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Comparative Contribution of the Aryl Hydrocarbon Receptor Gene to Perinatal Stage Development and Dioxin-Induced Toxicity Between the Urogenital Complex and Testis in the Mouse[1]

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ABSTRACT

TCDD (2,3,7,8-tetrachlorodebenzo-*p*-dioxin) requires the presence of the aryl hydrocarbon receptor (*Ahr*) gene for its toxic effects, such as reproductive disorders in male offspring of maternally exposed rats and mice. To study the involvement of the *Ahr* gene in producing the toxic phenotype with respect to testicular development, we administered a relatively high dose of TCDD to mice with three different maternally derived *Ahr* genotypic traits, and then compared several *Ahr*-dependent alterations among male reproductive systems on Postnatal Day 14. Reduction in anogenital distance and expression of prostatic epithelial genes in the urogenital complex (UGC) were detected in $Ahr^{+/+}$ and $Ahr^{+/-}$ mice exposed to TCDD, whereas no difference was observed in $Ahr^{-/-}$ mice. In situ hybridization revealed the absence of probasin mRNA expression in the prostate epithelium, despite the obvious development of prostatic lobes in TCDD-exposed mice. In contrast to obvious prostatic dysfunction and induction of cytochrome P450 (CYP) family genes in the UGC by TCDD, no alterations in testicular functions were observed in germ cell/Sertoli cell/interstitial cell marker gene expression or CYP family induction. No histopathological changes were observed among the three genotypes and between control and TCDD-exposed mice. Therefore, mouse external genitalia and prostatic development are much more sensitive to TCDD treatment than testis. Further, the *Ahr* gene, analyzed in this study, does not significantly contribute to testicular function during perinatal and immature stages, and the developing mouse testis appears to be quite resistant to TCDD exposure.

aryl hydrocarbon receptor, developmental biology, dioxin, knockout mouse, prostate, spermatogenesis, testis, toxicology

INTRODUCTION

TCDD (2,3,7,8-tetrachlorodebenzo-*p*-dioxin) is an extremely potent xenobiotic chemical. Maternal exposure to TCDD induces a wide range of physiological alterations and toxicities in the fetus and pups of laboratory animals and perhaps in humans [1]. TCDD induces various toxicological endpoints in male reproductive organs, such as decreased size of sex-accessory glands and reduced sperm counts in testis, epididymis, and ejaculate [2–10]. Although male rat and mouse offspring exposed to TCDD in utero can produce testicular androgen normally, the androgen responsiveness of the ventral prostate is lowered by unknown mechanisms of TCDD [9–12].

Interestingly, the effects of TCDD on testicular development and perinatal stage spermatogenesis are still unclear because the results are contradictory [2–10]. Some studies on testicular development that used conventional experimental animals reported the presence of slight reductions in testicular weight, daily sperm production, and steroidogenesis [2–5]. On the other hand, clear negative data concerning the TCDD effect on testicular development were presented in many other papers [7–10]. Taken together, the impairment in prostate development by in utero TCDD exposure appears to occur in many mammals, including rats and mice, but it is not understood whether or how susceptible the testicular development and perinatal stage spermatogenesis is to the TCDD exposure.

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mostly mediates inductions of drug-metabolizing enzymes such as members of the CYP1A family, and it appears to act as a sensor for environmental contaminants such as dioxins and polyaromatic hydrocarbons [13, 14]. Experimental studies with *Ahr* gene knockout mice have already revealed that AHR plays an essential role in the occurrence of multiple TCDD-induced adverse effects, including teratogenic cleft palate and hydronephrosis [15, 16]. Reproductive disorders, such as prostatic growth impairment in male offspring following maternal exposure to TCDD, were also shown to be dependent on the *Ahr* gene [17].

Some evidence also suggested that AHR was involved in developmental signaling in the immune and hepatic systems [18, 19]. In analyses using *Ahr*-null female mice, AHR was

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also required for normal ovarian germ cell dynamics, based on the observation that numbers of nonatretic primordial, primary, and small preantral follicles in ovaries of *Ahr*-null females at early postpartum stages were higher than those in wild-type mice [20–22]. Moreover, AHR cooperates with an orphan nuclear receptor, NR5A1 (also known as Ad4BP/SF-1), to activate P450 aromatase (CYP19) gene transcription in ovarian granulosa cells and modulate endogenous estrogen production in the female reproductive cycle [23]. AHR is also expressed in interstitial cells and male germ cells at specific stages [24]. More recently, AHR has been demonstrated to have a ubiquitin ligase activity, which enhances the degradation of estrogen and androgen receptors, suggesting that AHR may modulate androgen sensitivity in normal development [25]. To date, the possible involvement of AHR in testicular development during immature stages using AHR agonist administration or *Ahr*-null male mice has not been addressed.

To the best of our knowledge, this is the first study that used *Ahr*-null mice to investigate the involvement of the *Ahr* gene in early stages of testicular development and to assess the differences in susceptibility to in utero TCDD exposure between prostate and testicular development.

MATERIALS AND METHODS

Materials

TCDD was purchased from Cambridge Isotope Laboratory (Andover, MA). The purity was higher than 99.5%. Corn oil for dissolving TCDD or the control vehicle was obtained from Sigma-Aldrich (St. Louis, MO). TRIzol reagent, SuperScript III RNase H- Reverse Transcriptase, and oligo(dT)12–18 primer were purchased from Invitrogen (Carlsbad, CA). SYBR Premix Ex Taq (Perfect Real Time) was purchased from TAKARA BIO, Inc. (Otsu, Japan). The plasmid pGEM-TEasy vector was obtained from Promega Corp. (Madison, WI).

Animals and TCDD Administration

Ahr knockout mice were kindly provided by Dr. Yoshiaki Fujii-Kuriyama (Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba) [15]. They were bred in our own facility at the National Institute for Environmental Studies (NIES). All described procedures were approved by the NIES Institutional Animal Care and Use Committee and were performed in accordance with the Guidelines for Animal Experiments at the NIES. They were maintained in a controlled environment of temperature 24 ± 1°C, humidity 45 ± 5%, and a 12L:12D cycle and were given food and distilled water ad libitum. *Ahr* heterozygous male mice were back-crossed with wild-type female C57BL/6J mice (CLEA Japan, Tokyo, Japan) six times, and heterozygous offspring of both sexes were used in this study. The heterozygous female mice (7- to 10-wk-old) were mated 1:1 with the heterozygous males overnight, and the females that had a vaginal plug on the following morning were designated as being pregnant at Gestational Day 0 (GD0). Dams were housed individually in clear plastic cages with heat-treated wood chips as bedding. On GD13, pregnant mice were given a single dose of TCDD orally (10 μg/kg body weight, close to a lethal dose for a C57BL/6J fetus) or an equivalent volume of vehicle (95% corn oil, 4% n-nonane; 5 ml/kg) as control. Male pups were killed under diethyl ether anesthesia on Postnatal Day 14 (PND14).

Sample Collection

On PND14, immediately before euthanization, the anogenital distance, determined by the length from the base of the genital tubercle to the anterior edge of the anus, was measured with a digital caliper. We also measured the crown-anal length (the distance between the nose and anterior edge of the anus). The testis and epididymis on both sides were excised from the abdomen, and the surrounding adipose tissue was carefully removed. After removing urine from the bladder, the deferent ducts were cut at the base of the bladder. The urogenital complex (UGC), which is a small mass comprising all the lobes of the prostate and seminal vesicle, was then collected by cutting the anterior end of the urethra. All tissue samples were frozen in liquid nitrogen immediately after dissection and kept at −80°C until RNA extraction.

Real-Time RT-PCR

The protocols for real-time RT-PCR quantifications were described previously [26]. Briefly, total RNA was extracted from the UGC (n = 5) and testis (n = 3) using TRIzol reagent. RNA samples were reverse-transcribed with SuperScript III reverse transcriptase and oligo(dT)12–18 primer. Nineteen genes examined in this study: aryl hydrocarbon receptor (*Ahr*), cytochrome P450 1A1 (*Cyp1a1*), cytochrome P450 1A2 (*Cyp1a2*), cytochrome P450 1B1 (*Cyp1b1*), androgen receptor (*Ar*), steroid 5α-reductase type 1 (*Srd5a1*), steroid 5α-reductase type 2 (*Srd5a2*), probasin (*Pbsn*), Mp25 (*Sbp*), PSP94 (*Msmb*), calnexin-t (*Clgn*), Hsp70.2 (*Hspa2*), androgen-binding protein (*Shbg*), cytochrome P450 side chain cleavage (*Cyp11a1*), cytochrome P450 17α/C_{17-20} lyase (*Cyp17a1*), 3β-hydroxysteroid dehydrogenase type I (*Hsd3b1*), 3α-hydroxysteroid dehydrogenase type I (*Akr1c4*), 17β-hydroxysteroid dehydrogenase type III (*Hsd17b3*), and cyclophilin B (*Ppib*). All primer sets are shown in Supplemental Table S1 (available online at www.biolreprod.org). For the real-time RT-PCR, target genes were amplified with SYBR Premix Ex Taq (Perfect Real Time) system by using a LightCycler (Roche, Manheim, Germany). The relative expression level was calculated by normalizing with the average value of each control wild-type ($Ahr^{+/+}$) group. To determine the sequences, the PCR product for each gene was subcloned into pGEM-TEasy vectors or directly sequenced by the dideoxynucleotide chain termination method using the ABI Prism BigDye terminator cycle sequencing kit (PE-Biosystems, Foster City, CA).

In Situ Hybridization

Mouse probasin mRNA was detected in the UGC specimens by in situ hybridization. The UGCs were fixed with neutralized formalin for 48 h, embedded in paraffin, and then cut into 4-μm sections. A template was amplified from the pGEM-TEasy vector inserted with a 377-bp probasin (*Pbsn*) RT-PCR fragment by T7 and SP6 primers to generate sense and antisense transcripts. Digoxigenin-labeled riboprobes were used, and the hybridization was performed using an automated in situ hybridization instrument Gen II (Ventana Medical System, Tucson, AZ). Detection and counterstaining were done with the BlueMap Kit (Ventana Medical System) and Nuclear Fast Red (Sigma-Aldrich).

Immunohistochemistry

Anti-calnexin-t (CLGN), a male germ cell developmental stage-specific protein, was immunostained by the method described previously [27]. Briefly, testes (n = 3; left side) were fixed with Bouin solution and embedded in paraffin. Two different cross-sectional regions (4-μm thickness) from one testis were obtained (six sections from each group). Deparaffinized sections were incubated with anti-mouse calnexin-t antibody, followed by incubation with peroxidase-conjugated goat anti-mouse or rabbit IgG. After washing with PBS, immunoreactivity was detected with diaminobenzidine, followed by hematoxylin counterstaining. Morphometric measurement of the amount of CLGN-positive germ cells was carried out using AxioVision version 4.5 software (Carl Zeiss Co., Ltd., Oberkochen, Germany). The total area of seminiferous tubules within the 1-mm^2 area of the cross-sections from each genotype and treatment group was traced and summed. Then, the CLGN-positive cell numbers were counted and divided by the total area traced for the seminiferous tubules.

Testosterone Assay

Testicular testosterone levels were determined by the enzyme immunoassay (EIA) Kit (Cayman Chemical Co., Ann Arbor, MI). The frozen testis was homogenized in PBS, and the protein concentration was measured by the BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL). The homogenate was then extracted with diethyl ether, and the ether phase was air-dried. The dried lipophilic substances were resuspended in the appropriate volume of EIA buffer, and the measurements were done according to the manufacturer's instructions.

Statistical Analysis

For statistical analysis, StatView for Windows version 5.0 (SAS Institute, Cary, NC) was used. All data were expressed relative to the means of the control groups. All results are represented as the mean ± SE. Two-way ANOVA was used for comparison of a given parameter among three control groups ($Ahr^{+/+}$, $Ahr^{+/-}$, $Ahr^{-/-}$), followed by the Fisher PLSD post hoc test. $P < 0.05$ was considered significant.

TABLE 1. Reproductive outcomes of male mice exposed to TCDD in utero.[a]

Parameter	Genotype		
	$Ahr^{+/+}$	$Ahr^{+/-}$	$Ahr^{-/-}$
No. of male pups			
Control (n)[b,c]	17 (1.70)	14 (1.40)	6 (0.60)
TCDD (n)[b,d]	6 (0.40)	16 (1.07)	9 (0.60)
Body weight (g)			
Control	6.82 ± 0.41	7.16 ± 0.23	6.04 ± 0.73
TCDD	5.49 ± 0.59	6.59 ± 0.55	7.55 ± 0.27
Crown-anal length (mm)			
Control	54.1 ± 1.2	55.7 ± 0.9	53.0 ± 2.2
TCDD	50.4 ± 2.0	53.1 ± 2.0	56.3 ± 0.9
Anogenital distance (mm)			
Control	3.82 ± 0.11	4.05 ± 0.19	3.66 ± 0.31
TCDD	3.19 ± 0.28*	3.27 ± 0.12**	3.33 ± 0.09
Testicular testosterone level (pg/mg protein)			
Control	140 ± 72 (n = 8)	173 ± 112 (n = 8)	141 ± 86 (n = 5)
TCDD	179 ± 73 (n = 5)	124 ± 33 (n = 8)	197 ± 126 (n = 5)

[a] Data are expressed as means ± SEM, and significant differences were analyzed with ANOVA followed by Fisher PLSD test (versus control of the same genotype, $*P < 0.05$, $**P < 0.01$).
[b] n = The number of male pups per litter.
[c] The number of dams = 10.
[d] The number of dams = 15.

RESULTS

Reproductive Outcome

The number, body weight, and crown-anal length of male pups in each genotype on PND14 are represented in Table 1. There were no statistically significant differences in body weight or crown-anal length among the three genotypes, or between control and TCDD-exposed groups. The number of $Ahr^{+/+}$ male pups exposed to TCDD was only six, which was much lower than the number of control pups, probably due to the fetal death by TCDD exposure in this genotype. Anogenital distance of $Ahr^{+/+}$ and $Ahr^{+/-}$ male pups in the TCDD-exposed groups was significantly reduced compared to control groups ($P = 0.016$ and $P = 0.001$, respectively). In contrast, the anogenital distance of $Ahr^{-/-}$ mice was not significantly different between TCDD-exposed and control mice. ANOVA did not reveal any significant differences in the mean anogenital distance among the three genotypes.

Gene Expressions in UGC

Quantitative RT-PCR analysis of the UGC on PND14 showed that *Ahr* mRNA was detected in $Ahr^{+/+}$ and $Ahr^{+/-}$ mice but not in the $Ahr^{-/-}$ mice. The expression level in the $Ahr^{+/+}$ group was 2-fold higher than that in the $Ahr^{+/-}$ mice, suggesting *Ahr* is transcribed from both alleles in the wild-type mice (Fig. 1). *Cyp1a1*, *Cyp1a2*, and *Cyp1b1* mRNAs, biomarkers of dioxin exposure, were not induced by TCDD

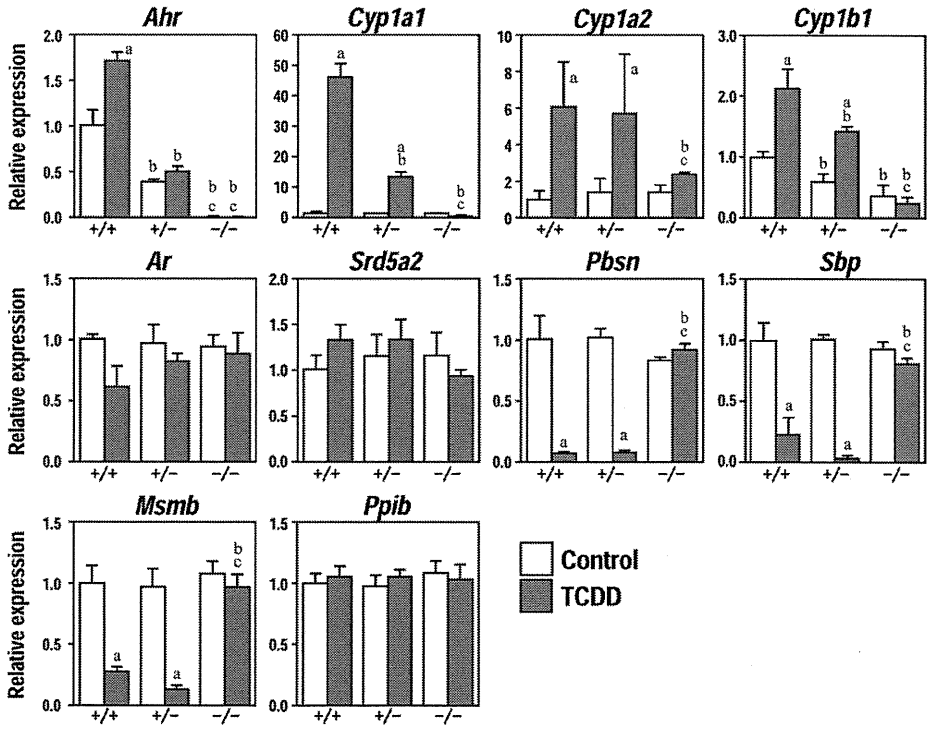

FIG. 1. Quantitative RT-PCR analysis of gene expressions in the urogenital complex of male mouse offspring of three *Ahr* genotypes ($Ahr^{+/+}$, $Ahr^{+/-}$, and $Ahr^{-/-}$) on PND14 with or without TCDD exposure in utero. The values are expressed as mean ± SE for three samples from each group. Note that in utero and lactational exposure to TCDD significantly upregulated *Cyp1a1* and *Cyp1b1* in $Ahr^{+/+}$ and $Ahr^{+/-}$ mice, but not in $Ahr^{-/-}$ mice and that it completely suppressed mRNA expression of the prostatic secretory protein markers *Pbsn*, *Sbp*, and *Msmb* in $Ahr^{+/+}$ and $Ahr^{+/-}$ mice. Significant differences were analyzed with ANOVA followed by the Fisher PLSD test (a, versus control of the same genotype; b, versus the same treatment of wild type; c, versus the same treatment of heterozygous; $P < 0.05$).

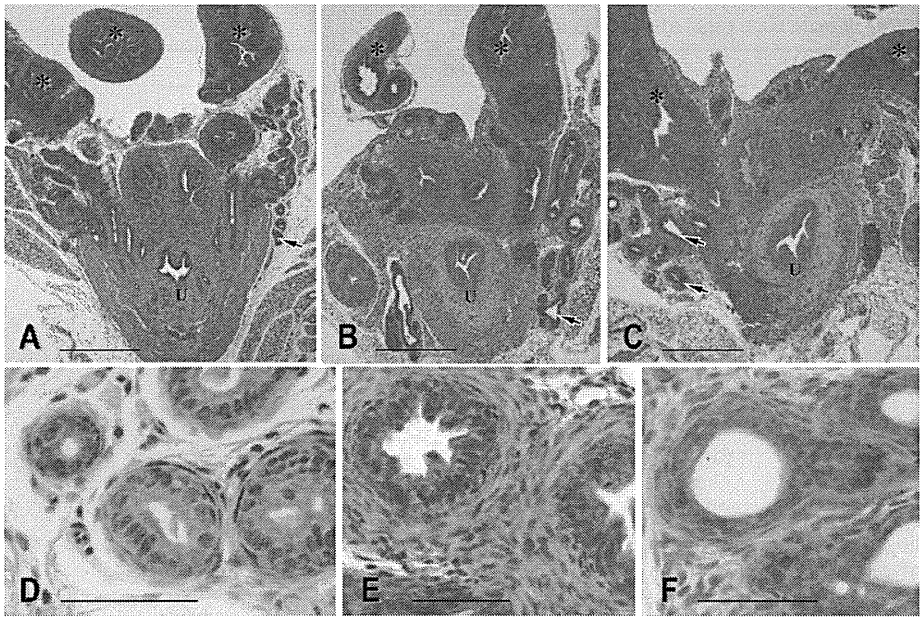

FIG. 2. Histological examinations of the UGC in male mouse pups on PND14. Hematoxylin-eosin staining of $Ahr^{+/+}$ UGCs (**A** and **D**, control; **B**, **C**, **E**, and **F**, TCDD-exposed). Note that control mice had well-developed prostatic lobes on PND14 (**A**). Fewer layers of epithelial cells and increased cell numbers in mesenchymal cells were observed in the dorsolateral prostate lobe of TCDD-exposed animals compared to controls (**E** and **F**). Asterisks, seminal vesicle; arrows, dorsolateral prostate; U, urethra. Bars = 500 μm (**A**, **B**, and **C**), 50 μm (**D**, **E**, and **F**).

in the $Ahr^{-/-}$ mice, but were significantly upregulated in $Ahr^{+/+}$ and $Ahr^{+/-}$ mice. In $Ahr^{+/+}$ and $Ahr^{+/-}$ mice, the *Cyp1b1* expression level in the TCDD-exposed group was higher than that in the control group (Fig. 1). Although a slight decrease of *Ar* mRNA was seen in the $Ahr^{+/+}$ mice (TCDD exposed), we did not detect any significant change in *Ar* and *Srd5a2* mRNA levels among the three TCDD-exposed genotypes. *Pbsn* (dorsolateral), *Sbp* (ventral), and *Msmb* (lateral) were used to investigate functional cytodifferentiation levels of each prostatic epithelia, as reported by others [28]. These three prostate markers were expressed in the control UGCs on PND14. They were barely detectable in TCDD-exposed $Ahr^{+/+}$ and $Ahr^{+/-}$ mice, while TCDD-exposed $Ahr^{-/-}$ mice had quantities of these three marker mRNAs that were very similar to control mice (Fig. 1).

Histopathology of the Urogenital Complex

Prostatic lobes were found to be well developed in the control animals (Fig. 2, A and D). In the TCDD-exposed $Ahr^{+/+}$ animals, the prostatic lobes with existing epithelial layers were clearly observed (Fig. 2, B, C, E, and F). In situ hybridization analysis of the tissue section adjacent to the sections used for histopathological examinations revealed that epithelial cells of dorsolateral prostate lobes had *Pbsn* mRNA signals (Fig. 3A). In accordance with the RT-PCR data, no signals were detected in the epithelia of TCDD-exposed dorsolateral prostates (Fig. 3C).

Gene Expressions in Testis

Consistent with the analysis of UGC (Fig. 1), the expression of *Ahr* mRNA was not detected in the $Ahr^{-/-}$ testis, whereas the *Ahr* expression level was 2-fold higher in the $Ahr^{+/+}$ than in the $Ahr^{+/-}$ mice. No differences were detected in *Ar* mRNA levels (Fig. 4). Among the three CYP1 genes tested, *Cyp1a1* mRNA was not detected in any of the *Ahr* genotypes (data not shown). Although *Cyp1a2* and *Cyp1b1* mRNA was detected in the testis, there were no statistically significant differences among the three genotypes and between the control and TCDD-exposed testes (Fig. 4). The mRNA of *CLGN* and *Hspa2*, male germ cell-specific markers expressed in the pachytene stage of spermatocytes [29, 30], was observed at the same levels among the three genotypes, regardless of TCDD exposure (Fig. 4). No difference was observed in the expression level of *Shbg*, a protein secreted from Sertoli cells [31]. RNA expression levels of four steroidogenic enzyme genes for testosterone synthesis, *Cyp11a1*, *Cyp17a1*, *Hsd3b1*, and *Hsd17b3*, were not affected in the three genotypes under the TCDD dosing regimen used (Fig. 4). Consistently, intratesticular testosterone levels in all genotypes and TCDD-exposed animals were not changed among the three genotypes, regardless of TCDD exposure (Table 1). Additionally the mRNA of *Akr1c4* and *Srd5a1* enzymes for synthesis of 5α-androstane-3α, 17β-diol, the major form of testicular androgen in immature mice [32], was not altered by TCDD exposure and showed no differences among the three genotypes in the testes (Fig. 4).

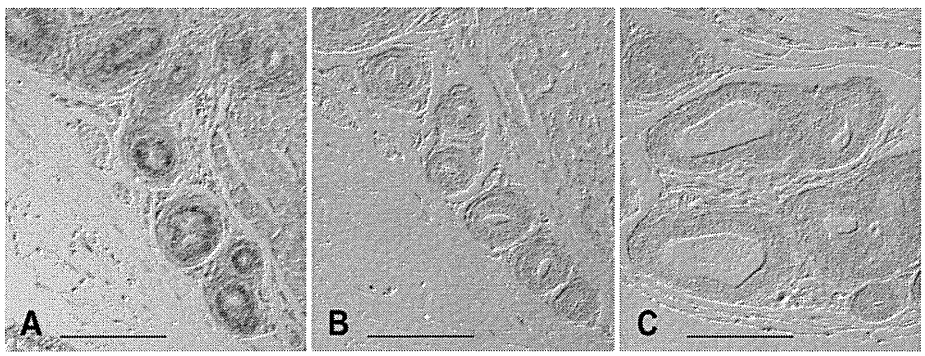

FIG. 3. In situ hybridization analysis of *Pbsn* mRNA expression in the dorsolateral prostate lobe of $Ahr^{+/+}$ mouse UGCs on PND14. **A**) Antisense probe for control mouse. **B**) Sense probe for control mouse. **C**) Antisense probe for TCDD-exposed mouse. Note that epithelial cells of the dorsolateral prostate lobes show *Pbsn* mRNA signals in control mice (**A**), but not in TCDD-exposed mice (**C**). Bar = 200 μm.

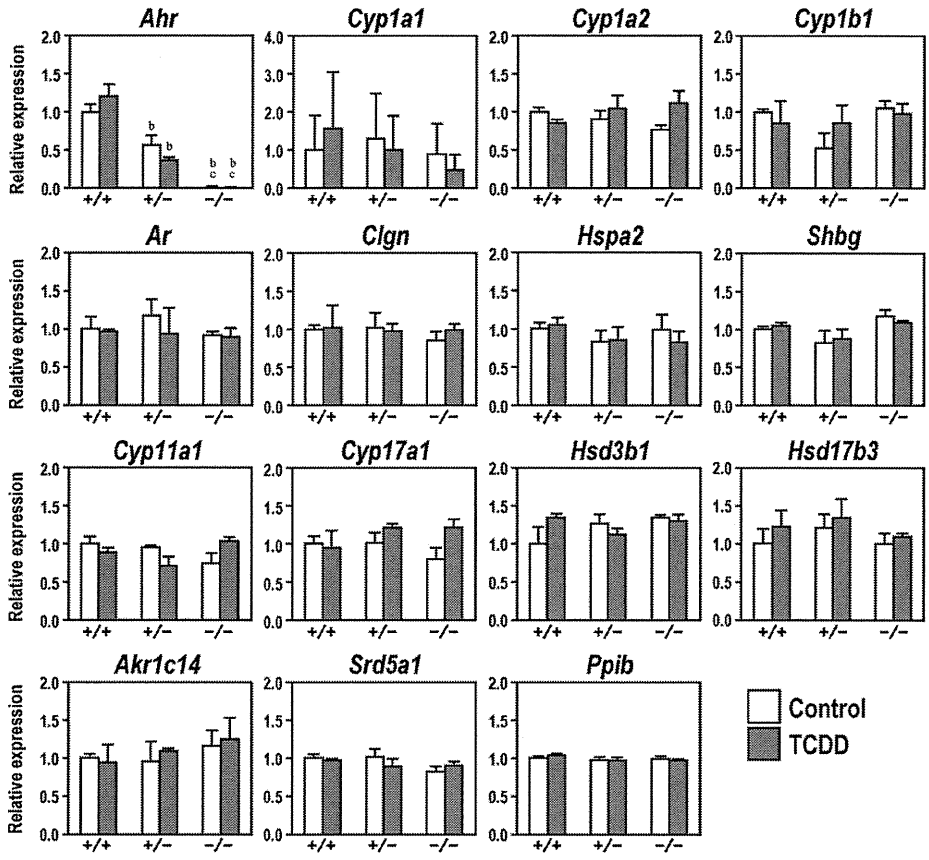

FIG. 4. Quantitative RT-PCR analysis of gene expression in the testes of male pups of three *Ahr* genotypes ($Ahr^{+/+}$, $Ahr^{+/-}$, and $Ahr^{-/-}$) on PND14 with or without TCDD exposure in utero. The values are expressed as the mean ± SE for three samples from each group. Significant differences were analyzed with ANOVA followed by the Fisher PLSD test (b, versus the same treatment of wild-type; c, versus the same treatment of heterozygous; $P < 0.01$).

Histopathology of the Testis

Spermatocytes at the pachytene stage proliferate from the spermatogonium on PND14, and calnexin-t is expressed at this stage [33]. In the present study, germ cells from the three mouse genotypes were immunostained for calnexin-t (Fig. 5, A–C). Positive cell populations were similar in control and TCDD-exposed testes from all genotypes (Fig. 5, D–F).

DISCUSSION

Ahr-*Dependent Reduction in Anogenital Distance and Impairment of Prostatic Development by In Utero and Lactational TCDD Exposure*

In this study, we demonstrated that in utero and lactational TCDD exposure caused reduction of the anogenital distance and impairment of prostatic development in an *Ahr*-dependent manner, because reduction of anogenital distance and disappearance of prostatic epithelial protein mRNAs were observed in $Ahr^{+/+}$ and $Ahr^{+/-}$ but not in the $Ahr^{-/-}$ offspring. The induction of *Cyp1a1* and *Cyp1b1* mRNAs was also observed in $Ahr^{+/+}$ and $Ahr^{+/-}$ mice but not in $Ahr^{-/-}$ mice. Taken together, impairment to the male reproductive system by in utero and lactational TCDD exposure was mainly dependent on the fetal *Ahr* gene.

A study using *Ahr* knockout mice that produced results similar to ours has been reported [17]. In that study, $Ahr^{+/-}$ female and $Ahr^{+/-}$ male mice were mated, and TCDD (5 μg/kg) was injected into the pregnant mice on GD13. The levels of prostatic protein markers were reduced in the TCDD-exposed $Ahr^{+/+}$ mice on PND90, but not in the $Ahr^{-/-}$ mice. Furthermore, those authors reported that TCDD administration on GD13 severely inhibited prostatic bud formation from the urogenital sinus in the fetus [28]. The use of in an vitro organ culture system as well as *Ahr* knockout mice revealed that the inhibition was mediated by AHR expressed in the mesenchy-

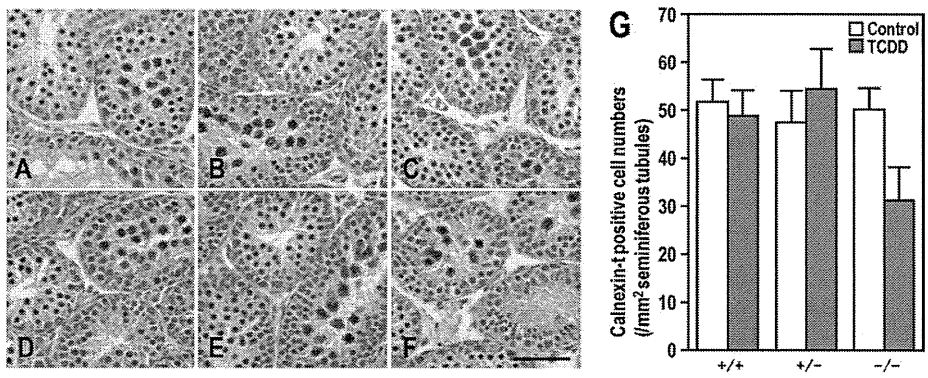

FIG. 5. Immunostaining of CLGN in the testes of male pups on PND14. Testis tissue preparations from each genotype (n = 3) were immunostained. **A**) Control $Ahr^{+/+}$ testis. **B**) Control $Ahr^{+/-}$ testis. **C**) Control $Ahr^{-/-}$ testis. **D**) TCDD-exposed $Ahr^{+/+}$ testis. **E**) TCDD-exposed $Ahr^{+/-}$ testis. **F**) TCDD-exposed $Ahr^{-/-}$ testis. CLGN-positive spermatocytes were observed in all genotypes and TCDD-treatment groups. **G**) Morphometric analysis. There were no statistical differences in the number of positive cells per testis cross section, for all genotypes and treatments. Bar = 100 μm.

mal cells of the urogenital sinus [34] and was not caused by interruption of androgen signaling in this tissue [35]. It is still unclear how TCDD impairs the responsiveness of the developing prostate to androgen.

In Utero and Lactational TCDD Exposure Decreased Androgen Responsiveness of the Prostate

In the histological examinations, the prostatic lobes developed with obvious epithelial layers in TCDD-exposed $Ahr^{+/+}$ mice, indicating TCDD-exposed prostates may function as normal exocrine glands (Fig. 2, B and C). However, using RT-PCR and in situ hybridization analyses, we could not detect prostatic epithelial secretory protein mRNAs in the TCDD-exposed UGCs (Figs. 1 and 3). Fewer layers of epithelial cells and increased cell numbers in mesenchymal cells were observed in the TCDD-exposed $Ahr^{+/+}$ animals, suggesting that in utero and lactational exposure to TCDD produced functional abnormalities (Fig. 2, E and F). The prostatic secretory proteins, PBSN, MSMB, and SBP, were reportedly upregulated via the androgen receptor [36–38]. Since no significant differences in intratesticular testosterone levels were found between control and TCDD-exposed animals on PND14, we speculated that abnormal development of prostate glands may be due to decreased androgen sensitivity or that TCDD disrupts mouse prostate epithelial cell differentiation into luminal epithelial cells. This notion is consistent with a previous study [9] in the sense that the ventral prostate of male rat offspring exposed to TCDD in utero and via lactation did not respond to the exogenous androgens testosterone, 5α-dihydrotestosterone (DHT), and 5α-androstane-3α, 17β-diol, in the organ culture system. Administration of the androgen receptor antagonist flutamide and the 5α-reductase inhibitor finasteride to rats in the late pregnancy period did not cause prostate growth in male offspring on PND60 [39], suggesting that DHT is an essential steroid hormone for prostate development. Since the inhibition was found specifically in the prostate but not in the seminal vesicle, the reduction in DHT production in the prostate was initially hypothesized to occur in males exposed to TCDD in utero and via lactation. However, 5α-reductase type 2 enzymatic activity and mRNA expression was elevated, compared to control groups [9, 10]. Thus, it is reasonable to speculate that decreased androgen responsiveness in the TCDD-exposed offspring was caused by other factors. Our previous study using Holtzman rats showed significantly reduced androgen receptor mRNA expression in the ventral prostate following in utero and lactational TCDD exposure, suggesting that the decreased androgen responsiveness might be due to reduced amounts of receptor molecules [10]. However, we could not detect a significant reduction in androgen receptor mRNA here, probably due to a difference in animal species or organs used for RT-PCR analysis.

Function of AHR in the Testis

AHR was reportedly responsible for apoptotic signaling, and the number of primordial cells in the ovarian germ line was not attenuated due to a defect in the apoptosis in *Ahr*-null female mice [20]. More recently, testicular dysfunction was reported in aged *Ahr*-null mice [40], and HSD3B1 expression in Leydig cells was significantly reduced at 24 wk, resulting in serum testosterone decline, lowered sperm number, and reduced size of seminal vesicles. AHR seems to play a role in maintaining normal steroidogenesis in aged animals. However, in that study, there were no significant differences in testicular functions between wild-type and *Ahr*-null mice during younger stages (10 wk old). In our present study, we were unable to find differences in testicular functions among the *Ahr* genotypes, including testosterone production, Sertoli cell differentiation, and spermatogenic cell differentiation. Therefore, it is reasonable to conclude that AHR has very little function in early stages of gonad development. If the AHR functions even at the early stage of development, the functional redundancy with other genes may also exist among *Ahr* and other genes during testicular development.

Resistance of Testicular Development to In Utero and Lactational TCDD Exposure

In our previous study, TCDD administration to pregnant Holtzman rats on GD15 did not alter the testicular weight or serum testosterone levels of male offspring on PND49 [10]. In our present study, we did not find any differences in testicular cell differentiation levels between control *Ahr*-carrying animals and TCDD-exposed animals that survived the high dose of TCDD exposure, including supporting cell marker and spermatogenic cell differentiation markers, which suggested that even in the TCDD-exposed animals testicular differentiation proceeded normally. 5α-androstane-3α, 17β-diol, the major form of testicular androgen in immature mice [32], was previously reported to be slightly reduced in PND21 mice perinatally exposed to TCDD [17]. However, in our present study, in the assayed testes from PND14, no difference was seen in both *Akr1c4* and *Srd5a1* mRNA expression levels among the three genotypes and TCDD treatment. Moreover, in utero and lactational exposure to TCDD did not alter the expression levels of steroidogenic enzyme genes in Leydig cells in $Ahr^{+/+}$ and $Ahr^{+/-}$ animals. At a relatively high dose, testicular CYP11A1 activity was reduced by TCDD exposure [41]. In our previous report, we also found that administration of 100 μg TCDD/kg to adult male mice reduced testicular *Cyp11a1* mRNA and protein levels [42], and in vitro co-planer PCB (3,3′,4,4′,5-pentachlorobiphenyl; PCB126) exposure to neonatal mouse testis downregulated *Cyp11a1* mRNA expression [33]. The reason why we could not detect the reduction in *Cyp11a1* mRNA in the present study is not clear, but it is speculated that intratesticular levels of TCDD in male pups born to dams given TCDD on GD13 was not sufficient to downregulate *Cyp11a1* by PND14.

Although testes and UGCs were collected from the same individual pups, both *Cyp1a2* and *Cyp1b1* mRNAs were not induced in the testes of mice with any of the three genotypes, whereas in the UGCs, *Cyp1a2* and *Cyp1b1* were significantly induced. Moreover, an approximately 30-fold increase in *Cyp1a1* was observed in the $Ahr^{+/+}$ UGCs. Thus, UGCs are much more sensitive to TCDD than testis in terms of *Cyp1a1* mRNA induction. Using a xenobiotic-responsive element connected to the β-galactocidase reporter gene, a transgenic mouse line was generated and then exposed to TCDD in utero and via lactation [43]. X-Gal staining analysis clearly demonstrated that fetal urogenital tracts showed significant induction of the reporter gene, but that the testis did not respond. Based on the above-mentioned results, we speculated that UGCs express modulating factors (modifiers) that enhance AHR-mediated transcription, and that these were lacking or present in small amounts in the testis. Although it cannot be excluded that tissue concentration of TCDD in the testis was lower than that in the UGC, it is more likely that the testis is much more resistant to TCDD exposure than the UGC.

In conclusion, using *Ahr* knockout mice, we confirmed that in utero exposure to TCDD caused *Ahr*-dependent impairment of prostate development and reduced anogenital distances in

male offspring, but that the testicular development seemed to be resistant to TCDD exposure. AHR was not associated with testicular development under physiological conditions.

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REPRODUCTION
RESEARCH

Induction of spermatogenic cell apoptosis in prepubertal rat testes irrespective of testicular steroidogenesis: a possible estrogenic effect of di(*n*-butyl) phthalate

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Abstract

Although di(*n*-butyl) phthalate (DBP), a suspected endocrine disruptor, induces testicular atrophy in prepubertal male rats, whether it exerts estrogenic activity *in vivo* remains a matter of debate. In the present study, we explored the estrogenic potency of DBP using 3-week-old male rats, and then examined the relationship between estrogen-induced spermatogenic cell apoptosis and testicular steroidogenesis. Daily exposure to DBP for 7 days caused testicular atrophy due to loss of spermatogenic cells, whereas testicular steroidogenesis was almost the same with the control values. A single exposure of DBP decreased testicular steroidogenesis in addition to decreasing the level of serum LH at 3 h after DBP treatment, with an extremely high incidence of apoptotic spermatogenic cells at 6 h after administration. To elucidate the estrogenic activity of DBP, we carried out an inhibition study using pure antiestrogen ICI 182,780 (ICI) in a model of spermatogenic cell apoptosis induced by DBP or estradial-3-benzoate (EB). Although both the DBP- and EB-treated groups showed a significant increase in spermatogenic cell apoptosis, ICI pretreatment significantly decreased the number of apoptotic spermatogenic cells in these two groups. In contrast, testicular steroidogenesis and serum FSH were significantly reduced in all the treated groups, even in the DBP+ICI and EB+ICI groups. Taken together, these findings led us to conclude that estrogenic compounds such as DBP and EB induce spermatogenic cell apoptosis in prepubertal rats, probably by activating estrogen receptors in testis, and that reduction in testicular steroidogenic function induced by estrogenic compounds is not associated with spermatogenic cell apoptosis.
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Introduction

Di(*n*-butyl) phthalate (DBP) is one of the most widely studied phthalate esters that disrupt the growth of normal reproductive organs, because of its wide use as a plasticizer in cosmetics, printing inks, and pharmaceutical coatings. The most prominent effect of DBP is testicular atrophy (Oishi & Hiraga 1980, Gray *et al.* 1982). To date, several mechanisms have been proposed to explain the induction of testicular atrophy by DBP, such as the depletion of zinc (Oishi & Hiraga 1980), increased oxidative damage of proteins, lipids, and DNA (Fukuoka *et al.* 1990), alteration of vimentin cytoskeleton organization (Kleymenova *et al.* 2005), or membrane alteration in Sertoli cells leading to sloughing of spermatogenic cells (Kleymenova *et al.* 2005). However, the cellular target of DBP and molecular mechanisms of DBP-induced spermatogenic cell apoptosis remain to be unknown.

The study on serum levels of DBP in thelarche patients (a premature breast development before age 8) showed that 28 of 41 (68%) thelarche patients displayed significantly higher (15–276 mg/l) levels of DBP (Colon *et al.* 2000). The high-serum level of DBP in thelarche patients is a matter of concern as an estrogenic xenobiotic. DBP and butyl benzyl phthalate were found to be capable of binding to estrogen receptor α (ERα) and then enhancing the proliferation of MCF-7 human breast cancer cells expressing ERα (Jobling *et al.* 1995, Harris *et al.* 1997, Zacharewski *et al.* 1998, Nishihara *et al.* 2000). In addition, DBP has been shown to exhibit an

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estrogenicity in an E-screen assay (Soto *et al.* 1995, Hong *et al.* 2005) and to prevent tamoxifen, an ER antagonist, from inducing apoptosis in MCF-7 cells (Kim *et al.* 2004). In contrast, phthalate esters including DBP displayed no biological activity in a rat uterotrophic assay, an *in vivo* screening test for estrogenicity (Milligan *et al.* 1998, Zacharewski *et al.* 1998). Therefore, the specific cellular target of DBP is still a matter of debate.

The role of estrogen in spermatogenesis has been reported that estrogen is essential for spermatogenesis (Eddy *et al.* 1996, O'Donnell *et al.* 2001), spermatogenic cells express ERs (Saunders *et al.* 1998, O'Donnell *et al.* 2001), and estrogen-like chemicals present in the environment adversely affect male reproductive health (Akingbemi & Hardy 2001). Such chemicals have the ability to affect gene expression and cellular function by binding to hormone receptors (Hall & Korach 2002). It is generally accepted that the hypothalamo-pituitary–gonadal (HPG) axis regulates spermatogenesis by controlling circulating levels of LH through the feedback regulation of steroid hormones, and this feedback loop can be intercepted by endocrine-disrupting chemicals (EDCs) binding to ERs in hypothalamus. Direct interference to spermatogenic cells is also possible, because these cells are known to express ERs (Saunders *et al.* 1998, 2002). Adult male hamsters given diethylstilbestrol showed a significant decrease in gonadotropin levels, leading to the increased spermatogenic cell apoptosis through the suppression of testosterone level (Nonclercq *et al.* 1996). In another study, administration of a single high dose of estradial-3-benzoate (EB) to 1-day-old male rats causes a reduction in GnRH secretion, resulting in the suppression of circulating level of LH and consequently testosterone (Tena-Sempere *et al.* 2000). Estrogen is directly involved in the suppression of testicular ER expression (Tena-Sempere *et al.* 2000). Therefore, we hypothesize that ERs present in testes may have a role in the induction of spermatogenic cell apoptosis by estrogenic compounds.

ICI 182,780 (ICI), a pure estrogen antagonist, binds to both ERα and ERβ (Kuiper *et al.* 1998, Howell *et al.* 2000), does not cross the blood–brain barrier (Wade *et al.* 1993), and is currently used as a first choice treatment for human breast cancer, because of its local peripheral action (Wiebe *et al.* 1993). In the present study, in order to reveal the estrogenicity of DBP, attempts were made to block the ERs on prepubertal rat testes with ICI prior to DBP or EB administration.

Results

Effects of repeated DBP exposure on testis morphology and testicular steroidogenesis

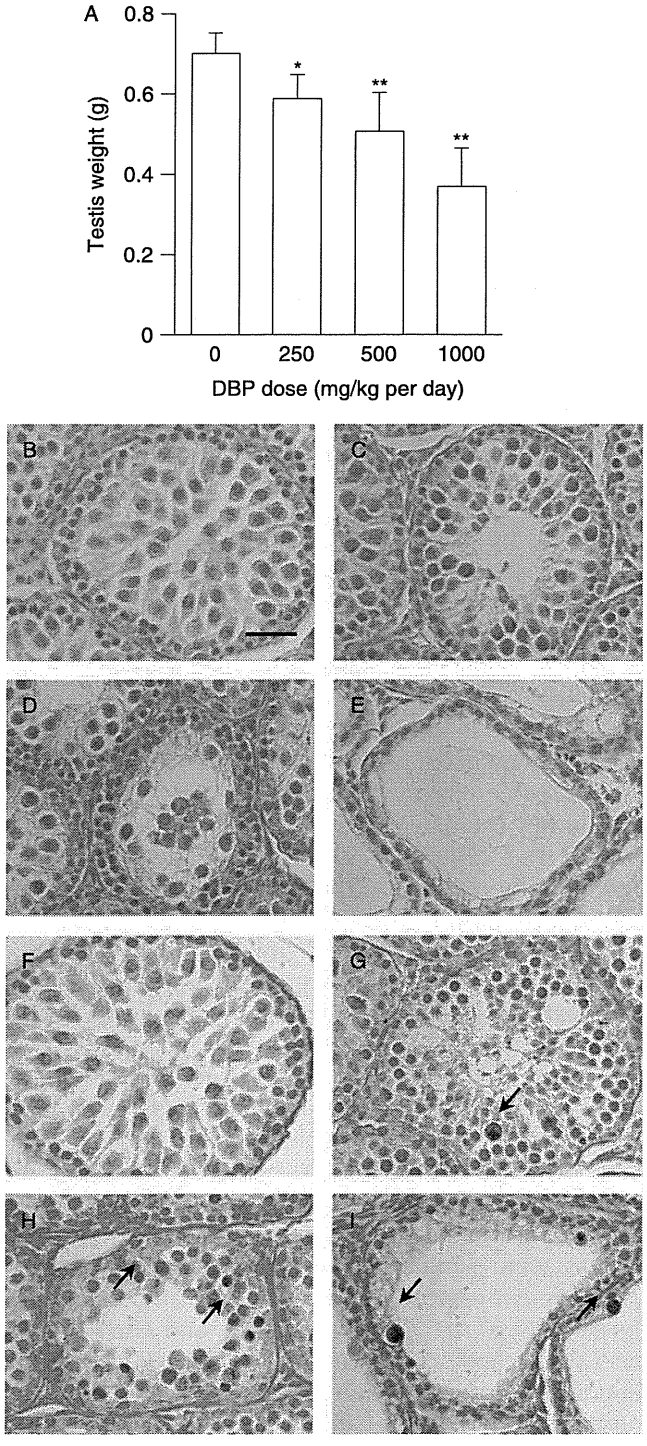
Daily administration of DBP for 7 days resulted in a significant decrease of testis weight in a dose-dependent manner (Fig. 1A). In histological observations, the most common lesion site was a seminiferous tubule. Lesions in seminiferous tubules were characterized by decreased tubular size, depletion of spermatogenic cells, wider tubular lumen, and ultimately a thin layer of seminiferous tubules depending on the dose. Detachment of spermatogenic cells, reduction in diameter of tubular size, and giant cells with cellular debris in tubular lumen were frequently observed in the group treated with 500 mg/kg per day DBP (Fig. 1D), while complete loss of spermatocytes in seminiferous tubules was seen in the group treated with 1000 mg/kg per day DBP, leading to irregularly shaped and sized tubules (Fig. 1E). We performed a TUNEL assay to examine whether degraded spermatogenic cells were undergoing apoptosis. A significant number of apoptotic (TUNEL-positive) spermatogenic cells were detected in the 500 mg/kg per day group (Fig. 1H) as compared with the control (Fig. 1F). In the 1000 mg/kg per day group, only a few apoptotic spermatogenic cells were found due to the complete loss of spermatocytes (Fig. 1I). The number of apoptotic cells per seminiferous tubule was higher in the 500 mg/kg per day group than in the 250 mg/kg per day group.

To examine the possible involvement of testicular steroidogenesis in spermatogenic cell apoptosis, the ultrastructure of Leydig cells was observed by transmission electron microscopy, and intratesticular testosterone (ITT) levels were analyzed by testosterone EIA assay. We selected 500 mg DBP/kg per day as a representative dose, based on the above observation. Several lipid droplets in the Leydig cell cytoplasm were distinctly observed in the treated group, whereas these droplets were not found in the control group (Fig. 2A and B). The average ITT concentration of the DBP-treated groups was almost the same with that of the control group (Fig. 2C). The mRNA expression levels of four steroidogenic enzymes were also analyzed by real-time RT-PCR. Although statistical significance was not detected, DBP exposure apparently increased the cytochrome P450 side chain cleavage (*P450scc, Cyp11a1*) and 3β hydroxysteroid dehydrogenase (*3βHsd, Hsd3b*) expressions (Fig. 2D and F). The cytochrome P450 17α/C_{17-20} lyase (*P450c17, Cyp17a1*) and 17β hydroxysteroid dehydrogenase (*17βHsd, Hsd17b*) expressions in the DBP-treated groups were almost the same with that of the control group (Fig. 2E and G). These findings indicate that the testes after daily exposure of DBP for 7 days shows normal or slightly enhanced steroidogenesis with a high rate of seminiferous tubule disruption.

Effects of an acute single exposure of DBP on spermatogenic cell apoptosis and testicular steroidogenesis

In a 7-day daily exposure of DBP, seminiferous tubules showed a depletion of spermatogenic cells, probably due to apoptosis. In order to determine the exact time point of maximum apoptosis and the exact target cell

type for DBP insult, we conducted a time-course study with exposure times of 3, 6, and 24 h after a single exposure of DBP. We found a significant increase in the number of apoptotic spermatogenic cells in the treated groups in comparison with the control (Fig. 3A–D). The maximal number of apoptotic spermatogenic cells was detected at 6 h after treatment (Fig. 3C and J).



At 24 h after administration, the number of apoptotic cells began to gradually decline, although it was still significantly greater than that in the control group (Fig. 3J).

In order to evaluate the spermatogenic cell types that underwent apoptosis, apoptotic cells were analyzed by light and transmission electron microscopies. At the light microscopic level, apoptotic cells were identified by their prominent basophilia and shrinkage of both cytoplasm and nucleus. Spermatocytes were the largest cells and showed typically dispersed chromatin, and spermatogonia were identified by their location (Fig. 3F and G). Similarly, at the electron microscopic level, apoptotic spermatocytes with chromatin clumping and shrinkage of cytoplasm and nucleus were clearly distinguishable (Fig. 3H and I).

ITT level was also measured by a testosterone EIA assay. In contrast to 7-day daily exposure, a single exposure of DBP (500 mg/kg) significantly decreased the level of ITT (Fig. 4A). To address whether DBP suppressed the ITT level by affecting the HPG axis, we analyzed the serum LH level by RIA. Inhibition of testicular testosterone production by estrogen administration is mediated by a decrease in serum LH level through the action of estrogen on the hypothalamo-pituitary axis (Jong *et al.* 1975). As shown in Fig. 4B, the serum LH concentration showed a tendency to decrease ($P=0.06$) in the DBP-treated groups, especially at 3 h after DBP administration. These data suggested that DBP might have blocked LH secretion shortly after administration, probably due to estrogenic activity in hypothalamus, and then diminished the stimulation of Leydig cells, thereby reducing the production of testosterone (Fig. 4). Real-time RT-PCR analysis also showed a statistically significant reduction in *Cyp11a1*, *Cyp17a1*, *Hsd3b*, and *Hsd17b* expressions by DBP treatment (Fig. 4C–F). The decrease in ITT nearly paralleled the decrease in the mRNA levels of all testicular steroidogenic enzymes (*Cyp11a1*, *Cyp17a1*, *Hsd3b*, and *Hsd17b*). These results illustrated that DBP decreased testicular steroidogenesis and that this decrease likely occurred via suppression of the HPG axis, suggesting that DBP acts as an estrogen agonist.

Figure 1 Effects of repeated DBP exposure on testis morphology. Testis weight after once daily administration of DBP for 7 days (A) is shown. Results are expressed as the means±S.E.M. ($n=6$). Statistically significant differences were determined by ANOVA followed by Fisher's PLSD test (*$P<0.05$; **$P<0.01$ versus control). Histological changes of testes after once daily administration of DBP for 7 days are shown using hematoxylin and eosin staining (B–E) and TUNEL labeling (F–I). Results are shown for the vehicle-treated (B), 250 mg/kg per day (C), 500 mg/kg per day (D), and 1000 mg/kg per day (E) groups. Note the reduction in diameter and irregularly shaped seminiferous tubules due to depletion of spermatogenic cells. TUNEL-positive (apoptotic) spermatogenic cells are indicated by arrows in testes. Vehicle-treated control (F), 250 mg/kg per day (G), 500 mg/kg per day (H), and 1000 mg/kg per day (I) treated groups. Scale bar, 20 μm.

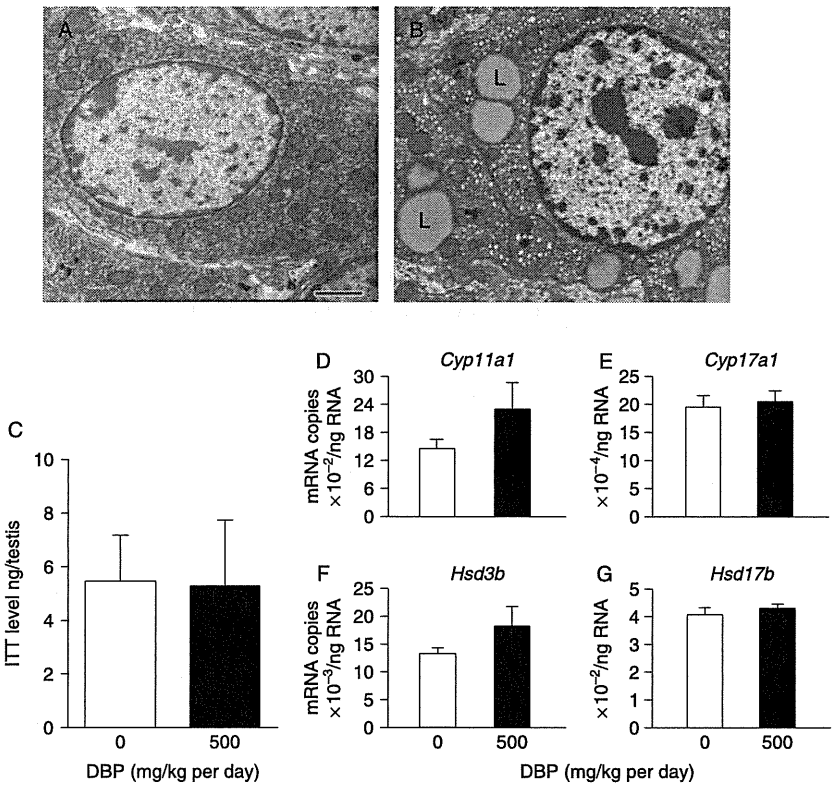


Figure 2 Effects of repeated DBP exposure on testicular steroidogenesis. Ultrastructural changes in Leydig cells of the control (A) and 500 mg/kg per day treated groups (B). L indicates lipid droplets. Scale bar, 1 μm. Intratesticular testosterone (ITT) level (C) and testicular mRNA expressions for *Cyp11a1* (D), *Cyp17a1* (E), *Hsd3b* (F), and *Hsd17b* (G). ITT level was measured by testosterone EIA assay, and mRNA expressions of testicular steroidogenic enzymes were determined by real-time RT-PCR. Results are expressed as the means±S.E.M. ($n=8$ for ITT and $n=3$ for testicular steroidogenic enzymes mRNA expressions). Statistically significant differences between means from the control and treated groups were determined by Student's *t*-test.

Antiestrogen ICI inhibited DBP- or EB-induced spermatogenic cell apoptosis

A single exposure of DBP resulted in decreased testicular steroidogenesis by suppression of LH level together with increased spermatogenic cell apoptosis. To examine the hypothesis that spermatogenic cell apoptosis is induced by estrogenic compounds through ERs, we further conducted an inhibition study with pure antiestrogen ICI. The effect of EB, a synthetic long-term acting estrogen which is converted to 17β-estradiol (E_2) in liver, was examined using male rats of the same age. As shown in Fig. 5B, 500 mg/kg of DBP significantly increased ($P<0.001$) the number of apoptotic spermatogenic cells compared with that in the control. Treatment with 200 μg/kg EB also significantly increased ($P<0.001$) the number of apoptotic spermatogenic cells as compared with that in the control group, although the number of apoptotic spermatogenic cells in the EB-treated group was lower than that in the DBP-treated group (Fig. 5D). In the rats treated with the above doses of DBP and EB, pretreatment with ICI decreased ($P<0.001$) the apoptotic spermatogenic cell number compared with that in the groups without pretreatment (Fig. 5C, E, and G).

Comparison of the effects of DBP and EB on testicular steroidogenesis and serum FSH level

In order to examine the possible correlation between inhibition of DBP- or EB-induced spermatogenic cell apoptosis by ICI and testicular steroidogenesis, the testicular steroidogenic capacity was examined in the samples obtained from the above inhibition study (Fig. 5). It is known that testosterone withdrawal results in spermatogenic cell apoptosis (Blanco-Rodriguez & Martinez-Garcia 1997, 1998, Creasy 2001, McLachlan *et al.* 2002). In our study, a single exposure of DBP caused a significant reduction in ITT as well as testicular steroidogenic enzymes expressions (Fig. 4). Therefore, we further examined whether ICI pretreatment would inhibit the reduction in testicular steroidogenesis induced by DBP or EB. However, ITT level was significantly lower in the DBP+ICI− ($P<0.001$) or EB+ICI− ($P=0.001$) treated group compared with that in the intact and control groups. The level in the pretreated groups was similar to that in the group treated with DBP ($P<0.001$) or EB ($P<0.001$) alone (Fig. 6A). The serum FSH level was also measured in the samples obtained from the above inhibition study (Fig. 5) to correlate between the apoptotic index and testicular steroidogenesis (Figs 5 and 6). The serum FSH level was significantly decreased in the DBP- ($P<0.001$) and EB- ($P<0.001$) treated groups. These levels in the pretreated groups were similar to those in the groups without pretreatment respectively (Fig. 7). Similarly, the mRNA levels of *Cyp11a1*, *Cyp17a1*, *Hsd3b*, and *Hsd17b* were also significantly decreased in the DBP, EB, DBP+ICI, and EB+ICI groups compared with those in the control and intact groups (Fig. 6B–E), whereas no significant

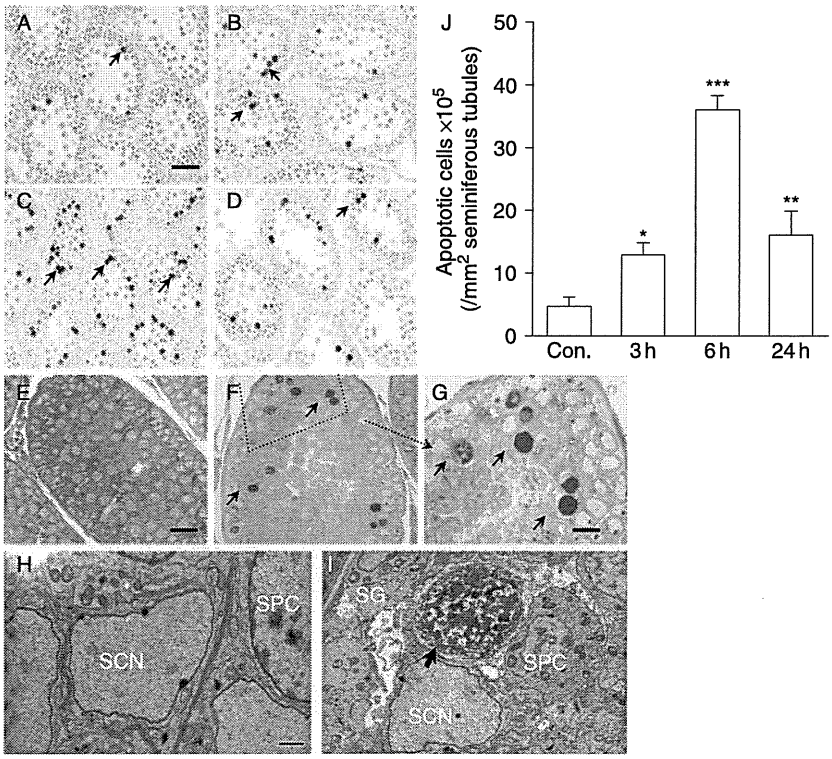


Figure 3 Spermatocytes apoptosis after a single exposure of 500 mg DBP/kg. TUNEL labeling of testes. Vehicle-treated control (A), and at 3 h (B), at 6 h (C), and at 24 h (D) after treatment. Note the maximal apoptotic spermatogenic cell number at 6 h after treatment. Arrows indicate apoptotic spermatocytes. Scale bar, 50 μm. Semi-thin sections of seminiferous tubules of the control (E), and at 6 h (F and G) after treatment. Toluidine blue staining. E–F, scale bar, 20 μm. G, scale bar, 10 μm. Transmission electron micrographs of control (H) and at 6 h (I) after treatment. Arrowhead, apoptotic spermatocyte; SCN, Sertoli cell nucleus; SG, spermatogonium; SPC, spermatocyte. Scale bar, 1 μm. Quantification of apoptotic spermatogenic cells (J). Values representing the number of apoptotic cells per 1 mm^2 seminiferous tubules areas are expressed as the means±S.E.M. ($n=8$). Statistically significant differences were determined by ANOVA followed by Fisher's PLSD test (*$P<0.5$, **$P<0.01$, ***$P<0.001$ versus control).

changes of ITT concentration, serum FSH level, and steroidogenic enzymes expressions were found in the ICI alone-treated group compared with those in the intact and vehicle groups.

Discussion

Repeated DBP exposure causes testicular atrophy without significant changes in testicular steroidogenesis

The present study demonstrated that repeated administration of DBP for 7 days increased the number of disrupted seminiferous tubules with spermatogenic cell depletion. Disturbance in seminiferous tubules and enhanced testicular steroidogenesis have been reported in prepubertal rats chronically exposed to DBP (Ryu *et al.* 2007). In our study, however, there was no significant change in testicular steroidogenesis, although excess lipid droplets were clearly observed in Leydig cells. The discrepancy in testosterone level between this study and Ryu *et al.* (2007) may be due to the difference in exposure period (7 vs 30 days). Our study, however, showed a similar level of disrupted seminiferous tubules to that by Ryu *et al.* (2007), indicating that testosterone production may not be associated with testicular atrophy induced by DBP.

Acute DBP exposure increases spermatogenic cell apoptosis and decreases testicular steroidogenesis

It is well established that testosterone synthesis is controlled by negative feedback regulation of HPG axis, and an increase in testosterone level causes a reduction in LH secretion from pituitary by activating androgen receptor (AR) in hypothalamus. A testosterone metabolite by aromatization, E_2, can also reduce LH secretion by binding to ERs in hypothalamus as well as androgens. Then, a subsequent reduction in LH pulse would lead to a suppression of testosterone production. Decreased testicular testosterone biosynthesis as well as decreased serum LH level and serum FSH level occurs after exogenous estrogen exposure, together with increased spermatogenic cell apoptosis (D'Souza *et al.* 2005). To our knowledge, the present study reveals for the first time that DBP suppresses testicular steroidogenesis with a correspondent decrease in serum FSH (Fig. 7) and serum LH level (Fig. 4B) shortly after treatment, probably by interacting with ERs in hypothalamus. In addition, the similar reduction in serum FSH level was found in the EB-treated group (Fig. 7). All of the data indicate that phthalate ester has an estrogenicity similar to that of E_2.

It is well established that testosterone synthesis depends on the frequency and amplitude of LH pulse, and disruption of LH pulse by administration of estrogen has been shown to cause the suppression of testosterone

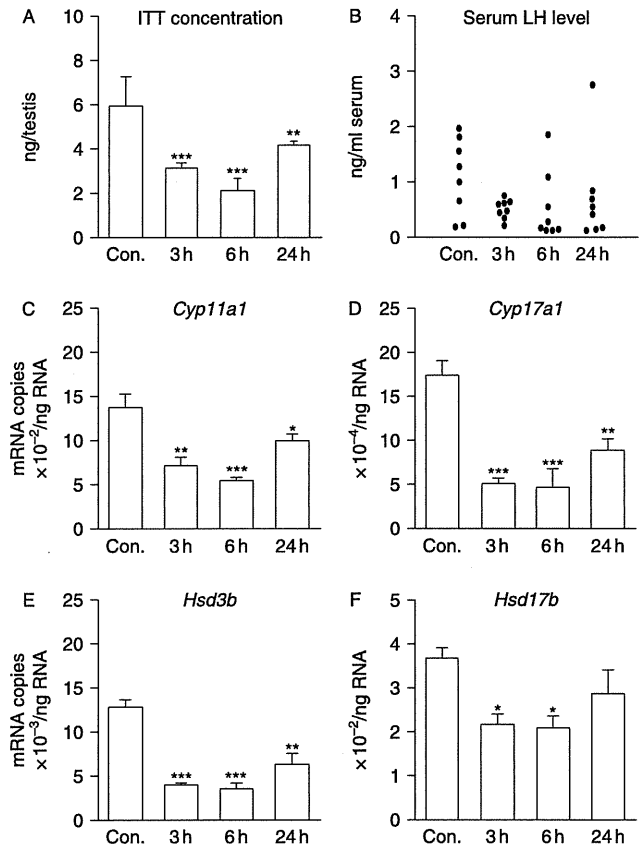


Figure 4 Testicular steroidogenesis and serum LH levels after a single exposure of 500 mg DBP/kg. Intratesticular testosterone (ITT) level (A), serum LH level (B), and testicular mRNA expressions for *Cyp11a1* (C), *Cyp17a1* (D), *Hsd3b* (E), and *Hsd17b* (F). ITT level was measured by testosterone EIA assay, serum LH was measured by RIA, and mRNA expressions of testicular steroidogenic enzymes were analyzed by real-time RT-PCR. Note that the decreased serum LH pulse led to a reduction in ITT level as well as a decrease in mRNA expressions in the treated rats. Results are expressed as the means ± S.E.M. ($n=8$ for ITT and serum LH, and $n=3$ for real-time RT-PCR). Statistically significant differences were determined by ANOVA followed by Fisher's PLSD test (*$P<0.05$; **$P<0.01$, ***$P<0.001$ versus control).

production (McGarvey *et al.* 2001). To demonstrate the effects of DBP on LH pulsatility, the serial blood sampling at several minute intervals from live animals and the appropriate pulse analysis should be required. But, this is difficult to detect in the present study because of a single spot blood sample. DBP might induce disruption of LH pulse and subsequently decrease testicular testosterone production. On this point, further studies are required in the future.

DBP-induced spermatogenic cell apoptosis is mediated via ERs

There is much evidence that DBP can interact with ERs. For example, several studies employing an ER-mediated yeast growth assay or a reporter assay with human breast cancer cell line MCF-7 have demonstrated the interaction between DBP and ERs (Jobling *et al.* 1995, Harris *et al.* 1997, Zacharewski *et al.* 1998, Andersen *et al.* 1999). Moreover, DBP exhibited an estrogenicity in an E-screen assay (Soto *et al.* 1995) and prevented tamoxifen (ER antagonist)-induced apoptosis in MCF-7 cells (Kim *et al.* 2004). However, it is still unclear whether DBP has an estrogenicity in an *in vivo* experimental model, because any phthalate esters, including DBP, displayed no significant changes in the female reproductive organ weight in the rat uterotrophic assay (Milligan *et al.* 1998, Zacharewski *et al.* 1998). In the present study, decreased testicular steroidogenesis was observed in a single exposure of DBP with the same spectrum of those of the EB-treated groups (Fig. 6). Administration of synthetic estrogens, including EB, is known to induce spermatogenic cell apoptosis. Here, for the first time, we have demonstrated that pure antiestrogen ICI inhibited EB-induced spermatogenic cell apoptosis. Similarly, DBP-induced spermatogenic cell apoptosis was significantly decreased by ICI pretreatment (Fig. 5), clearly demonstrating that, at least in immature male rats, DBP acts as an estrogen agonist. To our knowledge, this is the first report using an *in vivo* model to demonstrate the estrogenicity of DBP through ERs.

EB- and DBP-induced spermatogenic cell apoptosis was blocked by pretreatment with an ER antagonist, ICI, indicating that E_2 as well as DBP was functioning through ERs. Indeed, both receptors are present in testes (Saunders *et al.* 1998, Pelletier *et al.* 2000). In general, the classic ER-signaling pathway involves the binding of the ligand-bound ERs (either α or β) to the estrogen-responsive element (ERE) that regulates transcription of target genes. However, ERs also mediate gene transcription by binding to an AP-1 element together with the transcription factors, Fos and Jun (McEwen & Alves 1999). ERα and ERβ have been shown to transcript in opposite ways from the AP-1 site; when bound to E_2, ERα activates and ERβ inhibits transcription (Paech *et al.* 1997). In contrast to the endogenous estrogen, antiestrogens, including raloxifene, and ICI have been shown to effectively activate transcription from an AP-1 site when bound to ERβ (Paech *et al.* 1997). The intracellular mechanisms of ERs action in the present study and the possible involvement of both ERα and ERβ in spermatogenic cell apoptosis are not known, and further studies are required. More recent work has been shown that estrogen action in male reproductive system does not involve EREs pathway (Weiss *et al.* 2008). Moreover, many studies revealed a nongenomic signaling pathway through membrane-associated ERs (Hammes & Levin 2007) and cross-talk between genomic pathways (Revelli *et al.* 1998, Losel *et al.* 2003). Binding of E_2 to the membrane-associated ERs results in rapid (within minutes) activation of the MAP kinase, phosphatidylinositol 3-kinase, and protein kinase C and phosphatases, as well as the release of several cyclic amines

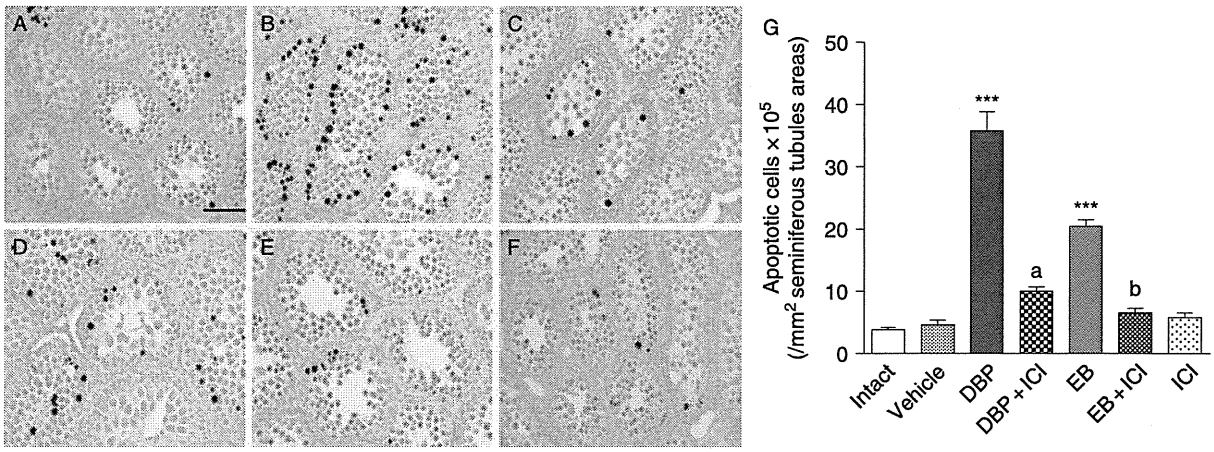


Figure 5 ICI inhibits DBP- or EB-induced spermatogenic cell apoptosis. Results are shown for the control (A), DBP alone (B), DBP+ICI (C), EB alone (D), EB+ICI (E), and ICI alone (F) groups. Rats (n=12) were i.p. administered with 3 mg/kg of ICI (5% ethanol and 95% corn oil) 4 h prior to administration of DBP or EB. The doses of DPB and EB were 500 mg/kg and 200 μg/kg respectively. Apoptotic spermatocytes are stained brown. Scale bar, 50 μm. Comparison of the numbers of TUNEL-positive cells in the testes of treated rats (G). Note that the numbers of both DBP- and EB-induced apoptotic spermatocytes were decreased by ICI pretreatment. Values representing the number of apoptotic cells per 1 mm^2 seminiferous tubules areas are expressed as the means±S.E.M. (n=12). Statistically significant differences were determined by ANOVA followed by Fisher's PLSD test (***P<0.001 versus vehicle or intact; ^{a}P<0.001 versus DBP; ^{b}P<0.001 versus EB).

(cAMP and cGMP) and calcium, in a variety of cell types (Losel *et al.* 2003, Levin 2005, Hammes & Levin 2007). Membrane-associated ERs have been shown to prevent chemotherapy or radiation-induced apoptosis in MCF-7 cell (Razandi *et al.* 2000). Since the reduction in spermatogenic cell apoptosis by pretreatment with ICI was seen after 6 h of DBP or EB treatment in the present study, it is possible that the spermatogenic cell apoptosis induced by estrogenic compounds is at least partly mediated by this rapid nongenomic ERs signaling pathway.

DBP-induced spermatogenic cell apoptosis is independent of testicular steroidogenesis

The number of apoptotic cells was drastically decreased in the rats treated with DBP+ICI or EB+ICI compared with the number in the rats treated with DBP or EB alone. Since ICI can bind to both ERα and ERβ (Kuiper *et al.* 1998, Howell *et al.* 2000), the effects of DBP as well as EB on testes may be mediated by ERs that expressed in hypothalamus–pituitary, or possibly in testis. One of the novel findings of our study is that ICI can abolish the DBP- as well as the EB-induced spermatogenic cell apoptosis, but does not rescue the decreased ITT level and testicular steroidogenic enzyme mRNA expression (Fig. 6). Similarly, decreased level of serum FSH induced by DBP and EB was not restored by pretreatment with ICI (Fig. 7), indicating that ICI had no biological effect on hypothalamic level. The increased rate of spermatogenic cell apoptosis induced by DBP may not be due to the reduction in testicular testosterone level. In some cases, a lowered level of ITT could maintain spermatogenesis. For example, Zirkin *et al.* (1989) demonstrated that an 80% reduction in the ITT level from the control values was sufficient for the maintenance of quantitatively complete spermatogenesis. They have concluded that there is far more testosterone present within the testis of intact rats than is required for the maintenance of normal spermatogenesis. Therefore, no induction of apoptosis was detected in the DBP+ICI-treated animals in which the number of apoptotic spermatogenic cells was almost the same as that in the control animals, although the ITT level was still much lower (Fig. 6). Therefore, it is suggested that DBP-induced spermatogenic cell apoptosis is not due to ITT reduction, but may be mediated by ERs expressed in testes. The discrepant finding that ICI inhibited spermatogenic cell apoptosis but did not rescue the testicular steroidogenesis and serum FSH level reduced by DBP or EB can be explained that ICI does not cross the brain barrier and fails to block uptake of [^{3}H]-estradiol into the nuclei of hypothalamic cells (Wade *et al.* 1993, Howell *et al.* 2000). The current study that ICI abolished the DBP- or EB-induced apoptosis but could not restore testicular steroidogenesis and serum FSH level is well correlated with Wade *et al.* (1993), suggesting that it cannot penetrate across the blood–brain barrier and fails to block ERs in the hypothalamus.

In the present study, we demonstrated for the first time that estrogenic compound-induced spermatogenic cell apoptosis is not associated with testicular steroidogenesis and that ERs are directly involved in spermatogenic cell apoptosis. Indeed, ERα and ERβ are both present in rat spermatocytes and round spermatids

(Saunders *et al.* 1998, Pelletier *et al.* 2000). Moreover, using an *in vitro* model with isolated spermatogenic cells, it has been reported that estrogen directly induces spermatogenic cell apoptosis by cytochrome *c* release from mitochondria and FasL up-regulation (Mishra & Shaha 2005). However, this apoptosis was inhibited by tamoxifen, an ER antagonist, indicating that an estrogen-induced change occurs through hormone receptor interaction in spermatogenic cells. Therefore, the present observations essentially suggest the possibility that the ERs present in testes, probably in spermatogenic cells, have a role in inducing spermatogenic cell apoptosis when binding to exogenous/endogenous estrogenic compounds. However, a direct action of estrogen or estrogenic compounds on spermatogenic cell apoptosis via ERs has to be clarified in the future.

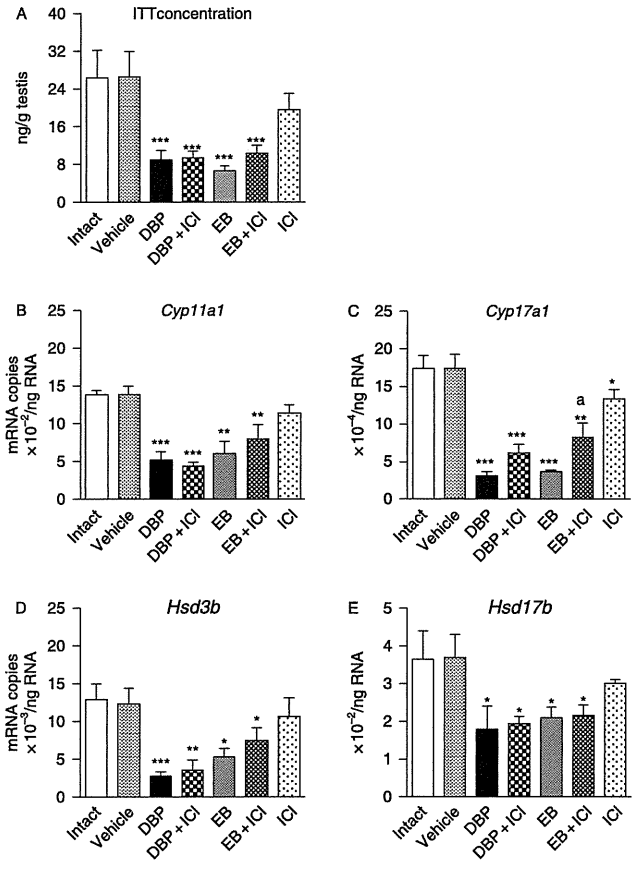


Figure 6 Comparison of ITT concentration and testicular steroidogenic enzyme gene expressions in the different treated groups. Intratesticular testosterone (ITT) level (A) and testicular mRNA expressions for *Cyp11a1* (B), *Cyp17a1* (C), *Hsd3b* (D), and *Hsd17b* (E). ITT level was measured by testosterone EIA assay, and mRNA expressions of testicular steroidogenic enzymes were analyzed by real-time RT-PCR. Results are represented as the means ± S.E.M. ($n=12$ for ITT and $n=9$ for testicular steroidogenic enzymes mRNA expressions). Statistically significant differences were determined by ANOVA followed by Fisher's PLSD test (*$P<0.05$; **$P<0.01$, ***$P<0.001$ versus vehicle or intact; $^{a}P<0.05$ versus EB).

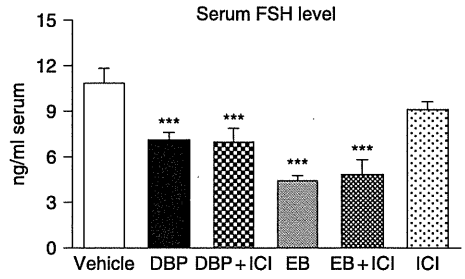


Figure 7 Serum FSH levels in the above inhibition study (Fig. 5) were measured by FSH ELISA assay. Results are represented as the means ± S.E.M. ($n=9$–12). Statistically significant differences were determined by ANOVA followed by Fisher's PLSD test (***$P<0.001$ versus vehicle).

In conclusion, this is the first report of estrogenic effects of DBP *in vivo* and essentially illustrates the possible role that ERs in testes play a role in spermatogenic cell apoptosis in response to environmental estrogenic compounds. We also demonstrated that testicular steroidogenesis was not associated with spermatogenic cell apoptosis induced by estrogenic compounds. Moreover, these data will be important for understanding the role of endogenous estrogen, which might induce spermatogenic cell apoptosis via ERs for the proper maintenance of normal testicular homeostasis.

Materials and Methods

Chemicals

DBP (purity >99.8%) and EB (purity >99.0) were purchased from Sigma–Aldrich. Antiestrogen ICI 182,780 was from Tocris Cookson, Ltd (Bristol, UK). Proteinase K and 3,3′-diaminobenzidine tetrahydrochloride were from TaKaRa (Otsu, Japan). Neutral buffer formalin and propylene oxide were from Wako (Osaka, Japan). Osmium tetroxide (OsO_4) and Araldite M were from Nisshin EM Co., Ltd (Tokyo, Japan). Testosterone EIA Kit was obtained from Cayman Chemical Company (Ann Arbor, MI, USA) and rat FSH ELISA kit from ALPCO Diagnostics (Windham, NH, USA). RNeasy Mini Kit was from Qiagen. TUNEL Kit, PrimeScript RT reagent Kit (Perfect Real Time), and Ex Taq polymerase with 10× Ex Taq buffer, SYBR Premix Ex Taq (Perfect Real Time) were purchased from TaKaRa.

Animals and treatments

Male Sprague–Dawley rats (3-week-old) were purchased from Charles River Laboratories Japan (Tokyo, Japan). The rats were housed five per one plastic cage, maintained on a 12 h light:12 h darkness cycle at constant temperature (22 ± 1 °C) and humidity (45–70%), and provided water and rodent pellets (Oriental Yeast, Tokyo, Japan) *ad libitum*. Animals were maintained and handled humanely in accordance with the guidelines of the animal experiments of the Institutional Animal Care and Use Committee (IACUC) of the University of Tokyo, Tokyo, Japan.

In the first experiment, 3-week-old male rats ($n=6$) were given once daily by oral gavage for 7 days at the dose of 250, 500, or 1000 mg/kg DBP in mixture of 5% ethanol and 95% corn oil or vehicle (5% ethanol and 95% corn oil). Rats were killed using diethyl ether on the day after completion of the treatment schedule, and testes were collected, weighed, and subjected to histopathology. In the second experiment, 500 mg/kg DBP as a representative dose was given to rats ($n=8$) for 7 days in the same way as used in the first experiment. Testes were collected on the day after completion of the treatment schedule for ultrastructural observations of Leydig cells by transmission electron microscopy and analysis of testicular steroidogenesis using a Testosterone EIA Kit and real-time RT-PCR. In the third experiment, an acute DBP exposure study, rats ($n=8$) were given a single exposure of DBP (500 mg/kg) or vehicle by oral gavage and killed at 3, 6, or 24 h after administration. Then, testes and sera were collected. In the last experiment, an inhibition study with pure antiestrogen, rats ($n=12$) were i.p. administered with 3 mg/kg ICI (5% ethanol and 95% corn oil) 4 h prior to administration of DBP or EB.

The chosen dose of DBP (500 mg/kg) was based on previous reports that adverse effects on male reproductive development occur between 100 and 500 mg DBP/kg per day without systemic toxicity (Mylchreest *et al.* 2002). And the dosage of ICI was 3 mg/kg based on the previous study in which 1.5 mg/kg dosage was found to be effective estrogen antagonism (Sibonga *et al.* 1998).

Histopathology

For histopathological observations with hematoxylin and eosin or TUNEL staining, testes were immersed in 10% neutrally buffered formalin for 48 h at room temperature. Then, the samples were washed in 0.1 M PBS for 3 h, dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin. The paraffin blocks were cut at 4 μm thickness. For transmission electron microscopy, rats were perfused with 5% glutaraldehyde in 0.1 M phosphate buffer, and then testes were immersed in the same fixative at 4 °C for 3 h and postfixed in 1% OsO_4 at 4 °C for 2 h. The samples were then dehydrated in ethanol, infiltrated in propylene oxide, and embedded in Araldite M. Semi-thin sections were cut at 1 μm thickness, stained with 1% toluidine blue, and observed by light microscopy. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined with a JEM-1010 transmission electron microscope at 80 kV (JEOL, Ltd, Tokyo, Japan).

TUNEL assay

In order to quantitatively assess the incidence of apoptotic spermatogenic cells after treatment, *in situ* TUNEL was performed by using an Apoptotic Detection Kit according to the manufacturer's instructions. Briefly, the tissue sections were deparaffinized and digested with 10 μg/ml proteinase K at 37 °C for 15 min. After being washed three to five times with 0.01 M PBS (pH 7.4), they were treated with terminal deoxynucleotidyl transferase (TdT) enzyme and labeling safe buffer, which were included in the kit. The TdT reaction was conducted at 37 °C for 90 min. After further washing three to five times with PBS, they were incubated with HRP goat anti-biotin at 37 °C for 30 min. The localization of HRP sites was determined by the application of diaminobenzidine. The sections were then counterstained with methyl green and mounted. Images of seminiferous tubules were obtained by using an OLYMPUS (BX50) light microscope connected to a digital camera (OLYMPUS, DP20, Tokyo, Japan). Under the microscope at 200× magnification, three fields in each section were randomly selected. The area of seminiferous tubules in all fields was measured by a computer-assisted system using Scion Image software (Scion Co., Frederick, MD, USA). Then, TUNEL-positive (brown-stained) spermatogenic cells in all selected area were counted. The number of TUNEL-positive cells per 1 mm^2 seminiferous tubules was calculated by dividing the total TUNEL-positive cell numbers by total seminiferous tubules area of each field and calculated accordingly in all of the fields. Data were obtained from 8–12 rats in each group and were given as mean ± S.E.M.

Hormone assay

The concentration of ITT was determined by using a Testosterone EIA Kit as previously described (Ohsako *et al.* 2003). To measure the concentration of ITT, the frozen testis was thawed and homogenized in PBS with a Polytron homogenizer (Kinematica, Luzern, Switzerland), and the testicular homogenate was extracted twice with an appropriate volume of diethyl ether. ITT level was measured according to the manufacturer's protocol. Serum LH concentration was analyzed by double antibody RIA, using materials supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). The reference standard for the LH assay was NIDDK-rLH-rp-3. The intra- and inter-assay coefficients of variation (CV) for the LH assays, which were calculated from five to seven replicated determinations for the

Table 1 Primers used for real-time quantitative RT-PCR.

	Primer sequences			
Gene	Forward (genomic position)	Reverse (genomic position)	**Product size** (bp)	**Gene ID**
Cyp11a1	TGAGATCCCTTCCCCTGGTG (exon 1)	TCGCTGCGTCCTTAGGGTC (exon 2)	179	J05156
Cyp17a1	GGTCCCATCTATTCTCTTCGC (exon 1)	CTGAACACCAACTTCCGGT (exon 2)	209	M22204
Hsd3b	CTCTGGACAAAGTCTTCAGACCAGA (exon 2)	GCCTGGGTAGGACATGTGAGAC (exon 3)	183	M38178
Hsd17b	GGTTCTCCCGGTACCTTTTT (exon 1)	GTCCGGCTGATAAGTACAACA (exon 3)	166	AF035156
Ppia	GGTCCTGGCATCTTGTCCATC (exon 4)	TTCCACAATGCTCATGCCTT (exon 5)	141	M19533

pool of rat serum containing 3.0 ng/ml of LH, were 6.8 and 7.1% respectively. The serum FSH levels were determined using a rat-specific FSH ELISA kit, 29-AER004, from ALPCO Diagnostics. The manufacturer has validated this kit for the measurement of FSH in rat serum samples. The FSH assays were carried out according to the manufacturer's protocol. The sensitivity of the assay was 0.2 ng/ml. The kit was highly specific for rat FSH, with <0.1% cross-reactivity to rat GH, LH, TSH, and PRL. The intra- and inter- assay CV were 4.73 and 8.47% respectively.

Real-time RT-PCR

Total RNAs were extracted from testes by using an RNeasy Mini Kit (Qiagen). Four micrograms of total RNA samples were reverse-transcribed by using a PrimeScript RT reagent Kit (Perfect Real Time) according to the standard protocol of the supplier. Real-time PCR was performed by using SYBR Premix Ex Taq and a Light Cycler rapid thermal cycler system (Roche Molecular Systems). Table 1 shows the primer sequences, PCR product sizes, and GenBank accession numbers for all genes examined in this study – i.e. the genes encoding cytochrome P450 side chain cleavage (*P450scc, Cyp11a1*), cytochrome P450 17α/C_{17-20} lyase (*P450c17, Cyp17a1*), *Hsd3b, Hsd17b,* and cyclophilin-A (*Cp, Ppia*). The denaturation step was performed at 95 °C for 15 min, and the PCR was carried out in separate glass capillary tubes over 40 cycles (95 °C for 15 s, 60 °C for 20 s, and 72 °C for 10 s). Detection of fluorescent products was carried out at the end of the 72 °C extension period. The copy numbers of target mRNA molecules in each total RNA sample were determined by the original methods as described previously (Sakata *et al.* 2007). The mRNA levels were expressed as copy number per ng total RNA.

Statistical analysis

Statistical analysis was performed using StatView software (SAS Institute Inc., Cary, NC, USA). All results are represented as the means$\pm$S.E.M. For the comparison of testis weight, apoptotic spermatogenic cell index, serum LH, serum FSH, testicular testosterone levels, and steroidogenic enzyme gene expression levels in the dose response, time-course and inhibition studies, one-way ANOVA were carried out followed by Fisher's PLSD as a *post hoc* test. Two-tailed Student's *t*-test was used to compare the mean values of the ITT and testicular steroidogenic enzyme gene expression obtained in the repeated DBP exposure study. Differences were considered to be statistically significant when the *P* value was <0.05. Significant differences between each group are indicated in the figures where detected.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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