Effects of Postnatal Exposure to Octylphenol on the Transcriptions of Steroidogenic Enzymes in Mouse Testis

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Abstract - The effects of postnatal exposure to octylphenol (OP) on the expressions of the steroidogenic enzymes and testosterone production were evaluated. Postnatal male mice (15-day-old) were injected with 2 or 20 mg kg^{-1} body weight (BW) of OP for 5 days and sacrificed on postnatal day 21. Testosterone concentration was measured by radioimmunoassay and the expressions of the testicular genes were determined by RT-PCR analyses. Significant reductions in the mean body and testis weight were observed in the OP treated animals. No marked alteration in the histological structure of the testis were observed, however, slight reduction in the seminiferous tubule diameter and the number of Leydig cells and several pyknotic cells could be identified in the 20 mg kg^{-1} BW of the OP treated animals. Serum testosterone concentration was dramatically reduced and the mRNA expressions of the steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (P450scc) and 17β-hydroxylase/C17-20 lyase ($P450_{17\alpha}$) were decreased. No significant changes of the gene expressions of the steroidogenic factor-1 (SF-1) and estrogen and androgen receptor after the OP treatment showed that the decreased expressions of the steroidogenic enzymes in the present study did not correlate with these genes. Altogether, the present study demonstrates that postnatal treatment of OP inhibits steroidogenesis by decreasing the transcriptional expressions of the StAR and steroidogenic enzymes. The alteration in steroidogenesis may adversely affect the normal development of the testis and spermatogenesis.

Key words : octylphenol, steroidogenesis, testis, testosterone

INTRODUCTION

Octylphenol (OP) is a primary alkylphenol breakdown product and the alkylphenols are one of the most common chemicals in our environment because they are used as diluents in pesticides and as components in paints, shampoos, and inks as well as cosmetics (Ying *et al.* 2002). OP is well-known as one of endocrine disrupting chemicals (EDCs). These man-made chemicals can enter the bodies by ingestion or adsorption and are known to mimic, block or alter the actions of hormones resulting in the alteration of the endocrine and reproductive systems (Guillette and Gunderson 2001; Mueller 2004).

Like other EDCs, the estrogenic nature of OP has been demonstrated well in cell cultures (White *et al.* 1994), in a recombinant yeast screen assay with human

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estrogen receptors (Kuiper *et al.* 1998), and in reporter cell lines (Paris *et al.* 2002). OP is known to display approximately 1/1000-1/10000 estrogenicity of estradiol-17β (White *et al.* 1994). Besides its estrogenic nature, the toxic effects of OP have also been well-documented by previous studies. In cultures of murine splenocytes, OP killed cells at concentrations as low as 10^{-12} M (Nair-Menon *et al.* 1996). OP has been shown to exert direct toxic effects on cultured spermatogenic cells and Sertoli cells isolated from male rats (Raychoudhury *et al.* 1999). OP may also have other receptor-independent metabolic actions depending on the exposure dose and target tissue. Exposure of OP in mouse Leydig tumor cells inhibited the hCG-stimulated cAMP and progesterone production while estradiol (10^{-9} M) did not inhibited them (Nukula *et al.* 1999). Moreover, many in vitro studies using different developmental stages of Leydig cells showed that OP changed their steroidogenic competences (Murono *et al.* 2000; Murono *et al.* 2001).

The Leydig cell of the testis has the capacity to synthesize testosterone from cholesterol (Payne and Youngblood 1995). The biosynthesis of testosterone is dependent on the various steroidogenic enzymes. Steroidogenic acute regulatory protein (StAR) is known to be required for the transport of cholesterol to the mitochondrial membrane and conversions from cholesterol to testosterone occur through mitochondria to smooth endoplasmic reticulum by cholesterol side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase/C_{17-20} lyase ($P450_{17\alpha}$) and 17β-hydroxysteroid dehydrogenase (17β-HSD). Leydig cells also express P450 aromatase, which catalyzes the aromatization of testosterone to estradiol (Payne and Youngblood 1995) and steroidogenic factor 1 (SF-1), which plays an important role in mediating transcriptional regulation of several steroid hydroxylase genes (Peter and Dubuis 2000).

Two populations of Leydig cells arise in the course of testis development. In the mouse, the "fetal" population arises soon after testis differentiation, at about 12.5 day postcoitum and is essential for masculinization of the fetus. The "adult" population, arises after birth, and testosterone secretion by this population is critical for initiation and maintenance of spermatogenesis and the expression of the male secondary sex characteristics (Habert *et al.* 2001). The expressions of steroidogenic enzymes and various transcriptional factors show steady increases during the postnatal development of Leydig cells and reach peaks around puberty (O'Shaughnessy *et al.* 2002).

Previous studies have demonstrated that exposure of the developing male fetus or neonate to estrogenic chemicals can result in reduced testicular size and sperm production in adult life (Sharpe *et al.* 1995) and chronic exposure to OP for adult rats reduced testosterone production and disrupted spermatogenesis (Blake and Boockfor 1997; Boockfor and Blake 1997). Because postnatally differentiated Leydig cells are major sites for testosterone production in the adult stage, it would be important to understand the effects of OP exposure during the developmental stage.

The aim of the present study was therefore to investigate the effects of postnatal treatment of OP on the testosterone production and testicular development *in vivo*. The present study also extended to identify the changes of the gene expressions of the steroidogenic enzymes and their related transcription factor in order to clarify whether the changes in the testosterone production coincide with the altered gene expressions of these enzymes in the testis.

MATERIALS AND METHODS

1. Chemicals

Corn oil was purchased from Sigma Chemicals Co. (St. Louis, MO); 4-*tert*-octylphenol (OP) was purchased from Fluka Chemica (Buchs, Switzerland; >90% pure); and 1,3,5 [10]-estratriene-3, β-diol 17-valerate (EV) was purchased from Sigma Chemical Co. (St. Louis, MO; 98% pure).

2. Animals and treatments

Mice were maintained and utilized in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Pregnant ICR mice [ILAR Code: Sam;Tac; ICR; gA (ICR)fBR] were purchased from Samtako BioKorea. Each pregnant mouse was housed per cage in a room with a controlled lighting (12L : 12D) and temperature (20-22°C), and given food and tap water *ad libitum*.

Male offspring on postnatal day (PND) 15 were either not treated (no injection; CON), injected intraperitoneally with 0.1 mL corn oil alone (vehicle control; CV) or 2 or 20 mg kg^{-1} body weight of OP. Injections were given from 15 to 19 PND for 5 days and the animals were sacrificed 2 days after the last injection.

3. Measurement of body and organ weight

Body and organ weights were measured two days after the last treatment. A blood sample was obtained by a cardiac puncture under diethyl ether anesthesia. Both sides of the testis and spleen were weighed.

4. Histological analyses

The testes were fixed in 10% neutral buffered formalin (NBF), dehydrated in graded ethanol, cleared in xylene, embedded in paraffin. Sections (4 μm thick) were mounted on glass slides and stained with hematoxylin and eosin (Sigma Chemical Co., St Louis, MO).

5. Radioimmunoassay (RIA) of the serum testosterone

The testosterone antibodies were produced by an immunization of 4-androsten-17β-ol-3-one 3-carboxymethyloxim: bovine serum albumin (Testosterone 3-CMO: BSA, Steraloids Inc., Wilton, NH) to rabbit (New Zealand White). Steroid hormones in the serum were extracted using diethyl ether and dissolved in gelatin phosphate buffered saline. Serum testosterone level was measured by the conventional radioimmunoassay (RIA) (Yoon *et al.* 1981; Yoon and Kim 1987). The cross-reactivity of the antibody is 41% for 5α-dihydrotestosterone, 27.48% for 5β-dihydrotestosterone, 0.14% for androsterone, and 0.054% for progesterone. The cross reactivity to other steroids including estradiol and cortisol was below 0.001%. The between assay variation for testosterone was 8.78% at 42.4±3.7 pmol L^{-1} and 7.8% at 10.3±0.8 pmol L^{-1}. The within assay variation was 4.1% at 45.6±2.8 pmol L^{-1} and 4.4% at 9.8 pmol L^{-1}.

6. RT-PCR analysis of the testicular gene expression

Total RNA from the whole testis was isolated by using TRIzol (Invitrogen; Carlsbad, Canada) and denatured at 65°C for 5 min. First-strand cDNA synthesis was carried out using 1 μg of the total RNA in a total volume of 20 μL containing 20U AMV reverse transcriptase (Roche, Penzberg, Germany) and the reaction mixture of 10 mM Tris (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dNTP mix, 2.5 μM oligo-$p(dT)_{15}$-primer, and 50U RNase inhibitor. The reaction was allowed to proceed at 42°C for 75 min followed by an increase to 95°C for 5 min. The cDNAs obtained were used as a template for a subsequent PCR amplification using 1U of Taq DNA polymerase (Roche, Penzberg, Germany). Primer sequences and their PCR conditions used in this study are summarized in Table 1. In these conditions, preliminary experiments demonstrated that the plateaus for the amplification were not reached. Finally, PCR products were analyzed by electrophoresis on 2% agarose gel. The gel was photographed under UV light and an analysis of the respective bands was performed using an image analyzer (Viber Louvmat, Marne-la-Vallée, France). The intensity of each mRNA band was normalized to the intensity of the corresponding β-actin band.

7. Statistics

Numerical values were expressed as the mean ± SEM. One-way ANOVA was performed when the analysis of the variance indicated a significant difference. A $p < 0.05$ was considered statistically significant.

RESULT AND DISCUSSTION

Significant reduction in the mean body weight was observed in the 20 mg kg^{-1} OP treated group compared to those in the control and vehicle treated groups (Table 1). Nagao *et al.* (2001) also reported that neonatal exposure to 25 mg kg^{-1} of OP reduced the body weight at 7 and 9 weeks of age in rat. It is well documented that estrogenic and antiestrogenic compounds alter the hypothalamic-pituitary-ovarian feedback and the secretion and regulation of growth hormone are related to this axis (Pons *et al.* 1993). Hence, Nagao *et al.* (2001) suggested that the measurement of levels of growth hormone (GH), insulin growth factor-1 (IGF-1), IGF-

Table 1. PCR primers used to analyze mRNA expressions by RT-PCR

Target mRNA	Primer sequence	Annealing Tm (°C)	Product size (bp)	Reference
β-actin	F 5′-GTGGGCCGCTCTAGGCACCAA-3′ R 5′-CTCTTTGATGTCACGCACGATTTC-3′	63	540	Wolkowicz *et al.* 1996
StAR	F 5′-GAC CTT GAA AGG CTC AGG AAG AAC-3′ R 5′-TAG CTG AAG ATG GAC AGA CTT GC	57	980	Manna *et al.* 2001
$P450_{scc}$	F 5′-AGT GGC AGT CGT GGG GAC AGT-3′ R 5′-TAA TAC TGG TGA TAG GCC ACC-3′	63	411	O'shaughnessy and Mannan 1994
$P450_{17\alpha}$	F 5′-CCC ATC TAT TCT CTT CGC CTG GGT A-3′ R 5′-GCC CCA AAG ATG TCT CCC ACC GTG-3′	68	743	Strömstedt and Waterman 1995
Aromatase	F 5′ -CCT GAC ACC ATG TCG GTC ACT-3′ R 5′- GGG CTT AGG GAA GTA CTC GAG -3′	56	312	Ivanova and Beyer 2000
AR	F 5′-CAG GAG GAA GGA GAA AAC-3′ R 5′-GAT AAC AAG GCA GCA AAG-3′	55	188	Gupta 1999
ERβ	F 5′-ACA GTC CTG CTG TGA TGA ACT-3′ R 5′-ACT AGT AAC AGG GCT GGC ACA-3′	59	273	Küppers and Beyer 1999
SF-1	F 5′-TGG TGT CCA GTG TCC ACC CTT AT-3′ R 5′-TCG GTG CAC GTG TAA TGC TTG T-3′	59	212	Strömstedt and Waterman 1995

binding protein 3 needs to be investigated for the mechanism of the reduced body weight after treatment of xenoestrogens. Therefore, we assumed the decreased body weights after the OP treatment in the present study might have resulted from the GH deficiency and/or other direct toxic effects of the treated chemicals.

Absolute testis weight was reduced both in the 2 and 20 mg kg^{-1} OP treated groups but the relative testis weight was reduced only in the 20 mg kg^{-1} OP treated group. In contrast, both the absolute and relative spleen weights were not changed significantly after the OP treatments (Table 1 and 2). Based upon the other results and our previous data that shrinkage of the testis and other male accessory sex organs were observed after OP administration (Boockfor and Blake 1997; Yoshida *et al.* 2001; Kim *et al.* 2004), it is thought that OP affects especially on the development of the reproductive organs. The reduction in testis weights was accompanied by a minor alteration of histological structure in the testis. Slight reduction in the seminiferous tubule diameter and the number of Leydig cells was observed in the 20 mg kg^{-1} OP treated groups (Fig. 1) and several multinucleated bodies and cells with pyknotic nuclei could be identified. However, no significant change in overall number of germ cells inside the tubules and in the lumen formation was observed in all the OP treated groups (Fig. 1). Other studies using high doses of OP treatment showed a marked reduction in the size and weight of the testis and a severe impairment in histological structure of the testis (Boockfor and Blake 1997; Yoshida *et al.* 2001) and moreover, in our previous study, increased apoptosis of testicular germ cells with severe shrinkage of the testes was observed after prepubertal exposure to high dose of OP treatment (Kim *et al.* 2004). In the present study, those marked alterations in histological structure of testis were not observed because of the relatively lower doses of OP treatment. However, alteration in normal development of testis still observed as could be seen in the reduction in the testis weight and the incidences of apoptosis in testicular germ cells.

Table 2. Body and absolute organ weights of mice treated with EV or OP

Body (g) or organ (mg)	Experimental group			
	CON	CV	OP (2 mg)	OP (20 mg)
Body	9.92±0.4	12.14±0.6	10.17±0.4	7.67±0.2*
Testis	24.63±0.9	28.00±2.2	21.50±1.6*	15.92±1.1**
Spleen	106.75±8.2	150.00±20.4	95.67±6.4	81.83±5.9

Each value represents the mean±SEM of data from the 5 mice/group.
Significantly differences from the control values are denoted as *$P<0.05$ and **$P<0.01$.

Serum testosterone concentration was markedly reduced in the 20 mg kg^{-1} of OP treated group (Fig. 2). Testosterone level in the 2 mg kg^{-1} OP treated group was similar to the control and vehicle treated group. Abnormal development of the testis after OP treatment

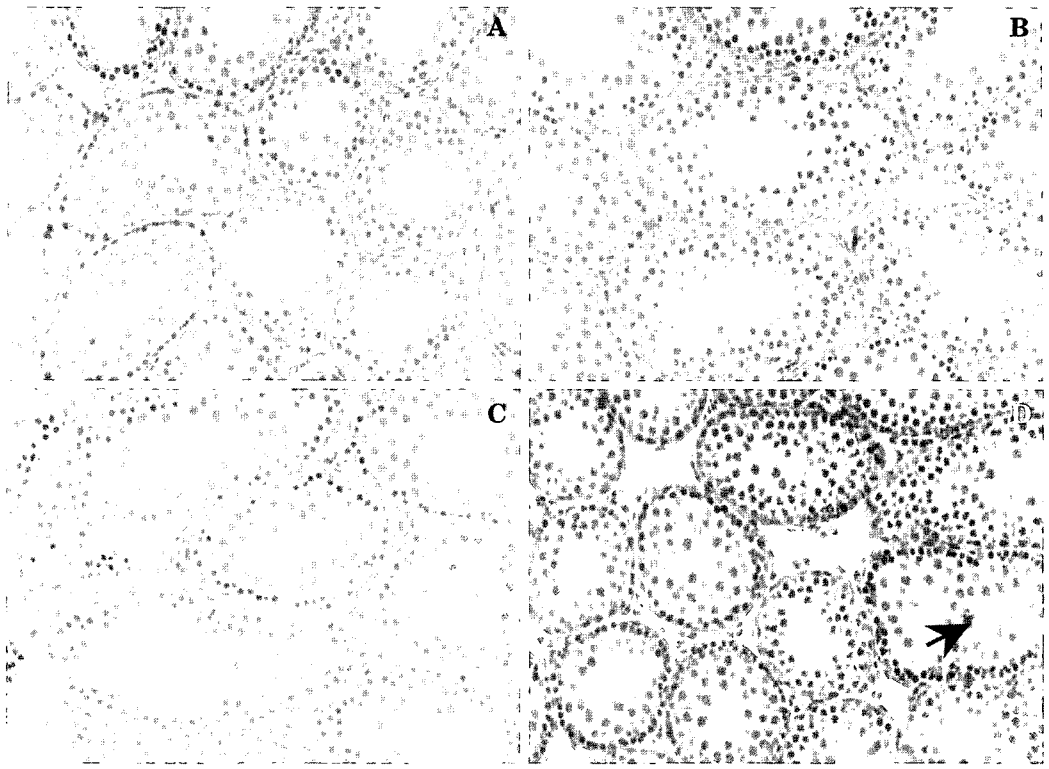

Fig. 1. Microphotographs of PND 21 testes after the treatment. The testes were sectioned with a thickness of 4 μm and stained with hematoxylin-eosin. Arrow indicates a testicular germ cell showing pyknotic nuclei. Magnification, × 200. A, Control; B, CV; C, 2 mg of OP; and D, 20 mg of OP.

in the present study could have resulted from the reduced testosterone production as this hormone is necessary to maintain the normal structure and function of the testis. Other studies with high doses of OP exposure during different developmental stages also revealed a reduced testosterone production (Blake and Boockfor 1997; Haavisto *et al.* 2003). Especially, Sharpe *et al.* (2003) reported the reduced testosterone concentration after neonatal treatment of OP, however, in this study, Leydig cell volume per testis and immunoexpression of 3β-HSD in Leydig cell were not changed in the OP treated group. Although a slight reduction in Leydig cell number was observed in the present study, further studies would be needed to clarify whether the decreased testosterone level resulted from the reduced number of Leydig cell and/or alteration in the function of these cells.

In the present study, the expression of StAR mRNA in the testis of mice treated with 20 mg kg^{-1} OP was significantly decreased while the expression of SF-1 mRNA was not changed compared with that of the control and the vehicle-injected group (Fig. 3). In utero exposure to 100 mg kg^{-1} OP, lipid droplet vacuolizations were observed in Leydig cells (Haavisto *et al.* 2003). As

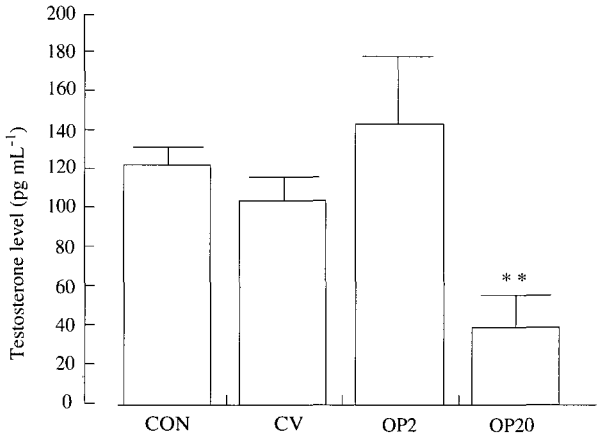

Fig. 2. Changes of the serum testosterone concentration of mice treated with EV or OP. Serum testosterone was determined by radioimmunoassay. Each value represents the mean ± SEM of data from the 5 mice/group. Significantly differences from the control values are denoted as **, $P<0.01$.

StAR protein has a role in the transport of cholesterol, which is mainly stored in the lipid droplet, to the mitochondria membrane (Payne and Youngblood 1995), it is thought that reduced expression of StAR after the OP treatment could have resulted in the accumulation of lipid droplet in Leydig cells.

The mRNA expression of P450scc also decreased in the 20 mg kg^{-1} OP treated group and in the case of the expressions of $P450_{17\alpha}$, both 2 and 20 mg kg^{-1} of OP treated groups showed decreased levels (Fig. 4). In contrast, no significant changes in the mRNA expression of the aromatase, androgen receptor (AR) and estrogen receptor β (ERβ) were observed in all the treated groups compared to the control and vehicle treated groups (Fig. 4 and 5). Majdic *et al.* (1996) also reported a decreased mRNA expression and enzyme activity of $P450_{17\alpha}$ in fetal rat testis after maternal exposure to 600 mg kg^{-1} of OP. Several *in vitro* studies have demonstrated that OP exposure alter the steroidogenic competence of Leydig cells (Murono *et al.* 2000; Murono *et al.* 2001), however, these studies did not identify the alteration in the expressions of the steroidogenic enzymes in Leydig cells. From our results, the decreased testosterone concentration after OP treatment was thought to have resulted from the decreased expressions of the steroidogenic enzymes in Leydig cell. Another previous study showed that maternal exposure to 600 mg kg^{-1} of OP altered the mRNA and protein expressions of SF-1 in fetal rat testis (Majdic *et al.* 1997). In the present study, a decreased mRNA expression of SF-1 was not observed, therefore, the decreased transcriptional exprressions of StAR and steroidogenic enzymes in the trea-

Table 3. Relative organ/BW ratios (mg g^{-1} BW) of mice treated with EV or OP

Body (g) or organ (mg)	Experimental group			
	CON	CV	OP (2 mg)	OP (20 mg)
Testis	2.49±0.13	2.31±0.19	2.11±0.09	2.07±0.17
Spleen	10.72±0.45	12.30±1.34	9.47±0.91	10.60±0.55

Each value represents the mean±SEM of data from the 5 mice/group.
Significantly differences from the control values are denoted as *P<0.05.

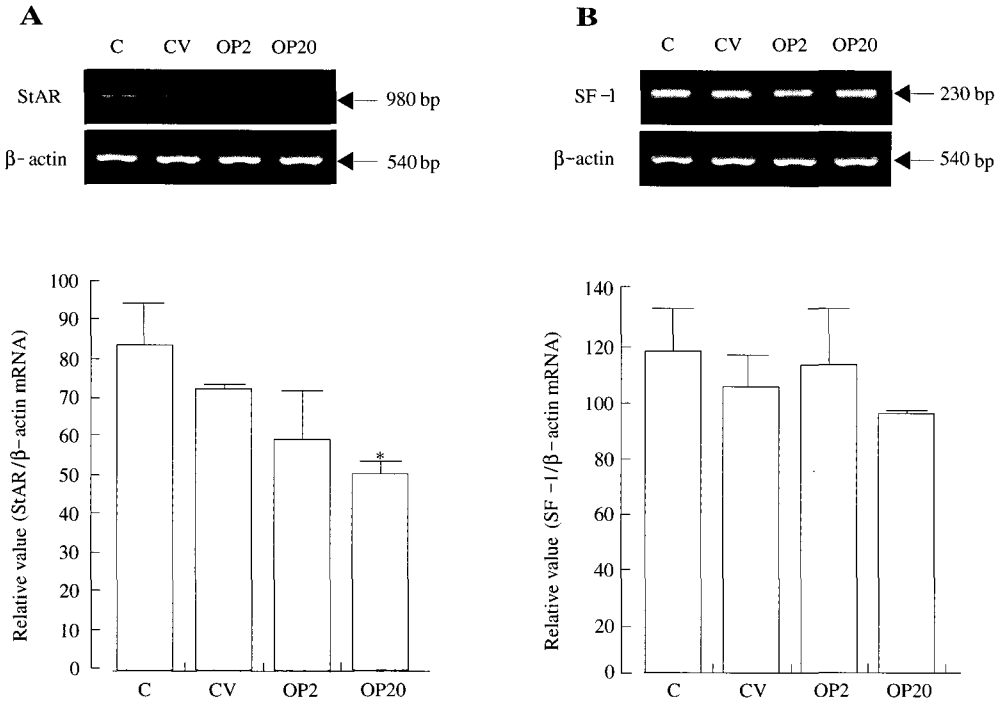

Fig. 3. Changes on the expressions of testicular StAR (A) and SF-1 (B) mRNA in 21 days old mice after the treatment. Total RNA were prepared from the testis of mice injected *i.p.* with 0.1 mL corn oil or containing 2 or 20 mg of OP from postnatal day 15 to 19. The upper panel shows the representative PCR products stained with ethidium bromide. A lower panel illustrates the relative ratios of StAR and SF-1 mRNA to β-actin, respectively. Significantly differences from the control values are denoted as *, P<0.01.

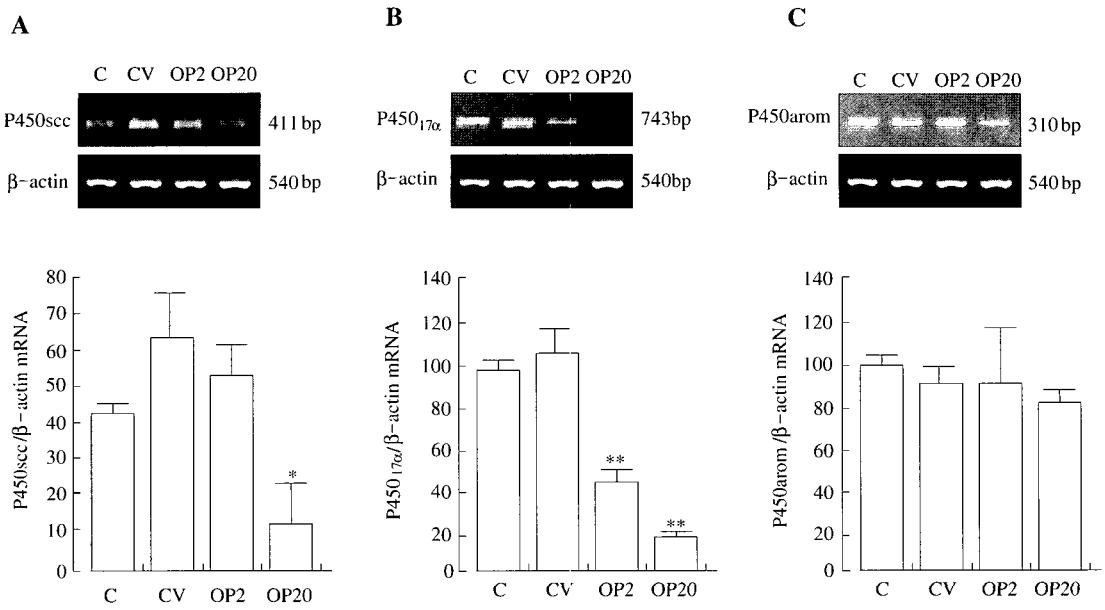

Fig. 4. Changes on the mRNA expressions of steroidogenic enzymes in 21 days old mice after the treatment. Total RNA were prepared from the testis of mice injected *i.p.* with 0.1 mL corn oil or containing 2 or 20 mg of OP from postnatal day 15 to 19. The upper panel shows the representative PCR products stained with ethidium bromide. A lower panel illustrates the relative ratio of P450scc (A), P45017α (B) and Aromatase (C) mRNA to β-actin, respectively. Significantly differences from the control values are denoted as *, $P<0.01$ and **$P<0.01$.

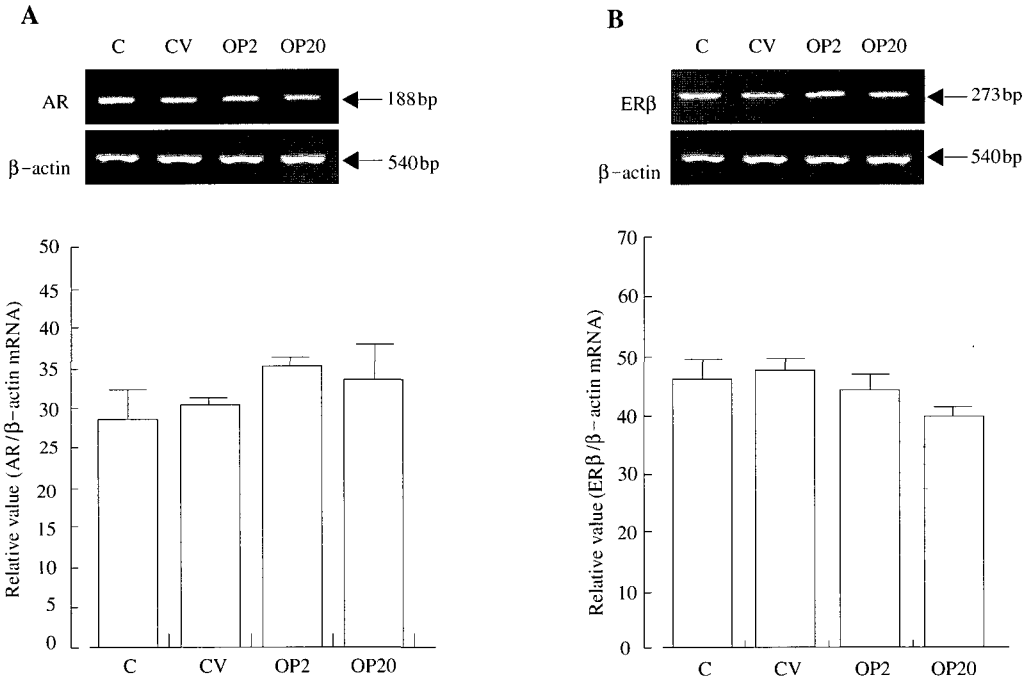

Fig. 5. Changes on the expression of testicular AR (A) and ERβ (B) mRNA in 21 days old mice after the treatment. Total RNA were prepared from the testis of mice injected *i.p.* with 0.1 mL corn oil or containing 2 or 20 mg of OP from postnatal day 15 to 19. The upper panel shows the representative PCR products stained with ethidium bromide. A lower panel illustrates the relative ratios of AR and ERβ mRNA to β-actin, respectively.

ted doses of OP in this study did not result from an alteration in the mRNA expression of SF-1.

ERs are present in the testis, efferent ductules and epididymis (Hess 2003) and many investigators have reported that exogenous estrogens may compromise the male reproduction system by binding to ERs (Nagoa *et al.* 1999; Toyama *et al.* 2001). From our results, it could be assumed that an impairment of the testis development after OP treatment was not at least caused by alteration in the expression of ER and AR in testis.

In conclusion, results obtained in the present study indicate that postnatal treatment of OP causes decreased transcriptional expressions of StAR and the steroidogenic enzymes in the differentiating Leydig cells. The reduced gene expressions of the steroidogenic enzymes result in a decreased testosterone level and hence disturb the normal development of the testis. Because the development and differentiation of Leydig cell in this treated period are important to the later adult life, the alteration in steroidogenesis by OP exposure may adversely affect the normal development of the testis and spermatogenesis.

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