Neonatal Exposure to Environmental Estrogen Suppresses Testis Weight and Steroidogenesis in Juvenile Rats, and Powerfully Suppresses Steroidogenesis in Puberty, Especially 17β-estradiol

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Abstract

Neonatal exposure to environmental estrogen induces developmental abnormalities in the male reproductive system. To investigate whether neonatal exposure affects spermatogenesis in juvenile and pubertal testis, Sprague-Dawley rat pups were given environmental estrogen or estrogen by a single injection on the day of birth at concentrations ranging between 2 mM to 40 mM, and sacrificed on day 21 (juvenile), 35 (prepuberty) or 50 (puberty). The testes were weighed and examined histologically at each stage. Further, the metabolites of steroidogenesis were analyzed using normal-phase high performance liquid chromatography. Neonatal exposure significantly reduced testis weights and steroidogenesis of juveniles, and additionally pubertal steroidogenesis. Neonatal exposure to 17β-estradiol completely prevented steroidogenesis during puberty.

Key Words: environmental estrogen; testis; estrogen; spermatogenesis; steroidogenesis.

Introduction

Exposure of rats to environmental estrogen during the neonatal period causes marked developmental abnormalities in the testis (Khan et al., 1998; Prins, 1992; Iguchi et al., 2001; Spearow et al., 2001). The neonatal developmental changes that occur within the testis are extremely sensitive to environmental endocrine disruptors, including androgens and estrogens (Iguchi et al., 2001; Spearow et al., 2001; Bullock et al., 1998; Amold et al., 1996; Schönesöfer and Dulce, 1979; Schatzman et al., 1988), but the mechanism of this neonatal endocrine disruptor imprinting is not known. The other hand, androgen is required for normal development of the male reproductive tract. Synthesis of testosterone occurs in the Leydig cells and

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depends on the expression of several enzymes, including cytochrome P-450 17α-hydroxylase/C17-20-lyase (P-450c17) and 5α-reductase, which is highly regulated within the testis. Environmental estrogen has been linked with recent increases in the incidence of testicular cancer as well as decreases in sperm counts in humans (London, 1993 ; Sharpe, 1993 ; Sharpe and Shakkebaek, 1993) ; however, the relationship of these trends to environmental estrogen has not been established (White et al., 1994 ; Mclachlan and Newbold, 1987). In the present study we examined the effects of neonatal exposure to environmental estrogen and estrogen at different stages, corresponding to juvenile testis and pubertal testis. Our results show that administration of environmental estrogen and estrogen resulted in a significant reduction in juvenile testis weight and steroidogenesis, and additionally pubertal steroidogenesis. In particular, neonatal exposure to 17 β-estradiol prevented steroidogenesis completely and powerfully suppressed testis weight during puberty.

Materials and Methods

Animals. Adult female Sprague-Dawley rats and rat pups were purchased from Japan SLC Inc. (Shizuoka, Japan). Rat pups were injected sc with 25 μl sesame oil containing 40 mM bisphenol A : p,p′isoprophylidenediphenol, 40 mM 17β-estradiol, 2 mM estrone, 40 mM genistin : 4′, 5, 7-trihydroxyisoflavone, 2 mM nafoxidine hydrochloride or sesame oil alone on day 1. Rat pups in various treatment groups (n=10, 20 and 30) were killed on days 21 (juvenile), 35 (prepuberty) and 50 (puberty). Testes were isolated from the rats, and their weights were recorded. Testosterone, 17-hydroxyprogesterone and androstene-3, 20-dione levels were also measured.

Preparation of testicular microsomes. Microsomes were prepared according to the method of Omura and Sato (Omura and Sato, 1964), with slight modification (Kuwada et al., 1996). Fifty mM potassium phosphate buffer, pH 7.4, containing 250 mM sucrose, 100 μM EDTA and 100 μM dithiothreitol, was used throughout. Rat testes were decapsulated and sliced. The tissues were resuspended in 3 volumes 50 mM potassium phosphate buffer and then homogenized with a blender at 3,000 rpm for 10 min in ice. The homogenate was centrifuged at 7,000×g for 20 min. This supernatant was centrifuged at 105,000×g for 90 min in 4°C. The precipitated microsomal pellets were resuspended in 2-3 volumes 50 mM potassium phosphate buffer and kept at-80°C until further use.

Incubation Procedure. Progesterone (5 mM) was introduced into 1×10 cm tubes and dissolved in 0.02 ml ethanol. To each tube, 0.5 ml of incubation buffer solution was added. The buffer solution consisted of 300 mM potassium phosphate buffer, pH 7.4, 60 mM nicotinamide, 2 mM $MgCl_2$, 500 mM G6P and G6PDH (0.5 U). The suspension, 0.5 ml of the microsone fraction was then introduced to make the total volume of 1 ml. The mixture was incubated for 5 min at 35°C before the addition of 0.02 ml of NADPH (100 mM). The incubation was stopped at 30 min by adding 3.5 ml of dichloromethane (Kuwada et al., 1996). Each sample was incubated in duplicate.

Histology. Tissue specimens from developing animals in each treatment group were examined. The tissues were fixed in 10% formaldehyde. Fixed tissues were embedded in paraffin, mounted on slides and stained with hematoxylin and eosin. The testicular tissue morphology was assessed by light microscopy.

Analytical methods. Each sample was extracted with 3.5 ml of dichloromethane. The organic phase was washed with 2 ml of 0.1 N HCl, evaporated to dryness in a stream of nitrogen in a 40°C water bath and dissolved with 1 ml of dichloromethane. Samples for HPLC were filtered through a Millipore HV 0.45 micron disk filter. The extracted metabolites were finally dissolved in n-hexane preparatory to HPLC. Metabolites were separated using normal- phase high performance liquid chromatography (HPLC) (Iguchi et al., 2001 ; Cupp and Skinner, 2001). We used a TSK gel OH-120 (DIOL) obtained from Tosoh (Tosoh, Tokyo, Japan) with a mobile phase of solvent A : solvent B (1 : 5) at a flowrate of 1.5 ml/min. Components of solvent A and solvent B were n-hexane and n-hexane : i-propanol＝70 : 30, respectively. We used an HPLC system (Shimadzu, Kyoto, Japan) equipped with LC-10 AD pump, an SPD-10 AUU-VIS detector, a TSK gel OH-120 column (4.6 mm×250 mm) and a CTO- 10A column oven. The column oven was maintained at 30°C. The UV wavelength for detection was 246 nm. Sample volume was 20 μl.

Statistics. The testes weights were subjected to Student's *t*-test to determine whether there were significant effects of treatment. Where these were indicated, subgroup comparisons between means for the control and individual treatment groups were then made using the variance from the experimental results as a whole as the measure of error.

Results and Discussion

Testis weight was affected by a variety of environmental estrogens. Estrogen (40 mM 17 β-estradiol or 2 mM estrone), nafoxidine hydrochloride (2 mM) as antiestrogen and bisphenol A (40 mM) or genistin (phytoestrogen) (40 mM) as environmental estrogens were used in this experiment (Khan et al., 1998 ; Takao et al., 1999 ; Mitchell et al., 2001). Neonatal administration of all five treatments suppressed the testis weights in the juvenile (21 days) rats (Fig. 1). At the pubertal stage (50 days), neonatal exposure to 17 β-estradiol (40 mM) or nafoxidine hydrochloride (2 mM) powerfully suppressed testis weight during puberty, although the testis weights of rats subjected to other environmental estrogens were restored to the level of the control animals (Fig. 1). Testis weight was extremely sensitive to nafoxidine hydrochloride (2 mM), and the higher concentration produced many deaths. Testis weights were significantly reduced in environmental estrogen-treated rats, but during adulthood this decrease was restored to that of the control animals (Khan et al., 1998 ; Prins, 1992 ; Iguchi, 1992 ; Iguchi et al., 2001 ; Cupp and Skinner, 2001). Testicular histology also revealed different susceptibilities to environmental estrogen in spermatogenesis and germ cell development (Fig. 2). Testicular tissue histology of neonatally bisphenol A-treated animals at the age of 21 days (Fig. 2, right upper panel) was different from untreated control animals (Fig. 2, left upper panel). The

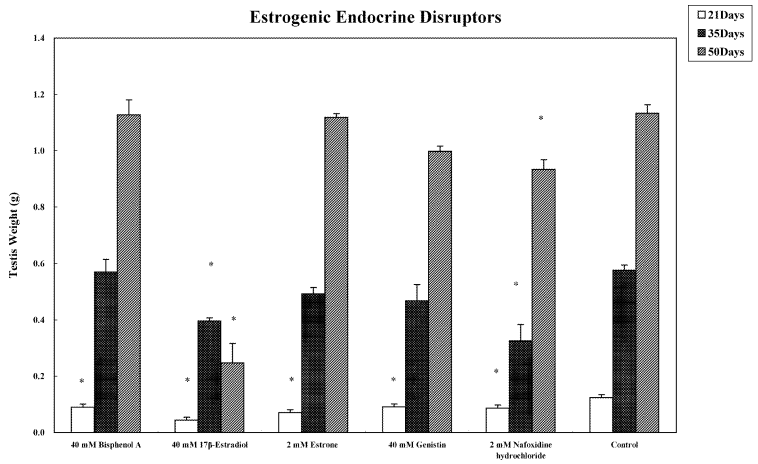

Fig. 1　All animals in a litter received a single injection of 25 μl sesame oil vehicle on the day of birth either alone (control) or containing 2 mM to 40 mM of an environmental estrogen. The animals in various treatment groups ($n=10$, 20 and 30) were killed at 21 days (juvenile), 35 days (prepuberty) and 50 days (puberty). Testes were collected. Each bar is the mean±SES. ＊, $P>0.01$ (compared with the control group).

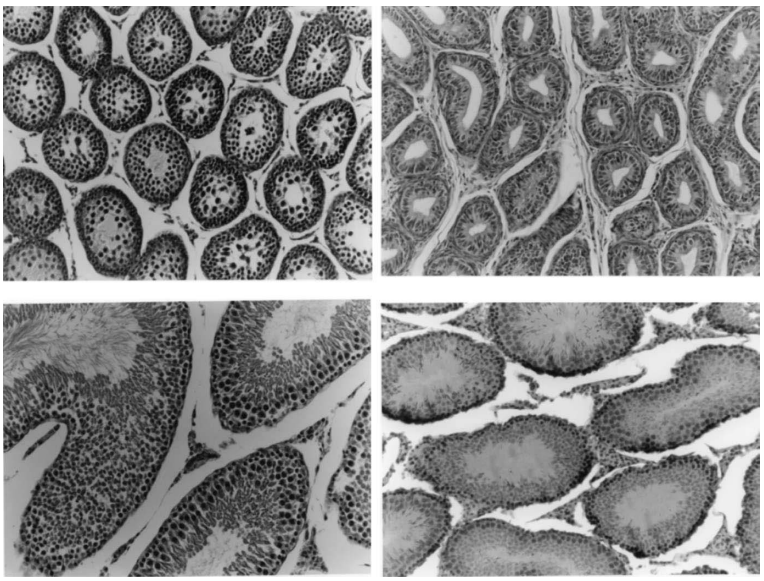

Fig. 2　Histology of rat testes killed at 21 days (upper panels) or 50 days (lower panels) of age. The animals were treated on the day of birth with either sesame oil (right panels) or 40 mM bisphenol A (left panels). The photomicrographs were taken a using×20 objectives.

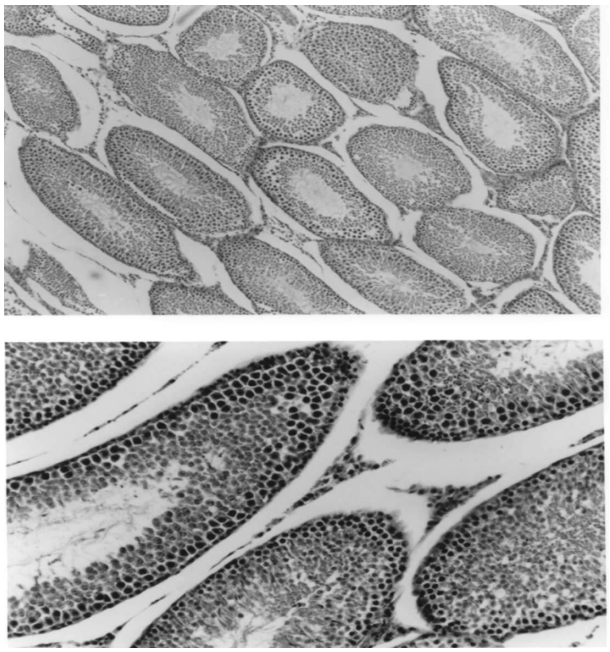

Fig. 3 Histology of rat testis killed 50 days of age. The animals were treated on the day of birth with either 40 mM 17β-estradiol (upper panel) or 40 mM genistin (phytoestrogen, lower panel). The photomicrographs were taken a using×10 objective (upper panel) or 20×objective (lower panel).

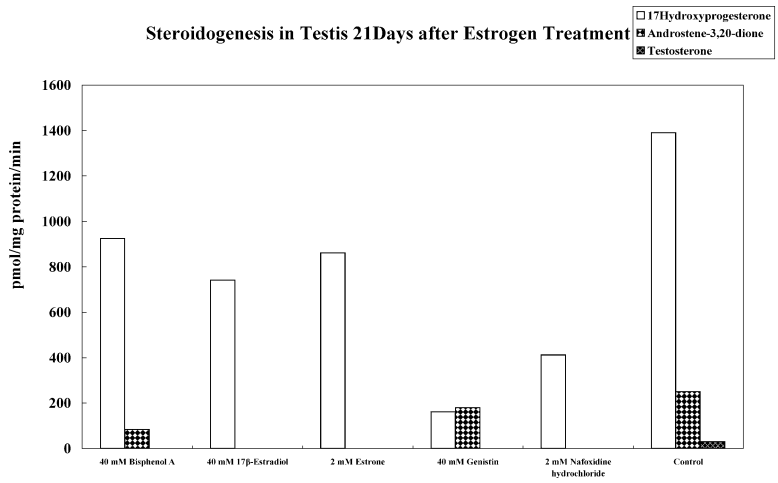

Fig. 4 Steroidogenesis in 21-day-old testis from rats neonatally treated with sesame oil (control) or an environmental estrogen. Testes catalyzed 17-hydroxyprogesterone of progesterone to androstenedione. Steroids were extracted, separated by HPLC, and analyzed as described in the methods.

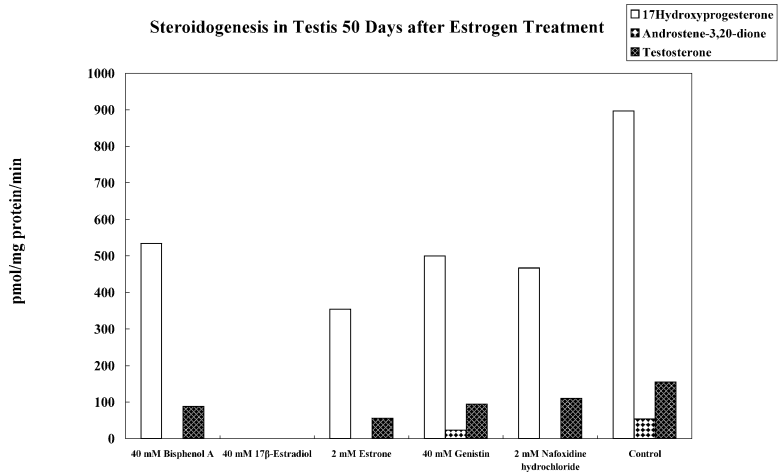

Fig. 5　Steroidogenesis in 50-day-old testis from rats neonatally treated with sesame oil (control) or an environmental estrogen. Testes catalyzed 17-hydroxyprogesterone of progesterone to androstenedione and further converted androstenedione to testosterone. Steroids were analyzed as described in the methods.

interstitial tissue was organized as a sheath around the seminiferous tubules instead of as the single isolated clusters of Leydig cell seen in the control testis. The tubules were diminished in size, and the tubular lumina were increased with empty space (Fig. 2, right upper panel). Neonatally bisphenol A-treated animals at the age of 50 days revealed few germ cells in advanced stages of development (round and elongated spermatids) (Fig. 2, right lower panel) (Prins, 1992 ; Spearow et al., 1999). The Leydig cell morphology appeared normal. Neither neonatally estrogen (17 β-estradiol)-nor genistin-treated animals at the age of 50 days had round or elongated spermatids, although Leydig cells appeared to be morphologically normal (Fig. 3). The control animals at the age of 50 days revealed elongated spermatids in all seminiferous tubules (Fig. 2, left lower panel). Steroidogenesis (androgen biosynthesis), which is essential for spermatid differentiation and maturation in the seminiferous tubules, occurred in the Leydig cells. These observations were supported by measurement of the metabolites of steroidogenesis (Fig. 4 and Fig. 5). Steroidogenesis in 21-day-old testis from all neonatally treated animals was reduced to half or less by environmental estrogens, compared to untreated control animals (Fig. 4). Steroidogenesis of many metabolites from neonatally estrogen (17 β-estradiol or estrone)-treated animals was powerfully reduced in 50-day-old testis. Neonatal exposure to 17 β-estradiol prevented steroidogenesis completely during puberty (Fig. 5). Compared with 17 β-estradiol, environmental estrogen responses seem to be less drastic, but environmental estrogen restrained steroidogenesis more powerfully than general endocrine disruptors. However, because steroid biosynthesis was not restored at 50 days, these data suggest that the mechanism of permanent estrogen imprinting is different from that of endocrine disruptors (Khan et al., 1998 ; Prins, 1992 ; Iguchi et al., 2001 ; Spearow et al., 2001 ; Bullock et al., 1988 ; Amold et al., 1996 ; Spearow et al., 1999 ; Ficher and Steinberger, 1971 ;

Coffey et al., 1971 ; Inano and Tamaoki, 1967 ; Rhind et al., 2001 ; Majdic et al., 1996 ; Sweeny et al., 2000). The postnatal developmental changes that occur within the testis are extremely sensitive to several different hormones, including estrogens. To further understand the hypothalamic—pituitary—testicular axis and to provide insight into the most powerful synthetic estrogenic disruptor imprinting and estrogen receptor inhibitor, investigation of spermatogenesis is now in progress.

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