Toxicology Letters 188 (2009) 192–200

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Chronic exposure to perfluorododecanoic acid disrupts testicular steroidogenesis and the expression of related genes in male rats

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ARTICLE INFO

Article history:
Received 15 January 2009
Received in revised form 16 April 2009
Accepted 20 April 2009
Available online 3 May 2009

Keywords:
PFDoA
Testosterone
Steroidogenic acute regulatory protein
Insulin-like growth factor I

ABSTRACT

Perfluorododecanoic acid (PFDoA), a synthetic perfluorinated chemical, has been detected in environmental matrices, wildlife, and human serum. Its potential health risk for humans and animals has raised public concern. However, the effects of chronic PFDoA exposure on male reproduction remain unknown. The aim of this study was to determine the effects of chronic PFDoA exposure (110 days) on testosterone biosynthesis and the expression of genes related to steroidogenesis in male rats. In this study, we examined the serum levels of sex hormones, growth hormone, and insulin in male rats. Testicular morphology and the expression of key genes and proteins in testosterone biosynthesis were also analyzed. Markedly decreased serum testosterone levels were recorded after 110 days of PFDoA exposure at 0.2 mg PFDoA/kg/day and 0.5 mg PFDoA/kg/day, and cast-off cells were observed in some seminiferous tubules in testes exposed to 0.5 mg PFDoA/kg/day. PFDoA exposure resulted in significantly decreased protein levels of steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage enzyme (P450scc), along with significantly reduced mRNA levels of insulin-like growth factor I (IGF-I), insulin-like growth factor I receptor (IGF-IR), and interleukin 1α (IL-1α) in rat testes at 0.2 mg/kg/day and 0.5 mg/kg/day. In addition, PFDoA exposure also affected the expression of some genes in the hypothalamo-neurohypophyseal system. However, PFDoA did not affect the expression of 5α-reductase, 3α-hydroxysteroid dehydrogenase, or aromatase in testis and liver. These findings demonstrate that chronic PFDoA exposure disrupts testicular steroidogenesis and expression of related genes in male rats. Multiple factors may be involved in the inhibition of testosterone by PFDoA.

1. Introduction

The perfluoroalkyl acids (PFAAs), including perfluorododecanoic acid (PFDoA, C12), perfluorodecanoic acid (PFDA, C10), perfluorooctanoic acid (PFOA, C8), and perfluorooctane sulfonic acid (PFOS, C8), are a family of perfluorinated chemicals that contain a carbon backbone. PFAAs are used as surfactants in industrial and consumer products, including firefighting foams, paint additives, and stain-resistant coatings for clothing (Renner, 2001). PFAAs have been detected in the environment, in wildlife and in human blood, testes, liver, and breast tissue in various countries (Lau et al., 2007). Studies determined that the total amounts of PFAAs transferred from nursing mothers to breast-feeding infants are approximately 200 ng/day and 23.5 ng/kg of bw/day in Sweden and the USA, respectively (Karrman et al., 2007; Tao et al., 2008). Although PFOA and PFOS are the two main PFAAs detected in samples worldwide, the existence of longer forms, such as PFDoA, has also been consistently reported in the recent scientific literature (Tao et al., 2008; Van de Vijver et al., 2007). For example, the highest PFDoA concentrations reported were 9.74 pg/ml in human milk in Massachusetts (Tao et al., 2008) and 9.5 ng/g (wet weight) in the liver of harbor porpoises from the Black Sea (Van de Vijver et al., 2007). The potential health risk of PFAAs to humans and wildlife has raised public concern.

PFAAs with chains of 8–10 carbons lead to hepatotoxicity, immunotoxicity, and lung toxicity in laboratory animals (Lau et al., 2007). In our previous study, PFDoA exposure also caused prominent hepatotoxicity in male rats (Zhang et al., 2008). Moreover, the reproductive toxicity of PFAAs has been reviewed in rodent models (Lau et al., 2007). PFDA targets the testes; exposure to PFDA results in obvious testicular toxicity, with decreased plasma testosterone and dihydrotestosterone levels in rats (Bookstaff et al., 1990; Olson and Andersen, 1983). PFOA exposure leads to decreased testosterone levels in serum and in testicular interstitial fluid, as well as increased serum estradiol levels in male rats (Biegel et al., 1995; Martin et al., 2007). Tumors in both Leydig cells and pancreatic acinar cells have been observed in male rats following chronic exposure to ammonium PFOA at 300 ppm in their diet (Biegel et al., 2001). An exclusive study examining PFDoA toxicological activity by our group demonstrated that 14 days of PFDoA exposure at a 5 mg/kg/day or 10 mg/kg/day dose resulted in testicular cell apop-

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doi:10.1016/j.toxlet.2009.04.014

tosis and a decline in serum testosterone (T) levels (Shi et al., 2007). Recently, PFAAs have been considered to be a potential endocrine disruptor, demonstrating effects on sex hormone levels (Jensen and Leffers, 2008).

PFDoA is likely to be more toxic than PFOA and PFDA, since PFAAs with longer carbon chains persist longer in the body than shorter chain PFAAs (Kudo et al., 2001; Ohmori et al., 2003). PFDoA has been shown accumulate more than PFOA in wildlife species (Senthilkumar et al., 2007). However, little is known about the toxic effects and mechanism of PFDoA on animals. Although an acute study suggests that testosterone inhibition by PFDoA is associated with cholesterol transport and steroidogenesis in the testes (Shi et al., 2007), it is unknown whether other signaling mechanisms are affected by PFDoA. Moreover, the toxic characteristics that occur in acute exposure studies with high doses may not be the same as those present during chronic exposure at low doses. In addition, since humans and animals are exposed to this chemical on a prolonged basis in normal environmental, chronic exposure provides a more relevant model to study the effects of PFDoA on reproductive function.

In the present study, we exposed male rats to PFDoA for 110 days to identify any effects on testicular function after chronic exposure. We analyzed the resulting changes in serum levels of sex hormones, growth hormone (GH), and insulin in male rats treated with PFDoA for 110 days. Real-time quantitative polymerase chain reaction (qPCR) and Western blot analyses were used to characterize the changes in gene and protein expression associated with cholesterol transport and testosterone biosynthesis. In addition, the expression of hypothalamo-neurohypophyseal genes responsible for regulating testicular function was also evaluated by qPCR. This study reveals the toxic effects of PFDoA on chronically exposed male rats and a possible molecular mechanism by which it disrupts steroidogenesis.

2. Materials and methods

2.1. Animals

Three-week-old male Sprague–Dawley rats were purchased from Weitong Lihua Experimental Animal Center, Beijing, China. Animals were housed separately and maintained in a mass air-displacement room with a 12-h light–dark cycle at 20–26 °C and a relative humidity of 40–60%. Animals had access to food and water *ad libitum*. All rats were acclimatized for 1 week before the experiment began.

2.2. Study protocol

PFDoA (CAS No. 307-55-1, 95% purity, Sigma–Aldrich) was dissolved in 0.2% Tween-20. Rats were divided into one control and four treatment groups. Each group contained six rats. The rats in the treatment group were given PFDoA orally at doses of 0.02 mg PFDoA/kg weight body/day, 0.05 mg PFDoA/kg weight body/day, 0.2 mg PFDoA/kg weight body/day, and 0.5 mg PFDoA/kg weight body/day for 110 days in a volume of 6 ml/kg of body weight. Control animals were treated with 0.2% Tween-20 (vehicle) delivered orally for 110 days in a volume of 6 ml/kg of body weight.

At the end of the experiment, all of rats from each group were weighed and euthanized by decapitation. Blood was collected and centrifuged at 2000 × *g* at 4 °C for 15 min. Serum was stored at −20 °C until analysis. The testes, prostate, seminal vesicle, and vas deferens of each animal were immediately isolated and weighed. One part of each testis was fixed in modified Davidson's fluid (mDF) containing 30% formaldehyde (concentration 37–40%), 15% ethanol, 5% glacial acid, and 50% distilled water for histological evaluation. Another part of the testis was immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

2.3. Histological analysis

After fixation in mDF for 48 h, testes were embedded in paraffin. Sections of 5 μm in thickness were serially cut from every testis and mounted on glass slides. Slides were stained with hematoxylin and eosin (H&E). Images were photographed using a BH2 microscope fitted with a DP-71 digital camera (Olympus, Tokyo, Japan).

2.4. Detection of serum hormone and total cholesterol levels

Serum testosterone concentrations were measured by the enzyme-linked immunosorbent assay (ELISA) using commercial rat ELISA kits (RapidBio Lab. Calabasas, CA, USA). Estradiol (E_2) concentrations were analyzed by chemiluminescence, while concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), growth hormone (GH), and insulin were determined by the radioimmunoassay (RIA) using commercial kits (Beijing North Institute of Biological Technology, China). Total cholesterol (TCHO) in serum was measured with a commercial TCHO kit (Biosino Bio-technology and Science Inc., Beijing, China). Total cholesterol was quantified by colorimetry (500 nm) using a UV1240 spectrophotometer (Shimadzu, Japan).

2.5. RNA extraction

Testicular RNA was isolated from the testis with TRIZOL reagent (Invitrogen Corp., Carlsbad, CA, USA), according to the manufacturer's instructions. Pituitary and hypothalamic RNA were extracted using the RNeasy kit (Qiagen, Germany). RNA was purified on an affinity resin (Qiagen, Germany). The concentration of total RNA was measured by absorbance at 260 nm using a UV1240 spectrophotometer (Shimadzu, Japan). The purity was estimated by the 260/280 nm absorbance ratio. The quality of total RNA was further confirmed based on the integrity of 28S and 18S rRNA using 1% agarose gel electrophoresis. All RNA samples were stored at −80 °C until analysis.

2.6. Real-time quantitative polymerase chain reaction (qPCR)

RNA samples from each rat were synthesized into cDNA by reverse transcription using an oligo-$(dT)_{15}$ primer (Promega, USA) and M-MuLV reverse transcriptase (Promega, USA), according to the manufacturer's instructions. Real-time PCR reactions were performed using SYBR Green PCR Master Mix reagent kits on the Stratagene Mx3000P qPCR system (Stratagene, USA). The detected genes included luteinizing hormone (LHR) (GenBank accession no. NM_012978), scavenger receptor class B type 1 (SR-B1) (GenBank accession no. AY451993), steroidogenic acute regulatory protein (StAR) (GenBank accession no. NM_031558), cholesterol side-chain cleavage enzyme (P450scc) (GenBank accession no. J05156), 3-beta-hydroxysteroid dehydrogenase (3β-HSD) (GenBank accession no. M38178), 17-beta-hydroxysteroid dehydrogenase (17β-HSD) (GenBank accession no. NM_054007), cytochrome P450c17 subfamily a (CYP17a) (GenBank accession no. NM_012753), aromatase (GenBank accession no. NM_017085), androgen receptor (AR) (GenBank accession no. NM_012502), insulin-like growth factor I (IGF-I) (GenBank accession no. NM_178866), insulin-like growth factor I receptor (IGF-IR) (GenBank accession no. NM_052807), insulin-like growth factor binding protein 3 (IGFBP3) (GenBank accession no. NM_012588), growth hormone receptor (GHR) (GenBank accession no. J04811), insulin receptor (IR) (GenBank accession no. M29014), interleukin-1 alpha

Table 1
Changes in body and reproductive organ weights in PFDoA-treated male rats.

	Doses (mg/kg/day)				
	0	0.02	0.05	0.2	0.5
BW (g)	556.33 ± 11.26	562.00 ± 11.80	571.33 ± 6.48	546.83 ± 1.47	525.50 ± 11.75*
TW (g)	3.65 ± 0.14	3.54 ± 0.09	3.75 ± 0.05	3.40 ± 0.11	3.15 ± 0.20
RTW (%)	0.65 ± 0.02	0.63 ± 0.02	0.66 ± 0.01	0.62 ± 0.02	0.61 ± 0.03
PW (g)	0.65 ± 0.03	0.58 ± 0.06	0.64 ± 0.02	0.65 ± 0.04	0.60 ± 0.02
RPW (%)	0.12 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.11 ± 0.01
SW (g)	1.47 ± 0.12	1.67 ± 0.16	1.75 ± 0.04	1.74 ± 0.07	1.83 ± 0.10
RSW (%)	0.26 ± 0.02	0.29 ± 0.03	0.31 ± 0.01	0.33 ± 0.02	0.30 ± 0.02
VW (g)	0.24 ± 0.01	0.22 ± 0.02	0.26 ± 0.01	0.25 ± 0.01	0.25 ± 0.01
RVW (%)	0.043 ± 0.002	0.039 ± 0.004	0.049 ± 0.002	0.045 ± 0.001	0.047 ± 0.001

Values represent the means ± S.E.M., $n = 6$. Asterisks indicate a statistically significant difference compared to the control, $p < 0.05$ (one-way ANOVA followed by Dunnett's post hoc two-sided *t*-test). BW, TW, PW, SW, and VW indicate body, testis, prostate, seminal vesicle, and vas deferens weights, respectively. RTW, RPW, RSW, and RVW indicate relative testis, prostate, seminal vesicle and vas deferens weights, respectively.

(IL-1α) (GenBank accession no. NM_017019), gonadotrophin releasing hormone (GnRH) (GenBank accession no. NM_012767), gonadotrophin releasing hormone receptor (GnRH-R) (GenBank accession no. NM_031038), follicle-stimulating hormone (FSH) (GenBank accession no. NM_001007597), luteinizing hormone (LH) (GenBank accession no. J00749), 5alpha-reductase (5α-R) (GenBank accession no. NM_017070), and 3alpha-hydroxysteroid dehydrogenase (3α-HSD) (GenBank accession no. AH009074). The nucleotide sequences for the primers used to test the indicated gene products are shown as supplementary data. The housekeeping gene β-actin (GenBank accession no. NM_031144) was used as an internal control. Cycling conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 94 °C for 5 s, 53–60 °C for 30 s, and 72 °C for 30 s. The expression level of the target gene is presented as sample versus control, compared to the β-actin gene, and was calculated as described previously (Barlow et al., 2003; Pfaffl, 2001).

2.7. Western blot

Protein was extracted from testes using lysis buffer containing the protease inhibitor phenylmethylsulphonyl fluoride (PMSF) (Applygen Technologies Inc., China). Protein concentrations were determined using the Bradford Kit (Applygen Technologies Inc., China). After 70 μg of protein was separated by electrophoresis in a 12% sodium dodecyl sulfate–polyacrylamide gel, proteins were transferred onto a polyvinylidene fluoride membrane (Hybond, Amersham). The blots were probed overnight at 4 °C, following by exposure to either rabbit anti-rat polyclonal StAR antibodies (Abcam) and P450scc antibodies (Chemicon) or a mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were further incubated for 2 h at room temperature in the presence of horseradish peroxidase-conjugated secondary antibodies against rabbit or mouse (BioRad, Hercules, CA, USA). Antibody binding was detected with an ECL Western Blot Detection Kit (Tiangen, Beijing, China) and visualized by exposure to X-ray film (Kodak, Rochester, NY, USA). The intensities of the scanned bands obtained with a Tanon Imager program (Tanon, China) were normalized to the β-actin signal.

2.8. Data analysis

All data were analyzed using SPSS for Windows 13.0 (SPSS Inc., Chicago, IL, USA). All values are expressed as means ± S.E.M. The ratio of testis, prostate, seminal vesicle, and vas deferens organ to body weight was calculated to yield relative weights. Differences in body weight, relative organ weight, serum hormone concentrations, and expression levels of genes and proteins between the control and treatment groups were analyzed by one-way ANOVA followed by Dunnett's post hoc two-sided *t*-test. Values of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Body, testis, prostate, seminal vesicle, and vas deferens weights

The body, testis, prostate, seminal vesicle, and vas deferens weights of male rats exposed to PFDoA for 110 days are shown in Table 1. PFDoA had no effect on the body weight of rats at doses of 0.02 mg/kg/day, 0.05 mg/kg/day, and 0.2 mg/kg/day compared to controls. For the group exposed to 0.5 mg/kg/day PFDoA, however, the body weight of rats significantly decreased by 7.2% ($p < 0.05$). Neither the absolute nor relative weights of the testis, prostate, seminal vesicle, or vas deferens of rats were statistically different between the control and PFDoA-exposed groups.

3.2. Histological features

The testes of vehicle-treated rats showed normal seminiferous tubules lined by both spermatogenic cells and Sertoli cells (Fig. 1A). In rats receiving 0.02 mg PFDoA/kg/day (Fig. 1B), 0.05 mg PFDoA/kg/day (figure not shown) or 0.2 mg PFDoA/kg/day (data not shown), the structure of seminiferous tubules was similar to that in controls. However, cast-off cells were found in some seminiferous tubules in the testes of rats treated with 0.5 mg/kg/day PFDoA (Fig. 1C), although the base membranes of seminiferous tubules were relatively normal compared with controls.

3.3. Total cholesterol and hormone levels in serum

Serum TCHO levels in PFDoA-treated rats were not significantly different from controls (Fig. 2A). Following 110 days of exposure

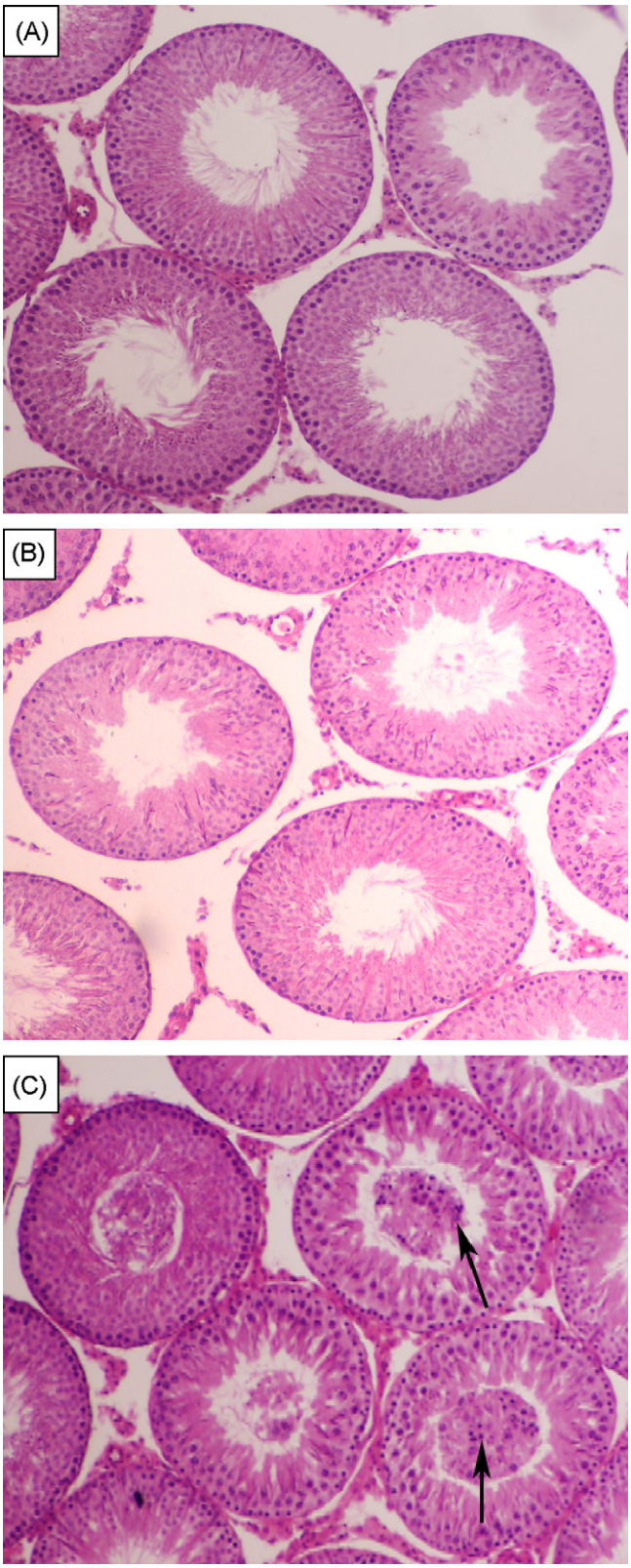

Fig. 1. Changes in the morphology of rat testes in control and PFDoA-treated groups. (A) Testis of control rat. (B) Testis of rat treated with 0.02 mg PFDoA/kg/day. (C) Testis of rat treated with 0.5 mg PFDoA/kg/day. The arrow indicates cast-off cells in the seminiferous tubule. Magnification: 400×.

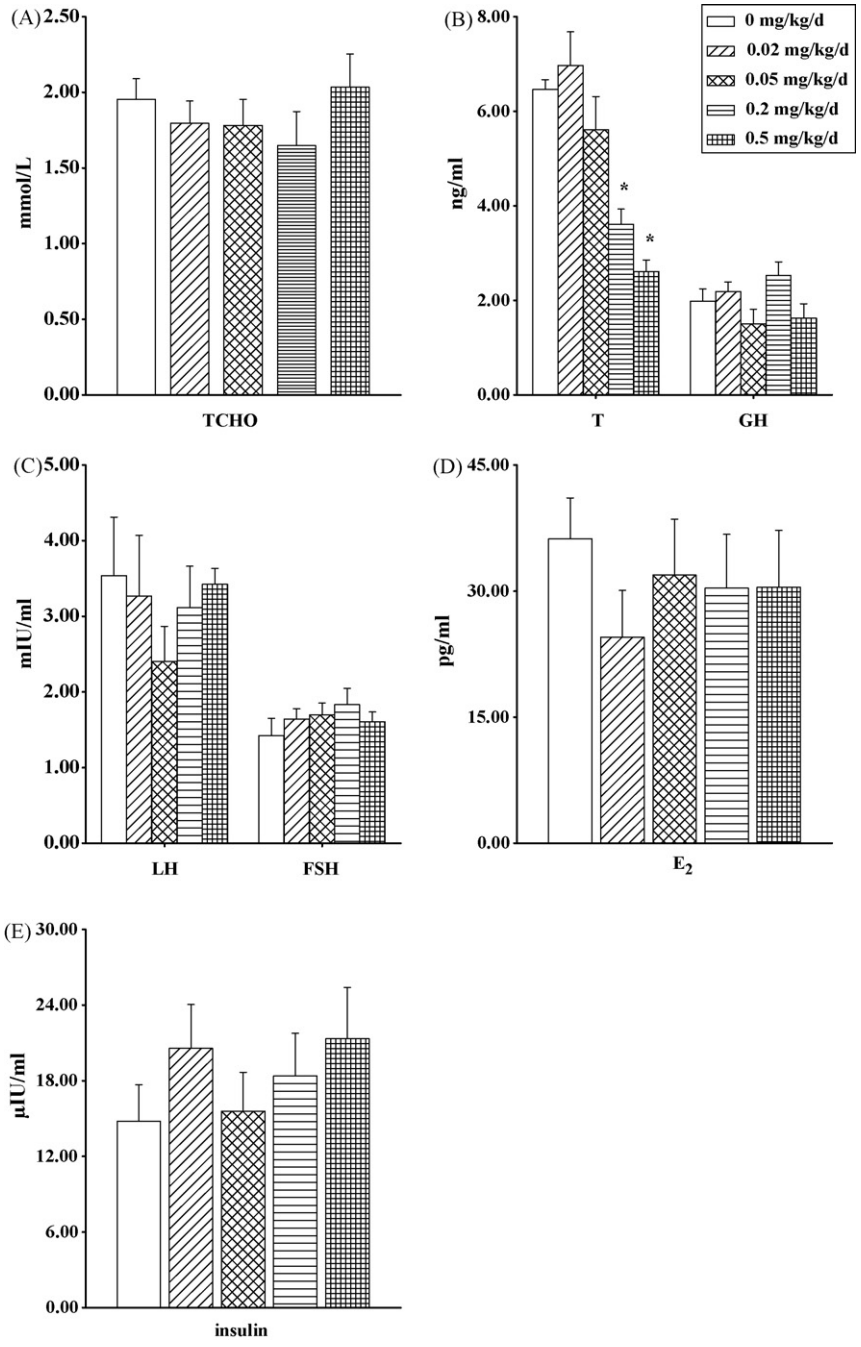

Fig. 2. Serum levels of TCHO, LH, FSH, T, E_2, GH, and insulin in control and PFDoA-exposed rats. Values represent the means ± S.E.M. for groups of six rats each. (A) TCHO levels in control and PFDoA-exposed rats. (B) T and GH levels in control and PFDoA-exposed rats. (C) LH and FSH levels in control and PFDoA-exposed rats. (D) E_2 levels in control and PFDoA-exposed rats. (E) Insulin levels in control and PFDoA-exposed rats. Asterisks indicate statistically significant differences, $p < 0.05$ (one-way ANOVA followed by Dunnett's post hoc two-sided *t*-test).

to PFDoA, serum testosterone levels of rats exposed to 0.2 mg PFDoA/kg/day or 0.5 mg PFDoA/kg/day decreased to 56% and 40% of the control levels, respectively ($p < 0.05$) (Fig. 2B). However, exposure to 0.02 mg PFDoA/kg/day or 0.05 mg PFDoA/kg/day did not change serum testosterone levels. In addition, PFDoA treatment of any tested amount did not significantly change FSH, LH, E_2, GH, or insulin serum levels (Fig. 2B–E).

3.4. qPCR analysis

All doses of PFDoA exposure significantly decreased levels of StAR expression (Fig. 3A). P450scc expression was markedly decreased only after exposure to 0.05 mg/kg/day PFDoA ($p < 0.05$) (Fig. 3A). Testicular IGF-I mRNA levels were significantly decreased by PFDoA at 0.05 mg/kg/day, 0.2 mg/kg/day, and 0.5 mg/kg/day ($p < 0.05$), along with decreased mRNA levels of IGF-IR and IL-1α at 0.2 mg/kg/day and 0.5 mg/kg/day ($p < 0.05$) (Fig. 3B). Testicular AR mRNA levels were significantly increased by PFDoA at 0.2 mg/kg/day ($p < 0.05$), but markedly decreased at 0.5 mg/kg/day ($p < 0.05$) (Fig. 3B). No level of PFDoA exposure affected the expression of GHR or IGFBP-3 in rat testis. Moreover, PFDoA exposure did not exhibit significant effects on the mRNA levels of aromatase, 5α-R, or 3α-HSD in testis and liver (Fig. 3C). In addition, the levels of hypothalamic AR were significantly decreased by PFDoA at 0.05 mg and 0.2 mg/kg/day ($p < 0.05$). However, PFDoA exposure at any dose did not significantly affect hypothalamic GnRH expres-

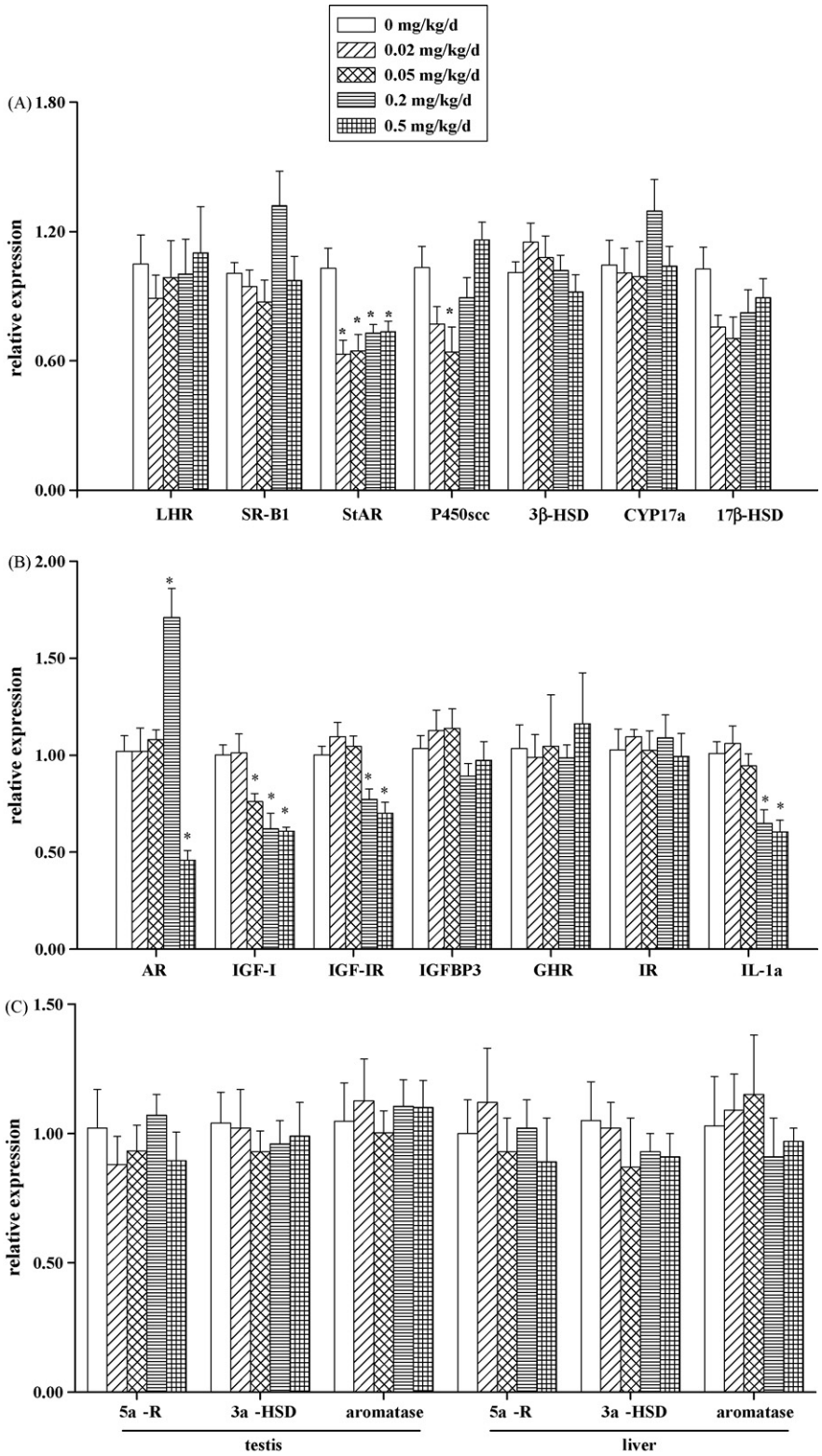

Fig. 3. Real-time quantitative RT-PCR analyses for control and PFDoA-exposed rats. (A) Testicular mRNA for LHR, SR-B1, StAR, P450scc, 3β-HSD, CYP17a, and 17β-HSD. (B) Testicular mRNA for AR, IGF-I, IGF-IR, IGFBP3, GHR, IR, and IL-1α. (C) Testicular mRNA and liver mRNA for 5α-R, 3α-HSD, and aromatase. Gene expression levels represent the mRNA expression levels relative to control levels. Values represent the means ± S.E.M. for six rats per group. Asterisks indicate statistically significant differences, $p < 0.05$ (one-way ANOVA followed by Dunnett's post hoc two-sided *t*-test).

sion (Fig. 4A). LH expression was reduced after PFDoA exposure at 0.05 mg/kg/day ($p < 0.05$) (Fig. 4B). Following 110 days of high-dose PFDoA treatment (0.5 mg/kg/day), the levels of pituitary GnRH-R and FSH expression were significantly reduced ($p < 0.05$) (Fig. 4B). PFDoA exposure at any dose did not affect the levels of pituitary AR.

3.5. Western blot analysis

To further investigate the effects of PFDoA on testicular steroidogenesis, we measured the protein levels of StAR (30 kDa) and P450scc (49 kDa) in PFDoA-treated rats (Fig. 5). Following 110 days

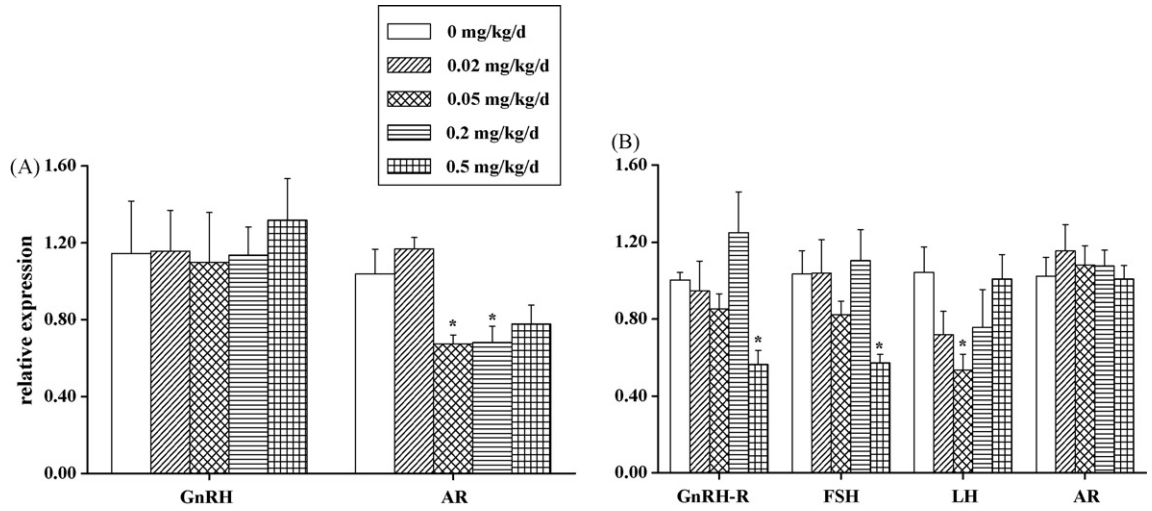

Fig. 4. Real-time quantitative RT-PCR analyses for control and PFDoA-exposed rats. (A) Hypothalamic mRNA for GnRH and AR. (B) Pituitary mRNA for GnRH-R, FSH, LH, and AR. Gene expression levels represent mRNA expression relative to controls. Values represent the means ± S.E.M. for six rats per group. Asterisks indicate statistically significant differences, $p < 0.05$ (one-way ANOVA followed by Dunnett's post hoc two-sided *t*-test).

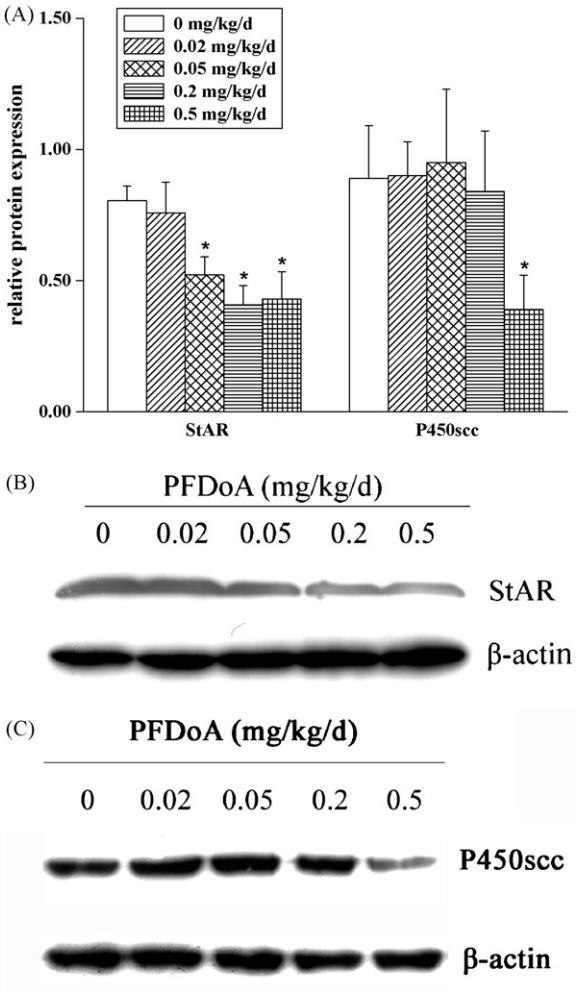

Fig. 5. Western blot analysis of testicular StAR and P450scc protein from control and PFDoA-exposed rats. (A) Quantification of the relative expression of StAR and P450scc in the control and treatment groups. Values represent the means ± S.E.M. for six rats per group. Asterisks indicate statistically significant differences, $p < 0.05$ (one-way ANOVA followed by Dunnett's post hoc two-sided *t*-test). (B) Representative Western blot of StAR and P450scc from control rats. (C) Representative Western blot of StAR and P450scc from PFDoA-exposed rats.

exposure to 0.05 mg PFDoA/kg/day, 0.2 mg PFDoA/kg/day, or 0.5 mg PFDoA/kg/day, 30 kDa StAR protein levels were markedly decreased to 62.6%, 50.6%, and 53% of those in the control rats, respectively ($p < 0.05$). Exposure to 0.02 mg PFDoA/kg/day had no significant effect on StAR protein levels. Only the highest level of PFDoA exposure (0.5 mg/kg/day) significantly decreased P450scc protein levels.

4. Discussion

In the present study, the highest dose (0.5 mg/kg/day) of PFDoA significantly reduced mean body weight. However, the absolute or relative weights of testis, prostate, seminal vesicle, and vas deferens were not affected by PFDoA at any dose, indicating that these endpoints may not be sensitive to PFDoA exposure.

Similar to our previous study investigating 14 days of PFDoA exposure, testosterone concentrations were significantly reduced after chronic exposure to 0.2 mg PFDoA/kg/day or 0.5 mg PFDoA/kg/day. This result is also consistent with a study that exposed male rats to PFOA and PFDA for 14 days or 7 days, respectively (Biegel et al., 1995; Bookstaff et al., 1990). Since serum testosterone level is considered to be an indicator of testicular function, the decreased serum testosterone indicates that chronic PFDoA exposure may affect testicular function and possibly lead to adverse effects on male reproductive function in rats.

Morphological observations revealed that after prolonged exposure to 0.5 mg PFDoA/kg/day, some cells were cast off from some seminiferous tubules in the testes, although the cell types were not determined. Since the seminiferous tubule plays an important role in spermatogenesis, this result implies that PFDoA may affect spermatogenesis in male rats exposed to 0.5 mg/kg/day. Testosterone and FSH are known to support normal morphology of seminiferous tubules and spermatogenesis via binding to their corresponding receptors (AR and FSHR, respectively) (Amann, 2008). Since serum FSH levels and testicular FSHR expression were not affected by PFDoA in the present study, FSH signaling may not be associated with the morphological changes observed in testes at 0.5 mg PFDoA/kg/day. Decreased testosterone levels and AR expression were observed at 0.5 mg PFDoA/kg/day, suggesting that changes in these two factors may be responsible for morphological changes seen in the testis after exposure to 0.5 mg PFDoA/kg/day. Although testosterone levels were also decreased at 0.2 mg PFDoA/kg/day, abnormal testicular morphology was not observed. One explanation for this specificity may be that the small amount of AR

expression enhanced the responsiveness of testicular cells to testosterone stimulation and compensated for its decline.

Testosterone production is highly regulated via negative feedback control of the hypothalamic–pituitary–testicular (HPT) axis. LH from the pituitary plays a crucial role in regulating testosterone synthesis in this feedback cycle. In the present study, the expression of some genes, including hypothalamic AR, pituitary GnRH-R, and pituitary FSH, were significantly decreased by PFDoA treatment. However, serum LH levels were stable across all exposure groups, suggesting that the reduction in testosterone may be due to a mechanism that is independent of serum LH levels. Moreover, levels of serum FSH and GH, two hormones secreted by the pituitary, were not affected by any level of PFDoA exposure in male rats, further suggesting that PFDoA does not affect testis activity by disrupting hypothalamic and pituitary function. Collectively, these results imply that PFDoA may inhibit testosterone production via direct disruption of testicular function.

Serum cholesterol is a major component of testosterone biosynthesis. In the present study, serum cholesterol levels were not significantly affected by chronic PFDoA exposure, indicating that PFDoA did not disrupt cholesterol synthesis in male rats. To further clarify the mechanism of testosterone reduction following chronic PFDoA treatment, we measured the expression levels of testicular genes responsible for cholesterol transport and steroidogenesis, including SR-B1, StAR, PBR, P450scc, 3β-HSD, 17β-HSD, and CYP17a. SR-B1 is responsible for cholesterol transport from the plasma into Leydig cells, where cholesterol is carried from the outer mitochondrial membrane to the inner mitochondrial membrane by StAR (Payne and Hales, 2004). In the inner mitochondrial membrane, cholesterol is converted to pregnenolone by P450scc. Pregnenolone is then converted to progesterone by 3β-HSD in the cytoplasm. Progesterone is converted to 17a-hydroxyprogesterone and androstenedione by CYP17a. Androstenedione, in turn, is converted to testosterone by 17β-HSD. In the current study, no significant changes in LHR, SR-B1, PBR, 3β-HSD, 17β-HSD, or CYP17a expression were observed, suggesting that these genes are not sensitive PFDoA targets or function as crucial factors in mediating PFDoA toxic action, although they play important roles in testosterone production in testicular Leydig cells.

Compelling evidence documenting the critical role of StAR in the regulation of steroidogenesis has been obtained from both basic and clinical studies (Caron et al., 1997; Hasegawa et al., 2000). Transcriptional and/or translational inhibition of StAR expression results in a dramatic decrease in steroid biosynthesis (Clark et al., 1997; Cooke, 1999). Thus, StAR has been viewed as a rate-limiting factor for testosterone biosynthesis in the testis (Clark et al., 1995; Stocco and Clark, 1996). Although StAR mRNA levels were significantly suppressed in rats following chronic PFDoA treatment at all tested doses, changes in StAR proteins were highly similar to changes in serum testosterone in male rats. Thus, it is possible that PFDoA decreased serum testosterone levels in male rats by affecting StAR protein levels. These results also suggest that StAR may be sensitive to PFDoA in male rats. Since StAR is crucial for cholesterol transport from the outer mitochondrial membrane to the inner mitochondrial membrane in Leydig cell (Stocco and Clark, 1996), PFDoA may disrupt this process and subsequently lead to testosterone inhibition. Interestingly, no clear correlation was observed between StAR mRNA transcription and protein abundance at 0.02 mg/kg/day. Since the pattern difference between mRNA and protein is associated with translational regulation and protein half-life *in vivo* (Greenbaum et al., 2003), it is possible that PFDoA disrupts the process of StAR translation or degradation, resulting in the observed discrepancy between mRNA and protein levels. In addition, LH may stimulate StAR expression through binding LHR. The result of stable serum LH levels and testicular LHR mRNA levels maintained across all groups demonstrates that, in the present study, PFDoA did not act via LH receptor-medicated signaling to affect StAR mRNA and protein levels.

In the inner mitochondrial membrane of Leydig cells, P450scc initiates pregnenolone production, the first step in steroidogenesis (Payne and Hales, 2004). Thus, this factor plays an important role in testosterone biosynthesis. We found that P450scc mRNA and protein expression significantly decreased after exposure to PFDoA at 0.05 mg/kg/day and 0.5 mg/kg/day, respectively. The discrepancy in the dose effects of PFDoA on mRNA and protein levels indicates that P450scc transcription and translation are regulated by complex mechanisms. Like StAR, we speculate that PFDoA may also disrupt P450scc translation and degradation *in vivo*. Since the decreased P450scc protein level was correlated with a decreased testosterone level at the 0.5 mg/kg/day dose, reduced P450scc protein rather than reduced P450scc mRNA may contribute to PFDoA's disruption of testosterone synthesis.

IGF-I is important for the control of Leydig cell numbers and the onset of steroidogenesis (Saez, 1994; Spiteri-Grech and Nieschlag, 1992). IGF-I may stimulate testosterone production in the testis via paracrine and autocrine pathways (Lin et al., 1986; Rouiller-Fabre et al., 1998; Saez, 1994). In the present study, testicular IGF-I mRNA levels were significantly decreased by PFDoA at 0.05 mg/kg/day, 0.2 mg/kg/day, and 0.5 mg/kg/day, suggesting that IGF-I, as a paracrine and autocrine factor, may participate in the PFDoA-mediated inhibition of testosterone. Moreover, testicular mRNA levels of IGF-IR, which binds to IGF-I and mediates its action, were also markedly decreased by PFDoA exposure at doses of 0.2 mg/kg/day and 0.5 mg/kg/day. This result further demonstrates that IGF-I signaling may play an important role in the testosterone suppression that results from chronic PFDoA exposure in male rats. Previous *in vitro* and *in vivo* studies clearly showed that IGF-I stimulates testosterone production by inducing StAR expression (Manna et al., 2006; Wang et al., 2003). Thus, IGF-I signaling may be indirectly involved in the PFDoA-mediated inhibition of testosterone in male rats through disruption of StAR expression at the mRNA and protein levels.

In addition, circulating GH may stimulate testosterone synthesis directly by binding to testicular GHR or indirectly by stimulating testicular IGF-I synthesis (Chandrashekar and Bartke, 1993; Hull and Harvey, 2000). The maintenance of serum GH levels and testicular GHR mRNA levels across all groups shows that GH signaling does not mediate the inhibition of testosterone by PFDoA. IGFBP-3, which predominantly binds to IGF-I in the testis, may regulate IGF-I activity and testosterone production in the testis (Lin et al., 1993; Rappaport and Smith, 1995). Our observation of the stable maintenance of IGFBP-3 mRNA levels across all groups demonstrates that IGFBP-3 is not associated with the inhibition of testosterone by PFDoA in male rats. Insulin has been shown to potentiate cAMP-mediated StAR expression and testosterone synthesis through its specific receptor, insulin receptor (IR) (Ballester et al., 2004; Lin et al., 1986; Manna et al., 2006). No significant changes in circulating insulin levels or testicular IR mRNA levels were observed in PFDoA-treated rats, suggesting that testicular insulin signaling is not involved in decreased StAR expression or in the subsequent inhibition of testosterone in male rats exposed to PFDoA.

Testicular Sertoli cells have been found to produce cytokine IL-1α, which is capable of stimulating Leydig cell testosterone production (Gustafsson et al., 2002; Sultana et al., 2000; Svechnikov et al., 2003). In the current study, after male rats were treated with PFDoA at 0.2 mg/kg/day or 0.5 mg/kg/day for 110 days, significant reductions in testicular IL-1α mRNA levels were observed in the two dosage groups. This result implies that decreased testicular IL-1α expression may contribute to testosterone reduction through the paracrine pathway in PFDoA-treated rats. Additionally, the role of IL-1α in stimulating testosterone synthesis has been shown to

be mediated by induction of StAR (Stocco, 2001; Svechnikov et al., 2003). Thus, depressed testicular IL-1α mRNA levels may be associated with decreased StAR expression in PFDoA-treated rats.

The serum testosterone level is not only associated with its synthesis pathway, but also with its catabolism. The liver is a primary organ for testosterone metabolism, and the testes also play a role in testosterone degradation. The 5α-reductase catalyzes conversion of testosterone to 5α-dihydrotestosterone, which is converted to 3α-androstanediol by reactive 3α-hydroxysteroid dehydrogenase (Jin and Penning, 2001). The cytochrome P450 aromatase enzyme, which is responsible for estradiol synthesis, catalyzes the conversion of testosterone to estradiol. We found that the mRNA levels of these genes in the liver and testes were not affected by PFDoA. Moreover, serum estradiol levels were also stable across all groups. These results imply that 110 days of PFDoA exposure may not affect testosterone degradation, although testosterone metabolites were not measured in this study. In another study, PFOA treatment of male rats for 14 days resulted in increased aromatase activity in the liver, but not in the testes (Biegel et al., 1995). In a 2-year study, PFOA also caused increased serum estradiol levels associated with enhancement of Leydig cell tumors in rats (Biegel et al., 2001). The difference in serum estradiol levels between PFOA and PFDoA exposures suggests the presence of different mechanisms by which PFOA and PFDoA exert their effects on estradiol levels in male rats. We conclude that serum testosterone decreases may be associated with the perturbation of testicular testosterone synthesis in PFDoA-treated male rats.

In summary, the present study reveals that PFDoA disrupts testicular steroidogenesis and the expression of related genes in male rats. Since changes in the protein levels of StAR and P450scc and mRNA levels of IGF-I, IGF-IR, and IL-1α exhibit were similar to serum testosterone fluctuations in PFDoA-exposed male rats, these factors may be important for testosterone suppression of PFDoA in male rats. Considering that IGF-I, IGF-IR, and IL-1α may regulate testosterone production indirectly by modulating StAR expression, it is possible that StAR plays a special role in PFDoA-mediated testosterone inhibition. In addition, the testosterone degradation process may not be involved in the inhibition of testosterone by PFDoA in male rats.

Conflict of interest

The author declares that there are no conflicts of interest.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (20777074 and 20677060).

Appendix A. Supplementary data

Supplementary data associated with this article can be found at doi:10.1016/j.toxlet.2009.04.014.

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