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Alterations in Gene Expression and Testosterone Synthesis in the Testes of Male Rats Exposed to Perfluorododecanoic Acid

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Perfluorododecanoic acid (PFDoA, C12), a synthetic perfluorinated chemical containing 12 carbons, has broad industrial applications and has been detected in sera from humans and other animals; however, few reports have addressed the effects of PFDoA exposure on male reproduction. In the present study, the effects of PFDoA exposure on testes ultrastructure, testosterone levels, and steroidogenic gene expression were investigated. Male rats were orally dosed for 14 days with 1, 5, or 10 mg PFDoA/kg/day or with vehicle. Absolute testis weight was diminished at the highest dose while relative testes weight was markedly increased at doses of 5 and 10 mg/kg/day. Total serum cholesterol levels were significantly increased at the highest dose. While luteinizing hormone was significantly decreased at the highest dose, testosterone was markedly decreased at doses of 5 and 10 mg PFDoA/kg/day. Serum levels of follicle-stimulating hormone were not significantly affected by PFDoA, and estradiol levels were markedly decreased only at 5 mg/kg/day. Leydig cells, Sertoli cells, and spermatogenic cells from rats that received 5 or 10 mg PFDoA/kg/day, exhibited apoptotic features including dense irregular nuclei, condensed chromatin, ill-defined nuclear membranes, and abnormal mitochondria. PFDoA exposure resulted in significant declines in mRNA expression of several genes involved in cholesterol transport and steroid biosynthesis at doses of 5 and 10 mg PFDoA/kg/day, while the gene expression of luteinizing hormone receptor and aromatase was not significantly changed. Our results demonstrate that PFDoA affects the reproduction function of male rats via alterations in steroidogenesis genes, testosterone levels, and testes ultrastructure.

Key Words: **perfluorododecanoic acid; testis; testosterone; ultrastructure; steroidogenesis gene.**

Perfluoroalkyl acids (PFAA), such as perfluorododecanoic acid (PFDoA, C12), perfluorodecanoic acid (PFDA, C10), perfluorooctanoate (PFOA, C8), and perfluorooctane sulfonate (PFOS, C8), are used in a variety of products including lubricants, paints, cosmetics, and fire-fighting foams. Commercial use of PFAA over the course of several decades has resulted in a broad distribution of stable precursors and metabolites in animals from terrestrial and aquatic environments (Armitage *et al.*, 2006; Martin *et al.*, 2004; Prevedouros *et al.*, 2006). Despite the widespread distribution of these compounds, relatively little is known about their environmental fate or their specific effects on animals (Hekster *et al.*, 2003; Kennedy *et al.*, 2004). Recently, PFAA were detected in multiple organisms from the Great Lakes area and in wildlife from the Canadian Arctic and East Greenland (Martin *et al.*, 2004; Smithwick *et al.*, 2006). The suspected toxicity of some PFAA along with their environmental persistence has raised concern among environmental agencies. The 3M Company, the major manufacturer of PFAA, announced a phase out of the production of sulfonyl-based perfluorochemicals beginning in December 2000.

PFOA has been shown to cause hepatomegaly, induce hepatic peroxisomes, and increase β-oxidation and necrosis of the liver in rodents (Griffith and Long, 1980; Ikeda *et al.*, 1985; Kennedy, 1987; Pastoor *et al.*, 1987). In addition to these effects on the liver, PFAA such as PFOA and PFOS have been found to adversely affect both prenatal and postnatal development as well as the reproductive system in laboratory animals (Lau *et al.*, 2003; Luebker *et al.*, 2005a,b; Thibodeaux *et al.*, 2003). In male rats, PFOA exposure resulted in decreased testosterone levels in serum and in testicular interstitial fluid, increased serum estradiol levels, and testis lesions (Biegel *et al.*, 1995). In a chronic mechanistic dietary study, PFOA exposure resulted in tumors in both Leydig cells and pancreatic acinar cells (Biegel *et al.*, 2001), indicating that PFOA may impact a range of reproductive tissues. Studies in male rats by Olson and Anderson (1983) indicated that PFDA targets the testis. In another experiment, PFDA markedly decreased plasma testosterone and dihydrotestosterone levels but did not affect plasma clearance of testosterone and plasma luteinizing hormone (LH) in male rats (Bookstaff *et al.*, 1990). These results suggested a direct effect of PFDA on testicular androgen production. Although previous studies clearly demonstrated that exposure to PFAA containing eight or 10 carbons led to alterations in testis function including androgen production, the possible mechanisms responsible for these toxicities were not thoroughly

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investigated. Until now, only one report describes a possible mechanism of PFDA inhibition of testosterone production. Boujrad *et al.* (2000) reported that PFDA decreased mRNA and protein expression of mitochondrial peripheral–type benzodiazepine receptor (PBR) *in vitro*. The resultant decrease in cholesterol transport and testosterone production suggested that PBR is a target of PFDA action in Leydig cells. In addition, studies with PFAA containing seven to 10 carbon atoms indicated that a shorter carbon chain length was more rapidly eliminated in the urine and was less bioaccumlative (Kudo *et al.* 2001; Ohmori *et al.* 2003). In another study, PFDA was shown to be more toxic than PFOA in rats based on LD_{50} values of 189 mg/kg body weight for PFOA and 41 mg/kg body weight for PFDA (Olson and Andersen, 1983). Taken together, these data indicate that PFDoA is likely more toxic than PFDA or PFOA, and thus, the effects of PFDoA on reproductive function should be examined carefully in both human and wildlife populations.

Thus, the aims of the present study were to determine whether PFDoA exposure produces a similar toxic effect as the eight or ten carbon PFAA in the male reproduction system and to determine the mechanism of PFDoA toxicity in the testis. As the objective of the present study was to evaluate the *in vivo* effects of PFDoA on reproductive function, serum levels of total cholesterol, testosterone, follicle-stimulating hormone (FSH), LH, and estradiol were measured in male rats following exposure to PFDoA. In addition, mRNA levels of genes responsible for cholesterol transport and steroid synthesis were examined. These genes included scavenger receptor class B type 1 (SR-B1), steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (P450scc), 3-beta-hydroxysteroid dehydrogenase (3β-HSD), 17-beta-hydroxysteroid dehydrogenase(17β-HSD), cytochrome P450c17 subfamily a (CYP17a), luteinizing hormone receptor (LHR), and aromatase. Changes in testes ultrastructure were also examined in the male rats as an additional marker of PFDoA exposure.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats with body weights of 230–240g were obtained from Weitong Lihua Experimentary Animal Central, Beijing, China. The animals were ranked by body weight and assigned to treatment and control groups (10 rats per group). Animals were housed two per cage and maintained in a mass air-displacement room with a 12-h light-dark cycle at 20°C–26°C with a relative humidity of 30–70%. Animals had access to food and water *ad libitum*. All rats were acclimatized for 1 week before experiments were begun.

Chemicals and treatments. PFDoA (CAS No.307-55-1, 95% purity) was purchased from Sigma Aldrich. All other chemicals and reagents were analytical grade. PFDoA was prepared in 0.5% Tween-20 (Beijing Chemical Reagent Co., Beijing, China) and orally given via gavage to rats in the treatment group for 2 weeks at doses of 1, 5, 10 mg/kg body weight/day. Control rats were treated similarly with the vehicle only. The doses chosen for this study were based on a preliminary trial in which all rats died during treatment for 14 days with a dose of PFDoA 20 mg/kg body weight/day. The PFDoA and the control Tween-20 solution were administered in a volume of 6 ml/kg of body weight. The gavage dosing was selected based on the accuracy of delivery of this volume. At the end of experiment, all rats were weighed, and six rats from each group were 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. Testes and epididymides were immediately isolated and weighed. One part of the testis was fixed in 2.5% gluteraldehyde and another part was frozen immediately in liquid nitrogen and stored at − 80°C for RNA isolation.

Ultrastructure observation. After testis and epididymis tissues were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) at 4°C for 24 h, the samples were washed with phosphate buffer (0.1M, pH 7.4) for 12 h and postfixed for 20 min in 1% OsO_4 in 0.1M phosphate buffer (pH 7.4). The samples were then washed with phosphate buffer (0.1M, pH 7.4) for 30 min, dehydrated in a series of increasing (20–100%) ethanol solutions, and embedded in Epon:alcohol mixture 1:1 for 2 h followed by 100% Epon for an additional 3 h. The samples then were incubated overnight in the oven. Thin sections (70 nm) were placed on copper grids and stained in 2% uranyl acetate solution in a 1% solution of lead citrate for 30 min. A JEM 100CX transmission electron microscope operated at 50–60 kV was used to visualize the ultrastructure of testes samples.

Serum hormone levels. Concentrations of serum testosterone and estradiol were measured by ELISA using commercial rat ELISA kits (RapidBio Lab, Calabasas, CA). Since FSH and LH levels of some rats were lower than the detection limit of the ELISA kits, these two hormones were detected by radioimmunoassay (RIA) using commercial kits from Beijing North Institute of Biological Technology, China. The assay detection limit was 1.0 mIU/ml for FSH and LH. Interassay and intraassay coefficients of variation were <10% and <15%, respectively. Both ELISA and RIA were performed according to the manufacturers' protocols.

Serum cholesterol levels. Total cholesterol (TCHO) in serum was measured with a commercial TCHO kit according to the manufacturer's recommendations (Biosino Bio-technology and Science Inc., Beijing, China). Interassay and intraassay coefficients of variation were < 3 and < 5%, respectively. Total cholesterol was quantitated colorimetrically (500 nm) using a UV1240 spectrophotometer (Shimadzu, Japan).

Reverse transcription-polymerase chain reaction. Total RNA was isolated from the testis using TRIZOL reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. 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. Reverse transcription was performed using oligo-$(dT)_{15}$ primer (Promega) and M-MuLV reverse transcriptase (New England Biolabs, United Kingdom) according to the manufacturers' instructions. Reverse transcription was achieved by incubation for 60 min at 37°C followed by 5 min at 95°C. Real-time PCR reactions were performed with the Stratagene Mx3000P qPCR system (Stratagene). SYBR Green PCR Master Mix reagent kits were used according to the manufacturer's instructions for quantification of gene expression. Rat-specific primers were designed for the genes of interest: SR-B1, StAR, P450scc, 3β-HSD, 17β-HSD, CYP17a, LHR, and aromatase (Table 1). Amplification products were 100–160 base pairs. The housekeeping gene β-actin was used as an internal control. Cycling conditions were as follows: 95°C for 15 min followed by 40 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. After PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product, which was displayed by a single peak (data not shown). Every sample was analyzed in triplicate. Amplification efficiencies were assumed to be 100% for both the gene of target and β-actin. The relative expression ration (R) of a target gene is expressed in a sample versus a control in comparison to β-actin gene and calculated based on the following equation (Pfaffl, 2001).

$$R = 2^{-\Delta\Delta C_t}$$

In the formula, C_t represents the cycle at which the fluorescence signal is first significantly different from background; $\Delta\Delta C_t = (C_{t,\text{target}} - C_{t,\text{actin}})_{\text{treatment}} - (C_{t,\text{target}} - C_{t,\text{actin}})_{\text{control}}$.

TABLE 1
Sequences of Primers Used for Real-Time RT-PCR Amplification

Target gene	GenBank accession number	Product length (bp)	Primer sequences	Temperature (°C)
SR-B1	AY451993	156	Sense: 5′-ACAGGTCCCAGGGCTCAG-3′	57.0
			Anti-sense: 5′-CGTGCGGTTCATAAAGG-3′	
LHR	NM_012978	130	Sense: 5′-CATTCAATGGGACGACTCTA-3′	53.0
			Anti-sense: 5′-GCCTGCAATTTGGTGGA-3′	
StAR	NM_031558	111	Sense: 5′-GGGCATACTCAACAACCAG-3′	57.0
			Anti-sense: 5′-ACCTCCAGTCGGAACACC-3′	
P450SCC	J05156	115	Sense: 5′-CTTTGGTGCAGGTGGCTAG-3′	57.0
			Anti-sense: 5′-CGGAAGTGCGTGGTGTTT-3′	
3β-HSD	M38178	145	Sense: 5′-TGTGCCAGCCTTCATCTAC-3′	53.0
			Anti-sense: 5′-CTTCTCGGCCATCCTTTT-3′	
CYP17a	NM_012753	114	Sense: 5′-CTCTGGGCACTGCATCAC-3′	53.0
			Anti-sense: 5′-CAAGTAACTCTGCGTGGGT-3′	
17β-HSD	NM_054007	140	Sense: 5′-GACCGCCGATGAGTTTGT-3′	53.0
			Anti-sense: 5′-TTTGGGTGGTGCTGCTGT-3′	
Aromatose	NM_017085	142	Sense: 5′-GCCTGTCGTGGACTTGGT-3′	57.0
			Anti-sense: 5′-GGTAAATTCATTGGGCTTGG-3′	
β-Actin	NM_031144	134	Sense: 5′-TCGTGCGTGACATTAAAGAG-3′	57.0
			Anti-sense: 5′-ATTGCCGATAGTGATGACCT-3′	53.0

Statistical analysis. All data were analyzed using SPSS for Windows 13.0 Software (SPSS, Inc., Chicago, IL). All values are expressed as mean ± SEM. The ratio of testis organ to body weight was calculated to yield relative testis weights. Body weight and relative weight of testis were analyzed using one-way ANOVA followed by Dunnett's *post hoc* two-sided *t*-test. Differences in testis weight, serum hormone concentrations, and gene expression levels between the treatment and control groups were analyzed using a general linear model. Body weight was used as a covariant factor in analysis of these indicators. Dunnett's *post hoc* two-sided *t*-test was used to confirm difference between the control and treatment group. A probability (p) of less than 0.05 was chosen as the limit for statistical significance.

RESULTS

Body and Testes Weight

The body and testes weights of male rats treated for 14 days with PFDoA are shown in Table 2. No significant changes in body weight were observed following exposure to PFDoA at 1 mg/kg/day compared with controls; however, for animals exposed to 5 and 10 mg/kg/day, body weight was significantly reduced by 25.7% and 38%, respectively ($p < 0.01$). Though testes weight exhibited a degressive trend at 1 and 5 mg/kg/day, no significant differences were observed between the control group and these two treatment groups. In the rats receiving 10 mg PFDoA/kg/day, the testis weight was significantly diminished ($p < 0.05$). Relative testis weights were markedly increased at doses of 5 and 10 mg/kg/day ($p < 0.05$).

Total Cholesterol in Serum

Total serum cholesterol levels were significantly increased by 35% at a dose of 10 mg PFDoA/kg/day ($p < 0.05$) (Fig. 1). No significant differences in cholesterol levels were observed for the 1- and 5-mg/kg/day groups compared to the control group.

Hormone Levels

The effects of PFDoA on hormone levels in male rat serum are shown in Figure 2. Testosterone was markedly decreased to 38 and 16% of the control rats at doses of 5 and 10 mg PFDoA/kg/day, respectively ($p < 0.05$). For 1 mg PFDoA/kg/day, testosterone levels were above the control level, but no statistical difference was observed. The LH levels were significantly decreased only for the 10-mg/kg/day group ($p < 0.05$). PFDoA exposure did not significantly affect the serum concentrations of FSH, although the levels were decreased by 28% in the 5-mg/kg/day group. The serum level of estradiol showed a decrease similar to FSH in rats following exposure to PFDoA, but this decrease was statistically different from the control only at a dose of 5 mg/kg/day ($p < 0.05$).

Ultrastructural Character in Testes

For vehicle-treated rats, Leydig cells, Sertoli cells, and spermatogenic cells in the testis showed normal ultrastructure with intact nuclei and peripherally arranged heterochromatin. The cytoplasm in these control cells contained varied numbers of lipid droplets and organelles (Figs. 3A and 3B). As compared with the control testes, the ultrastructure in the Leydig cells, Sertoli cells, and spermatogenic cells remained relatively normal in rats receiving 1 mg PFDoA/kg/day. In rats treated with 5 mg PFDoA/kg/day, the Sertoli cells had large clustered lipid droplets and enlarged mitochondria in the

TABLE 2
Body Weight and Testis Weight of Rats Treated with PFDoA for 14 Days

Doses (mg/kg/day)	0	1	5	10
Body weight (g)	292.30 ± 13.14	288.33 ± 10.55	218.70 ± 9.19**	184.50 ± 8.52**
Testis weight (g)	3.03 ± 0.18	2.74 ± 0.15	2.68 ± 0.19	2.35 ± 0.14*
Relative testis weight (%)[a]	0.97 ± 0.045	0.92 ± 0.056	1.32 ± 0.038**	1.32 ± 0.088**

Note. Data are given as mean ± SEM from 10 rats per group for weight or six rats per group for testis weight and relative testis weight.
[a]Percentage of total body weight.
Significant difference from control, *$p < 0.05$; **$p < 0.01$.

cytoplasm (Fig. 3C). The nucleus was altered with dense irregular nuclei and disintegrated membranes. In rat that received a dose of 10 mg/kg/day, the Sertoli cells exhibited large lipids droplets, and significant features of apoptosis including condensed chromatin, broken nuclei, and vacuolated mitochondria (Fig. 3D). The Leydig cells from rats that received 5 mg PFDoA/kg/day had large vacuoles and expanded mitochondria scattered throughout the cytoplasm (Fig. 3G). These cells exhibited dense irregular nuclei, ill-defined nuclear membranes, and slightly condensed chromatin. From rats receiving 10 mg PFDoA/kg/day, the Leydig cells also exhibited obvious features of apoptosis with condensed chromatin, broken nuclei, and vacuolated mitochondria similar to the Sertoli cells (Fig. 3H). The spermatogenic cells from rats treated with 5 mg PFDoA/kg/day had large number of vacuoles as well as dilated and vacuolated mitochondria in the cytoplasm (Fig. 3K); however, the nuclei remained as a relatively integrated structure. At doses of 10 mg PFDoA/kg/day, spermatogenic cells also demonstrated significant features of apoptosis similar to the Sertoli cells and the Leydig cells (Fig. 3L). Taken together, these observations indicate that features of apoptosis are common in Leydig cells, Sertoli cells, and spermatogenic cells in testes of rats treated with PFDoA.

Gene Expression

The effects of PFDoA exposure on mRNA expression of genes involved in cholesterol transport and steroidogenesis in male rats were determined (Figs. 4 and 5). Compared to the controls, expression of the gene for transporting plasma cholesterol to steroidogenic tissues, SR-B1, was significantly decreased by 40 and 37% in the 5- and 10 mg-PFDoA/kg/day groups, respectively ($p < 0.01$). Levels of StAR, which is responsible for cholesterol transport to the inner mitochondrial membrane, were also significantly reduced to 44 and 25% of the controls at doses of 5 and 10 mg PFDoA/kg/day, respectively ($p < 0.01$). Compared to the control, expression of P450scc, an enzyme that catalyzes cholesterol side-chain cleavage to form pregnenolone, was markedly reduced to 9 and 4% of the control at doses of 5 and 10 mg PFDoA/kg/day, respectively ($p < 0.01$). Similarly, CYP17a, which plays a significant role in steroid hormone synthesis, was also notably decreased by 22 and 4% of the controls at doses of 5 and 10 mg PFDoA/kg/day, respectively ($p < 0.05$).

Meanwhile, a significant reduction in mRNA levels was observed for 3β-HSD and 17β-HSD. Levels reached approximately 55–77% of the controls at doses of 5 and 10 mg PFDoA/kg/day ($p < 0.05$). Interestingly, no significant differences between the controls and the PFDoA treatment groups were detected for LHR and aromatase mRNA expression, although LHR decreased with increasing PFDoA exposure. At doses of 1 mg PFDoA/kg/day, no statistical differences in the expression of StAR, P450scc, or CYP17a were observed.

DISCUSSION

In this study, serum testosterone levels were markedly decreased in male rats that had been exposed to 5 or 10 mg PFDoA/kg/day. LH was decreased only in rats exposed to 10 mg PFDoA/kg/day, while estradiol was decreased only in rats exposed to 5 mg PFDoA/kg/day. In addition, an accompanying decline was observed for mRNA levels of genes

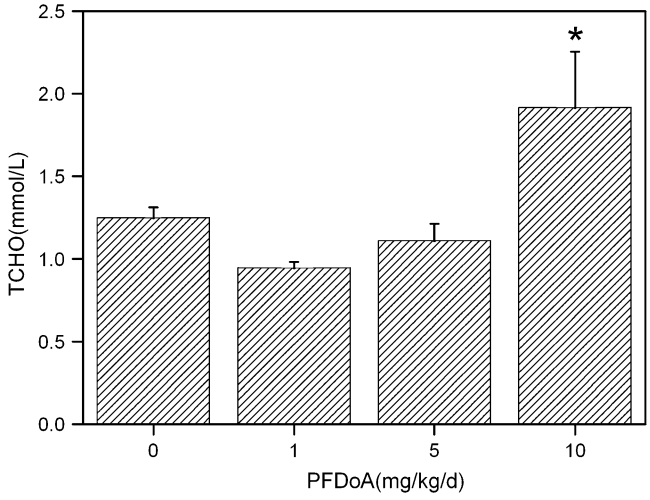

FIG. 1. Effects of PFDoA exposure on TCHO in serum in male rats. Values represent the average ± SEM from six rats per group. A statistically significant difference ($p < 0.05$) is indicated by an asterisk.

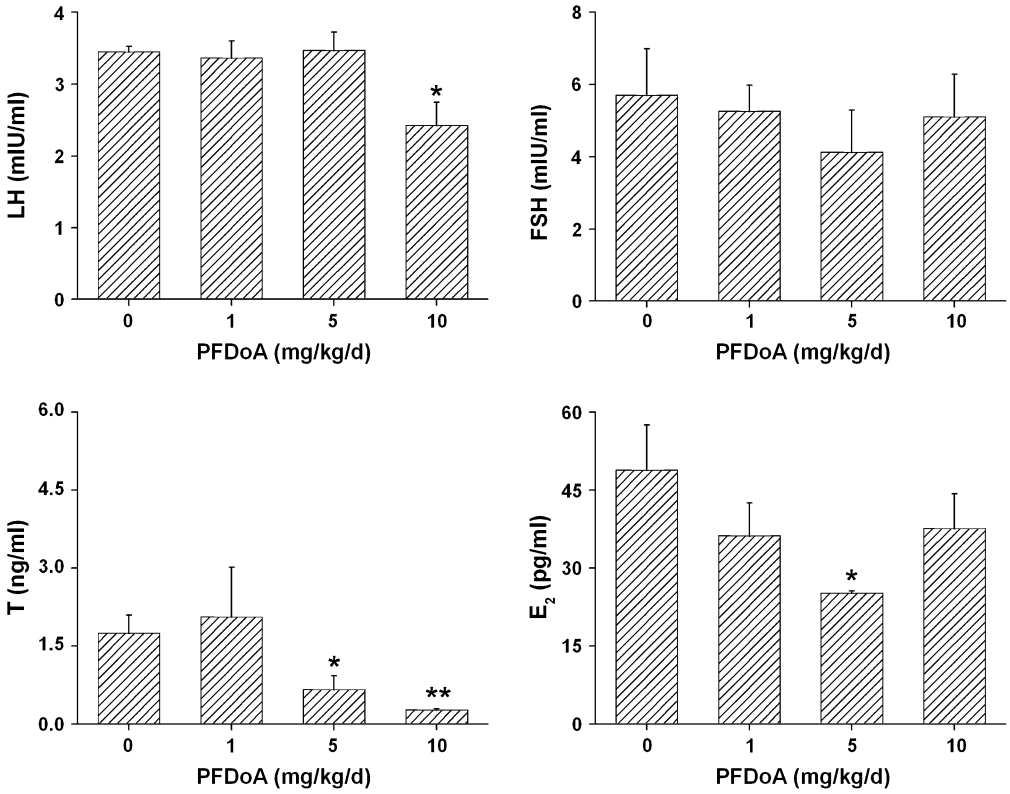

FIG. 2. Serum levels of LH, FSH, testosterone (T), and estradiol (E_2) from control- and PFDoA-exposed male rats. Values represent the average ± SEM from six rats per group. For T in the control group and 1 mg/kg/day and LH in the 10-mg/kg/day group, the mean represents only five rats. Asterisks indicate a statistically significant difference: $*p < 0.05$, $**p < 0.01$.

responsible for cholesterol transport and steroidogenesis including SR-B1, StAR, P450scc, 3β-HSD, 17β-HSD, and CYP17a. Total cholesterol levels were, in fact, decreased in rats exposed to 10 mg PFDoA/kg/day. Moreover, adverse changes in the ultrastructure of the testes were observed in the male rats exposed to PFDoA. The ability of PFDoA to disrupt steroidogenesis and the mechanisms by which the compound interferes with the function of steroidogenic enzymes is a relatively unexplored area of toxicology. This report is the first to thoroughly investigate the possible molecular mechanisms of serum testosterone reduction and to report alterations in ultrastructure in the testes following exposure to PFDoA.

Mean body weight was significantly reduced by 25.7 and 38% of the control for rats exposed to 5 or 10 mg PFDoA/kg/day, respectively. In similar studies, rats exposed to PFOA for 28 or 90 days, body weight decreased significantly at doses greater than 50 and 15 mg/kg/day, respectively (Griffith and Long, 1980). Moreover, some studies have shown that PFAA with a longer carbon chain length exhibit greater toxicity (Kudo *et al.*, 2001; Ohmori *et al.*, 2003). This idea was further supported as the LD_{50} of PFDA (C10) is far less than the LD_{50} of PFOA (C8) in rats (Olson and Andersen, 1983). Although the experiment was conducted with low doses of PFDoA and for a relatively short period of time, the documented decline in body weight in rats in this experiment suggests that PFDoA is more toxic than PFOA. The absolute testes weight remained unaltered at dosages of 1 and 5 mg PFDoA/kg/day but notably decreased only at 10 mg PFDoA/kg/day. These results suggest a reduced level of circulating testosterone may be responsible for testes weight as androgen exerts their major effects on testicular size and weight. Increases in the relative testes weight at doses of 5 and 10 mg PFDoA/kg/day, however, likely reflect the observed decrease in body weight.

With regard to male reproductive function, the level of serum testosterone in the male rats was significantly decreased to 38 and 16% of the control for the 5- and 10-mg PFDoA/kg/day groups, respectively. This result is in agreement with decreased testosterone levels in male rats following PFOA (Biegel *et al.*, 1995) and PFDA (Bookstaff *et al.*, 1990) exposure for 14 and 7 days. Since the testis has been implicated as a target organ for PFDA action (Olson and Andersen, 1983) and PFDA may directly affect testicular androgen production (Bookstaff *et al.*, 1990), the testis may also be directly affected by PFDoA. Testosterone production is mainly regulated by LH secreted from the pituitary gland (Wang and Stocco, 2005). In the current studies, LH levels were significantly decreased in the 10-mg PFDoA/kg/day rats, suggesting that the reduction in testosterone may be due, at least in part, to decreased LH levels.

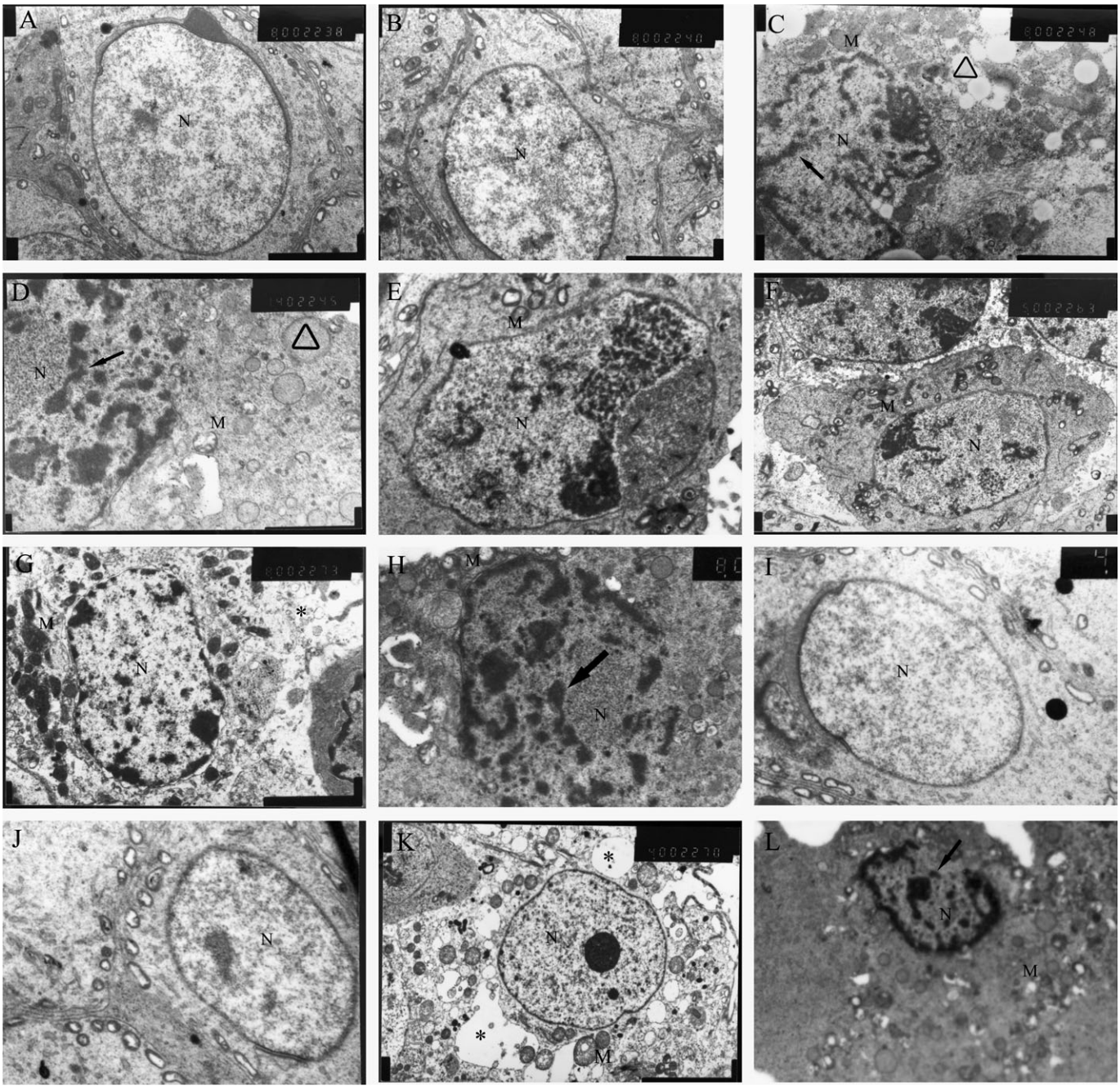

FIG. 3. Ultrastructure of Sertoli cells, Leydig cells, and spermatogenic cells from male rats exposed to PFDoA or vehicle. Sertoli cells from testis of rats exposed to vehicle (A, magnification ×8000), 1 mg PFDoA/kg/day (B, ×8000), 5 mg PFDoA/kg/day (C, ×8000), or 10 mg PFDoA/kg/day (D, ×14000). Leydig cells from testis of rats exposed to vehicle (E, ×5000), 1 mg PFDoA/kg/day (F, ×5000), 5 mg PFDoA/kg/day (G, ×8000), or 10 mg PFDoA/kg/day (H, ×8000). Spermatogenic cells from testis of rats exposed to vehicle (I, ×4000), 1 mg PFDoA/kg/day (J, ×4000), 5 mg PFDoA/kg/day (K, ×4000), or 10 mg PFDoA/kg/day (L, ×2700). N indicates nucleus; M indicates mitochondria; triangles indicate lipid droplets; arrows indicate condensed chromatin; asterisk indicates vacuoles.

Although these data together support the testes as a target for PFDoA action, the possibility that this compound affects the pituitary as well cannot be eliminated, although FSH, which is secreted from the pituitary, was not affected by PFDoA.

Examination of the testes ultrastructure in rats exposed to PFDoA revealed features of apoptosis in Leydig cells, Sertoli cells, and spermatogenic cells at doses of 5 and 10 mg PFDoA/kg/day. These cells exhibited dense irregular nuclei, condensed chromatin, and ill-defined nuclear membranes. Similar results were also observed following PFDA exposure (Olson and Andersen, 1983). Early degenerative changes in the rat testis were associated with a decrease in plasma testosterone levels

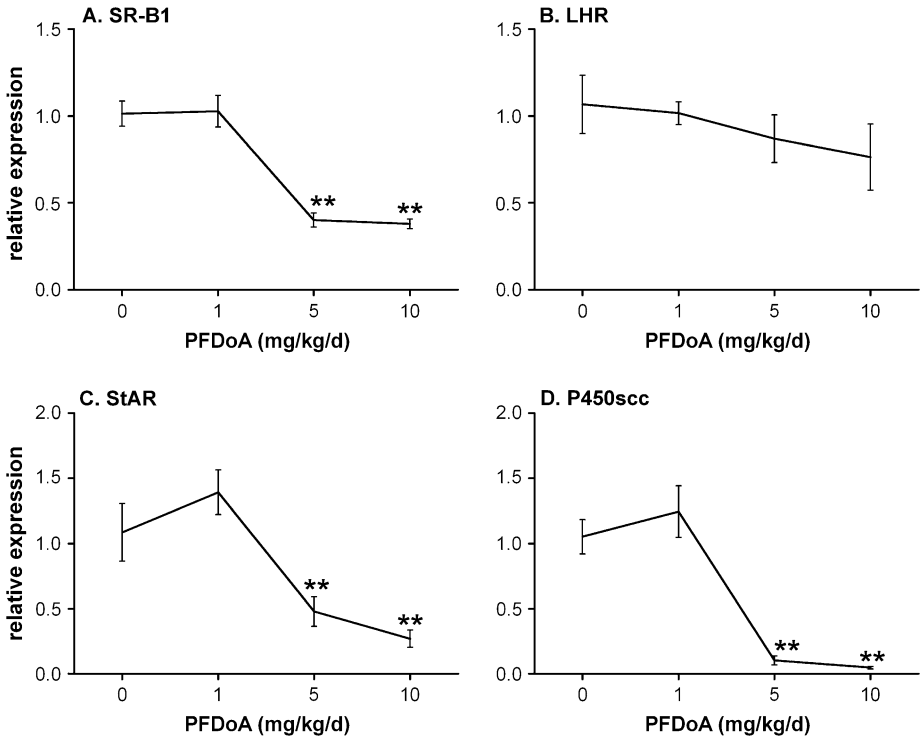

FIG. 4. Real-time quantitative RT-PCR analyses of testicular mRNA for SR-B1, LHR, StAR, and P450scc from control- and PFDoA-exposed rats. Gene expression levels represent the relative mRNA expression compared to controls. Values represent the mean ± SEM for six rats per group. Asterisks indicate a statistically significant difference, where $*p < 0.05$, $**p < 0.01$.

8 days after PFDA treatment and progressed to tubular atrophy, calcification, and necrosis by 16 days. Testosterone, which is considered a trophic factor for testis, plays a key role in the induction of apoptosis in the testis (Young and Nelson, 2001). In fact, testis cell apoptosis that resulted from decreased testosterone in male rats treated with endocrine disruptors were reversed once testosterone levels were restored (Bartlett *et al.*, 1986; Molenaar *et al.*, 1985; Nandi *et al.*, 1999). Collectively, dramatically decreased testosterone may contribute to changes in testes ultrastructure. Whether PFDoA directly induces cell apoptosis in testis remains to be determined.

Testosterone is synthesized in Leydig cells (Fig. 6; Barlow *et al.*, 2003; Payne and Hales, 2004). Genes directly responsible for cholesterol transport and testosterone synthesis in rat testes include SR-B1, StAR, P450scc, 3β-HSD, CYP17a, and 17β-HSD. SR-B1 is the predominant receptor that supplies plasma cholesterol to steroidogenic tissues in rodents (Cao *et al.*, 1999; Trigatti *et al.*, 2000a,b). Although cholesterol for steroidogenesis in the testis may be obtained from the conversion of intracellular acetate, the preferred source of cholesterol is via uptake of cholesteryl esters from high-density lipoprotein (HDL) by SR-B1 (Cao *et al.*, 1999). In this study, serum cholesterol levels remained relatively stable at 1 and 5 mg PFDoA/kg/day and increased following exposure to 10 mg PFDoA/kg/day; however, SR-B1 gene expression at doses of 5 and 10 mg PFDoA/kg/day were significantly decreased to 40 and 37% of control. Thus, these results suggest that the levels of cholesterol transported from the plasma into the cells or the total cholesterol within the cells was decreased, although the cholesterol in cells was not measured directly. Moreover, apoptosis of Leydig cells also indicates that cell functions including cholesterol transport are damaged following PFDoA exposure. Furthermore, reports that SR-B1 knockout mice have a two- to threefold increase in plasma HDL and a reduction in the selective uptake of cholesterol esters (Cao *et al.*, 1999) further supports the role of SR-B1 in this process.

StAR is necessary for the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane in Leydig cells, and this step is the rate-limiting regulated step in steroidogenesis (Hasegawa *et al.*, 2000; Manna *et al.*, 2001). The transcriptional and/or translational inhibition of StAR expression results in a dramatic decrease in steroid biosynthesis (Clark *et al.*, 1995; Stocco and Clark, 1996). In this study, expression levels of StAR were significantly decreased to 44 and 25% of the control following exposure to 5 or 10 mg PFDoA/kg/day. This reduction may affect cholesterol delivery across the mitochondrial membrane and thus contribute to the decrease in testosterone synthesis. StAR expression is stimulated via LH binding to its receptor (LHR). Therefore, LH also plays an indirect role in the delivery of cholesterol to the inner mitochondrial membrane (Omura and Morohashi 1995; Wang and Stocco, 2005). We observed

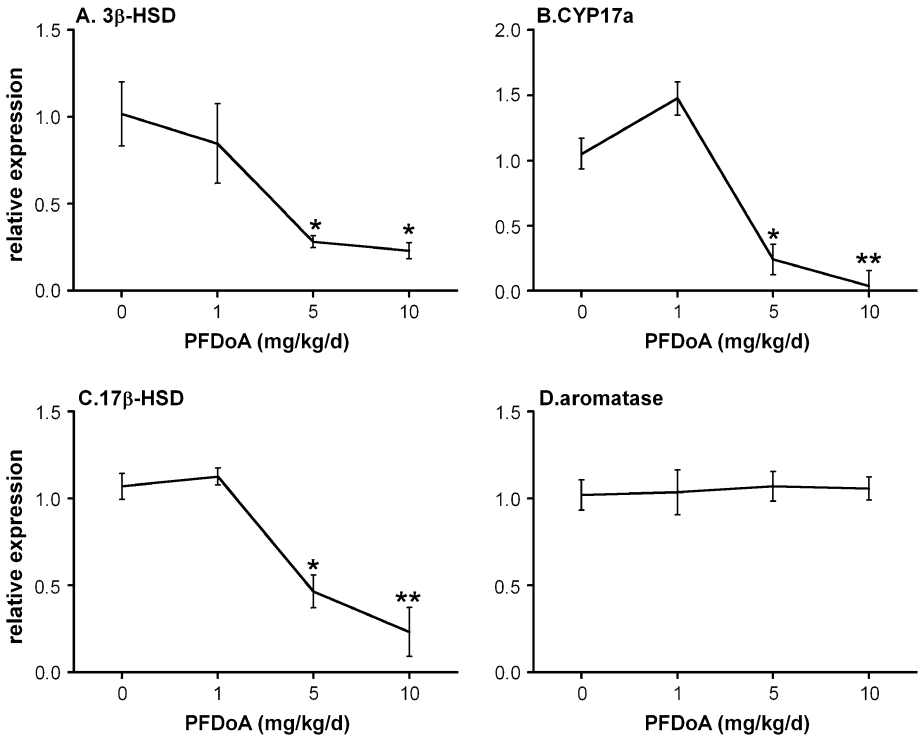

FIG. 5. Real-time quantitative RT-PCR analyses of testicular mRNA for 3β-HSD, 17β-HSD, CYP17a, and aromatase from control- and PFDoA-exposed rats. Gene expression levels represent the relative mRNA expression compared to controls. Values represent the mean ± SEM for six rats per group. Asterisks indicate a statistically significant difference, where $*p < 0.05$, $**p < 0.01$.

a significant reduction in serum LH levels in rats exposed to 10 mg PFDoA/kg/day, but LHR expression remained relative stable across all groups. These results suggest that a reduction in LH results in decreased StAR expression at levels of 10 mg PFDoA/kg/day, and therefore, PFDoA affects the transport of cholesterol into the inner mitochondrial membrane. Boujrad *et al.* (2000), however, reported that PFDA inhibits testosterone production *in vitro* via mitochondrial PBR and not via StAR. PBR, like StAR, mediates cholesterol delivery from the outer to the inner mitochondrial membrane. PFDA reduced Leydig cell PBR mRNA, protein, and ligand-binding levels and resulted in a reduced ability of the cells to synthesize steroids in response to human chorionic gonadotropin (hCG), an LH analog. In these studies, no changes in hCG-induced StAR levels were observed upon treatment of the cells with PFDA. In *in vivo* studies with 7-day PFDA exposure, no changes in circulating LH levels were observed (Bookstaff *et al.*, 1990). Although PBR expression was not determined in this particular experiment, these results suggest different mechanisms for the action of PFDoA and PFDA in inhibiting testosterone synthesis. These differences may correlate with the carbon chain length of the compounds or may result from differences between the *in vivo* and *in vitro* systems.

The conversion of cholesterol to pregnenolone by P450scc is a limiting enzymatic step in testosterone biosynthesis (Miller, 1988; Omura and Morohashi, 1995). The P450scc mRNA

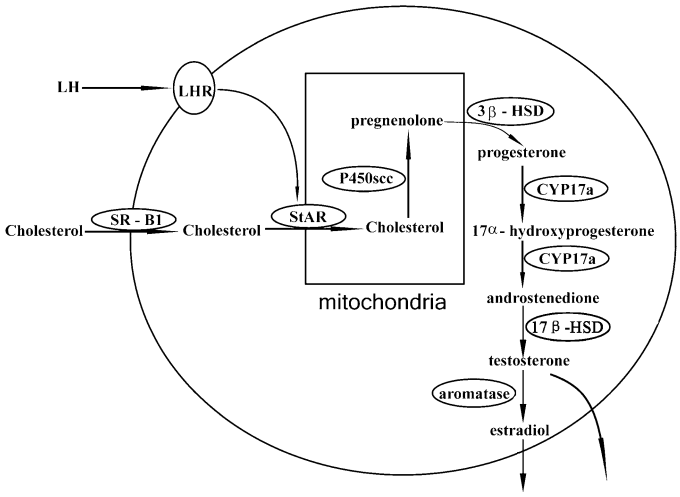

FIG. 6. Testosterone biosynthetic pathways in Leydig cells. Cholesterol is transported from the plasma into the cell by SR-B1. Then, the cholesterol is carried from the outer mitochondrial membrane to the inner mitochondrial membrane by StAR. The function of StAR is regulated by the binding of LH to the LHR. In the inner mitochondrial membrane, cholesterol is converted to pregnenolone by P450scc. Pregnenolone is then transported to the smooth endoplasmic reticulum in the cytoplasm, where it is converted to progesterone by 3β-HSD. Progesterone is then converted to 17α-hydroxyprogesterone and androstenedione by CYP17a. Androstenedione is converted to testosterone by 17β-HSD. Partial testosterone is catalyzed to estradiol by aromatase.

levels were decreased significantly to 9 and 4% of the control in rats exposed to 5 and 10 mg PFDoA/kg/day, respectively. Although alteration of SR-B1 and StAR appear to contribute to decreased testosterone synthesis, the significantly decreased level of mRNA expression for P450scc indicates an additional contributor. Decreased expression of P450scc may be partially due to the reduced expression of SR-B1 and StAR as the expression of the gene responsible for conversion of cholesterol to the next intermediate may have been downregulated. The considerable decrease of P450scc suggests that this gene is more sensitive to PFDoA than either StAR or SR-B1 in rats.

Expression of 3β-HSD, CYP17a, and 17β-HSD were significantly decreased in rats following exposure to 5 or 10 mg PFDoA/kg/day. These genes also play important roles in testosterone biosynthesis (Fig. 6; Barlow *et al.*, 2003; Payne and Hales, 2004). For example, 3β-HSD catalyzes the conversion of pregnenolone to progesterone in the smooth endoplasmic reticulum. Progesterone is then converted to 17α-hydroxyprogesterone and androstenedione by CYP17a. Finally, androstenedione is converted to testosterone by 17β-HSD. The decrease in gene expression of 3β-HSD, CYP17a and 17β-HSD indicates a possible contribution of these genes to the decrease in serum testosterone of male rats following PFDoA exposure. Interestingly, CYP17a was markedly decreased to 22% of the control at PFDoA levels of 5 mg/kg/day and further decreased to 4% of the control at 10 mg PFDoA/kg/day. As a result, this gene may also be sensitive to PFDoA exposure similar to P450scc. The reasons for these alterations in gene expression, however, remain to be elucidated.

The cytochrome P-450 aromatase enzyme, the rate-limiting and FSH-dependent factor in estradiol synthesis, catalyzes the conversion of testosterone to estradiol. The present study demonstrated that aromatase mRNA levels remained stable in all PFDoA treatment groups compared to the control group. Serum estradiol levels were reduced in rats exposed to 1 and 10 mg PFDoA/kg/day, but a statistically significant decline was observed only for rats receiving 5 mg PFDoA/kg/day. These changes in serum estradiol levels differ from those obtained in another study, in which increased serum estradiol levels were observed in adult male rats treated for 14 days with PFOA. This increase in estradiol was accompanied by a reduction in serum and testicular interstitial fluid testosterone levels. In addition, PFOA increased aromatase activity in the liver but not in the testes, suggesting that augmentation of serum estradiol levels in PFOA-treated rats resulted from an increase in aromatase activity in the liver (Biegel *et al.*, 1995). Since FSH stimulates the conversion of testosterone to estradiol, the finding that FSH was not significantly altered in PFDoA-exposed rats suggests FSH may not contribute to production of estradiol in present study. Taken together, these data indicate that changes in the serum estradiol levels in PFDoA-exposed male rats correlates with the decline in testosterone levels. In addition, although aromatase activity was not measured directly in this study, these data suggest that aromatase in the testes is not a target of PFDoA action.

The testis, which is an important endocrine organ for maintaining homeostasis for androgens, is a target organ for some endocrine disrupting chemicals (EDCs) such as di (*n*-butyl) phthalate and polychlorinated biphenyls (Fisher, 2005). EDCs can induce endocrine disruption via a number of routes that involve steroid receptor binding (agonists), blocking of steroid receptor binding (antagonists), or disrupting the biosynthesis and or metabolism of steroids (Sharpe and Irvine, 2004). In the present study, PFDoA was shown to disrupt testosterone biosynthesis and to cause adverse effects such as cell apoptosis in the testis. Collectively, these data indicate that PFDoA may function as an EDC as this compound interferes with the function of steroidogenic enzymes in male rats; however, the mechanism by which PFDoA affects these genes expression remains unknown. Further studies with particular attention to the status of enzymes involved in steroid metabolism are necessary to further understand the mechanism of PFDoA disruption of steroidogenesis.

In summary, this study demonstrates that PFDoA exposure leads to decreased testosterone biosynthesis in male rats with a concomitant reduction in expression of key genes responsible for cholesterol transport and steroidogenesis as well as apoptosis-like changes occurring in testes cells. Decreased testosterone synthesis was not a result of changes in the conversion of testosterone to estradiol or of cholesterol levels but most possibly a result of decreased steroidogenesis gene expression. The dramatic decreases in P450scc and CYP17a expression suggest these two genes are more sensitive to PFDoA exposure in male rats than other genes chosen in this trial.

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