenomenon remains to be elucidated. The combination of high levels of StAR with the exceptional high sensitivity of fetal-type leydig cells to LH/hCG (El-Gehani *et al*., 1998) could explain the elevated testosterone production. Compared to adult Leydig cells, surprisingly high testosterone production under extremely low cAMP levels has been demonstrated in neonatal rat Leydig cells (Huhtaniemi *et al.*, 1984).

Leydig cell hyperplasia/hypertrophy could, in part, explain the elevation of hCG-induced intratesticular testosterone levels, but due to a relative low incidence of enlarged Leydig cell islets this seems less evident. Hyperplasia/hypertrophy of Leydig cells has been described in adult rat testes exposed to FLU *in utero* (Mylchreest *et al.*, 1999) or in adulthood (O'Connor *et al.*, 2002). Some Leydig cell hyperplasia also occurs in the prenatal Leydig cells exposed *in utero* to100 mg/kg FLU (Mylchreest *et al.*, 2002). Hypertrophy apparently is due to the disturbance of endocrine hormonal homeostasis, a consequence of the absence of negative testosterone-mediated feedback of LH secretion. Transgenic mice with the overproduction of human chorionic gonadotropin also develop hypertrophy of fetal-type Leydig cells (Ahtiainen *et al.*, 2005). Whether hypertrophy of gonadotropin-sensitive fetal-type Leydig cells leads to the extended survival of the cell population is an intriguing question. Leydig cell adenomas have been described in rat testes exposed *in utero* to antiandrogen di(*n*-butyl)phathalte (Barlow *et al.*, 2004).

Octylphenol, a putative estrogen receptor agonist, had no or only marginal effects in neonatally exposed infant male rat testis. The only significant change was the observed elevation of hCG-induced progesterone production *ex vivo* at the highest dose of OP (100 mg/kg). The change was similar to that described in the prenatal rat testis exposed to OP (100 mg/l) *in vitro* (Haavisto *et al.*, 2003) and may be an indication of the reduced expression of P450c17 shown to occur in fetal rat testis maternally exposed to octylphenol and DES (Majdic *et al.*, 1996). Marginal changes observed in the present study may be a matter of dosing. Prolonged suppression of testosterone levels has been reported in male rats exposed neonatally to OP 100 mg/kg every other day until PND 15 (Yoshida *et al.*, 2001).

The purpose of the present study was to resolve the sensitivity of infant testis and its fetal-type Leydig cell population to DES, octylphenol, and antiandrogen FLU. Both DES and FLU induced acute changes in testis growth but via different steroidogenic mechanisms. While DES caused a strong downregulation of StAR protein expression and hCG-induced testosterone production, opposite effects were seen in the testes of FLU-treated rats. Further studies are needed to resolve the developmental maturation of androgen receptor–mediated autoregulation of testosterone synthesis in Leydig cells and its role in the neonatal regulation of testis growth and development.

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REFERENCES

Ahtiainen, P., Rulli, S. B., Shariatmadari, R., Pelliniemi, L. J., Toppari, J., Poutanen, M., and Huhtaniemi, I. T. (2005). Fetal but not adult Leydig cells are susceptible to adenoma formation in response to persistently high hCG level: A study on hCG overexpressing transgenic mice. *Oncogene* **24,** 7301–7309.

Anahara, R., Toyama, Y., and Mori, C. (2004). Flutamide induces ultrastructural changes in spermatids and the ectoplasmic specialization between the Sertoli cell and spermatids in mouse testes. *Reprod. Toxicol.* **18,** 589–596.

Ariyaratne, H. B., and Mendis-Handagama, S. M. (2000). Changes in the testis interstitium of Sprague Dawley rats from birth to sexual maturity. *Biol. Reprod.* **62,** 680–690.

Atanassova, N., McKinnell, C., Turner, K. J., Walker, M., Fisher, J. S., Morley, M., Millar, M. R., Groome, N. P., and Sharpe, R. M. (2000). Comparative effects of neonatal exposure of male rats to potent and weak (environmental) estrogens on spermatogenesis at puberty and the relationship to adult testis size and fertility: Evidence for stimulatory effects of low estrogen levels. *Endocrinology* **141,** 3898–3907.

Atanassova, N., McKinnell, C., Walker, M., Turner, K. J., Fisher, J. S., Morley, M., Millar, M. R., Groome, N. P., and Sharpe, R. M. (1999). Permanent effects of neonatal estrogen exposure in rats on reproductive hormone levels, Sertoli cell number, and the efficiency of spermatogenesis in adulthood. *Endocrinology* **140,** 5364–5373.

Barlow, N. J., McIntyre, B. S., and Foster, P. M. (2004). Male reproductive tract lesions at 6, 12, and 18 months of age following in utero exposure to di(n-butyl) phthalate. *Toxicol. Pathol.* **32,** 79–90.

Blake, C. A., and Boockfor, F. R. (1997). Chronic administration of the environmental pollutant 4-tert-octylphenol to adult male rats interferes with the secretion of luteinizing hormone, follicle-stimulating hormone, prolactin, and testosterone. *Biol. Reprod.* **57,** 255–266.

Casto, J. M., Ward, O. B., and Bartke, A. (2003). Play, copulation, anatomy, and testosterone in gonadally intact male rats prenatally exposed to flutamide. *Physiol. Behav.* **79,** 633–641.

Chandrasekharam, V. V., Srinivas, M., Das, S. N., Jha, P., Bajpai, M., Chaki, S. P., and Misro, M. M. (2003). Prepubertal human chorionic gonadotropin injection affects postpubertal germ cell maturation and androgen production in rat testis. *Urology* **62,** 571–574.

Delbès, G., Levacher, C., Duquenne, C., Racine, C., Pakarinen, P., and Habert, R. (2005). Endogenous estrogens inhibit mouse fetal Leydig cell development via estrogen receptor alpha. *Endocrinology* **146,** 2454–2461.

Döhler, K. D., and Wuttke, W. (1974). Serum LH, FSH, prolactin and progesterone from birth to puberty in female and male rats. *Endocrinology* **94,** 1003–1008.

Duckett, R. J., Wreford, N. G., Meachem, S. J., McLachlan, R. I., and Hedger, M. P. (1997). Effect of chorionic gonadotropin and flutamide on Leydig cell and macrophage populations in the testosterone-estradiol-implanted adult rat. *J. Androl.* **18,** 656–662.

El-Gehani, F., Zhang, F. P., Pakarinen, P., Rannikko, A., and Huhtaniemi, I. (1998). Gonadotropin-independent regulation of steroidogenesis in the fetal rat testis. *Biol. Reprod.* **58,** 116–123.

Fielden, M. R., Halgren, R. G., Fong, C. J., Staub, C., Johnson, L., Chou, K., and Zacharewski, T. R. (2002). Gestational and lactational exposure of male mice to diethylstilbestrol causes long-term effects on the testis, sperm fertilizing ability in vitro, and testicular gene expression. *Endocrinology* **143,** 3044–3059.

Fisher, J. S., Millar, M. R., Majdic, G., Saunders, P. T. K., Fraser, H. M., and Sharpe, R. M. (1997). Immunolocalisation of oestrogen receptor-alpha within the testis and excurrent ducts of the rat and marmoset monkey from perinatal life to adulthood. *J. Endocrinol.* **153,** 485–495.

Fritz, W. A., Cotroneo, M. S., Wang, J., Eltoum, I. E., and Lamartiniere, C. A. (2003). Dietary diethylstilbestrol but not genistein adversely affects rat testicular development. *J. Nutr.* **133,** 2287–2293.

George, F. W., and Ojeda, S. R. (1987). Vasoactive intestinal peptide enhances aromatase activity in the neonatal rat ovary before development of primary follicles or responsiveness to follicle-stimulating hormone. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 5803–5807.

Goyal, H. O., Robateau, A., Braden, T. D., Williams, C. S., Srivastava, K. K., and Ali, K. (2003). Neonatal estrogen exposure of male rats alters reproductive functions at adulthood. *Biol. Reprod.* **68,** 2081–2091.

Gray, L. E., Jr., Ostby, J., Monosson, E., and Kelce, W. R. (1999). Environmental antiandrogens: Low doses of the fungicide vinclozolin alter sexual differentiation of the male rat. *Toxicol. Ind. Health* **15,** 48–64.

Gutendorf, B., and Westendorf, J. (2001). Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. *Toxicology* **166,** 79–89.

Guyot, R., Odet, F., Leduque, P., Forest, M. G., and Le Magueresse-Battistoni, B. (2004). Diethylstilbestrol inhibits the expression of the steroidogenic acute regulatory protein in mouse fetal testis. *Mol. Cell. Endocrinol.* **220,** 67–75.

Haavisto, T., Nurmela, K., Pohjanvirta, R., Huuskonen, H., El-Gehani, F., and Paranko, J. (2001). Prenatal testosterone and luteinizing hormone levels in

male rats exposed during pregnancy to 2,3,7,8-tetrachlorodibenzo-p-dioxin and diethylstilbestrol. *Mol. Cell. Endocrinol.* **178,** 169–179.

Haavisto, T. E., Adamsson, N. A., Myllymäki, S. A., Toppari, J., and Paranko, J. (2003). Effects of 4-tert-octylphenol, 4-tert-butylphenol, and diethylstilbestrol on prenatal testosterone surge in the rat. *Reprod. Toxicol.* **17,** 593–605.

Houk, C. P., Pearson, E. J., Martinelle, N., Donahoe, P. K., and Teixeira, J. (2004). Feedback inhibition of steroidogenic acute regulatory protein expression in vitro and in vivo by androgens. *Endocrinology* **145,** 1269–1275.

Huhtaniemi, I., Warren, D. W., and Catt, K. J. (1984). Functional maturation of rat testis Leydig cells. In *Hormone Action and Testicular Function. Ann. N.Y. Acad. Sci.* (Catt, K.J, and Dufau, M.L., eds.), **438,** 283–303.

Korner, W., Vinggaard, A. M., Terouanne, B., Ma, R., Wieloch, C., Schlumpf, M., Sultan, C., and Soto, A. M. (2004). Interlaboratory comparison of four in vitro assays for assessing androgenic and antiandrogenic activity of environmental chemicals. *Environ. Health Perspect.* **112,** 695–702.

Lu, Y., McDonough, A., Farley, R. A., and Warren, D. W. (1991). Regulation of testicular P-450 cholesterol side-chain cleavage and P-450 C17-20 lyase/C17 hydroxylase enzymes in the neonatal and adult rat. *Acta Endocrinol. (Copenh.)* **124,** 449–454.

Majdic, G., Sharpe, R. M., O'Shaughnessy, P. J., and Saunders, P. T. K. (1996). Expression of cytochrome P450 17α-hydroxylase/C17-20 lyase in the fetal rat testis is reduced by maternal exposure to exogenous estrogens. *Endocrinology* **137,** 1063–1070.

Majdic, G., Sharpe, R. M., and Saunders, P. T. K. (1997). Maternal oestrogen/xenoestrogen exposure alters expression of steroidogenic factor-1 (SF-1/Ad4BP) in the fetal rat testis. *Mol. Cell. Endocrinol.* **127,** 91–98.

McKinnell, C., Atanassova, N., Williams, K., Fisher, J. S., Walker, M., Turner, K. J., Saunders., T. K., and Sharpe, R. M. (2001). Suppression of androgen action and the induction of gross abnormalities of the reproductive tract in male rats treated neonatally with diethylstilbestrol. *J. Androl.* **22,** 323–338.

Mendis-Handagama, S. M., Ariyaratne, H. B., Teunissen van Manen, K. R., and Haupt, R. L. (1998). Differentiation of adult Leydig cells in the neonatal rat testis is arrested by hypothyroidism. *Biol. Reprod.* **59,** 351–357.

Milligan, S. R., Khan, O., and Nash, M. (1998). Competitive binding of xenobiotic oestrogens to rat alpha-fetoprotein and to sex steroid binding proteins in human and rainbow trout (Oncorhynchus mykiss) plasma. *Gen. Comp. Endocrinol.* **112,** 89–95.

Miyata, K., Yabushita, S., Sukata, T., Sano, M., Yoshino, H., Nakanishi, T., Okuno, Y., and Matsuo, M. (2002). Effects of perinatal exposure to flutamide on sex hormones and androgen-dependent organs in F1 male rats. *J. Toxicol. Sci.* **27,** 19–33.

Mylchreest, E., Sar, M., Cattley, R. C., and Foster, P. M. (1999). Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol. Appl. Pharmacol.* **156,** 81–95.

Mylchreest, E., Sar, M., Wallace, D. G., and Foster, P. M. (2002). Fetal testosterone insufficiency and abnormal proliferation of Leydig cells and gonocytes in rats exposed to di(n-butyl) phthalate. *Reprod. Toxicol.* **16,** 19–28.

Nishikawa, J., Saito, K., Goto, J., Dakeyama, F., Matsuo, M., and Nishihara, T. (1999). New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. *Toxicol. Appl. Pharmacol.* **154,** 76–83.

Nordstedt, C., and Fredholm, B. B. (1990). A modification of a protein-binding method for rapid quantification of cAMP in cell-culture supernatants and body fluid. *Anal. Biochem.* **189,** 231–234.

O'Connor, J. C., Frame, S. R., and Ladics, G. S. (2002). Evaluation of a 15-day screening assay using intact male rats for identifying antiandrogens. *Toxicol. Sci.* **69,** 92–108.

Ohsako, S., Kubota, K., Kurosawa, S., Takeda, K., Qing, W., Ishimura, R., and Tohyama, C. (2003). Alterations of gene expression in adult male rat testis and pituitary shortly after subacute administration of the antiandrogen flutamide. *J. Reprod. Dev.* **49,** 275–290.

O'Shaughnessy, P. J., Johnston, H., Willerton, L., and Baker, P. J. (2002). Failure of normal adult Leydig cell development in androgen-receptor-deficient mice. *J. Cell Sci.* **115,** 3491–3496.

Pomerantz, D. K. (1980). Developmental changes in the ability of follicle stimulating hormone to stimulate estrogen synthesis in vivo by the testis of the rat. *Biol. Reprod.* **23,** 948–954.

Pomerantz, D. K. (1984). Mechanisms involved in the ability of human chorionic gonadotropin to increase the responsiveness of the infant rat's testis to follicle-stimulating hormone. *Biol. Reprod.* **30,** 119–125.

Rivas, A., Fisher, J. S., McKinnell, C., Atanassova, N., and Sharpe, R. M. (2002). Induction of reproductive tract developmental abnormalities in the male rat by lowering androgen production or action in combination with a low dose of diethylstilbestrol: Evidence for importance of the androgen-estrogen balance. *Endocrinology* **143,** 4797–4808.

Rulli, S. B., Ahtiainen, P., Makela, S., Toppari, J., Poutanen, M., and Huhtaniemi, I. (2003). Elevated steroidogenesis, defective reproductive organs, and infertility in transgenic male mice overexpressing human chorionic gonadotropin. *Endocrinology* **144,** 4980–4990.

Ruiz de Galarreta, C. M., Fanjul, L. F., Meidan, R., and Hsueh, A. J. (1983). Regulation of 3 beta-hydroxysteroid dehydrogenase activity by human chorionic gonadotropin, androgens, and anti-androgens in cultured testicular cells. *J. Biol. Chem.* **258,** 10988–10996.

Saunders, P. T. K., Fisher, J. S., Sharpe, R. M., and Millar, M. R. (1998). Expression of oestrogen receptor beta (ERβ) occurs in multiple cell types, including some germ cells, in the rat testis. *J. Endocrinol.* **156,** R13–R17.

Saunders, P. T. K., Majdic, G., Parte, P., Millar, M. R., Fisher, J. S., Turner, K. J., and Sharpe, R. M. (1997). Fetal and perinatal influence of xenoestrogens on testis gene expression. *Adv. Exp. Med. Biol.* **424,** 99–110.

Shan, L. X., Bardin, C. W., and Hardy, M. P. (1997). Immunohistochemical analysis of androgen effects on androgen receptor expression in developing Leydig and Sertoli cells. *Endocrinology* **138,** 1259–1266.

Sharpe, R. M. (1998). Natural and anthropogenic environmental oestrogens: The scientific basis for risk assessment. Environmental oestrogens and male infertility. *Pure Appl. Chem.* **70,** 1685–1701.

Sharpe, R. M., Rivas, A., Walker, M., McKinnell, C., and Fisher, J. S. (2003). Effect of neonatal treatment of rats with potent or weak (environmental) oestrogens, or with a GnRH antagonist, on Leydig cell development and function through puberty into adulthood. *Int. J. Androl.* **26,** 26–36.

Sohoni, P., and Sumpter, J. P. (1998). Several environmental oestrogens are also anti-androgens. *J. Endocrinol.* **158,** 327–339.

Sweeney, T., Nicol, L., Roche, J. F., and Brooks, A. N. (2000). Maternal exposure to octylphenol suppresses ovine fetal follicle-stimulating hormone secretion, testis size, and sertoli cell number. *Endocrinology* **141,** 2667–2673.

Tapanainen, J., Kuopio, T., Pelliniemi, L. J., and Huhtaniemi, I. (1984). Rat testicular endogenous steroids and number of Leydig cells between the fetal period and sexual maturity. *Biol. Reprod.* **31,** 1027–1035.

Tena-Sempere, M., Navarro, J., Pinilla, L., Gonzalez, L. C., Huhtaniemi, I., and Aguilar, E. (2000). Neonatal exposure to estrogen differentially alters estrogen receptor alpha and beta mRNA expression in rat testis during postnatal development. *J. Endocrinol.* **165,** 345–357.

Tsai-Morris, C. H., Knox, G., Luna, S., and Dufau, M. L. (1986). Acquisition of estradiol-mediated regulatory mechanism of steroidogenesis in cultured fetal rat Leydig cells. *J. Biol. Chem.* **261,** 3471–3474.

van Pelt, A. M., de Rooij, D. G., van der Burg, B., van der Saag, P. T., Gustafsson, J. A., and Kuiper, G. G. (1999). Ontogeny of estrogen receptor-beta expression in rat testis. *Endocrinology* **140,** 478–483.

Vinggaard, A. M., Christiansen, S., Laier, P., Poulsen, M. E., Breinholt, V., Jarfelt, K., Jacobsen, H., Dalgaard, M., Nellemann, C., and Hass, U. (2005). Perinatal exposure to the fungicide prochloraz feminizes the male rat offspring. *Toxicol. Sci.* **85,** 886–897.

White, R., Jobling, S., Hoare, S. A., Sumpter, J. P., and Parker, M. G. (1994). Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* **135,** 175–182.

Williams, K., McKinnell, C., Saunders, P. T. K., Walker, M., Fisher, J. S., Turner, K. J., Atanassova, N., and Sharpe, R. M. (2001). Neonatal exposure to potent and environmental oestrogens and abnormalities of the male reproductive system in the rat: Evidence for importance of the androgen-oestrogen balance and assessment of the relevance to man. *Hum. Reprod. Update* **7,** 236–247.

Yoshida, M., Katsuda, T., Takenaka, A., Watanabe, G., Taya, K., and Maekawa, A. (2001). Effects of neonatal exposure to a high-dose p-tert-octylphenol on the male reproductive tract in rats. *Toxicol. Lett.* **121,** 21–33.

You, L., and Sar, M. (1998). Androgen receptor expression in the testes and epididymides of prenatal and postnatal Sprague-Dawley rats. *Endocrine* **9,** 253–261.