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Differential Expression of 3β -Hydroxysteroid Dehydrogenase mRNA in Rat Testes Exposed to Endocrine Disruptors

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Abstract. Expression of 3β -hydroxysteroid dehydrogenase (3β -HSD) is mainly found in the Leydig cells from which steroid hormones are biosynthesized in the testes. To investigate whether endocrine disruptors affect the microenvironment of the testes, the mRNA expression of 3β -HSD as a molecular marker for androgen biosynthesis was analyzed in rat testes exposed to several endocrine disruptors using a reverse transcription-polymerase chain reaction technique. Testosterone [50, 200 and 1,000 μ g/kg body weight (BW)], flutamide (1, 5 and 25 mg/kg BW), ketoconazole (0.2, 1, 5 and 25 mg/kg BW), diethylhexyl phthalate (10, 50 and 250 mg/kg BW), nonylphenol (10, 50, 100 and 250 mg/kg BW), octylphenol (10, 50 and 250 mg/kg BW), and diethylstilbestrol (10, 20 and 40 μ g/kg BW) were orally administered to 4-week-old Sprague-Dawley rats for 3 weeks daily. Although testosterone at a low dose (50 μ g/kg/day) increased the expression of 3β -HSD mRNA, it was significantly decreased in the rats treated with 200 or 1,000 μ g/kg/day testosterone compared with the control group ($P < 0.05$). Furthermore, ketoconazole, diethylhexyl phthalate, nonylphenol, octylphenol and diethylstilbestrol caused significant downregulation of 3β -HSD mRNA in the testes at all doses ($P < 0.05$). However, flutamide remarkably increased the level of 3β -HSD mRNA in the testes ($P < 0.05$). These results suggest that endocrine disruptors may influence androgen biosynthesis in the testes by alteration of 3β -HSD mRNA expression.

Key words: 3β -hydroxysteroid dehydrogenase (3β -HSD), Endocrine disruptors, Leydig cells, Rat, Testis

(J. Reprod. Dev. 53: 465–471, 2007)

Steroid hormones are synthesized from cholesterol in the adrenal cortex and gonads in response to pituitary tropic hormones such as adrenocorticotrophic hormone (ACTH), follicle stimulating hormone (FSH) and luteinizing hormone (LH). Sex steroids induce and maintain secondary sexual characteristics and are essential for reproduction [1].

Development of the mature adult testis is initiated by the final generation of Leydig cells, which starts just before pubertal development in the male rat. The number and size of Leydig cells increases in conjunction with increased Leydig cell steroidogenesis [2]. Most studies of steroidogenesis during pubertal development have focused on the declining expression and activity of a steroid metabolizing enzyme, 3β -hydroxysteroid dehydrogenase/isomerase (3β -HSD), in Leydig cells which converts testosterone to biologically

Accepted for publication: December 2, 2006

Published online: January 17, 2007

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active compounds [3]. Little data exist concerning the tissue localization and temporal pattern of steroidogenic enzyme expression in the biosynthetic pathway to testosterone in the rat testis. A key enzyme in steroidogenesis is 3β -HSD, which is often used as a histochemical marker for Leydig cells. It converts pregnenolone to progesterone, an obligatory intermediate in testosterone biosynthesis [4]. Testosterone biosynthesis is also dependent on 17α -hydroxylase/ $17, 20$ desmolase (P450_{c17}), a single catalytic protein in the rat testis [5].

Endocrine disruptors can disturb development of the endocrine system and organs that respond to endocrine signals in organisms indirectly exposed to them during prenatal and/or early postnatal life [6]. Many studies have reported the detrimental effects of endocrine disruptors. For example, anti-androgenic endocrine disruptors inhibit spermatogenesis with additional antigonadotropic properties. Anti-androgens, such as flutamide (FM), have a slight and transient influence on spermatogenesis at most. FM blocks negative feedback of testosterone on the hypothalamo-pituitary axis [7] and inhibits production of the ventral prostate [8]. Ketoconazole (KC) decreases epididymal sperm number and motility, increases the proportion of abnormal sperm [9], and reduces steroidogenesis by inhibiting the cytochrome P-450 enzymes [10]. DeFelice *et al.* [11] reported that KC induced gynecomastia in male rats. Diethylhexyl phthalate (DEHP) treatment produces developmental and/or reproductive toxicity in a wide range of mammalian species with a period of susceptibility extending from the fetal to pubertal stages of life and induces reproductive tract malformations in androgen-dependent tissues in male rat offspring [12]. Octylphenol (OP) exerts estrogenic action to suppress LH, FSH and testosterone secretion and enhance prolactin secretion. OP greatly reduces sperm number and adversely influences the size of the rat testis [13]. Diethylstilbestrol (DES) induces a reduction in testis formation and abnormal semen, resulting in infertility in rats [14, 15].

In the present study, we investigated the effects of various endocrine disruptors on steroidogenesis by analyzing the 3β -HSD mRNA expression pattern as a biomarker for Leydig cells in the testes of rats.

Materials and Methods

Animals and chemical treatment

Four-week-old male Sprague-Dawley rats were purchased from Samtaco (Gyeonggi-do, Korea). The animals (n=10/group) were housed under standard laboratory conditions that included a 12-h light/dark cycle, temperature of 21 ± 2 °C, relative humidity of $50 \pm 10\%$ and were fed standard rat pellet and water *ad libitum*. All experiments were approved and carried out according to the "Guide for care and use of animals" (Chungbuk National University Animal Care Committee according to NIH #86-23). The endocrine disruptors, testosterone propionate [T; 50, 200 and 1,000 μ g/kg body weight (BW)], KC (0.2, 1, 5 and 25 mg/kg BW), DEHP (10, 50 and 250 mg/kg BW), OP (10, 50 and 250 mg/kg BW), DES (10, 20 and 40 μ g/kg BW), NP (10, 50, 100 and 250 mg/kg BW), and FM (1, 5 and 25 mg/kg BW), were orally administered to the rats daily for 3 weeks. The control group was treated with corn oil. The rats were euthanized at 7 weeks of age under pentobarbital anesthesia, and then their testes were removed. The testes were frozen using liquid nitrogen and stored at -70 °C until use. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Total RNA extraction and reverse transcription-polymerase chain reaction (PCR)

Total RNA was extracted from testes using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. The RNA pellet obtained in the final step was dissolved in 50 μ l of sterile diethylpyrocarbonate (DEPC)-treated water, and its concentration was determined using a UV spectrophotometer at 260 nm. RNA was kept in DEPC-treated water at -70 °C until use. Reverse transcription of mRNA and amplification of cDNA were performed using a Peltier Thermal Cycler (MJ Research, Waltham, MA, USA).

A cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany) was used to generate cDNA from 1.0 μ g total RNA according to the manufacturer's instructions. The following primer sets were used to amplify 3β -HSD (GenBank accession number GI388200) and GAPDH [16] as an internal control.

3β -HSD forward: 5'-TCTACTGCAGCACAG-TTGAC-3'

Fig. 1. Reverse transcription-polymerase chain reaction (RT-PCR) experiments showing the expression of 3β -hydroxysteroid dehydrogenase (3β -HSD) mRNA in the testes of testosterone-treated rats. GAPDH: the intrinsic control. Values represent means $\pm$ SD (n=10). *P<0.05: compared to the controls.

Fig. 2. The mRNA expression of 3β -hydroxysteroid dehydrogenase (3β -HSD) in the testes of rats exposed to flutamide for 3 weeks. GAPDH: the intrinsic control. Values represent means $\pm$ SD (n=10). *P<0.05: compared to the controls.

3 β -HSD reverse: 5'-ATACCCTTATTTTGTG-AGGGC-3'

GAPDH forward: 5'-AACCGGATTGTCG-TATTGG-3'

GAPDH reverse: 5'-AGCCTTCTCCAATGG-TGGTGAAGAC-3'

The PCR products were run on 2% agarose gel in Tris-borate-EDTA buffer. The expected PCR product sizes of 3β -HSD and GAPDH were 283 and 302 bp, respectively. The relative intensities of the 3β -HSD bands were normalized to the corresponding GAPDH band intensities.

Statistical analysis

The data were analyzed by analysis of variance and Duncan's test, as a post-hoc analysis, using the SPSS software. Statistical significance was set at P=0.05. All data were presented as means $\pm$ SD.

Results

The detrimental effects of endocrine disruptors in the testes of rats were studied through analysis of 3β -HSD mRNA expression as a biomarker. Treatment with endocrine disruptors resulted in a general decrease in 3β -HSD expression, except for treatment with FM. A dose of 50 $\mu\text{g/kg/day}$ T increased the expression of 3β -HSD mRNA.

Fig. 3. RT-PCR amplification of 3β -hydroxysteroid dehydrogenase (3β -HSD) mRNA in rat testes. Rats were exposed to ketoconazole for 3 weeks. GAPDH: the intrinsic control. Values represent means $\pm$ SD (n=10). *P<0.05: compared to the controls.

However, expression of 3β -HSD mRNA was significantly ($P<0.05$) decreased in the rats treated with 200 or 1,000 $\mu\text{g/kg/day}$ T (20 and 30%, respectively) compared with the control group (Fig. 1). Furthermore, while the levels of 3β -HSD mRNA significantly ($P<0.05$) increased to 220–280% in the FM-treated group compared with the control group (Fig. 2), KC caused downregulation of 3β -HSD

Fig. 4. RT-PCR analysis showing mRNA expression of 3β -hydroxysteroid dehydrogenase (3β -HSD) in diethylhexyl phthalate-treated testes. GAPDH: the intrinsic control. Values represent means $\pm$ SD (n=10). *P<0.05: compared to the controls.

Fig. 5. Photograph of the 3β -hydroxysteroid dehydrogenase (3β -HSD) mRNA expression in rat testes using RT-PCR. Rats were exposed to octylphenol for 3 weeks. GAPDH: the intrinsic control. Values represent means $\pm$ SD (n=10). *P<0.05: compared to the controls.

mRNA to less than 70% of the control group level (Fig. 3). In the case of DEHP, 3β -HSD mRNA was significantly ($P<0.05$) decreased to 48–70% of the control level (Fig. 4). OP inhibited 3β -HSD mRNA expression in the testes at all doses. In particular, the signal was remarkably decreased to 27% of the control level in the 50 mg/kg OP group ($P<0.05$; Fig. 5). In the NP-treated rats, 3β -HSD mRNA expression was significantly ($P<0.05$) reduced to 40–50% of the control level (Fig. 6). The testicular

Fig. 6. RT-PCR indicates the expression of 3β -hydroxysteroid dehydrogenase (3β -HSD) mRNA in nonylphenol-treated testes. GAPDH: the intrinsic control. Values represent means $\pm$ SD (n=10). *P<0.05: compared to the controls.

Fig. 7. Expression pattern of 3β -hydroxysteroid dehydrogenase (3β -HSD) mRNA in testes exposed to diethylstilbestrol as measured by RT-PCR. GAPDH: the intrinsic control. Values represent means $\pm$ SD (n=10). *P<0.05: compared to the controls.

level of 3β -HSD mRNA in the rats exposed to various concentrations (10, 20, and 40 μ g/kg/day) of DES was significantly ($P<0.05$) lower (35–42%) than that of the control group (Fig. 7).

Discussion

We investigated the changes of 3β -HSD mRNA expression in the rat testes using RT-PCR after

treatments with endocrine disruptors, including T, FM, KC, DEHP, NP, OP and DES. Our results demonstrate that endocrine disruptors have different effects on 3β -HSD mRNA expression in the testes.

The mRNA of 3β -HSD is known as a maker of steroidogenesis in Leydig cells. T, the principal circulating androgen in the adult male, is essential for maintenance of spermatogenesis and expression of secondary sex characteristics. Production of T is regulated in the testis by a negative feedback system [17].

Our study showed that high doses of T (200 and 1,000 $\mu\text{g/kg/day}$) decreased 3β -HSD mRNA expression, but that a low dose of T (50 $\mu\text{g/kg/day}$) increased the 3β -HSD mRNA level compared with the controls. This phenomenon could be explained by the fact that expression of 3β -HSD is under control of negative feedback mechanism that is regulated by testosterone.

FM is an anti-androgenic compound and a non-steroidal androgen receptor (AR) antagonist that interferes with endogenous androgen binding to ARs in target organs [18]. FM blocks the negative feedback of T in the hypothalamus and pituitary and induces overexpression of steroidogenic enzyme in the testis by increased T production [7]. In our study, the expression of 3β -HSD mRNA was significantly increased in all the FM-treated groups compared with the control group. These results suggest that the negative feedback of T was blocked by FM binding to the AR in the testis.

The KC-treated groups showed a decreased trend in the expression of 3β -HSD mRNA compared with the controls. KC, a broad-spectrum imidazole antimycotic agent, interferes with the cytochrome P-450 enzyme system in several organs [19]. It inhibits microsomal steroidogenesis in Leydig cells [20]. Therefore, our results suggest that KC may inhibit T production in the testis by the blocking 3β -HSD biosynthesis via inhibition of cytochrome P450 enzyme activity.

The DEHP treatment decreased 3β -HSD mRNA expression in the testes compared with the controls. This is in agreement with a previous report showing that DEHP has been recognized as an endocrine disruptor because it induces adverse effects in androgen responsive tissue following perinatal exposure of male rats [12]. It also reduces testicular T production *in vivo* and T levels in the testis [21]. Other studies have reported that

exposure of rats to DEHP (200 mg/kg/day) causes various effects, including a 77% decrease in the activity of the steroidogenesis enzyme 17β -hydroxysteroid dehydrogenase, a 50% reduction in T production in Leydig cells [22], enhanced testosterone 5 α -reductase (T5 α -R) activity in the testis [23], and reduced epididymal sperm density and motility [24].

OP is an alkylphenolic compound formed as a metabolite of some nonionic surfactants used widely in such things as industrial detergents, plastic and petrol additives and dispensers for insecticides [25]. It has been shown to reduce the expression of cytochrome P450 17α -hydroxylase/C17-20 lyase in fetal Leydig cells [26] and to decrease the expression of *steroidogenesis factor-1* (SF-1) mRNA in the fetal testis [27]. It has also been found to be weakly estrogenic *in vitro*, to bind to the estrogen receptor (ER), to stimulate estrogen-dependent growth of breast cancer cells and gene expression *in vitro* [28], and to decrease prenatal T production in the testis. In our study, treatment with OP (10, 50, 100 and 250 mg/kg/day) significantly decreased 3β -HSD mRNA expression. A significantly decreased trend of 3β -HSD mRNA expression was also shown in all the NP-treated groups (10, 50, 100 and 250 mg/kg/day). NP is a weakly estrogenic compound generated from alkylphenol ethoxylates that is widely used in the production of plastics, textiles, and agricultural chemicals and in household applications and cosmetics [25, 29, 30]. Several studies have reported the adverse effects of NP on development of the male reproductive tract when animals are parentally exposed to NP [13, 31]. Therefore, our results suggest that the inhibition of 3β -HSD mRNA production by NP and OP may be due to functional defects in Leydig cells such as a reduction in cytochrome P450 enzyme production.

DES is an anti-androgenic compound that has been used to prevent miscarriage and other pregnancy complications. It has been reported to reduce the activity of testicular P450c17 enzyme, 17α -hydroxylase, and SF-1 in 17.5-day-old fetuses [32–34]. In this study, treatment with DES inhibited 3β -HSD mRNA expression in the testis. Our results suggest that DES probably inhibited steroidogenesis via blockage of 3β -HSD and that it may reduce T production in the testis.

In conclusion, several endocrine disruptors changed the expression pattern of 3β -HSD mRNA

in the testes as determined by RT-PCR. These results suggest that endocrine disruptors may influence androgen biosynthesis in the testis through an abnormal change in *3 β -HSD* mRNA expression.

Acknowledgements

This work was supported by the Korea Research Foundation Grant funded by the Korea Government [MOEHRD, Basic Research Promotion Fund (KRF-2004-005-E00168 and KRF-2005-005-J15002)].

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